

S. Mohan Jain
H. Häggman
Editors

Protocols for Micropropagation of Woody Trees and Fruits



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PROTOCOLS FOR MICROPROPAGATION
OF WOODY TREES AND FRUITS

Protocols for Micropropagation of Woody Trees and Fruits

Edited by

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PREFACE

Micropropagation has become a reliable and routine approach for large-scale rapid plant multiplication, which is based on plant cell, tissue and organ culture on well defined tissue culture media under aseptic conditions. A lot of research efforts are being made to develop and refine micropropagation methods and culture media for large-scale plant multiplication of several number of plant species. However, many woody and fruit plant species still remain recalcitrant to *in vitro* culture and require highly specific culture conditions for plant growth and development.

The recent challenges on plant cell cycle regulation and the presented potential molecular mechanisms of recalcitrance are providing excellent background for understanding on plant cell totipotency and what is more development of micropropagation protocols. Today, the need for appropriate *in vitro* plant regeneration methods is overwhelming both for basic and applied research in order to overcome problems facing micropropagation such as somaclonal variation, recalcitrant rooting in woody species, hyperhydricity, high labour cost, contamination, loss of material during hardening, quality of plant material and polyphenols. For large-scale *in vitro* plant production the important attributes are the quality, cost effectiveness, maintenance of genetic fidelity, and long-term storage. Moreover, the useful applications of micropropagation in various aspects make this technology more relevant for example to production of virus-free planting material, cryopreservation of endangered and elite woody species, applications in tree breeding, afforestation and reforestation. Reforestation is important to prevent the loss of forest resources including timber, biodiversity and water resources, and would require continuous supply of planting material. The majority of world wood products still come from natural and semi-natural forests, but there is a clear trend towards more efficient plantation forestry. Generally, the development of vegetative propagation methods will yield additional profit for plantation forestry by the exploitation of non-additive genetic variation, by providing more homogenous planting material and by compensating potential shortage of improved seed stock.

The fruit trees and shrubs are grown to produce fruits to be consumed both as fresh and as processed forms including juices, beverages, and dried fruits. They are an important source of nutrition, e.g. rich in vitamins, sugars, aromas and flavour compounds, and raw material for food processing industries. Fruit trees have long juvenile periods and large tree size. Moreover, fruit trees are faced with agronomic and horticultural problems in terms of propagation, yield, appearance, quality, diseases and pest control, abiotic stresses and poor shelf-life. The available genetic information in fruit crops is very limited and their genetic improvement has heavily relied on classical breeding and on vegetative propagation of specific cultivars. Furthermore, micropropagation has increasingly been promoted in enhancing the total number of genetically modified fruit plants.

Our previous book entitled *Micropropagation of Woody Trees and Fruits* provided a comprehensive coverage on various aspects on micropropagation of economically important forest and fruit trees. However, it did not exclusively focus

on precise stepwise protocols for plant multiplication. The introductory chapter of this book will cover the present knowledge of plant cell totipotency in the context of the cell cycle and the potential mechanisms of gene silencing in competence and recalcitrance. The follow-up chapters will cover micropropagation protocols of diverse plant species, i.e. the practical examples of plant cell totipotency. The book will provide information on 'organogenesis' approach for plant multiplication, and various applications such as genetic transformation, cryopreservation and others. The chapters are easy to follow including step by step protocols for numerous woody plants. Therefore, the book can be used as a practical handbook in tissue culture laboratories. It will certainly benefit students, researchers, horticulturists, forest geneticists, and biotech companies.

This book has a total of 48 chapters on micropropagation protocols and is divided into three sections: Section A) contains 1–22 chapters on forest and nitrogen fixing trees, Section B) covers 23–40 chapters on fruit trees, and Section C) deals with 41–48 chapters on non-tree plants such as bananas and small fruits. All manuscripts have been peer reviewed and revised accordingly.

We appreciate very much all contributory authors for their contribution in compilation of this book, and for their co-operation in revising their manuscripts and sending them to us well in time. We are thankful to the reviewers for giving their precious time in reviewing manuscripts, and that has helped in improving the quality of the book. Springer publisher has given us the opportunity to edit this book, and we highly appreciate it.

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Section A

CHAPTER 1

TOTIPOTENCY AND THE CELL CYCLE

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Totipotency. The potential of an isolated undifferentiated plant cell to regenerate into a plant (Cassells & Gahan, 2006).

1. INTRODUCTION

In theory, each diploid plant cell contains the genetic information for the formation of an individual, and so each diploid nucleate cell should be capable of differentiating into a complete individual. Gurdon demonstrated this for animal cells (reviewed in Gurdon, 1974). Working with *Xenopus laevis*, nuclei from intestinal epithelial cells and skin epidermal cells were transferred to enucleated oocytes which were then initiated to develop into mature frogs. A parallel study by Steward showed that individual cells isolated from carrot-derived callus could be cultured to produce individual carrot plants (Steward, 1970). For this to be considered as universal for all plant cells rather than just intermediate callus cells, it needs to be demonstrated that each type of plant cell can give rise directly to whole plants by producing either shoots which can be rooted or roots which develop shoots or somatic embryos. Clearly, the ease with which this can be shown will depend upon the degree of differentiation undergone by each cell type and the degree of gene silencing that pertains together with the readiness with which these aspects can be reversed. Given that xylem elements lose their nuclei on differentiation eliminates them from this possibility, as is likely with sieve elements and their modified structure. Nevertheless, in *Solanum aviculare*, xylem parenchyma cells in cotyledons can give rise to somatic embryos (Alizdah & Mantell, 1991) whilst the mesophyll cells of both cotyledons and first leaves can give rise to roots though it is not clear if these arise from single cells as is the case of the somatic embryos.

There are a number of cases where the production of plants from single cells can be demonstrated. Thus, the basal cells from the hairs of *Kohleria* will develop into plants (Geier & Sangwan, 1996) whilst adventitious shoots have been reported

to form from single epidermal cells of a range of species such as *Streptocarpus* (Broertjes, 1969) and *Nicotiana* (De Nettancourt et al., 1971). Equally, somatic embryos can be derived from single cells in either explanted tissues, callus and suspension cell cultures, protoplasts and mechanically isolated cells (reviewed in Gahan, 2007).

At least two factors appear to influence the ability of cells to express this capacity namely, the degree of differentiation and specialization and the impact of one tissue on gene expression in an adjacent tissue. As meristematic cells are left behind by the advancing meristem, they are considered to differentiate in order to form cells with special functions within an organ. Differentiation implies an irreversible state and is suitable to describe changes in most vascular tissue, cork tissue and the development of the woody state. However, in many non-woody plants, roots and shoots this is not necessarily an irreversible process, in which case, the term specialization is, perhaps, more apt. Clearly, in the case of, e.g., cortical parenchyma and collenchyma the ability to enter mitosis is not lost (Esau, 1953; Hurst et al., 1973). Equally, mesophyll cells, epidermal and hypodermal cells can all revert to the mitotic state. Thus, the relative degree of specialization will involve the relative degree of gene silencing in relation to mitosis and the expression of the gene sequences for developing into an individual plant. The second point concerns the impact on the adjacent tissue. This is seen in the studies of Chyla (1974) on *Torenia fourieri* in which the presence of an epidermal layer influenced the subepidermal layers. Culturing the epidermis together with the subepidermal layers resulted in the production of shoots whilst the culturing of the subepidermal layers in the absence of the epidermis resulted in the production of roots.

In many ways, the ability of a single cell to form a shoot or somatic embryo on the way to producing a whole plant will depend upon whether it is competent or recalcitrant. Competence may be defined as the state of a cell in which it is able to respond to epigenetic signals. Determination may then be defined as the state of a – previously competent – cell that has responded to that (those) signal(s) so committing the cell to a particular pathway which will include organogenesis and the production of a somatic embryo. Such epigenetic factors include plant bioregulators, and RNAi. Whether such cells are in a position to respond to epigenetic signals may depend upon the phase of the cell cycle in which they are held. Thus, it is possible that for recalcitrant cells, which may well be specialized, they may be non-cycling and held in G0 in which phase they are unlikely to be able to perceive an epigenetic signal. In contrast, those cells which are cycling and are held in G1, could be susceptible to epigenetic signals.

2. THE CELL CYCLE

The cell cycle is comprised of four major periods termed G1, S, G2 and M where S is the period of DNA synthesis, M is mitosis (Howard & Pelc, 1953) and G1 and G2 refer to gaps in our knowledge (S.R. Pelc priv. comm.). It is now clear that there are many events occurring in G1 and G2 in preparation for S and M, respectively (Alberts et al., 2002). A fifth period, G0, is when the cell leaves the cell cycle for a period of time, e.g. on specialization. For cells to progress round the cycle, there are

a series of checkpoints which enable the cell to monitor its progress before moving to the next step. Such checkpoints include the monitoring of cell size and the environment prior to proceeding from G1 to S, that all DNA has been synthesized before moving from S to G2, cell size and correct environment before leaving G2 to enter mitosis and a further check on the alignment of the chromosomes at the mitotic plate and their attachment to the spindle fibres. Clearly there are additional controls that will be discussed later and in particular how they might affect the states of competence and recalcitrance. Once a cell has passed a specific point at the end of G1, it will enter S and must complete the cycle before being able to enter G1 again. Some cells will be blocked in G2 presumably because the all aspects of the cell and its environment are not adequate for it to pass into M. Lack of carbohydrate substrate is a typical feature causing a both a G1 and a G2 block (Van't Hof & Kovacs, 1972).

According to the studies of milk production by breast cells (Vonderhaar & Topper, 1974) there is a phase within G1 in which hormonal signals could be received by the cells to initiate milk production. This would imply that there is only a very short G1 phase between early and late G1 when the signal might be perceived by plant cells since on leaving M, cells would have an adjustment period prior to electing either to recycle or to enter G0. They could then have a window of time to receive any epigenetic signals prior to reaching the START phase which sees them either differentiate/specialize or enter S (Figure 1).

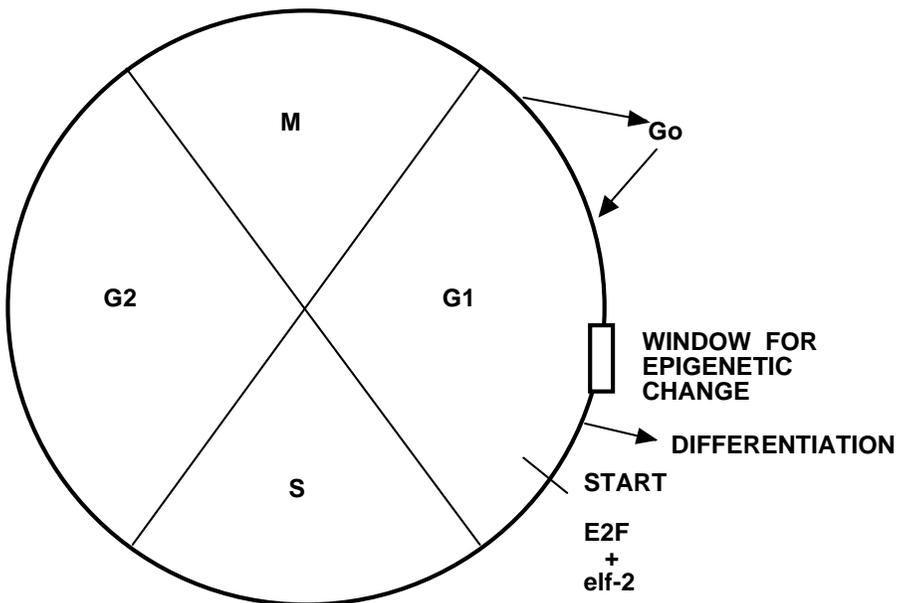


Figure 1. Diagrammatic representation of the cell cycle with events in G1. M = mitosis; S = DNA synthesis; G1 and G2 – gaps in our knowledge; Go = quiescent phase.

Two important periods occur prior to entry into S and M providing that the cell is ready to enter these phases. The entries depend upon two complexes being formed and comprising of a cyclin and cyclin-dependent protein kinase (CDK) the product of the gene *cdc2*. There are a number of cyclins of which cyclin B is necessary for entry to M. Of the cyclin Ds, when the gene for cyclin D1 from *Antirrhinum majus* was tested in *N. tabacum*, the cyclin D1 interacted with CDKA and, in contrast to animal cells, appeared to promote both Go/G1/S and S/G2/M progression (Koroleva et al., 2004). In addition, cyclinD2 appears to control the length of G1 whilst cyclin D3:1 appears to be important for the passage from G1 to S in *Arabidopsis thaliana* (Menges et al., 2006). Of the CDKs, CDKF has been found to be plant-specific in addition to CDKD that is homologous with that of vertebrates (Umeda et al., 2005).

Although the cyclinD3:1-CDK complex is necessary to pass from G1 to S, there is also the need for the gene regulatory protein E2F. The E2Fs are conserved transcription factors, of which six have been identified in *A. thaliana* (Sozzani et al., 2006), and which bind to specific gene sequences in the promoters of genes encoding proteins needed for entry to S and to M. The inhibition of E2F can be achieved with retinoblastoma protein (Rb protein) that binds to E2F so preventing it from binding to the promoters and resulting in an inhibition of the progress of the cell cycle. This inhibition can be reversed by the phosphorylation of Rb protein when the latter is released from the E2F. Phosphorylation of the Rb protein and histone H1 appears to be under the control of cyclinD1 associated CDK (Koroleva et al., 2004). The Rb protein-E2F complex can act either by sequestering transcription factors or by recruiting histone deacetylases or repressor proteins. Two forms of E2F have been found in plants, namely E2FA and E2FB. E2FB appears to be more important in Bright Yellow 2 (BY-2) cells from *N. tabacum* for passage from G1 to S (Magyar et al., 2005). The mechanism for the regulation of E2F in plants is not clear. However, in human cells, it has been proposed that the proto-oncogene *c-MYC* encodes a transcription factor that regulates cell proliferation, growth and apoptosis (O'Donnell et al., 2005). E2F1 is negatively regulated by two miRNAs from a chromosome 13 cluster at which *c-Myc* acts.

2.1. *Quiescent Cell*

Cells which are not cycling either can spend a prolonged period in G1 or can leave the cycle and enter a quiescent phase, Go, where they remain until receiving a signal to re-enter G1. A depression of protein synthesis is one feature resulting in the movement from G1 into Go and a non-proliferative state. This passage to Go is assisted by regulation of the gene *eIF-2*. The product of these gene complexes with GTP to mediate the binding of the methyl initiator of t-RNA to the small ribosomal subunit, that binds to the 5' end of the m-RNA and starts scanning (Alberts et al., 2002). Thus, regulation of this gene will impact on translation and hence the overall level of protein synthesis.

2.2. Plant Bioregulators and the Cell Cycle

The correlation between the cell cycle progression and endogenous levels of plant bioregulators was studied in synchronized *N. tabacum* BY-2 cell suspension cultures (Redig et al., 1996). No significant correlation was found for IAA and ABA. However, there were sharp peaks of zeatin and dihydrozeatin at the end of S and during mitosis. Other cytokinins such as N- and O-glucosides of zeatin remained low implying that there was a *de novo* synthesis of zeatin and dihydrozeatin. The role of zeatin in the G2-M transition was further confirmed when the addition to the cultures of lovastatin affected both cytokinin biosynthesis and blocked mitosis. Lovostatin is a competitive inhibitor of HMG-CoA reductase and blocks the mevalonic acid pathway (Metzler, 2001). Of eight different aminopurines and synthetic auxin tested, only zeatin could override the lovastatin inhibition of mitosis (Laureys et al., 1998).

Murray et al. (1998) proposed that cyclin Ds responded to specific signals and that cyclinD3 was induced by cytokinin. This was further confirmed by the response of cyclinD3 to cytokinin (Riou-Khamlichi et al., 1999). It is clear that passage from G1 to S requires a CDK-cyclin complex and E2F at adequate concentrations which processes appear to be controlled, at least in part, by auxin and cytokinin. Murray et al. (1998) proposed that auxin was able to induce CDK homologues (Figure 2).

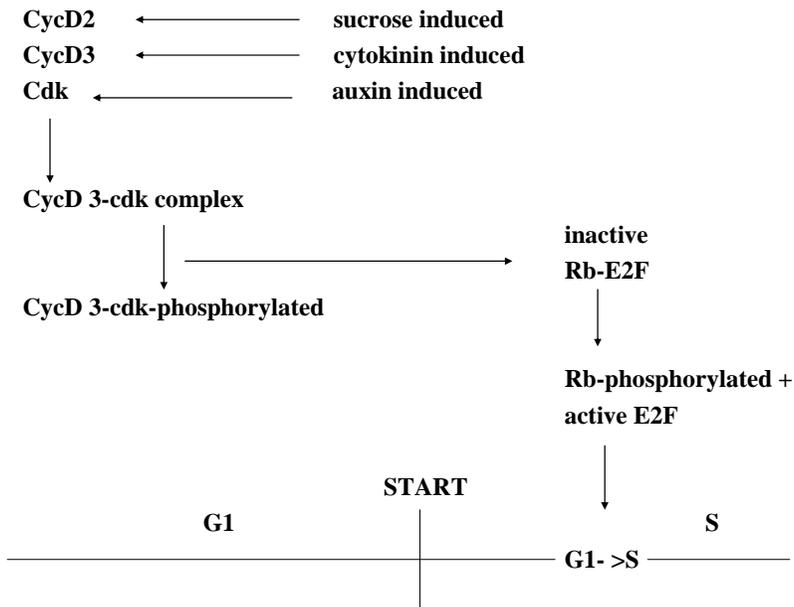


Figure 2. A speculative model for the control of the G1-S transition. (After Murray et al., 1998.)

Although jasmonic acid (JA) is better known for its involvement in plant fertility and defense, it has also been linked to a negative regulation of the cell cycle (Swiatek et al., 2004). JA prevents the accumulation of B-type CDKs and the expression of cyclinB1:1 in synchronized *N. tabacum* BY-2 cells, so causing G2 arrest and blocking entry to M. Hence JA could be affecting an early checkpoint in G2.

3. GENE SILENCING IN COMPETENCE AND RECALCITRANCE

It is generally accepted that actively transcribed genes are present in the euchromatin and that genes in the heterochromatin are not (Alberts et al., 2002). Whether the genes are located in either the eu- or the heterochromatin, they will be silenced at specific times. The activation or silencing will be influenced by epigenetic signals and can occur in a number of ways such as (a) complexing into heterochromatin, (b) through methylation, acetylation phosphorylation glycosylation, ADP ribosylation, carbonylation, sumoylation, biotinylation and ubiquitination of the histones (Loidli, 2004), methylation and deacetylation of the DNA, (c) RNA interference (RNAi) and (d) the action of retinoblastoma protein.

3.1. Heterochromatin Silencing

The complexing of genes into heterochromatic regions of the chromosomes generally result in gene silencing. In order to protect the euchromatin from being further linked into the heterochromatin, the nucleosome between the heterochromatin and euchromatin becomes modified. Instead of being composed of two pairs each of histones H2A, H2B, H3 and H4, H2A/H2B histones are replaced by H2AZ/H2b molecules. This histone exchange is mediated by the Swr1 complex (Alberts et al., 2002). This prevents the spread of silencing information regulator (Sir) proteins into the euchromatin from, e.g., the telomeres; the Sir proteins (Sir2, Sir3, Sir4) binding to the nucleosomes to transcriptionally silence the chromatin. Euchromatin H3 and H4 tails are usually acetylated, but heterochromatin H3 and H4 tails tend to be under-acetylated and are thought to complex with Sir proteins. Sir2 binds initially and helps to form new binding sites for the other Sir protein complexes.

3.2. Methylation and Acetylation

Although methylation, acetylation phosphorylation glycosylation, ADP ribosylation, carbonylation, sumoylation, biotinylation and ubiquitination (Zhang, 2003) of the histones can occur in modifying gene activity, little is known about many of these events. The better known include the methylation and deacetylation processes with more known about the former than the latter (reviewed in Loidli, 2004).

Methylation and acetylation of the core histones, H2A, H2B, H3, H4 and the histone variants H2AZ and H3.3 are implicated in gene regulation. Many of the modifications are specific for either euchromatin or heterochromatin, e.g. methylation of histone H3lysine4 for euchromatin and H3lysine9 for heterochromatin. The methylated residues on H3 histone are recognized by special chromo-domain proteins

including HP1, a highly conserved heterochromatin protein. DNA is also methylated at the cytosine residue of triplets CNG and CNN where N can be C, T, A or G. Hence the methylation of both the DNA and the histones can lead to gene silencing with DNA methylation in the heterochromatin having been identified before that of histone methylation and the role siRNA (Lippman & Martienssen, 2004). Methylation of H3 and H4 histones by histone methyl transferases leads to transcriptional activation and repression, depending upon the level of methylation (di- or trimethylation). To date, although DNA demethylation has been proposed to occur via a family of DNA glycosylases as proteins that can remove DNA methylation and so alleviate silencing (Gong et al., 2002; Chan et al., 2005), no histone demethylases have been identified in plants (Loidli, 2004)

Acetylation is the most extensively characterized type of histone modification. Core histones can be post-synthetically acetylated by histone acetyltransferases and deacetylated by histone deacetylases. However, little is known about acetylation in plants (Loidli, 2004).

The importance of methylation is seen in the studies of tree ageing where the quantification of genomic DNA methylation is being used to identify putative markers of ageing (Fraga et al., 2002a), phase change in trees (Fraga et al., 2002b) and reinvigoration (Fraga et al., 2002c). Indeed, global DNA methylation has been defined as a marker for forestry plant production so permitting an association between culture conditions and a specific epigenetic status.

3.3. siRNA

Short interference RNA (siRNA) is a class of double-stranded RNAs 21-24 nucleotides long. They are formed from dsRNA (double-stranded RNAs) and silence genes in one of three ways. The first is by initiating cleavage of mRNAs with the exact complementary sequences. The second method is by modifying the DNA directly by either complementary RNAi sequences or recruiting inhibitory proteins (Meister & Tuschli, 2004; Novina & Sharp, 2004; Jover-Gil et al., 2005). Finally, they compromise one of the more abundant classes of gene regulatory molecules in multicellular organisms and likely influence the output of many protein-coding genes (Bartel, 2004). They have a number of roles in plants (Baulcomb, 2004) including heterochromatic gene silencing (Lippman & Martienssen, 2004; Jia et al., 2004; Pal-Bhadra et al., 2004; Verdal et al., 2004).

Double-stranded RNAs appear to induce post-transcriptional gene silencing in several plant species apparently by targeting CpG islands within a promoter and inducing RNA-directed DNA methylation (see in Kawasaki & Taira, 2004). In addition, Lippman et al. (2004) have also indicated that siRNAs correspond to sequences of transposable elements in *A. thaliana* in which it is possible that the heterochromatin is composed of transposable elements (McLintock, 1956). Some 90–95% of endogenous siRNAs correspond to either transposons or repeats that are heavily methylated. Transposons can regulate genes epigenetically though only when inserted within or close to the gene. This could account for the regulation of the chromatin remodelling ATPase DDM1 (Decrease in DNA Methylation 1) and DNA methyltransferase (Lippman et al., 2004), siRNA silencing linked to DNA

methylation and suppression of transcription (Wassenger et al., 1994; Mette et al., 2000; Jones et al., 2001).

3.4. Heterochromatin Formation

Heterochromatin formation has been considered in *A. thaliana* where DNA methylation, H3 methylation, H4 acetylation are implicated (Loidli, 2004). However, such a model does not explain all of gene silencing in the heterochromatin and it is clear that siRNA also has a significant role.

3.5. Recalcitrance and Heterochromatin

It is clear that in competent cells, eIF-2 genes can be upregulated in order to permit a move from G₀ to G₁ and phosphorylation of Rb protein will result in the release of E2F to permit a move from G₀ to S. Evidently, these events can be triggered by treatment with auxin and cytokinin (Figure 2). The problem arises with recalcitrant cells that fail to respond to plant bioregulator treatments. A possible explanation for this may be found in an extension of the model proposed by Williams & Grafí (2000). As discussed earlier, Rb protein can inhibit E2F so blocking the passage from G₁ to S. This process will affect E2F in the euchromatic region of the chromosome, an apparently easily reversible situation. However, it is also possible that the Rb protein, on binding to E2F, brings the euchromatin closer to the heterochromatin. The heterodimer DF-E2F anchors the Rb protein into the promotor region (Figure 3). A direct connection can occur between the Rb protein and a region containing heterochromatin-associated proteins such as CLF (curly leaf) and HP1 (heterochromatic protein 1) proteins from *A. thaliana*. HP1 is found to contain an Rb protein binding motif located at the loop between B-3 short end and the α -helix structure (Figure 4). This loop is a variable region among the different chromodomain proteins which might not affect its 3-D structure. Maize Rb protein has been demonstrated to react with both HP1 and CLF proteins (Williams & Grafí, 2000). Such an interaction can result in the euchromatic E2F target gene being located in close proximity to the heterochromatin. This could result in a packaging into condensed, transcriptionally inactive chromatin (Figure 3).

Such a packaging could lead to recalcitrance which in some cases may be overcome by treatment with plant bioregulators, e.g. an auxin shock induced rooting in York M9 stems (Auderset et al., 1994). Normally, the nucleosome between the heterochromatin and the euchromatin will be modified, histone H2A.Z replacing histone H2A. However, if a closer integration of the portion of euchromatin with the heterochromatin occurs, this would lead to a modification of this nucleosome with H2A replacing H2A.Z again. This would result in the euchromatin becoming more closely integrated into heterochromatin and its genes transcriptionally silenced by Sir proteins binding to the nucleosomes after they have been deacetylated. Thus, E2F genes could be silenced in a way that cannot be readily reversed by plant bioregulators. At present it is not clear how such a reversal could be easily achieved and a variety of new strategies need to be developed.

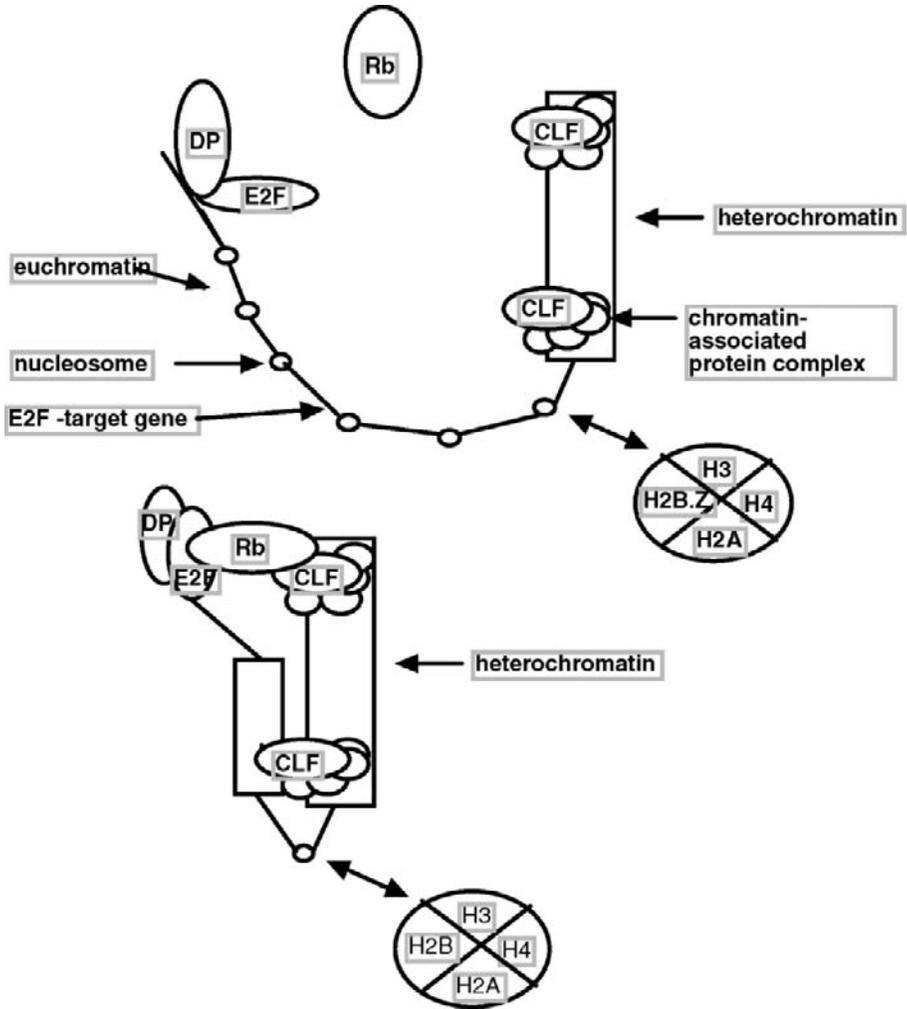


Figure 3. Diagrammatic representation of possible mechanism by which recalcitrance occurs. Upper figure shows E2F without Rb protein, so activating the target gene in the euchromatin. Lower figure shows an effect of the dephosphorylation of Rb protein which binds to the E2F site and is also linked to CLF protein as a part of the chromatin-associated protein complex on the heterochromatin. This results in the E2F protein being linked to the heterochromatin so drawing the target gene associated nucleosome to be complexed to another chromatin-associated protein complex. CRL = curly leaf protein. (After Williams & Graffi, 2000.)

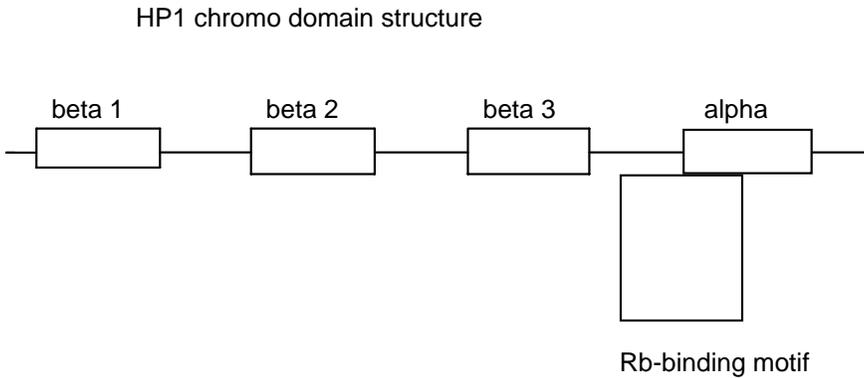


Figure 4. Model of HP1 chromodomain secondary structure in relation to the Rb-binding motif in *Arabidopsis thaliana* SET-domain CURLY LEAF protein. This is similar to that from other eukaryote HP1 proteins. (After Williams & Graffi, 2000.)

4. CONCLUDING REMARKS

In theory, each diploid plant cell is totipotent and contains the genetic information for the formation and differentiating into a complete individual. The degree of differentiation and specialization of the cells as well as the impact of one tissue on gene expression in an adjacent tissue appear to influence the ability of cells to express totipotency. In many ways, the ability of a single cell to form a shoot or somatic embryo on the way to producing a whole plant will depend upon whether it is competent or recalcitrant. Competence may be defined as the state of a cell in which it is able to respond to epigenetic signals such as plant bioregulators and RNAi. Whether such cells are in a position to respond to epigenetic signals may depend upon the phase of the cell cycle in which they are held. Thus, it is possible that for recalcitrant cells, which may well be specialized, they may be non-cycling and held in Go in which phase they are unlikely to be able to perceive an epigenetic signal. In contrast, those cells that are cycling and are held in G1, could be susceptible to epigenetic signals. This chapter has summarized the present knowledge of plant cell totipotency in the context of the cell cycle and the potential mechanisms of gene silencing in competence and recalcitrance. The follow-up chapters will cover micro-propagation protocols of diverse plant species, i.e. the practical examples of plant cell totipotency.

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CHAPTER 2

MICROPROPAGATION VIA ORGANOGENESIS IN SLASH PINE

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1. INTRODUCTION

Highly efficient and reproducible *in vitro* regeneration systems via somatic embryogenesis or organogenesis are a prerequisite for clonal propagation of elite genotypes of specific plant species and for production of transgenic plants (Becwar et al., 1990; Attree & Fowke, 1993; Tang & Newton, 2003). Although plant regeneration via somatic embryogenesis has been reported in a number of coniferous species, plant regeneration via organogenesis from callus cultures has been obtained in only a few conifers (Hakman & Fowke, 1987; Nørgaard & Krogstrup, 1991; Tang et al., 2004). Routine methods of transformation are still hampered by the lack of readily available, highly efficient, and long-term regenerable cell and tissue culture systems in conifers (Handley et al., 1995; Tang & Newton, 2004).

Currently, a variety of explants have been successfully used for obtaining morphogenesis *in vitro* in conifers (Nagmani & Bonga, 1985; Gladfelter & Phillips, 1987; Tremblay, 1990; Guevin & Kirby, 1997; Salajova et al., 1999; Zhang et al., 1999), of which the most common are immature and mature embryos (Attree & Fowke, 1993; Find et al., 2002; Vookova & Kormutak, 2002). However, developmental progression has been limited to cultures capable of somatic embryogenesis and plant regeneration directly from the explant or via a callus phase using immature embryos (Krogstrup, 1990; Harry & Thorpe, 1991; Jalonen & von Arnold, 1991; Nørgaard, 1997; Klimaszewska et al., 2000). The successful regeneration of somatic embryos and plantlets is achieved using immature embryos (Campbell et al., 1992; Attree & Fowke, 1993; Guevin et al., 1994) as the target tissues in Fraser fir and Nordmann fir. Nevertheless, these explants require that their collection be limited to a special season of the year. In addition, there is a strong genotype dependency involved in tissue culture and efficient regeneration with embryogenesis. Furthermore,

regeneration efficiency is still low, especially in commercial cultivars, due to various factors affecting the frequency of plant regeneration after transformation and selection (Find et al., 2002; Vookova & Kormutak, 2002). Therefore, a highly efficient regeneration system is needed for the genetic transformation of conifers.

Because of its rapid growth rate, slash pine (*Pinus elliottii* Engelm.) is a valuable southern pine for reforestation projects and timber plantations throughout the south eastern United States. Slash pine is also widely planted in the tropical and subtropical regions over the world. Slash pine is naturally found in wet flatwoods, swampy areas, and shallow pond edges. It can occur in the low sandy soils that are poor in nutrients. Millions of acres of slash pine have been planted and grown in the south eastern United States, where younger trees are harvested for pulpwood. Plant regeneration via somatic embryogenesis from embryogenic callus initiated from immature embryo explants of different slash pine genotypes has been reported (Jain et al., 1989; Newton et al., 1995). However, the development of a significantly improved plant regeneration system through multiple shoot differentiation from callus cultures derived from mature embryos would be valuable to clonal propagation and to genetic transformation in slash pine. In this study, we report the establishment of an efficient plant regeneration system via organogenesis from callus cultures in slash pine. The method presented here will be most useful for future slash pine clonal propagation and genetic transformation programs.

2. EXPERIMENTAL PROTOCOL

2.1. *Explant Preparation*

Mature seeds of genotypes 1177, 1178, 7524, 7556 of slash pine (*Pinus elliottii* Engelm.) are provided by Penny Sieling and Tom Byram (Texas Forest Service Forest Science Laboratory, Texas A&M University, College Station, TX 77843-2585, USA). All seeds are stored in plastic bags at 4°C before they are used for callus induction. Seeds are washed in tap water for 20 min, then disinfected by immersion in 70% w/w ethanol alcohol for 30 s and in 75% house bleach for 15 min, followed by five rinses in sterile distilled water. Mature zygotic embryos are aseptically removed from the megagametophytes and placed horizontally on a solidified callus induction medium in 15 × 100 mm Petri dishes (Fisher Scientific) with 20 ml medium. Make sure the whole embryos are touching the medium. Plates with embryos are incubated in the dark at 23°C.

2.2. *Culture Medium*

Basal media used in this investigation included BMS (Boulay et al., 1988), DCR (Gupta & Durzan, 1985), LP (von Arnold & Eriksson, 1979), MS (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1972), and TE (Tang et al., 2004) media (Table 1). Plant growth regulators (Table 2) used in callus induction medium include α -naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and

Table 1. The basal media used in tissue culture of slash pine. The basal media used for callus induction, adventitious shoot formation, shoot elongation, and rooting included BMS (Boulay et al., 1988), DCR (Gupta & Durzan, 1985), LP (von Arnold & Eriksson, 1979), MS (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1972), and TE (Tang et al., 2004) medium.

Chemical formula	BMS	DCR	LP	MS	SH	TE
Ca(NO ₃) ₂ ·4H ₂ O	0	556	0	0	0	556
KNO ₃	2,500	340	1,900	1,900	2,500	340
CaCl ₂ ·2H ₂ O	200	85	1,760	440	200	85
NH ₄ NO ₃	0	400	1,200	1,650	0	400
MgSO ₄ ·7H ₂ O	400	370	370	3,70	400	720
KCl	0	0	0	0	0	1,900
KH ₂ PO ₄	0	170	340	170	0	170
NH ₄ H ₂ PO ₄	300	0	0	0	300	0
ZnSO ₄ ·7H ₂ O	8.6	8.6	0	8.6	1.0	25.8
MnSO ₄ ·H ₂ O	16.9	22.3	2.23	16.9	10.0	25.35
H ₃ BO ₃	6.2	6.2	0.63	6.2	5.0	6.2
KI	0.83	0.83	0.75	0.83	1.0	0.83
Na ₂ MoO ₄ ·H ₂ O	0.25	0.25	0.025	0.25	0.1	0.25
CoCl ₂ ·6H ₂ O	0.025	0.025	0.025	0.025	0.1	0.025
CuSO ₄ ·7H ₂ O	0.025	0.025	0.025	0.025	0.2	0.025
FeSO ₄ ·7H ₂ O	27.8	27.8	13.9	27.8	15.0	27.8
NaEDTA	37.3	37.3	0	37.3	20.0	37.3
Myo-inositol	1,000	1,000	1,000	1,000	1,000	1,000
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5	0.5	0.5	0.5
Thiamine HCl	0.1	0.1	0.1	0.1	0.1	0.1
Glycine	0.1	0.1	0.1	0.1	0.1	0.1
Sucrose	30,000	30,000	30,000	30,000	30,000	30,000
Glutamine	0	0	0	0	0	500
Casein hydrolyzate	0	0	0	0	0	500
Gelrite	0	0	0	0	0	3,000
pH	5.7	5.7	5.7	5.7	5.7	5.7

2-isopentenyladenine (2iP). The pH is adjusted to 5.8 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121°C for 20 min. All media are adjusted to pH 5.8 prior to autoclaving for 20 min at 121°C. All tissues are cultured at 23°C. Adventitious shoot induction is conducted in the dark, and adventitious shoot differentiation and proliferation and rooting are conducted at 23°C under a 16-h photoperiod with cool fluorescent light (100 μmol m⁻² s⁻¹). Each experiment is replicated three times, and each replicate consisted of 50–200 embryos for callus induction, 30–50 pieces of calli (0.5 × 0.5 cm in size) for adventitious shoot formation, and 30–45 elongated shoots for rooting. For shoot proliferation and maintenance, the multiplied shoots of

each clump are cultured in the same shoot formation medium for 6 additional weeks. All cultures are subcultured every 3 weeks.

Table 2. Procedure for plantlet regeneration in slash pine. The basal media used for callus induction, adventitious shoot formation, shoot elongation, and rooting include BMS (Boulay et al., 1988), DCR (Gupta & Durzan, 1985), LP (von Arnold & Eriksson, 1979), MS (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1972), and TE (Tang et al., 2004).

Plant growth regulators	Stage of plantlet regeneration			
	Induction	Differentiation	Elongation	Rooting
α -Naphthaleneacetic acid (NAA)	12 μ M	0	0	0
Indole-3-acetic acid (IAA)	0	0	2 μ M	0.01 μ M
Indole-3-butyric acid (IBA)	0	2 μ M	0	0.01 μ M
2,4-Dichloroxyacetic acid (2,4-D)	15 μ M	0	0	0
6-Benzyladenine (BA)	0	3 μ M	1 μ M	0
Thidiazuron (TDZ)	0	9 μ M	0	0
2-Isopentenyladenine (2iP)	6 μ M	0 μ M	0	0
L-Glutamine	500 mg/l	500 mg/l	400 mg/l	400 mg/l
Myo-Inositol	500 mg/l	500 mg/l	250 mg/l	250 mg/l
Sucrose	30,000 mg/l	30,000 mg/l	20,000 mg/l	10,000 mg/l
Phytigel	4,500 mg/l	4,500 mg/l	5,000 mg/l	5,000 mg/l
PH	5.8	5.8	5.8	5.8
Culture time	6 weeks	6–12 weeks	6 weeks	6 weeks

2.3. Shoot Regeneration and Maintenance

The procedure of plant regeneration involving callus induction, adventitious shoot formation, shoot elongation, and rooting is shown in Table 2. Basal media used for callus induction include DCR, BMS, LP, MS, SH, and TE media. The frequency of callus formation is determined 6 weeks after culture. After calli are transferred onto adventitious shoot regeneration medium consisting of DCR, BMS, LP, MS, SH, and TE media for 6 weeks (Table 1), differentiation is evaluated by the percentage of calli forming adventitious shoots on the medium for a 6-week period.

1. Subculture calli every 3 weeks before the induction of shoot formation.
2. Transfer calli onto shoot formation medium supplemented with IBA, BA, and TDZ for 2–3 subcultures. If more calli are needed, subculture calli 4–6 times.
3. Make sure the whole calli are touching the medium.
4. Culture calli at 23°C under a 16-h photoperiod with cool fluorescent light (100 μ mol m⁻² s⁻¹).
5. Subculture calli with adventitious buds in LifeGuard plant growth vessels (Sigma) every 3 weeks on fresh shoot formation medium.
6. Determine the frequency of calli forming shoots, 6 weeks after calli are transferred onto shoot formation medium.

Among 6 basal media (BMS, DCR, LP, MS, SH, and TE) used in this study, higher frequency (34%–46%) of callus induction is obtained on BMS, SH, and TE, compared to DCR, LP, MSG, and MS. Similar callus induction frequency is obtained in four genotypes of slash pine. The frequency of callus formation increased during 4–6 weeks on fresh callus induction medium supplemented with NAA, 2,4-D, and 2iP. The highest frequency of callus formation is obtained on TE medium. After callus cultures (Figure 1A) are transferred onto shoot formation medium for 6 weeks, frequency of calli forming adventitious shoots is evaluated. Adventitious shoots (Figure 1B, C) are regenerated from callus cultures of four slash pine genotypes on BMS, SH, and TE media, with higher frequency (26%–35%) on SH and TE media and lower frequency (6%–9%) on BMS medium. The frequency of adventitious shoot formation increased during 6–12 weeks on fresh shoot formation medium supplemented with IBA, BA, and TDZ. The highest frequency of callus forming shoots is obtained on TE medium.

2.4. Rooting

Elongated, well-developed individual shoots with more than 8 needles are separated from the mother clumps and transferred onto rooting medium for 6 weeks. After elongated shoots are transferred onto rooting medium, rooting (Figure 1D, E) is evaluated by the percentage of shoots forming roots on the test medium for 6 weeks. Higher rooting frequency (26%–35%) is obtained in four genotypes on SH and TE media, compared to BMS medium (7%–9%).

1. Transfer shoots onto shoot elongation medium supplemented with IBA and BA.
2. Subculture shoots every 3 weeks.
3. Culture shoots at 23°C under a 16-h photoperiod with cool fluorescent light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$).
4. Subculture shoots every 3 weeks on fresh shoot elongation medium for 6 weeks.
5. Transfer elongated shoots 3–5 cm in height onto rooting medium supplemented with IAA and IBA.
6. Culture the elongated shoots for 6 weeks.
7. Rooting is conducted at 23°C under a 16-h photoperiod with cool fluorescent light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$).
8. Determine the frequency of shoots forming roots, 6 weeks after shoots are transferred onto rooting medium.
9. Plantlets with roots 2–5 cm in length can then be hardened.

2.5. Hardening

After rooting of adventitious shoots, regenerated plantlets from organogenic calli are treated at 4°C for 1 week. Regenerated plantlets are then transferred from culture in 125 ml Erlenmeyer flasks into a perlite:peatmoss:vermiculite (1:1:1 v/v/v) soil mixture. For acclimatization, plantlets are covered with glass beakers for 1 week. After acclimatization by decreasing relative humidity to ambient condition over a period of 1 week, plantlets are exposed to greenhouse conditions (Figure 1F).

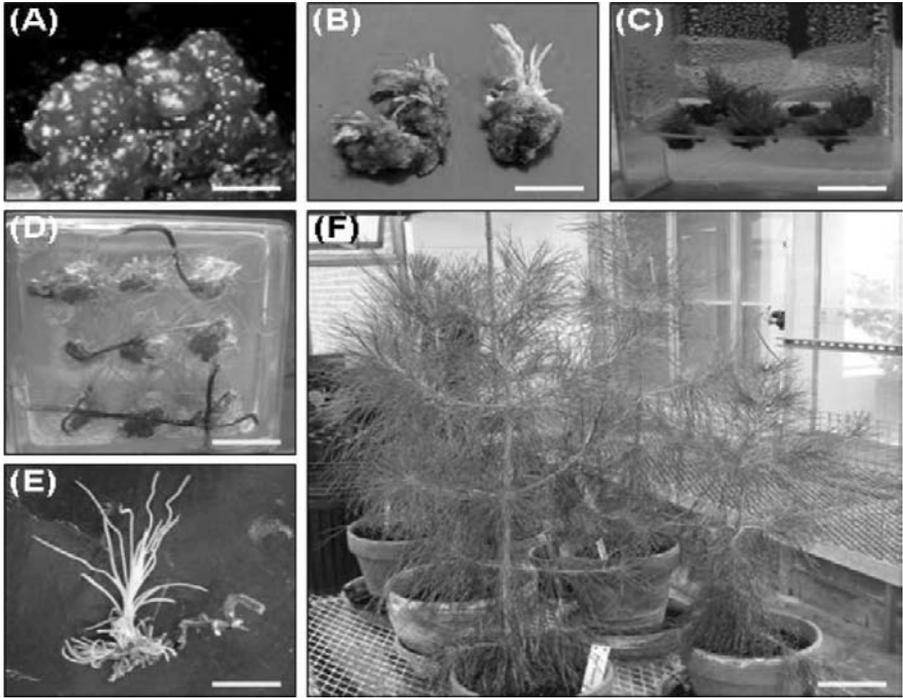


Figure 1. Plantlet regeneration via organogenesis from callus cultures in slash pine. A) Callus cultures induced from mature embryos cultured for 3 weeks on callus induction medium. B) Clusters of adventitious shoots 6 weeks after callus cultures are transferred into shoot formation medium. C) Clusters of adventitious shoots 9 weeks after callus cultures are transferred onto shoot formation medium. D) Rooting of elongated shoots on rooting medium for 6 weeks. E) Plantlets before transferring into potting soil. F) Regenerated plants established in potting soil in greenhouse for 18 months. (A, bar = 0.5 cm; B, bar = 0.8 cm; C and D, bars = 1.1 cm; E, bar = 2 cm; F, bar = 8 cm.)

2.6. Field Testing

After acclimatization, plantlets are taken out from the LifeGuard plant growth vessels (Sigma) and washed completely in tap water to remove the medium. The washing takes about 30 min. Plantlets are then planted into potting soil. In the first week, plantlets are watered two times a day. After that, they are watered once a day. Survival rate of regenerated plantlets is evaluated 6 weeks after their transfer to soil. More than 90% of the acclimatized plantlets survived in greenhouse.

3. CONCLUSION

The protocol established here is highly reproducible for the production of plantlets via organogenesis in four genotypes of slash pine. Plant growth regulators and the physiological activity of the explants are very important for successfully inducing plant regeneration via organogenesis in pine species. Mature zygotic embryos are good explants for the establishment of highly regenerable multiple shoot cultures of slash pine. The procedure presented here has several advantages over previously published reports of successful embryogenic callus induction from immature embryos. First, seeds of slash pine can be easily provided at any time throughout the year, but immature embryos are only available at the specific season of the year. Second, the process from callus to plant regeneration takes only a few months (8–10 months) which is less than plant regeneration via somatic embryogenesis. Third, plant regeneration from organogenic calli is a simple and highly efficient short-term in vitro regeneration system. There is no difference in the survival rate regenerated plantlets among different genotypes (genotypes 1177, 1178, 7524, 7556) used in this study. Regenerated plantlets produced from six basal media (DCR, BMS, LP, MS, SH, and TE) have very similar survival rates. The plant regeneration protocol established in this investigation may facilitate future research in genetic transformation in slash pine and other conifers.

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CHAPTER 3

MICROPROPAGATION OF COAST REDWOOD (*SEQUOIA SEMPERVIRENS*)

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1. INTRODUCTION

Sequoia sempervirens (Lamb.) Endl., coast redwood, is a long-lived evergreen gymnosperm belonging to the family Taxodiaceae. This species is endemic to the coastal regions of California and Oregon, USA (Srinivasan & Friis, 1989; Ma et al., 2005). The fossil records of the genus *Sequoia* can be traced back to the Jurassic Period, in China (Endo, 1951). The tree is characterized by a thick, fibrous, and deeply furrowed bark along with a fire-resistant reddish-brown heartwood. Leaves of *S. sempervirens* are dimorphic, including linear and scale-like leaves. The linear leaves are spirally arranged or occasionally sub-opposite (Ma et al., 2005). The tree is highly valuable not only for ornamental purposes as trees can grow up to 110 m in length, but also for industrial purposes as it grows quite vigorously, rarely suffers from disease or insect attack, and it is resistant to strong winds and other poor climatic conditions. It is the high longevity and size of Sequoia trees that allow for its substantial biomass accumulation (Busing & Fujimori, 2005). In some stands, it exceeds 3500 metric tons/hectar. Thus, *S. sempervirens* can be used in the timber industry (plywood), paper industry, as well as pulp industry. It is well suited for short rotation coppicing.

Mature trees will bloom between November and early March in the Northern hemisphere, and produce male and female cones at 15 years of age. Seeds are brown in color, weighing ~4 mg, elliptical in shape, and bordered by a small wing (Arnaud et al., 1993). Like most forest species, seed propagation is common for *S. sempervirens*, although vegetative propagation via root and stump sprouts also occurs. Seed germination is highly variable as many seeds are often empty, and the embryos are either malformed or infected with various parasites. Moreover,

viable seeds are difficult to store, and frequency of germination is also variable. When seed trees are used, genetic gain can be made by maintaining the best phenotypes as seed producers.

There are few *in vitro* studies on micropropagation of this coniferous plant using different sources of explants (Boulay, 1987; Fouret et al., 1988; Thorpe et al., 1991; Sul & Korban, 1994, 2005). Other studies have indicated that induction of adventitious shoots or somatic embryos in *S. sempervirens* is possible, but only from callus tissues derived from zygotic embryos or from cotyledons and hypocotyls of *in vitro* germinated seedlings (Ball, 1987; Bourgard & Favre, 1988), and then at low frequencies (ranging from 3 to 14%).

In general, most reports on induction of organogenesis and/or embryogenesis in conifers involved culture of zygotic or seed tissues (Attree & Fowke, 1993; Pullman et al., 2003; Stasolla & Yeung, 2003). These sources of explants are highly heterozygous, and therefore regenerants are likely to exhibit variability. In order to maintain trueness-to-type of elite clones or superior genotypes of conifer species having desirable characters (e.g., resistance to diseases or insects, wood quality, or growth characteristics, among others), explants for micropropagation should be derived from somatic tissues of trees old enough to have demonstrated their value, and not from zygotic tissues. Moreover, the micropropagation protocol should involve minimal or no callus development in order to reduce the likelihood of induction and recovery of variants.

In this chapter, protocols for *in vitro* micropropagation of *S. sempervirens* is described using nodal stem segments as well as needles as sources of explants. A brief description of using seed tissues for micropropagation is also presented.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Explants from Juvenile Material

Seeds. Open-pollinated seeds are collected and/or purchased from elite seed-producing *S. sempervirens* trees. Seeds weigh around 4 mg. These seeds can be used either for *in vitro* culture or they can be germinated and allowed to grow into seedlings, and then used as mother plants as described below.

Nodal stem segments. Open-pollinated seeds from elite seed-producing *S. sempervirens* trees are germinated in 20 cm plastic pots containing 1:1:1:1 (peat, sand, vermiculite, and sand) mixture, and grown in a greenhouse at $24 \pm 1^\circ\text{C}$. Germination rate is highly variable as many seeds are often empty or embryos are malformed or infected with parasites (Arnaud et al., 1993). When seeds successfully germinate, and young plantlets develop, these are watered daily using a drip irrigation system, and fertilized weekly with a 250 ppm of a 20-20-20 NPK Peter's fertilizer solution. These mother plants can then serve as sources of explants for as long as they are well maintained and continue their growth, and providing succulent new vegetative material. Stem segments (~10 cm in length) with 5 to 10 axillary buds are collected from young growth, and used as explants for establishing *in vitro* cultures.

In vitro-grown needles. Fully-expanded green healthy needles (~1 cm in length and ~0.2 cm in width) are collected from *in vitro*-grown proliferating shoot cultures. These are placed in 100 × 15 mm petri plates containing regeneration medium (as described below). Approximately 10–15 needles can be placed in each plate. Plates are wrapped with parafilm.

2.1.2. Explants from Adult Material

It is very difficult to utilize vegetative tissues from adult trees for micropropagation. Although attempts have been made to utilize vegetative tissues from adult trees of different ages (5 to almost 100 year-old), none of these have been successful (Arnaud et al., 1993). However, sprouts or suckers that arise from these adult trees can be successfully used as sources of explants. Stem segments are collected from apical shoots from these suckers, and used for establishing *in vitro* cultures (Arnaud et al., 1993).

2.1.3. Disinfection of Plant Material

Disinfection of explants is an important step to establish effective shoot cultures. In time, effective methods of disinfection have been developed for the various typologies of explants.

Seeds. Seeds are soaked in water for a period of 12–24 h. Then they are treated in either 80% ethanol or 10% hydrogen peroxide for 1–2 min, followed by 10 min in 0.75–1.0% sodium hypochlorite (15–20% commercial bleach, Clorox®). Then, seeds are rinsed three times with sterile deionized water. In some studies, seeds were surface-sterilized by soaking in full-strength commercial bleach (Ball, 1987) or 6% of a medical disinfectant consisting of mercurbutol and sodium lauryl sulphate, and then dipped in 3% hydrogen peroxide (Boukgard & Favre, 1988).

Nodal stem segments. Healthy stem segments containing 3 to 4 nodes are disinfected in 0.525% sodium hypochlorite (10% commercial bleach, Clorox®) solution containing a few drops of Tween 20 (used as a surfactant) for 10 min. These are rinsed three times with sterilized–deionized water (10 min per rinse) with continuous shaking (80 rpm) of glass jars (baby food jars) by placing them on a gyratory shaker. Cut end portions (0.5 cm) of each stem segment on a sterilized paper towel, and discard of these ends. Excess water is removed by blotting explants on a dry sterilized paper towel.

2.2. In Vitro Culture

2.2.1. Culture Media and Materials

All media stocks are stored at 4°C, and plant growth regulators (PGRs) are frozen at –20°C until they are used. The following is a brief description of the three steps that are required (along with media) to establish shoot cultures of stem segments from greenhouse-grown plants. The complete description of the media used is listed in Table 1.

1. *In vitro* establishment: Wolter and Skoog (WS) (1966) basal medium (4.4 g/l WS salts, 20 g/l sucrose, and 7 g/l agar) and Staba vitamins.
2. *In vitro* shoot proliferation: WS basal medium + Staba vitamins + kinetin (4.7 μ M) + 6-benzyladenine (BA; 4.4 μ M) + zeatin (15 μ M).
3. Shoot elongation and rooting: $\frac{1}{2}$ WS salts + Staba vitamins + activated charcoal.

Table 1. List of media components for micropropagation of *Sequoia sempervirens*.

Medium component	Culture establishment (per liter)	Shoot proliferation (per liter)	Shoot elongation and rooting ^a (per liter)
WS salts	4.4 g	4.4 g	2.2 g
Staba vitamins	10 ml	10 ml	10 ml
Myoinositol	10 mg	10 mg	10 mg
6-benzyladenine		1 mg	
kinetin		1 mg	
zeatin		3.3 mg	
Sucrose	20 g	20 g	20 g
Agar (Difco-bacto)	7 g	7 g	7 g
Charcoal			2 g
pH	5.6	5.6	5.6

^aSpontaneous rooting is observed on this medium; however, it may be necessary to transfer cultures to a fresh similar medium, but containing an auxin such as indolebutyric acid (IBA) at 0.5 mg/l to increase the frequency of rooted shoots.

2.2.2. Regeneration via Shoot Organogenesis

The overall protocol of micropropagation of *S. sempervirens* is maintained by continuous *in vitro* shoot proliferation and subsequent *ex vitro* rooted shoot production. The protocol used in our laboratory can be divided into three stages as follows: 1) establishment of explants; 2) shoot proliferation; and 3) shoot elongation and spontaneous rooting (Figure 1).

1. Establishment of explants: Greenhouse- or field-grown shoots are cut into 10 cm stem segments, and transferred to a WS medium without PGR for 4 weeks. Any contaminated shoots are discarded, and clean stem segments, about 1–2 cm in length, are maintained for shoot proliferation.
2. Shoot proliferation: sterilized shoot segments containing 2–3 axillary buds are cultured horizontally on WS medium containing zeatin (15 μ M) for 4 weeks. Depending on the genotype, it is expected that variations in shoot proliferation rate will be observed. Although a range of 5 to 15 μ M zeatin promotes shoot proliferation, 15 μ M zeatin showed the best frequency of shoot proliferation for our own tested genotypes. Therefore, it is important to determine the optimum zeatin concentration for the genotype used.

3. Shoot elongation and spontaneous rooting: Healthy shoots with healthy needles are cut into 1 cm and culture in the jars containing $\frac{1}{2}$ WS without PGR for 8 weeks. Spontaneous rooted shoots (about 20 to 30%) are transferred to the greenhouse for further shoot elongation and the rest of shoots can be segmented for *in vitro* proliferation (repeat step 2). Elongation of shoots can be achieved when shoots are grown on WS basal medium only, however adding activated charcoal can help promote shoot elongation.

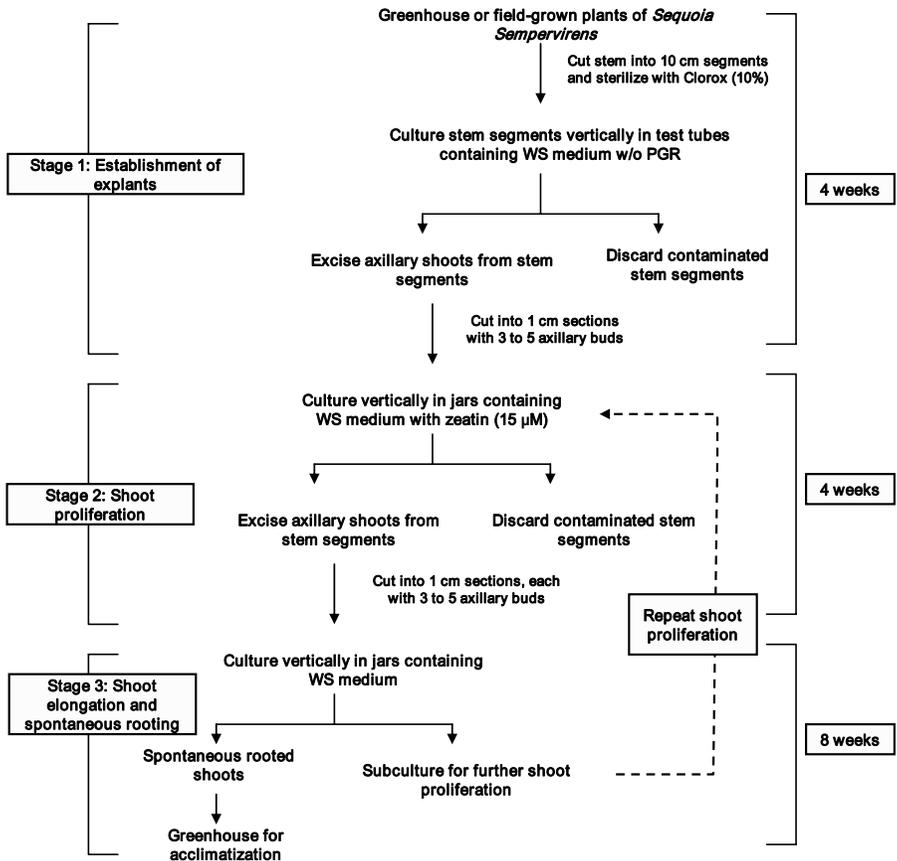


Figure 1. A schematic diagram of the overall micropropagation protocol for *S. sempervirens* using nodal stem segments.

Seed. Following surface-sterilization, testae are removed, and a thin layer of axenic female gametophytes are excised from the embryo (Ball, 1987). These are introduced into petri plates containing a modified Murashige & Skoog (MS) medium containing 2 μM BA and 2 μM kinetin. Within 2 months, organogenic callus is formed, and following monthly subcultures, shoots are observed.

Nodal stem segments. Each nodal stem explant is placed into a test tube containing Wolter & Skoog (WS) (1966) basal medium without any PGRs. Contaminated explants are discarded, and elongated healthy axillary shoots are excised, and cultured on a WS basal medium for further establishment as described below.

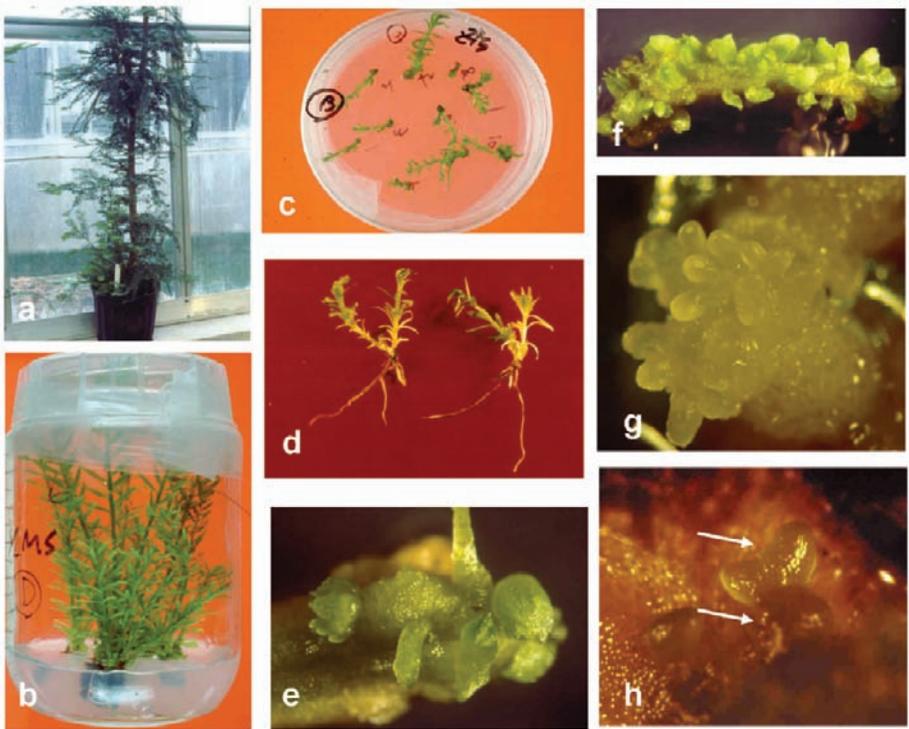


Figure 2. Micropropagation of *S. sempervirens*. Using greenhouse-grown mother plants as sources of nodal stem segments (A), these are then introduced in vitro, established, and proliferated (B). Following shoot proliferation medium, these are transferred to a fresh medium to elongate (C), and root spontaneously (D). Shoot buds (E, F) as well as somatic embryos (G, H) can also be induced on in vitro-grown needles.

Needle. Fully-expanded green healthy needles (~1 cm in length and ~0.2 cm in width) are collected from *in vitro*-grown shoots of *S. sempervirens*. A basal medium containing Wolter & Skoog (1966) (WS) salts, Staba vitamins (Staba, 1969), 100 mg·l⁻¹ myoinositol, and 20 g·l⁻¹ sucrose is supplemented with 5 µM BA and 0.1 µM 2,4-dichlorophenoxyacetic acid (2,4-D). The medium is solidified with 6 g·l⁻¹ Difco Bacto-agar. The pH of the medium is adjusted to 5.6 with 0.5 N KOH or 0.5 N HCl prior to autoclaving for 15 min at 121°C. Cultures are maintained in the dark for 2–3 weeks, and then transferred to low-light conditions (15–20 µmol·m⁻²·s⁻¹). Adventitious shoot buds are clearly visible 1 week following transfer of explants to light conditions (4 weeks after *in vitro* culture) (Figure 2). It is important to indicate that Liu et al. (2006) have also been able to induce shoot organogenesis from needles of Sequoia when these *in vitro*-derived needles are incubated on Schenk & Hildebrandt (SH) (1972) medium containing 2.22 µM BA, 0.93 µM kinetin, and 0.98 µM indolebutyric acid (IBA) which also effectively promoted adventitious bud regeneration (Sul & Korban, 2005).

2.2.3. *In Vitro* Shoot Establishment

Shoots induced from *in vitro*-grown stem segments are cut into 1 to 2 cm sections, each having 3 to 5 axillary buds, under sterile conditions. Each of these nodal shoots is subcultured horizontally in jars containing 30 ml of WS basal medium supplemented with 15 µM zeatin.

2.2.4. Shoot Proliferation

After 2 months in culture, elongated shoots from axillary buds are transferred to jars containing WS basal medium (without PGR), but with activated charcoal. Proliferated shoots were cut into 1 to 2 cm with 3 to 4 nodes and cultured on the same medium for continuous *in vitro* proliferation.

2.2.5. Shoot Elongation and Spontaneous Rooting

Elongated shoots over 2 cm were detached from the original stems and transferred to jars containing ½ WS basal medium without any regulators. As shoots continue to elongate, spontaneous roots (mostly 3 to 6 roots) are developed along the basal parts of these shoots.

2.3. Regeneration via Somatic Embryogenesis

Several efforts have been made to induce somatic embryogenesis in *S. sempervirens* using various tissues (Arnaud et al., 1993). In our laboratory, we have induced somatic embryogenesis on *in vitro*-grown needles incubated on a medium consisting of WS salts, Staba vitamins (Staba, 1969), 100 mg·l⁻¹ myo-inositol, 20 g·l⁻¹, 5 µM thidiazuron (TDZ), and 0.5 µM 2,4-D (Figure 2). The medium was solidified with 6 g·l⁻¹ Difco Bacto-agar. The pH of the medium was adjusted to 5.6. Unfortunately, we have not followed up on conversion of these somatic embryos into plantlets. Recently, Liu et al. (2006) reported successful somatic embryogenesis induction from needle tissues. Needles from *in vitro*-grown shoots are incubated on a medium containing

SH medium supplemented with 2.22 μM BA, 2.32 μM kinetin, and 4.9 μM IBA. The medium also contained 30 g/l sugar, 0.65% (w/v) agar, and 0.05% (w/v) casein hydrolysate (CH). The pH of the media was adjusted to 5.6–5.8 with 0.1 N NaOH. All cultures were maintained in the dark for 4 weeks, and then later transferred to light conditions (16-h photoperiod providing 55 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for somatic embryo development. When embryogenic callus is transferred to SH medium supplemented with 5.67 μM BA and 4.9 μM IBA, this promotes embryo development. It is reported that within 12 weeks of incubation, cotyledonary embryos with complete two cotyledons are generated from embryogenic callus (Liu et al., 2006). Although, these authors have indicated that somatic embryos are converted into plantlets, it is not clearly described how this is done.

2.4. Hardening

Gently remove plantlets with well-formed root systems from the culture vessel, and wash the medium off the roots using lukewarm tap water. Washing the medium from the roots reduces likelihood of bacterial and fungal growth that may kill these plantlets once they are transferred to soil. Transfer each plantlet to a 15-cm plastic pot containing thoroughly wet soil mix. Pots with plantlets should be covered with either a clear plastic bag or a clear plastic covering, placed in trays to maintain high moisture, and moved to the greenhouse. Transfer plants to shaded area of the greenhouse. If new transplants are placed under direct sunlight, heat will build up under the cover killing transplants. After 3 to 4 days, cut a few small holes in the plastic bag or slightly raise the plastic container. Repeat this each day for a period of 1 week to promote gradual acclimatization. Remove the cover entirely on the 8th day. A survival rate of over 90% can be easily obtained. Continue to properly water and fertilize all plants to promote healthy growth.

2.5. Field Testing

Well-established young micropropagules can then be transplanted to the field. As these plants are clones and thus are genetically identical, it is anticipated that these micropropagules will have similar growth characters. However, it will not be surprising to observe some differences in vigor among these plants. Other morphological differences may be observed as well, and they should be noted. These morphological variations are likely to be transient in nature; however, stable variants may be observed as well. The length of duration of proliferating shoots in the *in vitro* culture environment (i.e., number of passages) may influence the recovery of stable variants. Therefore, it is important to document and observe all micropropagules for any stable variations, and if these variants are undesirable, then these can be eliminated.

2.6. Molecular Marker Analysis

Using chloroplast DNA (paternal origin), a small number of restriction fragment length polymorphisms (RFLPs), previously identified in petunia, have been found

useful in studying genetic variation in Sequoia (Ali et al., 1991). Of six *pstI* cpRFLP markers, three markers, designated P3, P8, and S8, are polymorphic in redwood, and can be used as probes to assess genetic variability. Recently, two RFLP probes from *Pinus taeda* (loblolly pine) cross-hybridize to genomic Sequoia DNA, although it is not clear if these probes are useful for detecting genetic variability in Sequoia (Ahuja et al., 2004).

Rogers (1999) has also identified 10 allozyme loci that can be used to distinguish among clones in natural populations. Whether or not, these allozyme systems will be useful to assess genetic variability among clones of in vitro-derived propagules remains to be seen. Nevertheless, this provides an alternative approach for genotyping Sequoia.

2.7. Cytology/Flow Cytometry

All conifers, including *S. sempervirens*, are characterized by having large chromosomes; however, *S. sempervirens* is the only hexaploid ($2n = 66$). Thus, this polyploidy nature of Sequoia contributes to difficulty in breeding. The nuclear DNA content of *S. sempervirens* is reported as 32.14 pg/1C (Hizume et al., 2001).

2.8. Storage of in Vitro Cultures

In vitro cultures of Sequoia can be maintained under controlled environmental conditions as described above for long durations provided they are transferred to fresh media. There are no reports on cold storage of *in vitro* cultures of Sequoia, although it is likely that they can be successfully stored and maintained at low temperatures.

3. CONCLUSIONS

Micropropagation of *S. sempervirens* using nodal stem segments collected from young trees provides a successful means of maintaining and multiplying desirable clones of this important and tallest of trees on earth. The overall protocol involves three stages, including explant maintenance, shoot proliferation, shoot elongation and rooting, for a duration period of 16 weeks. Moreover, inducing shoot organogenesis and/or somatic embryogenesis from *in vitro*-grown needles also provide an efficient system of clonal micropropagation. These regenerants/plantlets can be proliferated, elongated, and rooted as described for nodal stem segments.

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CHAPTER 4

MICROPROPAGATION OF *PINUS PINEA* L.

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1. INTRODUCTION

Stone pine (*Pinus pinea* L.) is an economically important tree in the Mediterranean area, and has a significant role in soil conservation, landscape architecture, and for its edible seeds. This makes many aspects of its management similar to an agronomic tree. The wide potential for improvement and the great economic value of the pine nuts requires utmost attention to develop genetic breeding programs. These programs are based on the identification of excellent genotypes by establishing clonal banks of different provenances. Due to the fact that conventional techniques of asexual propagation do not work with *P. pinea*, grafting is the only method available to propagate and genetically evaluate individual clones. However, grafting is far from being optimal. Evaluating the same clone grafted on different rootstocks generates high variability due to scion–rootstock interaction that varies production levels (Mutke et al., 2000). The use of clonal rootstocks could allow to even this interaction, enabling a much more reliable evaluation of each clone.

The heritability of seed characters such as length, number per cone and cone weight is high (Oliveira et al., 2003) and that makes the main objective of its genetic improvement to enhance the quantity and quality of seed production per tree. Therefore, the production of clonal plants from selected seeds by micropropagation would be a desirable tool to improve genetic breeding programs and a means to establish high yield plantations.

In vitro micropropagation of this coniferous species via organogenesis has been extensively studied (García-Férriz et al., 1994; Capuana & Giannini, 1995; González et al., 1998; Oliveira et al., 2003; Sul & Korban, 2004). The micropropagation system is based on the induction of shoot buds in cotyledonary explants dissected from non-germinated embryos (Valdés et al., 2001) and cultured in the medium

supplemented with a cytokinin, usually N⁶-benzyladenine (BA), which has been proven to be the most effective (Moncaleán et al., 2003, 2005).

The routine clonal propagation of stone pine via adventitious bud stimulation from cotyledons has not yet been established. The low efficiency of the rooting process remains a bottleneck of stone pine micropropagation, reducing the possibilities of applying this technique for large-scale propagation. In this chapter, we present an improved plant regeneration method of *P. pinea* that reduces the culture time, increases the rooting rate and shows a successful proliferation procedure.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

Explant source. Cotyledons from non-germinated embryos of stone pine (*Pinus pinea* L.) were used. One year old seeds were obtained from selected open-pollinated trees in natural stands. The seed coat was cracked with a nut cracker and discarded.

Sterilization. After removal of the seed coat, seeds were surface sterilized by immersion in 7.5% H₂O₂ for 45 min, followed by three rinses in sterile double-distilled water. All of the following steps are carried out under aseptic conditions into a laminar flow hood. Seeds were then imbibed in moistened sterile paper for 48 h at 4°C in darkness to facilitate embryo dissection. The embryos were excised from the megagametophyte by making a longitudinal incision with a scalpel and by gently pulling the edges of the cleft with two forceps. Finally, the cotyledons were excised from the embryo axes with a cut at their base. It is recommended to put the cotyledons in the medium immediately after excision to avoid dehydration.

2.2. Culture Media

Media composition. All media used consisted of Le Poivre medium as modified by Aitken-Christie et al. (1988) (Table 1) with half-strength of the major salts (½ LP). Required modifications for the different culture steps are listed in Table 2.

Media preparation. Culture media were prepared using stock solutions of the different components. Plant growth regulators were dissolved separately with a few drops of 1N NaOH before diluting with water. After adjusting the pH to 5.8, the agar was added and dissolved. While stirring carefully, the medium was distributed with a dispenser pump to suitable culture vessels. Baby-food jars (125 mL) were filled with 20 mL of medium and closed with magentaTM B-Caps. Glass culture tubes (95 mm long with 28 mm diameter) were filled with 10 mL and closed with SerotapTM aluminium caps. Vessels with the medium were autoclaved at 121°C (pressure: 105 kPa) for 20 min.

Table 1. Composition of *Le Poivre* medium as modified by Aitken-Christie et al. (1988).

Component	mM	mg L ⁻¹
Major		
KNO ₃	17.8	1800
Ca(NO ₃) ₂ ·4H ₂ O	5.08	1200
NH ₄ NO ₃	5	400
MgSO ₄ ·7H ₂ O	1.46	360
KH ₂ PO ₄	1.99	270
Iron		
FeSO ₄ ·7H ₂ O	0.11	30
Na ₂ ·EDTA	0.11	40
Minor		
MnSO ₄ ·4H ₂ O	0.09	20
ZnSO ₄ ·7H ₂ O	0.03	8.6
H ₃ BO ₃	0.1	6.2
CuSO ₄ ·5H ₂ O	0.0014	0.25
Na ₂ MoO ₄ ·2H ₂ O	0.001	0.25
KI	0.0005	0.08
CoCl ₂ ·6H ₂ O	0.0001	0.025
Vitamins		
Thiamine HCl	0.001	0.4
Inositol	5.55	1000

Table 2. Composition of media used for *Pinus pinea* micropropagation. ½ LPB, caulogenic induction medium; ½ LPC caulogenic expression medium; ½ LPRI root induction medium; ½ LPRE root expression medium.

	½ LPB	½ LPC	½ LPRI	½ LPRE
Carbon source	30 gL ⁻¹ sucrose	30 gL ⁻¹ sucrose	21 gL ⁻¹ glucose	10.5 gL ⁻¹ glucose
Hormone	44.4 µM BA		2 mg L ⁻¹ NAA	
Activated Charcoal		5 gL ⁻¹		
Agar	8 gL ⁻¹	8 gL ⁻¹	8 gL ⁻¹	8 gL ⁻¹
Vessel	Baby-food jar	Baby-food jar	Tube	Tube

2.3. Shoot Regeneration and Maintenance

Bud induction. Cotyledons were placed horizontally in baby-food jars with ½ LPB. Cotyledons were maintained for 6 days [induction time based on Moncaleán et al. (2005)] in a growth chamber at 25 ± 1°C with a 16 h photoperiod at a photon flux of 40 ± 5 µmol m⁻² s⁻¹ provided by white fluorescent tubes (TLD 58 W/33, Phillips, France). This period is enough to obtain a 100% organogenic response in most cases. During this period the cotyledons become green and increase in size (Figure 1A).

Shoot development. Cotyledons were transferred and cultured onto $\frac{1}{2}$ LPC for two subcultures of 35 days each. These cultures were maintained in a growth chamber at $25 \pm 1^\circ\text{C}$ with a 16 h photoperiod at a photon flux of $80 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by white fluorescent tubes. After one subculture, small buds primordia appear along the surface of the cotyledon (Figure 1B). After 70 days in $\frac{1}{2}$ LPC, the shoots were separated from the cotyledonary explants and elongated by sequential subculturing in $\frac{1}{2}$ LPC (Figure 1C). The excision should be as close to the cotyledon as possible, taking care not to excise too small buds (≤ 0.5 cm) because its size is critical for survival.

Shoot proliferation. The shoots were subcultured monthly in the expression medium until they reached 2 cm (microshoots). During this phase, the axillary buds can also be excised and multiplied (Figure 1D). The axillary buds do not show plagiotropic growth. The extent of this phase is variable because of the asynchronic bud development. Shoot growth and multiplication rates can be increased by successive subcultures on $\frac{1}{2}$ LPC medium. After 22 weeks of culture in $\frac{1}{2}$ LPC, 200 microshoots per seed can be expected.

2.4. Rooting

Root induction. Basal calli were cut and removed from elongated microshoots (>1 cm) grown on $\frac{1}{2}$ LPC. The microshoots were transferred and placed vertically on $\frac{1}{2}$ LPRI, inserting only the basal part. During this period shoots were incubated for 1 week in darkness at 19°C , and 1 week at 19°C under 16 h photoperiod (photosynthetic flux is $100 \mu\text{mol}^{-2} \text{s}^{-1}$ provided by fluorescent lamps), as indicated by Potes (personal communication).

Root development. After induction phase, the explants were transferred to $\frac{1}{2}$ LPRE and incubated at $25/19^\circ\text{C}$ with 16 h photoperiod at a photon flux of $100 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by white fluorescent tubes. After 3–6 weeks under these conditions roots appeared (Figure 2A). Although rooting rate depends on seed genotype, up to 70% of rhizogenesis can be obtained with seeds from open-pollinated trees. Based on a 70% rooting success rate, at least 140 plantlets per seed can be expected after 29 weeks.

2.5. Acclimatisation and Hardening

Acclimatisation. After 6 weeks on expression medium, rooted shoots with at least 1 cm roots were ready to be grown in *ex vitro* conditions. The agar was removed from rooted shoots by washing in tap water. The rooted shoots were transferred to a wet peat-perlite (1:4 v/v) mixture into multipots and cultivated under decreasing high humidity conditions according to Cortizo et al. (2004). During these acclimatisation weeks, rooted shoots were irrigated with eight-fold diluted LP medium three times a week.

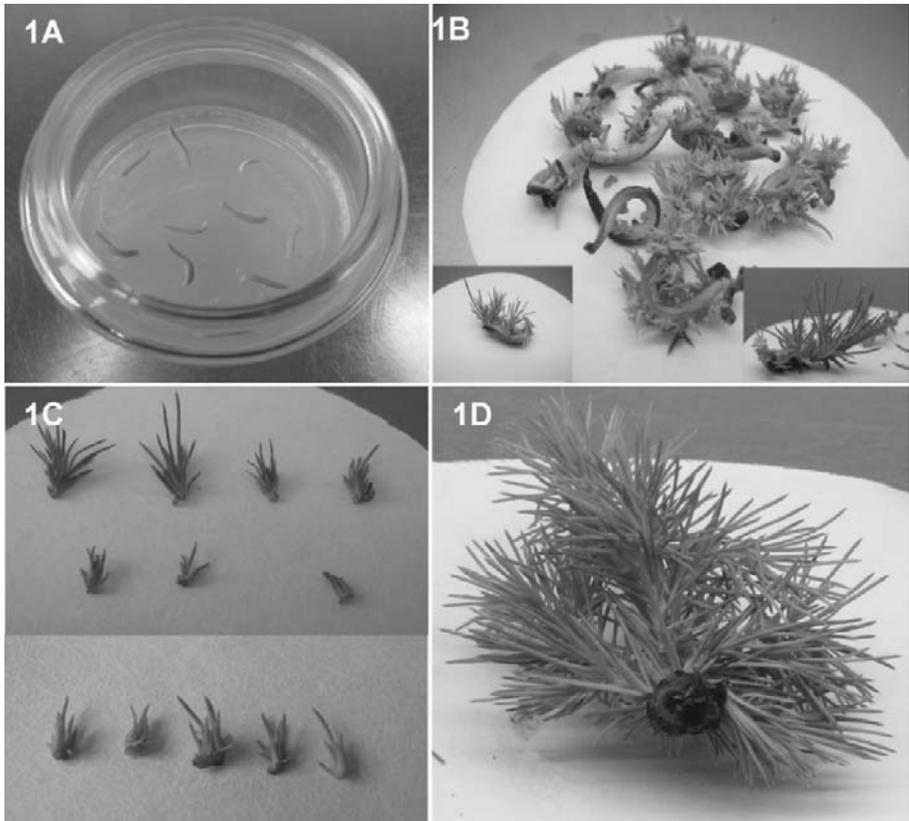


Figure 1. A) Isolated cotyledons of *Pinus pinea* after 6 days on caulogenic induction medium. B) Cotyledons after 6 days on caulogenic induction medium and 35 days on caulogenic expression medium. C) Buds isolated from cotyledons after 70 days on caulogenic expression medium. D) Shoot multiplication after 16 weeks on caulogenic expression medium.

Hardening. After 3 weeks, plantlets were transferred to greenhouse in normal humidity conditions (Figure 2B). Plantlets were fertilized every three months using Basacote 3M (Compo). The temperature in the greenhouse ranged between 18–28°C. After two months in nursery, the survival rate of microplants can reach 98%. No plagiotrophic growth was observed and plants showed a well-developed root system capable of sustaining further shoot outgrowth.

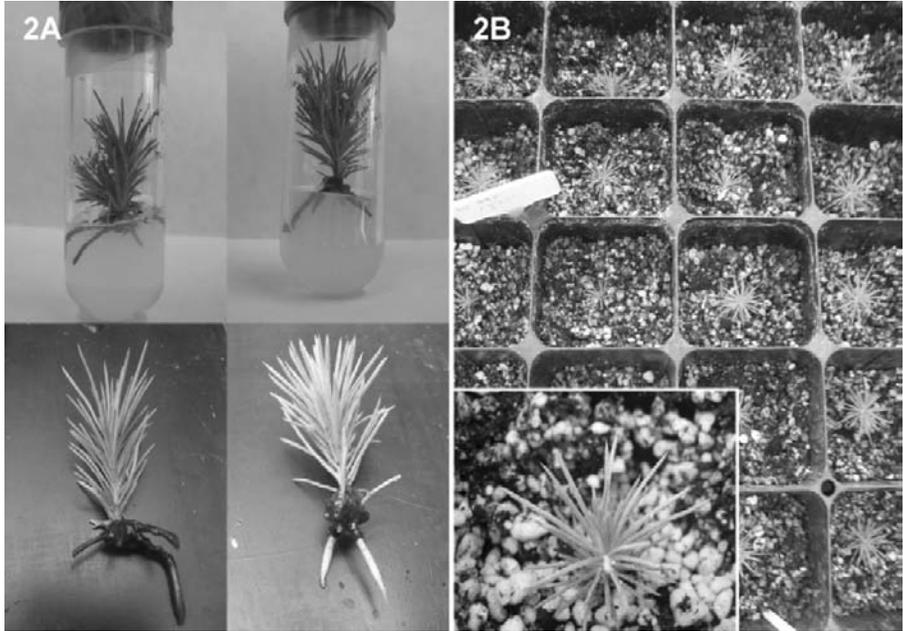


Figure 2. A) Rooted shoots of *Pinus pinea* after 4 weeks on root expression medium. B) Acclimatised *Pinus pinea* microplants after one month in the greenhouse.

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CHAPTER 5

MICROPROPAGATION OF *PINUS ARMANDII* VAR. *AMAMIANA*

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1. INTRODUCTION

In the survey conducted by the Environmental Agency on endangered species in Japan in the year 2000, 1665 species were listed as endangered one among 7087 vascular plants (Environmental Agency of Japan 2000). Collection for ornamental use, natural succession, and deforestation are the three major causes for threatening the species. To recover the endangered species, propagation of such plants for *ex situ* or *in situ* conservation is important. Among them, micropropagation by tissue culture is considered effective and useful method.

There were several reports on micropropagation of endangered trees (Okochi et al., 2003; Sugii & Lamoureux, 2004). Here we describe protocols for micropropagation of an endangered five needle pine, *Pinus armandii* Franch. var. *amamiana* (Koidz.) Hatusima for preserving it *ex situ*, and supply plants for rehabilitation.

Pinus armandii var. *amamiana* is an endangered tree inhabiting only in the south western islands of Japan, Yakushima and Tanegashima (Yahara et al., 1987). Recent survey showed that there are only about 2000 trees remaining in the both islands. Pine wilt disease by nematodes is suggested as one of the causes of the decline of this species (Akiba & Nakamura, 2005). Because of its decreasing number in recent years in the field populations, it was claimed as IB(EN) in the new Japanese Red List (Environmental Agency of Japan 2000) which denotes a high possibility of extinction in the near future. Five needle pine group which include this species are widely used as timber resources and ornamental bonsai trees in the world.

2. EXPERIMENTAL PROTOCOL

2.1. Organ Culture

2.1.1. Materials

1. Mature seeds collected from late August to early September from remaining trees (Figure 1A) of *Pinus armandii* var. *amamiana* in Yakushima island.
2. Laminar-flow chamber, Petri dishes, forceps, scalpel, pipettes.
3. Ethanol, sterile distilled water, culture tubes, culture flasks.
4. Dissecting microscope.
5. Media (see Table 1).

2.1.2. Methods

The regeneration method can be divided into three main steps: initial culture, shoot elongation, and rooting.

Initial culture. For elimination of empty seeds caused by inbreeding depression, only submerged seeds in 100% ethanol were used for further experiments in mature seeds.

1. Remove the seeds from the cones.
2. Sterilize the seeds with 70% ethanol for 3 min.
3. Wash the seeds two times with distilled water.
4. Mature embryos were excised from the seeds.
5. Transfer the sterile embryos on to the induction medium in the test tubes. Mainly a half strength DCR (Gupta & Durzan, 1985) medium with different concentration of plant growth regulators (2 or 10 μM BAP plus 0.1 μM NAA, 0.4, 2 and 10 μM BAP) was used. Culture tubes (18 mm i.d. \times 160 mm) containing 15 ml of agar solidified media were used for initial culture, and 200 ml culture flasks containing 70 ml agar-solidified medium were used for subculture.

Shoot elongation

1. Regenerated buds with embryos are transferred to shoot elongation medium containing 2g/l activated charcoal (Figure 1B) (Table 1).
2. Elongated shoots are divided and further subcultured on the same medium for 1–2 months interval.

Rooting

1. Shoots more than 1.5 cm length are cut and transferred to the rooting medium.
2. For rooting of shoots, RIM medium (Abo El-Nil & Milton, 1982) containing indole butyric acid (IBA) was used.
3. Rooted shoots (Figure 1C) are subcultured to the hormone free floralite[®] (Nisshinbo, Japan) medium containing 0.1% hyponex[®].

Table 1. Media for organ culture of *Pinus armandii* var. *amamiana*.

Chemicals (mg/l)	Initial culture (1/2 DCR)	Shoot elongation (1/2 DCR + AC)	Rooting (RIM)
NH ₄ NO ₃	200	200	
KNO ₃	170	170	187.5
Ca(NO ₃) ₂ × 4H ₂ O		152	
MgSO ₄ × 7H ₂ O	185	185	200
CaCl ₂ × 2H ₂ O	42.5	42.5	
NaH ₂ PO ₄ × H ₂ O	138		
KH ₂ PO ₄	85	85	170
K ₂ SO ₄		860	
Na ₂ SO ₄		200	
KCl		65	
FeSO ₄ × 7H ₂ O	13.9	13.9	27.8
Na ₂ EDTA	18.65	18.65	37.3
MnSO ₄ × 4H ₂ O	11.15	11.15	5
ZnSO ₄ × 7H ₂ O	4.3	4.3	0.5
H ₃ BO ₃	3.1	3.1	10
KI	0.415	0.415	1
Na ₂ MoO ₄ × 2H ₂ O	0.125	0.125	0.1
CuSO ₄ × 5H ₂ O	0.0125	0.0125	0.1
CoCl ₂ × 6H ₂ O	0.0125	0.0125	0.1
NiCl ₂	0.0125	0.0125	
Myo-inositol	100	100	100
Nicotinic acid	0.5	0.5	5
Pyridoxine HCl	0.1	0.1	0.5
Thiamine HCl	1	1	5
Glutamine	100		
Glycine	1	1	
Coumarin		0.0146	
Riboflavin		0.2	
BAP	0.045–2.25		
NAA	0–0.0186		
IBA		1–3	
Activated charcoal	2000		
Sucrose	15000	15000	15000
Agar	8000	9000	8000

All culture conditions were conducted at 25°C under a 16-h photoperiod (fluorescent light, 70 $\mu\text{Mm}^{-2}\text{s}^{-1}$). Regenerated and habituated plantlets from organ culture of mature embryos were transferred to the nursery field made of black soil loam (Figure 1D).



Figure 1. Micropropagation of *Pinus armandii* var. *amamiana* by organ culture. A) Remaining *Pinus armandii* var. *amamiana* in Yakushima island. B) Multiple shoots on the shoot elongation medium. C) Rooting of the shoot. D) Field grown plantlets obtained by organ culture of *Pinus armandii* var. *amamiana*.

2.2. Somatic Embryogenesis

2.2.1. Materials

1. Mature and immature seeds collected from early July to early September from remaining trees of *Pinus armandii* var. *amamiana* in Yakushima island.
2. Laminar-flow chamber, Petri dishes, forceps, scalpel, pipettes.
3. Ethanol, sterile distilled water, multi well plate, plastic Petri dish, culture flasks.
4. Dissecting microscope. Inverted microscope.
5. Media (see Table 2).

2.2.2. Methods

The regeneration method can be divided into four main steps: initiation of embryogenic cultures, proliferation of embryogenic cultures, maturation of somatic embryos, and germination.

Initiation of embryogenic cultures

1. For propagation via somatic embryos, embryogenic cell suspensions were induced from immature and mature seeds on modified 1/2MS (Murashige & Skoog, 1962) or 1/2EM (Maruyama et al., 2000) medium supplemented with different concentration of 2,4-D and BAP.
2. Disinfect cones by 15 min immersion in 70% ethanol containing few drops of neutral detergent and then wash in tap water before dissection.
3. Disinfect excised seeds with 3% (w/v chlorine) sodium hypochlorite solution for 30 min then rinse five times with sterile distilled water.
4. For induction of embryogenic cells, culture whole seed explants in 24-well tissue culture plates (one per well) containing induction medium as shown in Table 2.
5. Seal culture plates with Novix-II[®] (Iwaki Co. Tokyo) film and incubate in the dark at 25°C.
6. The presence or absence of distinct early stage of somatic embryo characterized by embryonal head (dense cells) with suspensor system (elongated cells) from the explant is observed weekly under the inverted microscope, up to 3 months.

Proliferation of embryogenic culture

1. Induced suspension cells were transferred to ammonium free (just by omitting ammonium nitrate) 1/2MS liquid medium supplemented with 2,4-D, BAP and L-glutamine and subcultured every 2–3 weeks.
2. For continuously proliferation routines, subculture embryogenic cells to fresh medium using transfer pipette (about 0.5 ml suspension culture in 30–40 ml fresh medium) and incubate in 100 ml flask on rotatory shaker at 100 rpm in the dark.

Table 2. Media for somatic embryo culture of *Pinus armandii* var. *amamiana*.

Chemicals (mg/l)	Induction & proliferation (ammonia free 1/2 MS)	Maturation (1/2 DCR + AC)	Germination (ammonia free MS)
KNO ₃	950	950	1900
MgSO ₄ × 7H ₂ O	185	185	370
CaCl ₂ × 2H ₂ O	220	220	440
KH ₂ PO ₄	85	85	170
FeSO ₄ × 7H ₂ O	13.9	13.9	27.8
Na ₂ EDTA	18.65	18.65	37.3
MnSO ₄ × 4H ₂ O	22.3	22.3	22.3
ZnSO ₄ × 7H ₂ O	8.6	8.6	8.6
H ₃ BO ₃	6.2	6.2	6.2
KI	0.83	0.83	0.83
Na ₂ MoO ₄ × 2H ₂ O	0.25	0.25	0.25
CuSO ₄ × 5H ₂ O	0.025	0.025	0.025
CoCl ₂ × 6H ₂ O	0.025	0.0125	0.025
Myo-inositol	100	100	100
Nicotinic acid	0.5	0.5	5
Pyridoxine HCl	0.5	0.5	0.5
Thiamine HCl	0.1	0.1	5
Glutamine		1 500	100
Glycine	2	2	
ABA		13.22	
PEG (M.W. 6,000)		100000	
Maltose		60000	
BAP	0.675		
2,4-D	0.663		
IBA		1–3	
Activated charcoal		2000	
Sucrose	15000	30000	
agar		8000	
gelrite	3000		

Maturation of somatic embryos

1. In order to develop somatic embryos, the suspension cells were transferred to ammonium free MS medium supplemented with 10 µM ABA, 0.2% activated charcoal, 10% polyethylene glycol (PEG, MW 6000), 30 mM L-glutamine and 6% maltose.
2. Collect embryogenic cells on 100 µm nylon screen.
3. Resuspend embryogenic cells in fresh medium (about 500 mg FW per 10 ml cell suspension medium).

4. Dispense as 2 ml aliquots on filter paper disk over each Petri dish containing maturation medium as specified in Table 2.
5. Seal Petri dish and culture in the dark.

Germination

1. Collect somatic embryos from maturation medium and transfer to filter paper disk over each Petri dish containing germination medium as described in Table 2.
2. Obtained cotyledonary embryos were transferred on ammonium free MS agar-solidified medium in culture flasks under a 16 h photoperiod.
3. Plantlets were transferred to vermiculite containing modified MS (ammonium and sugar free) liquid medium in 200 ml culture flasks, then out planted after habituation procedure of 2 weeks in 100% moisture content.
4. The cultures were incubated under daily 16/8 h light-dark photoperiods of fluorescent lamp at 25°C.

3. CONCLUDING COMMENTS

3.1. Organ Culture

Adventitious buds were induced on the surface of the mature embryos on 1/2 DCR medium containing 0.4 μM to 2 μM BAP (Table 3), and they grew to shoots after subculturing to medium containing 2 g/l activated charcoal. Cotyledon development was observed in the medium containing 0.1 μM NAA and green callus was prevalent at the higher concentrations of BAP in the medium in the initial culture (Table 3). From the elongated shoots, root primordia and roots were induced in RIM medium containing 4.9 to 14.8 μM IBA. Regenerated plantlets were in the pots with the floralite® containing 0.1 % hyponex® for 2 weeks under 100% humidity, then 13 plantlets were planted out successfully to the field (Ishii et al., 2004) (Figure 1D). Survival rate of the plantlets was 92% after 1 year in the field condition.

3.2. Somatic Embryogenesis

Embryogenic cell suspensions were induced better from immature seeds of *Pinus armandii* var. *amamiana* on modified MS (half strength in major elements and ammonium free) liquid medium supplemented with 3 μM 2,4-D and 3 μM BAP (Table 4). However, it seems that effects of hormonal combination was not so determinative because somatic embryogenic cells were also obtained in other combinations. Physiological and genetic conditions of immature embryos might be also important for somatic embryogenesis. Induced suspension cells were subcultured successfully every 2–3 weeks (Figure 2A). After 1 to 2 months culture on maturation

Table 3. Effects of plant growth regulators (PGR) on culture of mature embryos from seeds of *Pinus armandii* var. *amamiana*.

PGR	μM	No. of responded embryos/No. of embryos (%)		
		Adventitious buds	Green callus	Cotyledon development
BAP	2			
NAA	0.1	2/20 (10)	4/20 (20)	6/20 (30)
BAP	10			
NAA	0.1	0/20 (0)	14/20 (70)	2/20 (10)
BAP	0.4	6/10 (60)	2/10 (20)	0/10 (0)
BAP	2	4/10 (40)	4/10 (40)	0/10 (0)
BAP	10	0/10 (0)	6/10 (60)	0/10 (0)

Ten to twenty mature embryos were used for each treatment.

medium, differentiation of embryos progressed and cotyledonary embryos were obtained (Figure 2B). Transplanting of somatic embryos further to ammonium free MS solidified medium for 3 weeks was necessary for developing plantlets with roots and green cotyledons. Plantlets transplanted to vermiculite in 200 ml culture flasks survived (Figure 2C).

Embryogenic cells were also induced from mature seeds of *Pinus armandii* var. *amamiana* on 1/2 EM medium (Maruyama et al., 2000) containing 10 μM 2,4-D and 5 μM BAP. The supplement of L-glutamine into media enhanced embryo maturation and prevented somatic embryos from browning (Hosoi & Ishii, 2001). Forty seven regenerated plantlets showed normal growth in the greenhouse (Figure 2D).

For *ex situ* conservation of endangered *Pinus armandii* var. *amamiana*, *in vitro* culture methods will help propagate rootstocks for grafting or seedlings from seed orchard (Ishii et al., 2005). *In vitro* culture itself might be used as the *ex situ* conservation method.

Table 4. Effects of combination of 2,4-D and BAP on induction rate of somatic embryo forming cells from immature embryos of *Pinus armandii* var. *amamiana*.

BAP (μM)	2,4-D (μM)			
	0.3	1	3	10
0	*0/20 (0)	2/18 (11.1)	2/18 (11.1)	2/14 (14.3)
1	2/18 (11.1)	2/19 (10.5)	2/19 (10.5)	1/13 (7.7)
3	3/17 (17.6)	2/19 (10.5)	4/19 (21.1)	1/12 (8.3)

Twelve to twenty immature embryos were used for each treatment. * No. of embryos inducing somatic embryos/No. of embryos (%)

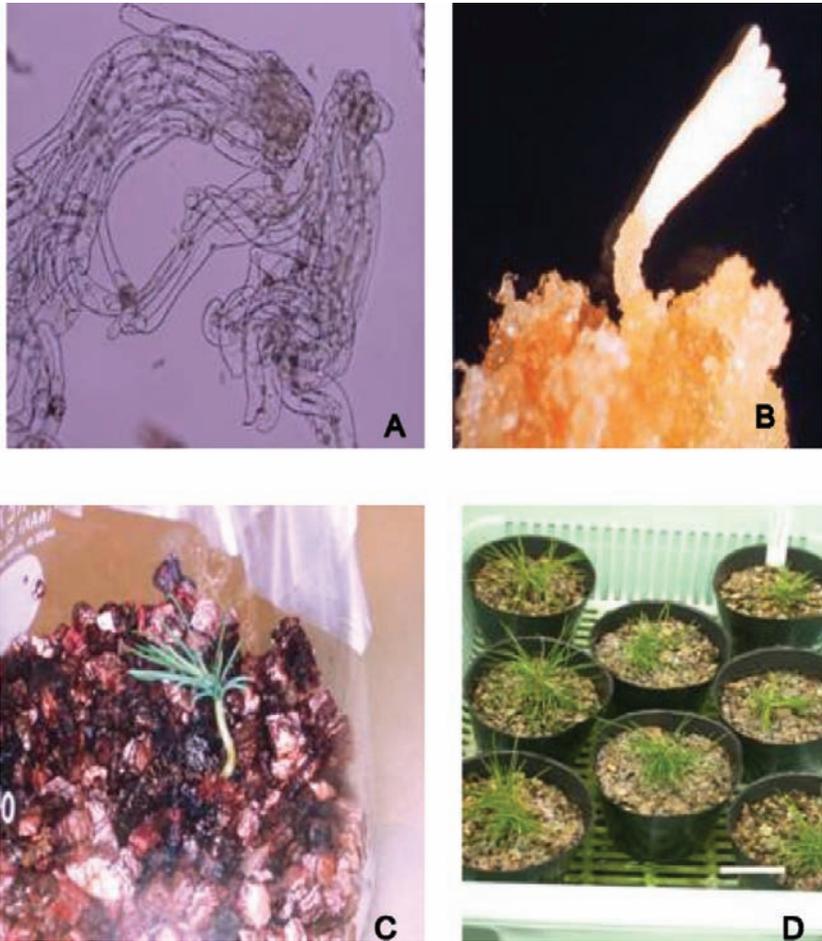


Figure 2. Micropropagation of *Pinus armandii* var. *amamiana* by somatic embryogenesis. A) Suspension culture of somatic embryogenic cells of *Pinus armandii* var. *amamiana*. B) Maturation of somatic embryo of *Pinus armandii* var. *amamiana*. C) Regenerated plantlet of *Pinus armandii* var. *amamiana* from somatic embryo. D) Habituated plantlets of *Pinus armandii* var. *amamiana* from somatic embryos.

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CHAPTER 6

ORGANOGENESIS AND CRYOPRESERVATION OF JUVENILE RADIATA PINE

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1. INTRODUCTION

New Zealand has 1.81 million hectares of plantation forests and 89% of this is radiata pine (*Pinus radiata* D. Don) (Ministry of Agriculture and Forestry 2006). Initial stocking rates vary but are typically in the range of 800–1100 plants per hectare. New planting and replanting of harvested areas was about 50,200 ha in 2004 (Ministry of Agriculture and Forestry 2006), requiring around 50 million nursery plants.

Sufficient open- and control-pollinated seed-orchard seed is produced for New Zealand requirements. The more expensive control-pollinated seed is used to improve genetic gain, and this has led to greater use of vegetative propagation by cuttings and tissue culture to provide more than 25% of current planting stock (Menzies et al., 2001). Tissue culture methods developed to amplify control-pollinated seed include organogenic methodologies (Reilly & Washer, 1977; Aitken et al., 1981; Horgan & Aitken, 1981; Smith et al., 1982) and somatic embryogenesis from immature zygotic embryos (Smith et al., 1994; Smith, 1996, 1997).

From control-pollinated seed, selections may also be made of outstanding individuals that can then be propagated and tested as clones. Clones from within top families have demonstrated marked improvements in performance when compared with family averages (Johnson, 1988). However, propagules generated clonally from mature radiata pine (more than 4 years old) have a lower initial growth rate than those from younger material (Menzies & Klomp, 1988). Conversely, many characteristics of commercial interest, such as wood properties and resistance to some diseases, can be identified only when trees are 8 to 12 years old. Therefore, at an age by when elite trees can be identified, they are mature and clonal propagules exhibit reduced growth rate. Options to maintain juvenile propagules while field testing takes place include cool storage of *in-vitro* grown shoots (Aitken-Christie & Singh, 1987; Horgan et al., 1997) and maintenance of stool beds in the nursery

(Menzies et al., 1985; Menzies & Aimers-Halliday, 1997). More recent advances in the area of juvenility maintenance include the development of cryogenic methods in combination with somatic embryogenesis (Hargreaves & Smith, 1992, 1994; Hargreaves et al., 2002). Methods for organogenic material have been more difficult to develop though significant progress has been made with zygotic embryos and recently, shoot tip meristems (Hargreaves et al., 2004, 2005).

Somatic embryogenesis was originally seen as the way forward in terms of delivering clonal forestry strategies to commercial forest operators, because the technique results in tissue that can be easily cryopreserved (thus ensuring juvenility while field testing takes place) and has the potential to produce millions of plants quickly (Durzan & Gupta, 1988; Attree & Fowke, 1991). However, major problems have been low genotype capture and a high labour cost of *in vitro* procedures (Park et al., 1998; Timmis, 1998).

Genotype capture using organogenesis methods with zygotic embryos (mature and immature) or seedling/stool bed material is high and the *in vitro* culture period short. Media formulations include few plant growth regulators, and less skilled observation through developmental stages is required than with somatic embryogenesis (Hargreaves et al., 2005).

Propagation techniques for radiata pine which utilise organogenic approaches are showing great versatility for a range of applications, including arresting of juvenile growth via cool storage and more recently developed cryogenic methods. These also extend to the preservation of genetic resources in the case where native populations may be under threat from human interference, including climate change and disease. The potential is promising for amplifying valuable seed that may be both limited in supply and less fit, in the case of historic seed collections and where new and novel hybrids may have been bred (Hargreaves et al., 2007). Other uses of the approach are to facilitate the safe (contamination free, pre-screened) dissemination of proven/novel genetic material internationally as shoot cultures. This material arrives in a ready-to-amplify condition.

This chapter sets out to describe the current methods for organogenic multiplication and cryogenic storage of radiata pine. Details are also given for the transition of material from the laboratory to the nursery and field.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.0. Culture Media

Constituent name	Constituent formula	Shoot initiation	Shoot multiplication	Root initiation
		Medium* (1/2 LP5)	Medium* (LPch)	Medium** (GD)
Major elements		mg l⁻¹	mg l⁻¹	mg l⁻¹
Ammonium sulphate	(NH ₄) ₂ SO ₄			200.00
Ammonium nitrate	NH ₄ NO ₃	200.00	400.00	

Constituent name	Constituent formula	Shoot initiation	Shoot multiplication	Root initiation
Calcium nitrate	$\text{Ca}(\text{NO}_3)_2 \times 4\text{H}_2\text{O}$	600.00	1200.00	
Magnesium sulphate	$\text{MgSO}_4 \times 7\text{H}_2\text{O}$	180.00	360.00	250.00
Potassium chloride	KCl			300.00
Potassium dihydrogen orthophosphate	KH_2PO_4	135.00	270.00	
Potassium nitrate	KNO_3	900.00	1800.00	1000.00
Sodium dihydrogen orthophosphate	$\text{NaH}_2\text{PO}_4 \times 2\text{H}_2\text{O}$			100.00
Disodium hydrogen orthophosphate	$\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$			75.00
Dodecahydrate				
Minor elements		mg l⁻¹	mg l⁻¹	mg l⁻¹
Manganous sulphate	$\text{MnSO}_4 \times 4\text{H}_2\text{O}$	5.0000	10.000	20.00
Boric acid	H_3BO_3	3.1000	6.200	5.00
Zinc sulphate	$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$	4.3000	8.600	1.00
Potassium iodide	KI	0.0400	0.080	1.00
Cupric sulphate	$\text{CuSO}_4 \times 5\text{H}_2\text{O}$	0.1250	0.250	0.20
Sodium molybdate	$\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$	0.1250	0.250	0.20
Cobaltous chloride	$\text{CoCl}_2 \times 6\text{H}_2\text{O}$	0.0125	0.025	0.20
Calcium				mg l⁻¹
Calcium chloride Dihydrate	$\text{CaCl}_2 \times 2\text{H}_2\text{O}$			150.00
Vitamins				mg l⁻¹
Thiamine HCl	$\text{C}_{12}\text{H}_{17}\text{ClN}_4\text{OS} \times \text{HCl}$	0.200	0.400	5.00
Nicotinic acid	$\text{C}_6\text{H}_5\text{NO}_2$			5.00
Pyridoxine HCl	$\text{C}_8\text{H}_{11}\text{NO}_3 \times \text{HCl}$			0.50
Iron		mg l⁻¹	mg l⁻¹	mg l⁻¹
Ethylenediaminetetraacetic acid	Na_2EDTA	15.00	30.00	30.00
Ferrous sulphate	$\text{FeSO}_4 \times 7\text{H}_2\text{O}$	20.00	40.00	40.00
NAA				mg l⁻¹
1-Naphthalene acetic acid (NAA)	$\text{C}_{12}\text{H}_{10}\text{O}_2$			0.50
IBA				mg l⁻¹
Indole-3-butyric acid (IBA)	$\text{C}_{12}\text{H}_{13}\text{NO}_2$			1.00

Constituent name	Constituent formula	Shoot initiation	Shoot multiplication	Root initiation
BA		mg l⁻¹		
Benzylaminopurine		5.00		
Sucrose		30000mg	30000mg	20000mg
Inositol		1000mg	1000mg	1000mg
Agar-Germantown		8000mg	7500mg	8000mg
Activated charcoal			3500mg	
pH		5.7	5.7	5.7

Preconditioning medium for cryopreservation (PGD)

Medium is a modification after Ryyänen (1996).

10% polyethylene glycol (PEG) (MW 4000) w/v

10% glucose w/v

10% dimethylsulphoxide (DMSO) v/v

Liquid LP 0.1 M sucrose to make up volume

Filter sterilise

Note:

* Medium is a modification of Quoirin and Lepoivre (1997).

** Medium is a modification of Gresshoff and Doy medium (as modified by Sommer et al., 1975 and Horgan & Holland 1989).

2.1.1. Organogenesis from Mature Zygotic Embryos

Culture initiation (epicotyl-axillary). Seeds are sterilised in a solution of 50% Chlorodux (calcium hypochlorite, 5% v/v) plus a surfactant (0.1 mL Silwet L-77·L⁻¹; alternately Tween 80 can be substituted) for 20 min followed by rinsing under running water overnight to facilitate imbibition.

Following imbibition, seeds are resterilised using a 6% hydrogen peroxide solution with 0.1 mL Silwet L-77·L⁻¹ (alternately Tween 80 can be substituted) for 10 min and then rinsed three times in sterile water. Extra rinses can be given if seed continues to form bubbles over seed coat surface, indicating some continued hydrogen peroxide activity. Embryos are dissected from seeds, with operators' fingernails, which have been dipped in 70% ethanol, to break open the seed coat and split the endosperm. The zygotic embryo is then carefully removed with sterilised forceps.

2.1.2. Organogenesis from Cotyledons of Mature Zygotic Embryos

Culture initiation (adventitious-axillary). Seeds are sterilised and embryos dissected from seeds as described earlier. Dissected-out embryos are left for a minimum of

3 hours on LPch medium to facilitate opening of the cotyledons from around the epicotyls. More commonly, the dissected-out embryos are put on LPch and then put into a domestic fridge (4°C, dark) overnight for cotyledon removal the following day. This overnight storage seems to firm up the embryos, making them easier for subsequent handling. Forceps and scalpels are used to snap each cotyledon at the point where it is attached to the hypocotyl (6–10 cotyledons are common in *Pinus radiata*) (Figure 1A). The scalpel is not used to ‘cut’ tissue but to collect the snapped off cotyledons. These are transferred to a half strength LP medium (including microelements, iron stock, and vitamins) containing 5 mg benzylaminopurine (BA) L⁻¹ ($22.22 \times 10^{-5} \text{ mol L}^{-1}$) and 3% sucrose in deep Petri dishes. Charcoal is excluded from this medium. Petri dishes are placed in a light incubator in the same conditions as described earlier for germinating zygotic embryos. Shade cloth is also used to reduce light intensity for the first week of culture. After 21–24 days, cotyledons are transferred to LPch in deep dishes, and shoots begin to elongate from nodular meristematic tissue which had formed on the BA medium. Following 6 weeks of growth, elongated shoots can be cut from the cotyledon tissue and transferred to jars, as described previously (Figure 1B) Remaining cotyledon tissue with small shoots can be cut to improve tissue contact with medium and returned to LPch in deep Petri dishes for further shoot initiation and elongation. Higher rates of vitrification/hyperhydricity are observed in adventitious-axillary shoot in the early phases of shoot initiation from cotyledons as compared to epicotyl-axillary material, possibly due to the proximity of the newly-formed meristems to the surface of the medium. Once sufficient shoots have been obtained, all remaining cotyledonary tissue is discarded and shoot multiplication continues as described for the epicotyl-axillary material. Vitrified tissue (i.e. hyperhydric tissues) is discarded at subculture (see below). Slower rates of shoot elongation have been observed in tissue of adventitious-axillary origin and transfer programmes can be modified to allow for this (Hargreaves et al., 2005).

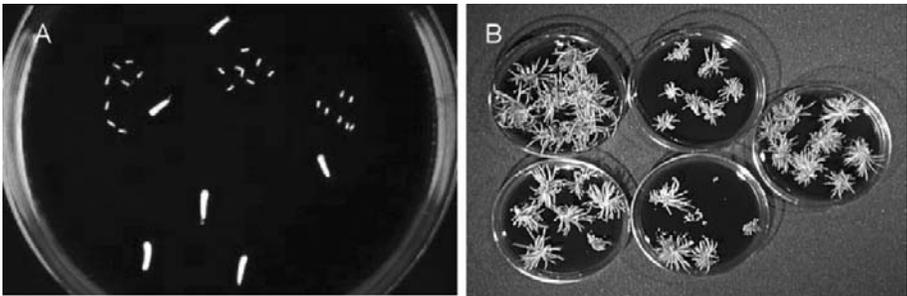


Figure 1. A) Zygotic embryo showing snapped off cotyledons and hypocotyls. B) Cotyledons with adventitious shoots elongating; at this stage shoots do not have clearly visible shoot tip meristems.

2.1.3. Organogenesis from Field-grown Material

Culture initiation. Shoot cultures can be initiated from nursery- or field-grown material, such as seedlings, which have been topped to induce new axillary shoots. Ideally, shoot material should be freshly elongated and sprayed with broad spectrum fungicides while in the field. The following procedure has been used successfully to disinfest field-collected explants. Excised shoot tips (approximately 50 mm) are given the following treatment:

1. Pre-wash field-collected material in sterile water with surfactant (Silwet or Tween 80): 150 μL in 200 ml of sterile water for 20 min with intermittent agitation.
2. Chlorodux (calcium hypochlorite, 5% v/v)/sterile water, (40:60) for 15 min with intermittent agitation.
3. Rinse three times in sterile water to remove Chlorodux.
4. Explants are then placed in 6% hydrogen peroxide solution with 0.1 mL Silwet L-77 L^{-1} (alternately Tween 80 can be substituted) for 10 min and then rinsed three times in sterile water.

Following rinsing, shoot tips are placed on sterile paper towels to remove excess moisture. The base of the shoot tip is discarded and the remaining explant is cut into segments approximately 10–15 mm in length. These are placed on LP medium in deep Petri dishes (25 \times 90 mm) without charcoal to make detection of contamination easier. Petri dishes are placed in standard growing conditions for tissue-culture shoots, with shade cloth to reduce light intensity from the standard photosynthetic photon flux density of 80 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ by approximately 50% for the first week of culture. Cultures are inspected for contamination and clean material is transferred to new dishes of LP medium for 4–6 weeks to insure the material is contamination-free before standard LPch multiplication methods are applied (see epicotyl-axillary methods).

2.1.4. Shoot Regeneration and Maintenance

Shoot multiplication (epicotyl-axillary). The zygotic embryos are placed on a modified Quoirin and Lepoivre medium (LP) (Quoirin & Lepoivre, 1977, modified by Aitken-Christie et al., 1988) containing 5 g activated charcoal (Merck) L^{-1} (LPch) in deep Petri dishes (90 mm diameter \times 25 mm depth). After 10 days on LPch, each embryo has its developing apical shoot (epicotyl) removed with up to 10 mm of hypocotyl and “planted” back into the LPch for further elongation. The remainder of the hypocotyl and root radical are discarded. All cultures are then placed in a light incubator with a photoperiod of 16 h light (photosynthetic photon flux density 80 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 24°C and 8 h dark at 18°C. Shade cloth is placed over the Petri dishes to reduce the light intensity by approximately 50% for the first week of culture.

Table 1. Example of shoot amplification in genotypes from a group of control-pollinated crosses following 5 months of culture and transfer.

Cross	Genotype no. tested	Explant total at transfer			
		January	February	April	May
1	6	16	22	92	216
2	9	19	37	140	368
3	9	18	40	127	301
4	9	18	29	109	273
5	9	20	34	109	272
6	9	22	33	157	346
7	9	20	33	101	214
8	9	21	30	105	242
9	5	10	17	56	144
Total explants		164	275	996	2376
Shoots/explants per initial (Jan.) explant		1	1.67	6.07	13.87

Following 4 weeks growth, shoots are transferred to LPch (80 mL) in 600 mL glass Agee jars (Australian Glass Company, Penrose, Auckland) with clear lids. The elongated epicotyl shoot is cut from the hypocotyl at this stage so that fresh stem tissue is in contact with the medium. Following a further 4–6 weeks growth, these shoots are sufficiently elongated (30–50 mm) to be divided into shoot tips and stem segments (Figure 2). Shoot tips (approximately 20 mm) are transferred to LPch in jars for further elongation. Stem segments (approximately 10 mm) are put in fresh deep Petri dishes of LPch. Stem segments frequently produce side shoots, which further enhance amplification (Figure 3). This transfer process is repeated every 4 to 6 weeks until sufficient shoots are obtained for rooting. Amplification rates vary between crosses and genotypes within crosses (Table 1). From initiation through all subsequent subculturing stages, any shoots that appeared “wet” or hyperhydric, indicating reduced cuticular wax, are discarded (Aitken-Christie et al., 1985; Debergh et al., 1992). In general, free water should be avoided in culture vessels throughout the multiplication stages to ensure optimal growth of cultures. Sources of free water include very fresh media and condensation (Petri dishes, jars, and plastic bottles).

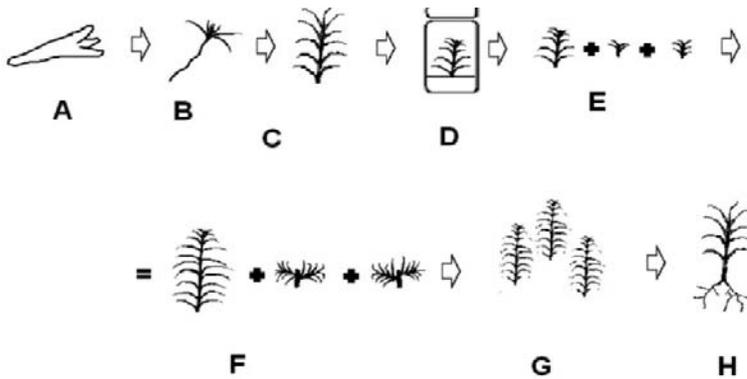


Figure 2. A) Zygotic embryo removed from seed; B) germinated zygotic embryo; C) elongated epicotyl shoot; D) epicotyl shoot removed from hypocotyls and elongated; E) elongated shoot post transfer, divided into shoot tip and stem segments; F) following elongation, shoot tip has elongated, stem segments have produced side shoots; G) serial amplification continues until shoots are set in rooting medium; H) rooted shoot.

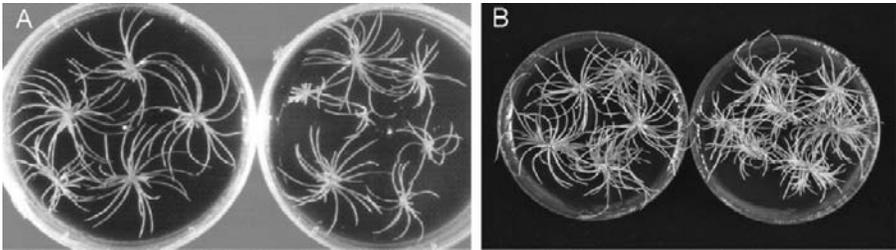


Figure 3. A) Elongated shoots, divided into shoot tips and stem segments. Stem segments are shown in Petri dishes; shoot tips are transferred to jars for further elongation (not shown). B) The stem segments shown in A) after 4 weeks' growth. Side shoots have formed at the needle stem axils from axillary meristems present. Reproduced by kind permission, from the *Canadian Journal of Forestry Science* (Hargreaves et al., 2005).

2.1.5. Rooting and Hardening

Root initiation and subsequent growth. When sufficient shoot multiplication has been achieved with serial culture, shoots are prepared for root initiation. Shoot tips are given a fresh base cut to remove any callus and cut to a length of 20–30 mm, followed by an *in vitro* auxin treatment for 12–14 days on a Greshoff and Doy medium (as modified by Sommer et al., 1975 and Horgan & Holland, 1989), containing 2% sucrose, 0.5 mg naphthalene acetic acid-L⁻¹, and 1.0 mg indole butyric acid-L⁻¹. Following auxin treatment, shoots are individually transferred (set) into trays of peat:perlite:sand (1:1:1) (Figure 4A). High humidity (90 +/- 10%) is maintained for 1–2 weeks with a combination of vented plastic lids and hand-misting twice daily (Figure 4B). Set shoots are held under light and temperature conditions similar to those described earlier for *in vitro* growth. After 1–2 weeks, shoots are gradually hardened off to ambient humidity conditions (60 +/- 10%). Four to six weeks after setting, shoots are assessed for root formation (Figure 4C) and potted up, using the same potting mix as used for setting, with the addition of 5g Osmocote-L⁻¹ (Figure 4D). The plant container is a rigid plastic container, such as HIKO V90 or Lannen 63F if the plants are going to be lined out later in a bare-root nursery bed, or into larger containers, such as HIKO V150 for growing on as a container plant. Plants are transferred to a greenhouse and after 8 weeks placed in a nursery shade house until planted out into the nursery bed (15 × 15 cm spacing) (Figure 5A). Depending on season and site where plants are to be established in the field, plants may undergo root cutting treatments while in the nursery bed to improve root mass prior to planting (Figure 5B).

Root initiation and subsequent growth (adventitious-axillary). Adventitious-axillary shoots are treated in the same way as epicotyl-axillary shoots for root initiation and through all phases of nursery handling. Rooting tends to be 1–2 weeks behind that of epicotyl-axillary shoots and this is accommodated in the setting environment. Transfer to containers occurs only after shoots have roots at least 10 mm long.

2.1.6. Field Testing

Growth in the nursery. If the plantlets are being raised full-term in containers, they are treated like a seedling or cutting crop, with regular watering, and application of fertilisers, fungicides, and pesticides as required through the overhead boom watering system (Menzies et al., 2001). If the plantlets are raised initially in smaller containers

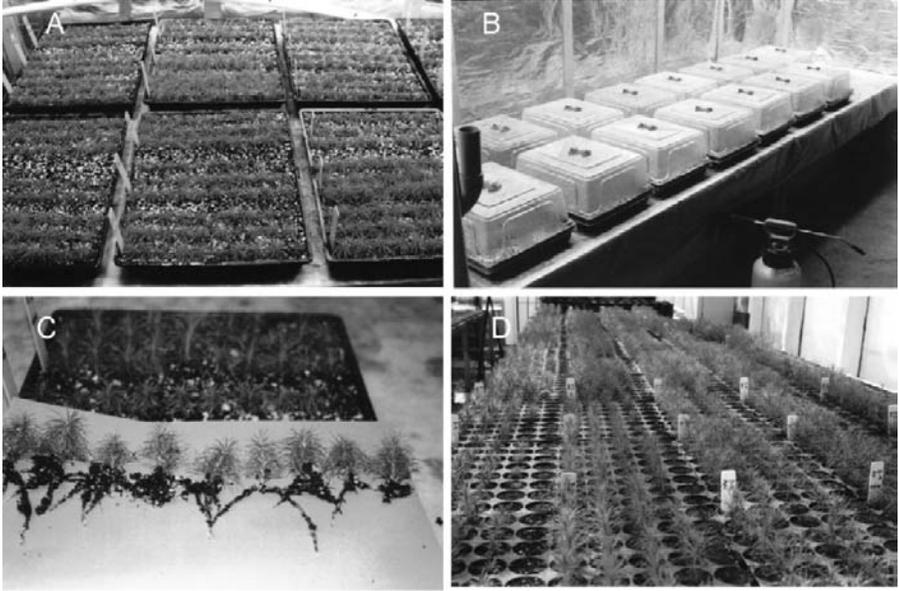


Figure 4. A) Auxin treated shoots set into propagation trays. B) Propagation trays with vented lids in place. C) Rooted tissue-culture shoots 4 weeks from setting. D) Rooted shoots 8 weeks after transfer to Hiko trays.

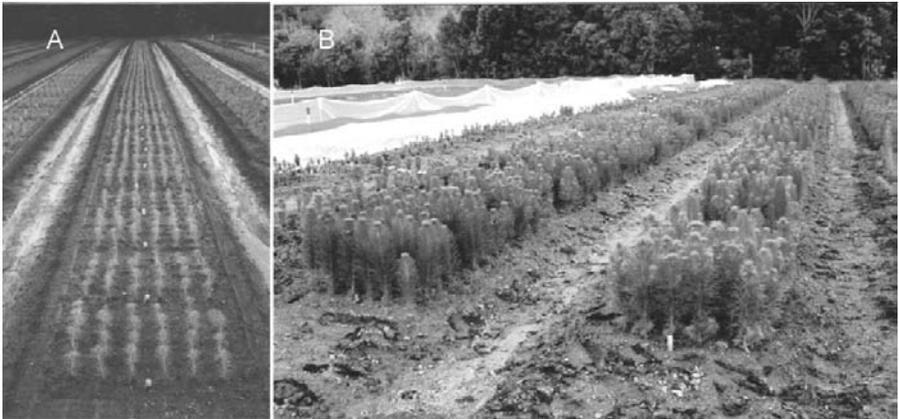


Figure 5. A) Rooted shoots lined out in the nursery bed. B) Tissue-cultured plants after 7 months in the nursery bed and prior to field planting.

and then lined out bare-root, they are transplanted into the nursery bed in late spring/early summer (November/December), once the roots have bound the container plugs. They are then raised like a seedling or cutting crop, with root conditioning by undercutting, wrenching and lateral root pruning, and may also be topped if plant height exceeds 35–40 cm (Menzies et al., 2005). After one growing season, they will reach a plantable size of at least 30–40 cm (Hargreaves et al., 2005).

Growth in the forest. In an early trial planted on five sites in 1983, tissue-cultured plantlets performed as if they were physiologically aged (Menzies et al., 2001), despite being initiated from seed. This was possibly because the organogenesis protocols were still being developed when the plants were produced. Further experience and commercialisation of the tissue-culture systems has improved the protocols, and ensured more uniform and fast early growth (Gleed, 1993). A recent assessment of a field trial established in 1984 (now aged 22 years) has shown that tissue-cultured plantlets were not significantly smaller in diameter than similar genetic quality seedlings and juvenile cuttings (Menzies & Low, unpublished data). Current protocols for organogenesis using axillary shoot multiplication have produced very juvenile plants (Hargreaves et al., 2005), and these are now being evaluated in a field trial.

2.1.7. Storage of *in-vitro* Cultures (Cryopreservation)

Cryopreservation of shoot tip meristems. Successful methods for cryopreservation of radiata pine shoot tip meristems have been difficult to develop. The method presented here was the most successful in a series of 7 preliminary experiments which included a variety of pre-conditioning treatments and regrowth media. The plant material used was a single commercial clone (Christmas Star), there were from 8–65 meristems in each experiment, and all meristems were exposed to liquid nitrogen. The best result was where 12.5% of cryopreserved meristems re-established shoot cultures. This research has subsequently provided a platform for improved results which will be published at a later date (Hargreaves, Towill & Bonnart, 2007).

Shoot tip meristems were isolated from *in-vitro* cultures of Christmas Star (Longview Horticulture, VIC, Australia). Shoots were growing in standard conditions described earlier for shoot multiplication. Meristems were dissected with a stereo microscope, with needles being plucked off until the apical dome of the primary meristem was visible, surrounded by a few needle primordia. Explants were approximately 2×1 mm.

Isolated shoot tip meristems were collected on dishes of LP medium (no charcoal) and then placed in cryoprotectant preconditioning medium (PGD, modified after Ryyänen, 1996) in 25 ml flasks, at room temperature. Following 2 hours exposure to preconditioning medium, individual meristems were placed into 3 separate drops (5–10 μ l) of the PGD solution on 5×10 mm strips of aluminium foil. Nunc cryogenic vials were filled with liquid nitrogen and placed in a polystyrene box containing liquid nitrogen. The aluminium foil strips with adhering droplets containing meristems were then plunged vertically into these pre-cooled cryogenic vials. In this manner, meristems were rapidly frozen to -196°C .

For regrowth, the meristems fixed to the aluminium foil were immediately thawed by plunging the foil into liquid LP medium at room temperature. The meristems remained immersed for 30 min to allow diffusion of the PGD from the tissue. The thawed meristems were then placed on sterile paper towels for 2 min to remove excess liquid, before being placed on $\frac{1}{2}$ strength LP medium plus 1 mg L^{-1} BA and returned to standard growing conditions with reduced light (approximately $5\text{--}10 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) provided by the addition of 4 layers of shade cloth for a minimum of 7 days. After two weeks, meristems were transferred to LPch medium and given the standard conditions of transfer described earlier. Early assessment of meristems showed a variety of responses; in some cases meristem outgrowth was directly from the apical meristem, while in others it was apparent that the surviving meristems were axillary meristems at the base of the few needle primordia which were left on the initial explant. Figure 6B shows a Christmas Star cryopreserved meristem 4 weeks following thawing. Shoot cultures were established and *in-vitro* performance was similar to non-cryopreserved material.

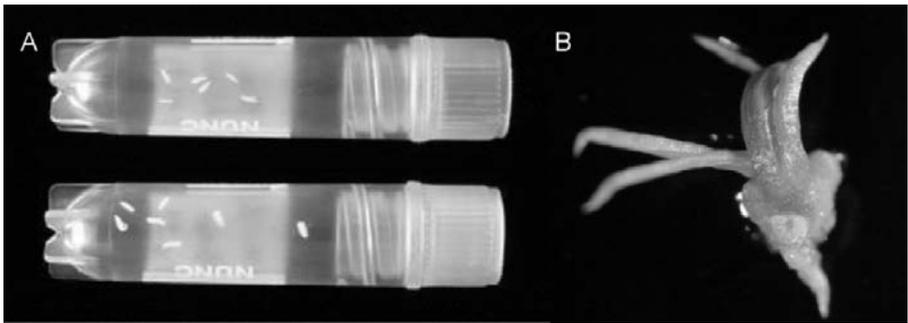


Figure 6. A) Cotyledons in Nunc cryovial, ready for freezing in liquid nitrogen. B) Regrowth of a Christmas Star meristem 4 weeks following thawing.

Cryopreservation of cotyledons. Cotyledons are prepared as described earlier, and following removal from the hypocotyl, are placed in groups on nybolt cloth (Madison Filters, Auckland, New Zealand) in a sterile Petri dish base. They are then left to dry for 2 hours on a laminar flow bench. The air flow is $0.46 \text{ m}\cdot\text{s}^{-1}$ and optimum relative humidity is $40 \pm 5\%$, and air temperature is $22 \pm 2^\circ\text{C}$, with the aim of an optimum moisture content of approximately 7% for the cotyledons. Cotyledons are then carefully removed from the nybolt cloth and placed into Nunc cryovials (Figure 6A) and plunged directly into liquid nitrogen for storage. Since this method was first published (Hargreaves et al., 2004, 2005), technique improvements have been made. A foil strip, as used in the meristem cryopreservation method, is used for each group of cotyledons and this is put into the cryovial. This has served to greatly reduce inadvertent damage to cotyledons with forceps in both insertion and extraction from cryovials. Care needs to be taken with regard to humidity, ambient weather patterns, activity within the transfer lab and quality of the cryovials, as these can affect

moisture content adversely. If the optimum moisture content is not realised, cotyledon response post-cryopreservation may be uneven.

Following storage in liquid nitrogen, vials are thawed rapidly in sterile water heated to 40°C for 2 min (please note safety considerations with regard to thawing material from the liquid phase). They are then treated in the same way that non-cryopreserved cotyledons are treated, as described earlier for adventitious-axillary shoot production. It has been observed that cryopreserved cotyledons do take approximately 24 hours longer to begin to green in comparison to control cotyledons.

3. CONCLUSION AND FUTURE PROSPECTS

The methods described here for organogenesis have been developed over several decades and are used successfully to amplify elite clonal material for afforestation. In general, genotype capture is high, and organogenic approaches can be used to amplify material from zygotic embryos, embryos from embryogenic tissue and field-grown material. The in-vitro shoots readily initiate roots and form good root systems. Improving methods of meristem cryopreservation will ensure the juvenility of selected material while field testing takes place. Hybrid approaches, combining somatic embryogenesis, organogenesis and nursery cuttings, offer possibilities of reducing costs and increasing effectiveness of existing propagation methods.

There are also wider implications of this work, including investigating fundamental aspects of maturation in *Pinus*. Some maturation is beneficial for improved stem form, while excessive maturation compromises root initiation and early growth rate. The ability to control maturation *in vitro* would be very beneficial. Accelerated maturation would be very useful in breeding programmes, especially where marker-aided selection was employed. Importantly, some induced maturation may help ascertain wood quality attributes, which only become apparent in trees with increased age, and also shorten breeding cycles if early flowering was induced.

We also need to ask hard questions with regard to our methodologies if we are to continue using organogenesis in combination with adventitious shoot induction, embryogenesis and nursery propagation. We know that shoots of cotyledonary adventitious-axillary origin show increased maturation in comparison to epicotyl-axillary, but why? If shoots are induced adventitiously from primary needles instead of cotyledons, do they show signs of increased maturation? Is the increased maturation simply a factor of cell division? In radiata pine, the origin of embryogenic tissue is the early zygotic embryo, which is encouraged into a state of prolonged cleavage polyembryony before further media modifications induce embryo maturation. If we apply organogenesis protocols to embryos from embryogenic tissue, there has already been a massive amount of cell division beyond the 'rejuvenating' meiotic event of fertilisation. These organogenesis protocols provide powerful tools to investigate these issues further.

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CHAPTER 7

GENETIC FIDELITY ANALYSES OF *IN VITRO* PROPAGATED CORK OAK (*QUERCUS SUBER* L.)

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1. INTRODUCTION

In vitro propagation methods, such as somatic embryogenesis (SE), are very interesting approaches when compared with traditional propagation, which presents serious drawbacks. SE is frequently regarded as the best system for propagation of superior genotypes (Kim, 2000) mostly because both root and shoot meristems are present simultaneously in somatic embryos. Cork oak (*Quercus suber* L.), as other woody species, is recalcitrant concerning somatic embryogenesis (SE). Most of the successful studies regarding SE within this species used juvenile materials (Gallego et al., 1997; Hernandez et al., 1999; Toribio et al., 1999; Hornero et al., 2001a; Pinto et al., 2001). Only recently, SE was successfully and reproducibly induced from adult cork oak trees (Pinto et al., 2002; Lopes et al., 2006), opening perspectives for breeding programmes of selected genotypes of this species.

Plantlets derived from *in vitro* culture might exhibit somaclonal variation (Larkin & Scowcroft, 1981), which is often heritable (Breiman et al., 1987). Some reports claim that morphological, cytological, and molecular variations may be generated *in vitro* (Larkin et al., 1989) due to the genotypes (Breiman et al., 1987) and to the protocols used in *in vitro* culture and plant regeneration. Main genetic variations may be divided in changes in chromosome structure and number and in changes involving DNA structure.

Flow cytometry (FCM) has increasingly been chosen for analysis of major ploidy changes in genetic stability assays. It thereby replaces other methods such as chromosome counting being that FCM provides unsurpassed rapidity, ease, convenience and accuracy. Until this moment very few reports used this technique to assay somaclonal variation in woody plants (e.g. Bueno et al., 1996; Endemann et al., 2002; Conde et al., 2004; Pinto et al., 2004). In *Q. suber*, only recently the first study on the assessment of ploidy stability of the SE process using FCM was presented (Loureiro et al., 2005).

This delay may be related, besides the difficulty of establishing the SE process, with the difficulty of analysing woody plant species using FCM. These species, usually present some compounds (e.g. tannins) that are released during nuclear isolation procedures, and that are known to interact with nuclei, hampering in some situations the FCM analysis (Noirot et al., 2000; Loureiro et al., 2006a).

Other genetic variations can be detected by molecular techniques such as RFLPs (restriction fragment length polymorphisms), RAPDs (randomly amplified polymorphic DNAs), AFLPs (amplified fragment length polymorphisms) or microsatellites/SSRs (simple sequence repeats). Both RFLPs and AFLPs are highly reproducible techniques but more costly and time-consuming than SSRs, while RAPDs show a lack of reproducibility either within or between laboratories (Jones et al., 1997). In addition, microsatellites have high levels of polymorphism (Glaubitz & Moran, 2000), being extremely useful for fine-scale genetic analyses. There are several reports concerning the use of these molecular markers in micropropagated plants. With respect to the *Quercus* genus, RAPD markers have been used in *Q. serrata* Thunb. (Ishii et al., 1999; Thakur et al., 1999) and *Q. robur* L. (Sanchez et al., 2003) somatic embryos and no aberrations were detected in the banding pattern. In *Q. suber*, no somaclonal variation in several embryogenic lines obtained from zygotic embryos has been detected by RAPD analyses (Gallego et al., 1997). This result was later confirmed by AFLP markers (Hornero et al., 2001a), while in embryogenic lines from leaves of mature trees, the same authors were able to detect somaclonal variation (Hornero et al., 2001a).

Until recently, *Quercus suber* combined recalcitrance in *in vitro* plant regeneration and in genetic analysis (e.g. this species was reported as being very difficult to analyse by FCM, at least when nuclear DNA content of mature leaves was estimated, see Loureiro et al., 2005). We have optimized both methodologies and this species is now an excellent model for genetic stability assessment within woody species micropropagation studies. The protocols here presented are focused on the successful genetic analysis (using FCM and microsatellites) of this species, highlighting strategies to overcome putative troubleshooting that may arise when dealing with this or other recalcitrant species.

2. PROTOCOL

2.1. Flow Cytometry

Methods for FCM measurement of DNA content have been developed for individual plant cells, protoplasts, and intact plant tissues, the latter being the most successful approach. The basis of the protocol here presented was developed by Galbraith et al. (1983) and consists in a rapid and convenient method for isolation of plant nuclei by chopping plant tissues in a lysis buffer.

Several recommendations on the behalf of best practices should be taken in consideration. Major issues are reported above and several advices are referred in each protocol step. A troubleshooting section is also provided.

Plant material. Intact plant tissues should be disease and stress-free if this is not the interest of the study (e.g. toxicological assays). Young and rapidly growing tissues usually give the best results. *Ex vitro* leaves should be transported or sent by post enclosed in moistened paper and kept at low temperatures.

Flow cytometers. The flow cytometers currently used in plant DNA flow cytometry can be divided in three major brands: Partec[®], Beckman-Coulter[®] and Becton-Dickinson[®]. According with the FLOWER database presented by Loureiro et al. (2007a), cytometers of these brands were used in 94.1% of the publications analysed in this database, with Partec[®], being the leading brand in plant science with 44.1% of publications. The prominent position of this brand was justified as being due to its suitability for analysis of plant materials and/or to a relatively low price of their products. When acquiring a flow cytometer attention is needed to the wavelength of the laser source (for details on fluorochromes characteristics see section below).

Nuclear isolation buffers. Due to different chemical composition and diversity of plant tissues, no single nuclear isolation buffer is universally optimal. This was clearly shown by Loureiro et al. (2006b), who systematically compared four of the most common lysis buffers for DNA analysis by FCM. Also, Loureiro et al. (2006a) studied the effect of tannic acid on plant nuclei and estimation of DNA content, and found that different nuclear isolation buffers granted samples with different resistance to the negative effect of this compound. Based on the results of these two studies, a new buffer was developed by the authors (Loureiro et al. 2007b). This buffer provided very satisfying results with more than 30 plant species, most of them woody plants, and was therefore called “woody plant buffer” (WPB). Nuclei isolated with this buffer are stabilised with **MgCl₂** (chromatin stabilizer) and **EDTA** (chelator agent), the pH of the solution is maintained at 7.5 with **TRIS buffer**, **NaCl** ensures proper ionic strength, and a high concentration of **Triton X-100** (surfactant) reduces adhesion of cellular debris and removes and hinders cytoplasmic remnants. **Metabisulfite**, as reducing agent of phenolic compounds and polyvinyl pyrrolidone (10 000 MW, **PVP-10**), as a competitor with amide groups were also added to the buffer. Phenols and other compounds are very common in woody plants, like *Q. suber*, and the addition of these protectants is mandatory for obtaining good results (Loureiro et al. 2007b).

Standardization. The fluorescence units of a FCM DNA histogram are presented in arbitrary units of channel numbers. Therefore for genome size and DNA ploidy analyses, a reference standard with known nuclear DNA content and/or ploidy level should be added. The nuclear DNA content estimation is made by comparing sample and standard G₀/G₁ peak positions. From the two types of standardization (**external**, nuclei from sample and standard are analysed separately; and **internal**, nuclei from sample and standard are isolated, stained and analysed simultaneously), internal standardization is considered the most reliable method as nuclei from sample and standard are exposed to the same conditions. An ideal DNA reference standard should have a genome size close but not overlapping to the targeted species (Greilhuber et al. 2007). A set of reference standards with genome size distributed at appropriate intervals is already available from some sources. We recommend those provided free of

charges by the Laboratory of Molecular Cytogenetics and Cytometry, Olomouc, Czech Republic (Doležel & Bartoš, 2005, Table 1). These standards are genetically stable with constant genome size, seed propagated, easy to use and available in sufficient quantities as elite lines from breeders. For DNA ploidy level analyses, reference standards can also be an individual from the same species with known ploidy level (e.g. tetraploid). Following the recommendations of the Genome Size Workshop, held at Kew Royal Botanical Gardens in 1997 (see <http://www.rbgekew.org.uk/cval/pgsm/>), the use of chicken red blood cells (CRBCs) as internal standard is discouraged.

Table 1. DNA reference standards available from the Laboratory of Molecular Cytogenetics and Cytometry, Olomouc, Czech Republic (adapted from Doležel & Bartoš, 2005).

<i>Species</i>	<i>Cultivar</i>	<i>2C DNA Content (pg)*</i>	<i>1C Genome Size (Mbp)**</i>	<i>Reference</i>
<i>Allium cepa</i>	Alice	34.89	17,061	(Doležel et al., 1998)
<i>Vicia faba</i> ssp. <i>faba</i> var. <i>equina</i>	Inovec	26.90	13,154	(Doležel et al., 1992)
<i>Secale cereale</i>	Dankovske	16.19	7,917	(Doležel et al., 1998)
<i>Pisum sativum</i>	Ctirad	9.09	4,445	(Doležel et al., 1998)
<i>Zea mays</i>	CE-777	5.43	2,655	(Lysák & Doležel, 1998)
<i>Glycine max</i>	Polanka	2.50	1,223	(Doležel et al., 1994)
<i>Solanum lycopersicum</i>	Stupické	1.96	958	(Doležel et al., 1992)
<i>Raphanus sativus</i>	Saxa	1.11	543	(Doležel et al., 1992)

* Nuclear DNA content was established using human male leukocytes (2C = 7.0 pg DNA; Tiersch et al., 1989) as a primary reference standard.

** 1 pg DNA = 978 Mbp (Doležel et al., 2003).

Fluorochromes. The fluorochromes used to stain DNA must be chosen according to their resolution, stability, DNA stoichiometry and most importantly the excitation wavelength available on the flow cytometer. DAPI (4',6-diamidino-2-phenylindone) and propidium iodide (PI) are the most popular fluorochromes used in plant DNA FCM. Whereas DAPI is AT-specific, inexpensive, staining is readable in 5 min and excitation occurs at 340 nm, PI intercalates with double stranded DNA, stains in 10 min and is excited at 535 nm. The excitation wavelength of both fluorochromes restricts their use; DAPI needs lamp-based machines (UV excitation) whereas PI requires laser-based flow cytometers. It should also be noticed that DAPI, due to its binding properties, should not be used for absolute nuclear DNA content estimations, at least if the AT:GC ratio of the sample and standard DNA is unknown (which is often the case) (Doležel et al., 1992). When PI is chosen, RNase should

be added to samples, as this fluorochrome also binds to double stranded RNA. The protocol here presented describes the use of the simplest instrument configuration, i.e. an argon laser operating a 488 nm, with one fluorochrome, PI.

Quality assessment. The quality and precision of DNA histograms obtained by FCM is usually evaluated by the coefficient of variation (CV) value, which should be <5% (Galbraith et al., 2002). Excellent analyses with high degree of resolution will have CV = 1–2%, and routine analyses CV values of 3%. Also, recent literature (Loureiro et al., 2006a) has alerted for the importance of measuring CV values of scatter parameters (in this case in the form of full peak coefficient of variation, FPCV), as higher values (>50%) may be an indication of the negative effect of cytosolic compounds, released upon chopping. It should be noticed that the CV value does not tell anything about the reproducibility of the DNA content estimation. It is therefore important to perform sufficient number of independent measurements. For genome size estimations, it is suggested that each measurement is repeated at least three times on three different days to uncover any unexpected variation (e.g. instrumental shifting).

2.1.1. Materials

Plant material for analysis. Intact plant tissues (leaves of *in vitro* and *ex vitro* plants) and plant tissue culture or callus (somatic embryos at different stages, embryogenic/organogenic/non-differentiated callus tissue). In *Q. suber*, plants were grown according to the protocols developed by Lopes et al. (2006) and Pinto et al. (2002).

Internal reference standard. Plant with known nuclear content. In the case of *Q. suber*, *Glycine max* cv. Polanka (2C = 2.50 pg of DNA, Doležel et al., 1994) was used as an internal reference standard.

Nuclear isolation buffer. WPB (Woody Plant Buffer) – 0.2 M Tris.HCl pH 7.5, 4 mM MgCl₂.6H₂O, 2 mM EDTA Na₂.2H₂O, 86 mM NaCl, 10 mM metabisulfite, 1% (w/v) PVP-10, 1% (v/v) Triton X-100. Store at 4°C.

Prepare 1 mg/ml propidium iodide (PI) stock solution with caution because it may cause health risks. Store in 1.5–2.0 ml aliquots at –20°C.

Prepare 1 mg/ml RNase stock solution. Store in 1.5–2.0 ml aliquots at –20°C.

Sheath fluid: use either distilled water or commercial sheath fluid solution.

Fluorescent microspheres (Flow-Check[®], Beckman-Coulter).

Ice in a polystyrene box.

Glass Petri dishes

New double-edged razor blades

50 µm nylon filters

Micropipettes and tips (200 µl and 1 ml)

Cytometer sample tubes

Flow cytometer with 488 nm light source

2.1.2. Methods

1. Weigh approximately 50 mg of plant material and place it in a glass Petri dish. A leaf of the internal reference standard should also be weighed (approx. 50 mg) and added to the same Petri dish. Some initial experiments should be performed to determine the weight of sample and internal standard necessary to obtain similar amounts of isolated nuclei. At a low speed flow cytometer configuration, a flow rate of 40–80 particles/s should be obtained.
2. Add 1.0 ml of WPB nuclear isolation buffer and chop tissues using a new double-edged razor blade.
3. Filter nuclear suspension through 50 μm nylon filters into an ice-cold cytometer sample tube. This step will remove large debris. Cut the 1 ml micropipette tip to help suction of the nuclear suspension liquid.
4. Add 1 mg/ml PI stock to a final concentration of 50 $\mu\text{g/ml}$ and 1 mg/ml RNase stock to a final concentration of 50 $\mu\text{g/ml}$.
5. Incubate sample on ice for 10 min. The time of incubation should be sufficient for a stable fluorescence staining.
6. Turn the computer and flow cytometer on. This process should take approximately 15 min. In the meantime, follow instrument starting instructions, fill the sheath fluid tank with commercial sheath fluid solution (or distilled water) and empty the waste tank.
7. Load a protocol for analysis of fluorescent beads. Run a sample tube with approximately 100 μl . Collect at least 5,000 beads. If the flow cytometer is correctly aligned, CV values must be $<2\%$ for fluorescence and scatter parameters.
8. Load the protocol for nuclear DNA content analyses. The discriminator should be set at a fluorescence signal of 50. The FCM analysis should include the following graphics (Figure 1): histogram of PI fluorescence (in our cytometer, using the photomultiplier tube n° 3, PMT3, Figure 1A), cytograms of forward-angle light scatter (FS) vs. side-angle light scatter (SS) both in logarithmic scale (Figure 1B), PI fluorescence vs. time (to monitor fluorescence stability of nuclei, Figure 1C), SS in logarithmic scale vs. PI (to monitor for possible effect of tannic acid, for details see Loureiro et al., 2006a, Figure 1D) and PI fluorescence pulse integral vs. PI fluorescence pulse height (Figure 1E). The discriminator is used to eliminate particles with autofluorescence and/or low fluorescence values.
9. Run samples at room temperature at a data rate of 40–80 particles/s (it usually stands for the lowest flow rate configuration).
10. Define the necessary regions for obtaining statistics from the flow cytometric data. Usually the following regions are defined: in PI fluorescence histogram (Figure 1A), linear regions are defined for each peak; in FS log. vs. SS log. cytogram (Figure 1B), a region is defined around the population of nuclei; in fluorescence pulse integral vs. fluorescence pulse height (Figure 1E), a region is defined around individual nuclei. By this way doublets of 2C that can be erroneously assess as 4C are eliminated.

11. Determine the mean channel number of the sample G_0/G_1 nuclear DNA peak and that of internal reference standard.
12. Estimate the nuclear DNA content of the sample using the formula:

$$\text{sample 2C DNA content (pg)} = \frac{\text{sample } G_0/G_1 \text{ peak mean}}{\text{standard } G_0/G_1 \text{ peak mean}} \times \text{standard 2C DNA content}$$

(1)

If needed, the DNA content values can be converted in number of base pairs (bp), taking in consideration that 1 pg DNA = 978 Mbp (Doležel et al., 2003). The genome size in base pairs is usually shown in terms of haploid size (1C) of the genome, whether genome size in mass values are typically shown per 2C value.

13. Using the regions that were defined, record the full peak coefficient of variation (FPCV) of both FS and SS and the half peak coefficient of variation (HPCV) of sample and standard G_0/G_1 peaks.
14. Carefully and critically interpret the results. Small differences in the peak position of both samples and internal standard should be interpreted with caution as they may be due to instrumental drift or to the possible effect of cytosolic compounds. Whether in the first case, these differences may be reduced by planning the experience in at least three different days, to eliminate the second hypothesis and ascertain the real occurrence of DNA differences, a new sample containing the tissues where the differences were obtained may be prepared. If only one peak is observed it is a strong indication that the observed differences were probably artefactual.

2.1.3. Troubleshooting

1. Empirical experience has strongly advised the chopping procedure instead of slicing for obtaining a high output of nuclei and little debris. Before the chopping step it is important that the plant tissue does not dry. Also, the chopping procedure should be fast (60 s of chopping should be enough). However, in some plant tissues containing compounds that affect histograms quality, chopping should be less intense. If only few nuclei are isolated, the amount of plant material can be increased. As an example, in *Q. suber* leaves chopping must consist of only several cuts and nuclear suspension should be quickly filtered. Callus tissue and somatic embryos of *Q. suber* are easier to be analysed than leaves. Friable callus (e.g. from *Pinus pinaster*) may be difficult or in some cases inappropriate for FCM analysis, as the method is only able to isolate a reduced number of nuclei.
2. After using the suggested buffer, if the obtained histograms present low resolution, other lysis buffers should be tested. A review on nuclear isolation buffers can be found in Greilhuber et al. (2007).

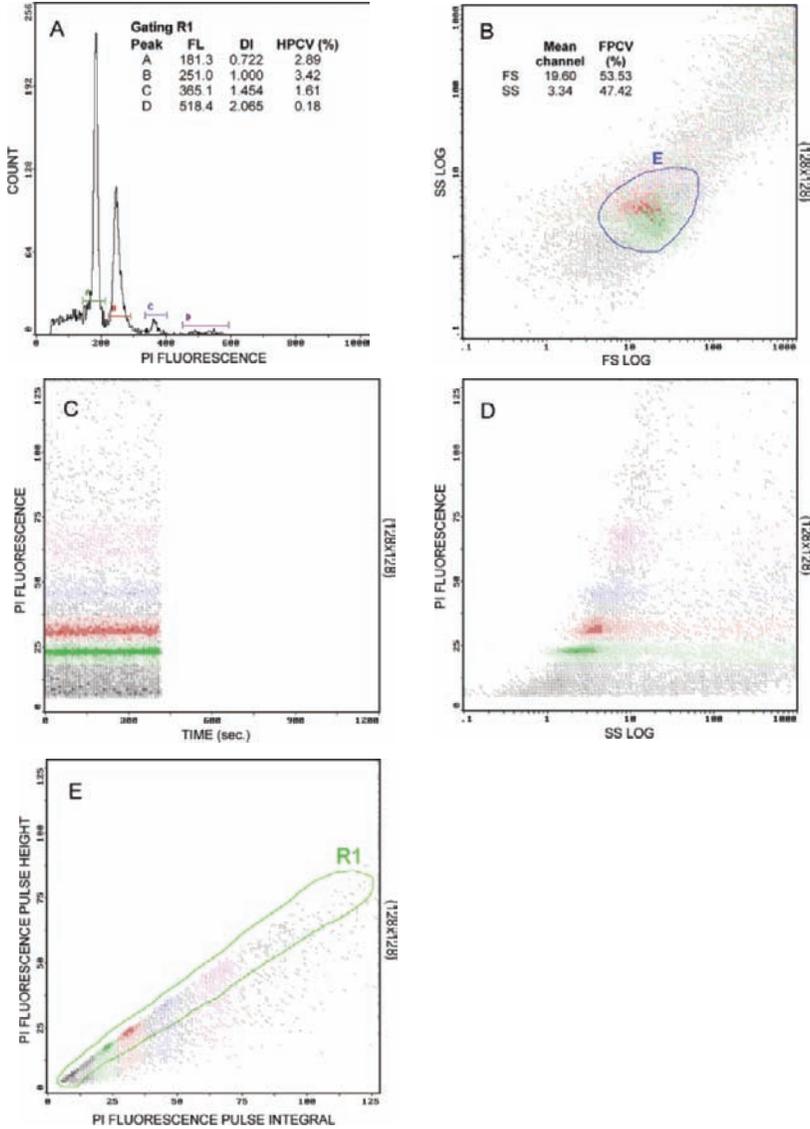


Figure 1. A) Histogram of PI fluorescence and B) cytochrome of forward-angle light scatter (FS LOG) vs. side-angle light scatter (SS LOG) both in logarithmic scale, C) PI fluorescence vs. time, D) SS LOG vs. PI and E) PI fluorescence pulse integral vs. PI fluorescence pulse height of nuclei of *Quercus suber* (peaks: A – G_0/G_1 nuclei, coloured in green, and C – G_2 nuclei coloured in blue) and Glycine max (as internal reference standard with peaks B – G_0/G_1 nuclei, coloured in red, and D – G_2 nuclei, coloured in purple) simultaneously isolated in WPB and stained with PI. Mean FL channel (FL), DNA index (DI=mean channel of *Q. suber*/mean channel of *G. max*) and HPCV % of each peak are given in the histogram A and mean channel of FS and SS and (Mean channel) FPCV % of each parameter are given in cytochrome B.

3. In addition to testing various buffers, selection of tissues with lower or no phenolic compounds may enable unbiased estimations (Suda, 2004). If no tissue/buffer provides acceptable results, changing the type of buffer additives (in WPB metabisulfite acts as an antioxidant, and PVP-10 as a tannin-complexing agent) or their concentration may reduce the negative effect of cytosolic compounds, and is therefore recommended.

However, the absence of major ploidy changes does not exclude the possible existence of genetic differences such as DNA polymorphism. Molecular methods like microsatellite marker analysis can be the ideal tool to assess DNA polymorphism, and a protocol developed for *Q. suber* by Lopes et al. (2006) is presented thereafter.

2.2. Microsatellite Markers

Genotyping (PCR analysis) of SSR fragments can be achieved in various ways: radioactive detection (incorporation of labelled nucleotides and end-labelling of one of the PCR primers), non-radioactive detection (high resolution gel electrophoresis, silver staining, blotting and hybridization and fluorescent dyes on automated sequencers).

The protocol given here is for genotyping using fluoro-labelled primers. The forward primers are synthesized with a fluorescent label attached to the 5' end (ABI dyes: 6-FAM, JOE, HEX and TET) to allow detection of the polymerase chain reaction (PCR) products (see Table 2). About 0.01 μmol of each primer are ordered, which is sufficient to perform at least 2,500 PCR runs under the standard conditions described in this protocol. Primers can generally be purified by standard liquid chromatography (e.g. the HPSF[®] method, MWG Biotech) or by high performance liquid chromatography (HPLC). For this individual application, we recommend the use of standard liquid chromatography for unlabelled primers and the use of HPLC purification for labelled primers, as it is more sensitive. We resuspend the primers in sterile deionized water or sterile buffer (i.e. TE; 10 mM Tris pH 8, 1 mM EDTA). The standard concentration for stock solution of PCR primers is 100 μM . To obtain a concentration of 100 μM the synthesis report of the vendor should provide you with the appropriate diluent volume.

The protocol described below refers only to genotyping with previously known markers and was adapted from Lopes et al. (2006). Protocols for isolation of microsatellite markers in *Quercus* species can be found elsewhere (Isagi & Suhandono, 1997; Steinkellner et al., 1997a; Kampfer et al., 1998). From the available nuclear microsatellites (nSSRs) developed in the *Quercus* genus that have been transferred with success to *Q. suber*, eight were chosen for study according to their degree of polymorphism (heterozygosity and number of alleles) and the quality of the PCR product. Briefly, of the eight nSSRs assayed, QM58TGT and QM50-3M were first described by Isagi & Suhandono (1997) in *Q. myrsinifolia* Blume, and QpZAG9, QpZAG15, QpZAG36 and QpZAG110 were first described in *Q. petraea* (Matts.) Liebl. (Steinkellner et al., 1997a,b). The transferability of these loci to *Q. suber* has previously been reported (Gomez et al., 2001; Hornero et al., 2001b). The other two nSSRs, QrZAG7 and QrZAG11 were first described in *Q. robur* (Kampfer et al., 1998) and their transferability to *Q. suber* was reported by Hornero et al. (2001b).

To amplify the selected microsatellites by PCR, the primers designed by the authors are used. It should be referred that more recently Borges et al. (2003, GenBank: <http://www.ncbi.nlm.nih.gov>) have developed SSR markers specifically for *Q. suber*.

Table 2. Characteristics of the microsatellite loci amplified in *Q. suber* that were primarily developed for *Q. myrsinifolia* (Isagi & Suhandono, 1997), *Q. robur* (Steinkellner et al., 1997a, b) and *Q. petraea* (Kampfer et al., 1998). Repeat structure, primer sequences and variable PCR conditions used.

Locus	Repeat structure	Primer sequences	Annealing temp. (°C)	Primer conc. (μM)
QM58TGT	(CAA) ₁₁	GGTCAGTGTATTTGTGGT AAATGTATTTGCTTGCTCA	55	0.5
QM50-3M	(CCT) ₃ (CCG) (CCT) ₂ (CCA)(CCT) ₂ + (CCA) ₇	CCCGATTCCCTTCCCTGCT CGGGCTTTGGATACGGATTC	55	0.3
QpZAG9	(AG) ₁₂	GCAATTACAGGCTAGGCTGG GTCTGGACCTAGCCCTCATG	55	0.3
QpZAG15	(AG) ₂₃	CGATTTGATAATGACACTGG CATCGACTCATTGTTAAGCAC	55	0.5
QpZAG36	(AG) ₁₉	GATCAAAATTTGGAATATTAAGAGAG ACTGTGGTGGTGAAGTCTAACATGTAG	50	0.2
QpZAG110	(AG) ₁₅	GGAGGCTTCCTTCAACCTACT GATCTCTGTGTGCTGTATTT	50	0.2
QrZAG7	(TC) ₁₇	CAACTTGGTGTTCGGATCAA GTGCATTTCTTTTATAGCATTAC	55	0.5
QrZAG11	(TC) ₂₂	CCTTGAACCTCGAAGGTGTCCTT GTAGGTCAAACCATTGGTTGTGACT	55	0.5

2.2.1. Materials

Plant material for analysis: leaves of *in vitro* and *ex vitro* plants, embryogenic and undifferentiated calli and somatic embryos at different stages of development. In *Q. suber* and for comparative purposes, the same plant material was used for microsatellite and FCM analyses. This is a recommended practice.

Other materials:

Taq polymerase (5 units/μl). Store at -20°C

10× PCR buffer (75 mM Tris-HCl pH 9, 50 mM KCl, 20 mM (NH₄)₂SO₄, 0.001% BSA-bovine serum albumin). Store at -20°C

Sterile ultra pure water

Forward primer. Store at -20°C in aliquots of 10 μM

Reverse primer. Store at -20°C in aliquots of 10 μM

dNTP mix: 2 mM of each dATP, dCTP, dGTP and dTTP. Store at -20°C

GeneScan internal size standards: 400 HD/500 labelled with ROX or 2500 labelled with TAMRA. Store at 4°C

De-ionized formamide. Store at -20°C

Thermal cycler

Automated sequencer

Micropipettes and sterile tips (2 µl, 20 µl, 200 µl and 1 ml)

Sterile thin-walled PCR tubes (200 or 500 µl)

2.2.2. Methods

1. Label thin-walled PCR tubes and add 1 µl (10–20 ng) of genomic DNA to each tube. Keep the samples on ice.
2. Preparation of master mix. Add the following components of the reaction to the bottom of a 1.5 ml tube kept on ice (where n denotes the number of PCR reactions):

$n \times 2.5 \mu\text{l}$ 10× PCR buffer

$n \times 1.25 \mu\text{l}$ MgCl₂ (2.5 mM)

$n \times 2 \mu\text{l}$ dNTP mix (0.2 mM each)

$n \times 0.5 \mu\text{l}$ each primer

$n \times 0.2 \mu\text{l}$ *Taq* polymerase (1 unit)

Sterile ultra pure water up to $n \times 24$

Remember to include a volume of master mix with no DNA for a negative control of the PCR reaction. Also, when a large number of samples need to be prepared, an extra 10% of master mix should be prepared to remedy pipetting errors.

3. Mix gently. Pulse spin for a maximum of 5 s.
4. Add 24 µl of master mix to each labelled thin-walled tube. If necessary pulse spin for a maximum of 5 s.
5. Place the tubes in a thermal cycler and perform the PCR amplification with the following profile (according to Hornero et al., 2001b):

5 min at 94°C, as initial denaturing step

followed by 10 cycles of:

– 94°C for 15 s

– 65 to 56°C (decreasing 1°C per cycle) for 30 s

– 72°C for 30 s

followed by 25 cycles of:

– 94°C for 15 s

– variable annealing temp °C for 30 s (see Table 2)

– 72°C for 30 s

final extension step at 72°C for 5 min.

6. Optional: it may be useful to run some if not all PCR products on an agarose gel prior to analysing them on an automated sequencer (see 2.2.3, Troubleshooting section). In this way one can check if the amplification was successful and even try to quantify the PCR product.
7. After PCR amplification, mix 1 μ l of PCR product with 0.5 μ l of GeneScan internal size standard and 25 μ l of formamide. Vortex the mixture briefly, spin it down for a few seconds, then incubate it at 95°C for 3–5 min and finally place it on ice for 3–5 min.
8. The PCR products can then be visualized by capillary electrophoresis on an automated sequencer.
9. Remember to run each sample about three times to minimise error, this is particularly important when trying to detect rare mutations.
10. Following capillary electrophoresis the computer generates a gel image showing the bands that were detected. DNA fragment size (in base pairs) can be estimated with precision for all the peaks in a lane, either by the use of internal (e.g. Applied Biosystems) or external lane standards (e.g. PHARMACIA and LI-COR systems). The computer programs recognize the standard peaks and construct a standard curve. The sizes of the products are then estimated based on their migration relative to the known standard. All peaks are labelled, including microsatellite alleles (the most intense peaks; intensity is measured in fluorescence units), stutter bands, and stray bands arising from non-specific PCR amplifications. Stutter bands are usually smaller than the original alleles and are most likely the result of *in vitro* DNA slippage during PCR amplification.
11. Figure 2 depicts a series of electropherograms of *Q. suber* microsatellite data analysed on an Applied Biosystems 310 system where fragment sizes were automatically calculated to two decimal places using the Local Southern Method option of the GeneScan v.3.1 software. Data were then imported into Genotyper program where the peaks were filtered to remove stutter bands. Since SSRs are co-dominant markers, a homozygous individual is represented by the presence of only one allele and a heterozygous individual is represented by two alleles, in the case of a diploid species such as *Q. suber* (see Figure 2). The differences in allele sizes between individuals are indicative of polymorphism/genetic variation.
12. A careful manual analysis should be done by the investigator to ignore stray peaks arising from non-specific PCR amplifications and to check the possibility of allelic deletion. Also, the investigator has to keep in mind that identical alleles can generally migrate within 0.5 bp of each other on the same sequencing run (gel). Larger variations could be observed when comparing data from the same sample on different sequencing runs (gels). Therefore, allele binning is of utmost importance.

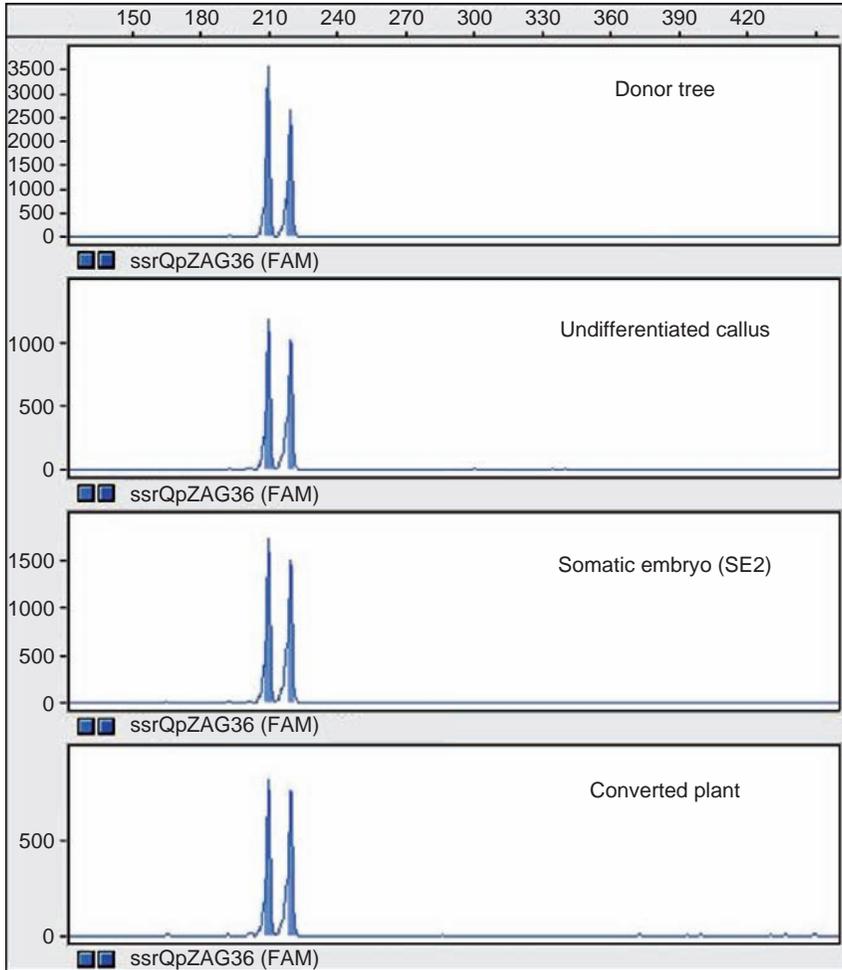


Figure 2. Amplification of the dinucleotide *ssrQpZAG36* (FAM) locus in *Quercus suber* (from top to bottom): donor tree, an undifferentiated callus, a normal dicotyledonary somatic embryo (SE2) and a converted plant. All electropherograms show heterozygous individuals with two alleles of approximately 209 and 219 bp. Top scale indicates fragment size in nucleotides. Left scale indicates fluorescence intensity measured in relative fluorescence units (adapted from Lopes et al., 2006).

Microsatellite markers/loci that can be amplified with minimum non-specific annealing, and that even have overlapping size ranges, can be separated efficiently and simultaneously in automated sequencers as there are dyes that fluoresce at different wavelengths (Karp et al., 1997). In this case, several PCR products can be pooled

(i.e. multiple loading/multiloading). Alternatively, several loci/markers can be co-amplified during PCR (i.e. multiplexing); this can be particularly useful when a great number of loci are to be amplified and/or when limited amounts of DNA are available. Optimizing a multiplex PCR system may present many difficulties, although there are now some multiplex PCR commercial kits available. For oak species PCR multiplexing procedures are available in the literature.

2.2.3. Troubleshooting

1. Although rare with this protocol, the problem of disappearing microsatellites, whereby previously scorable samples fail to appear when a PCR is repeated, can sometimes be avoided by pipetting the master mix onto each sample individually and ensuring it is mixed with the template DNA.
2. When analysing the fragment size in the automated sequencer, the fluorescence intensity of a specific sample may change dramatically when amplifying different microsatellite markers (using different fluoro-labels). Therefore, after PCR it is advisable to run samples on an agarose gel to try and quantify the PCR product. Alternatively, at first it is better to run only a few samples on the automated sequencer to estimate whether scorable readings are obtained or not. If fluorescence is too intense that it prevents the obtaining of a result, a water-dilute must be done to the PCR sample. Conversely, if fluorescence intensity is too low, it is very likely that the PCR was not successful. In this case a repetition of the PCR run and/or optimization of PCR conditions are needed.
3. Many microsatellites show, besides stutter bands, an additional band above the expected allele size. This is due to the activity of the terminal transferase of *Taq* DNA polymerase which adds an adenine to the PCR product (the plus-A phenomenon described by Clark, 1988). Note that this terminal transferase activity is polymerase- and PCR primer-dependent and if it cannot be surpassed by PCR optimization, the microsatellite in question must be discarded.

3. CONCLUSION

Once a micropropagation protocol is well established and performed routinely (as is the case of *Quercus suber* somatic embryogenesis) it is of utmost importance to verify the fidelity of the *in vitro* derived material, particularly, when the objective is to use the genetic material in a plant breeding programme. Due to the existing constraints of the current genetic screening tools and to the different types of genetic modifications, the combined use of different techniques able to provide complementary data is recommended.

In the present chapter, detailed protocols for the application of the highly sensitive and reproducible techniques of flow cytometry and nuclear microsatellites are given. Also, some best practices concerning plant fidelity using the FCM and microsatellite analysis in *Q. suber* are highlighted here, and can be transposed to regeneration processes of other species. Both techniques can be used at different stages of any

micropropagation protocol, requiring reduced amounts of plant material and their combination provides more accurate information on the fidelity of the obtained plants.

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CHAPTER 8

PROTOCOL FOR MICROPROPAGATION OF *QUERCUS* spp.

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1. INTRODUCTION

Oaks are important broad-leaved hardwood species of forest ecosystems, valuable from economical and ecological point of view. The preferred means of oak propagation is from seeds, what favours broadening of genetic diversity of these species. However, the possibilities of generative reproduction are limited by long life span necessary for achievement of physiological maturity, by irregular occurrence of mast years, by low crop and quality of seeds and difficulties in acorn storage. Seeds of *Quercus* species show different degrees of recalcitrance and can be stored only for a short period because of their sensitivity to desiccation. In addition, the conventional method of vegetative propagation of mature trees by cuttings is associated with difficulties in rooting.

The problems in oak propagation from juvenile or mature material could be solved by the use of *in vitro* techniques. *In vitro* propagation from dormant buds collected from mature trees is important especially for the commercial forestry, for clonal propagation of selected genotypes with valuable wood quality, resistant or tolerant to diseases and to conditions with increased pollution. *In vitro* propagation from juvenile material enables production of large number of vital plants of diverse genotypes necessary for the maintenance of genetic diversity in forest ecosystems. Immature and mature zygotic embryos are potentially suitable sources of explants for oak micropropagation. The cultures of excised embryos are practical and effective techniques of *in vitro* propagation. Using them the premature abortion of zygotic embryos, derived from open or controlled pollination, can be overcome and also their dormancy can be reduced or broken. In this chapter, the micropropagation protocol for *Quercus* spp. using explants from juvenile material (embryos and seedlings), as well as from selected mature trees is described.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

In vitro regeneration was tested in such *Quercus* spp., as *Q. robur* L., *Q. virgiliana* Ten., *Q. cerris* L., *Q. rubra* L. resulting in the optimal experimental protocol assessment.

2.1.1. Growing Conditions of Mother Plants

Q. robur L., *Q. virgiliana* Ten. and *Q. cerris* L. are the autochthonous species, naturally widespread in Slovakia. *Q. rubra* is an introduced species, which also grows well in conditions of Slovakia. The acorns and stem cuttings with dormant buds, which were used as source of explants, were collected from selected mature trees of the natural populations in Slovakian territory or from arboretum.

1. *Q. robur* L. acorns were collected from locality Gabčíkovo, situated in southern Slovakia.
2. *Q. virgiliana* Ten. acorns were collected from trees of natural population in Čankov, situated in southern Slovakia. Locality is *Corneto-Quercetum* forestation on neogene eruptive rocks.
3. *Q. rubra* L. and *Quercus cerris* L. acorns were obtained from collection of woody plants in Arboretum Mlyňany, dendrological park situated in southern Slovakia at the foothills of the Western Carpathian Mountains at an altitude 160–206 m above sea level.

Seedlings, used as a source of dormant buds, were produced outside from acorns obtained after open pollination. The acorns were collected from different localities in Slovakia.

2.1.2. Explant Excision and Sterilisation

Embryo cultures. The mature seeds were collected during October and November. Seeds were washed under tap water for 5 min. After pericarp removal the acorns containing the embryonic axes were surface disinfected in 70% ethanol for 10 min followed by 15–20 min treatment in 0.1% solution of mercuric chloride and finally washed with sterile distilled water under aseptic conditions for 3×15 min. Embryonic axes were aseptically isolated from the surrounding cotyledons with preservation of cotyledonary nodes and plumule. Isolated explants were treated in $100 \text{ mg}\cdot\text{l}^{-1}$ solution of ascorbic acid to prevent oxidation for 30 min. After removing the radicular pole, the embryos were placed upright on the culture medium.

Cultures of dormant buds. The stem cuttings with dormant buds were collected from 2 month to 1.5 year-old seedlings, as well as from selected mature trees during February to mid March. Apical and nodal segments with dormant buds were used for initial culture establishment. The material was sterilised by washing under tap water for 1 h, treating for 5–10 min in 70% ethanol and 10–15 min in 0.1% solution

of mercuric chloride with 3 drops of Tween, followed by rinsing in sterile distilled water (3×15 min) under aseptic conditions. One-node segment carrying dormant bud was cut horizontally and placed on the culture medium by the cutting face.

2.2. Culture Medium and Conditions of Cultivation

2.2.1. Embryo Cultures

For the cultivation of embryonic axes in *Quercus* spp. WPM medium (Lloyd & McCown, 1980) with $20 \text{ g}\cdot\text{l}^{-1}$ sucrose and $6 \text{ g}\cdot\text{l}^{-1}$ Difco-Bacto Agar, supplemented with $1 \text{ mg}\cdot\text{l}^{-1}$ BAP and $0.01 \text{ mg}\cdot\text{l}^{-1}$ NAA was used (Table 1). In all experiments the medium pH was adjusted to 5.5–5.7 using HCl and KOH before autoclaving at 121°C and 108 kPa for 20 min. Cultures were maintained in the growth chamber at $23 \pm 2^\circ\text{C}$ under 16/8 light and dark photoperiod and light intensity $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by white fluorescent lamps. The same culture conditions were applied also during the shoot multiplication and rooting stages. Cultures were transferred to the fresh medium at 4–5 week intervals. The number of shoots per explant formed during subculture was recorded. The mean number of shoots per explant from three subcultures was evaluated. Results were computed using variance analysis and multiple range analysis. The optimal composition of shoot regeneration medium and concentration of plant growth regulators for majority of tested *Quercus* species is presented in Table 1.

2.2.2. Cultures of Dormant Buds

Nodal segments with dormant buds collected from seedlings or mature trees were cultivated on WPM medium (Lloyd & McCown, 1980) of the same composition as the medium used for embryonic axis cultivation. The medium was supplemented with plant growth regulators $0.5\text{--}1.0 \text{ mg}\cdot\text{l}^{-1}$ BAP and $0.1\text{--}0.5 \text{ mg}\cdot\text{l}^{-1}$ NAA (Table 1). The multiplication effect – intensity of shoot proliferation was calculated after three subcultures as a mean number of shoots per primary explant.

2.3. Shoot Regeneration and Maintenance

2.3.1. Shoot Regeneration from Zygotic Embryonic Axes

Efficient system of micropropagation, especially for material originated by control pollination, is that represented by mature embryos as primary explants. Experiments showed that embryonic axes are suitable for effective *in vitro* propagation and production of plantlets in oak species. Multiple shoots developed from cotyledonary nodes and plumule (Figure 1A, B). In some cases also adventitious bud formation was observed in the hypocotylar zone (Figure 1C). The highest shoot proliferation was achieved on WPM medium with BAP in concentration $1.0 \text{ mg}\cdot\text{l}^{-1}$ in combination with $0.01 \text{ mg}\cdot\text{l}^{-1}$ NAA (Table 1). The species *Q. cerris* L. exhibited higher morphogenic potential when compared with other *Quercus* species. The more intensive bud regeneration and significantly higher shoot proliferation was observed in this species. On average, 6.5 shoots/explant were formed in *Q. cerris* L., as compared with only 4.5 shoots/explant in *Q. robur*.

Table 1. WPM culture medium composition.

WPM (Lloyd & McCown, 1980)	Shoot regeneration from embryonic axes (mg·l ⁻¹)	Shoot regeneration from dormant buds (mg·l ⁻¹)	Microshoot proliferation (mg·l ⁻¹)	<i>In vitro</i> rooting of microshoots (mg·l ⁻¹)
Macroelements				
CaCl ₂	72.50	72.50	72.50	72.50
KH ₂ PO ₄	170.00	170.00	170.00	170.00
MgSO ₄	180.54	180.54	180.54	180.54
NH ₄ NO ₃	400.00	400.00	400.00	400.00
Ca(NO ₃) ₂	386.80	386.80	386.80	386.80
K ₂ SO ₄	990.00	990.00	990.00	990.00
Microelements				
CuSO ₄ ·5H ₂ O	0.25	0.25	0.25	0.25
FeNaEDTA	36.70	36.70	36.70	36.70
H ₃ BO ₃	6.20	6.20	6.20	6.20
MnSO ₄ ·2H ₂ O	22.30	22.30	22.30	22.30
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	0.25	0.25
ZnSO ₄ ·2H ₂ O	8.60	8.60	8.60	8.60
Vitamins				
Glycine	2.00	2.00	2.00	2.00
Myo-Inositol	100.00	100.00	100.00	100.00
Nicotinic acid	0.50	0.50	0.50	0.50
Pyridoxine HCl	0.50	0.50	0.50	0.50
Thiamine HCl	1.00	1.00	1.00	1.00
Glutamine	2.00	2.00	2.00	2.00
Plant growth regulators and other components				
BAP	1.00 mg·l ⁻¹	0.5–.0 mg·l ⁻¹	0.50 mg·l ⁻¹	–
NAA	0.01 mg·l ⁻¹	0.1–.5 mg·l ⁻¹	0.01 mg·l ⁻¹	0.10 mg·l ⁻¹
IBA	–	–	–	0.30 mg·l ⁻¹
Charcoal	–	–	3 g·l ⁻¹	3 g·l ⁻¹
PVP	–	–	10 g·l ⁻¹	–
Sucrose	20 g·l ⁻¹	20 g·l ⁻¹	20 g·l ⁻¹	20 g·l ⁻¹
Difco-Bacto	6 g·l ⁻¹	6 g·l ⁻¹	6 g·l ⁻¹	6 g·l ⁻¹
Agar				
pH	5.5–5.7	5.5–5.7	5.5–5.7	5.5–5.7

¹In case, when brown colour appears in the medium during cultivation as the consequence of phenolic substances, one subcultivation on medium with polyvinylpyrrolidone (10 g·l⁻¹ PVP) or activated charcoal (3 g·l⁻¹) was done.

2.3.2. Shoot Regeneration from Dormant Apical and Axillary Buds

Results confirmed that successful plant regeneration and rapid propagation in tested *Quercus* species is readily achieved through cultures of dormant apical buds and axillary buds from both explant types (buds from seedlings and from mature trees). The best shoot regeneration was observed on the WPM medium supplemented with 0.5–1.0 mg·l⁻¹ BAP and 0.1–0.5 mg·l⁻¹ NAA in dependence on the species. The multiple vigorous shoot cultures were formed on the above mentioned combinations of plant growth regulators (Figure 1D, E). Differences in intensity of shoot proliferation were species dependent ranging from 3.13 to 6.34 shoots/explant, the lowest being in *Q. robur* and the highest in *Q. rubra* (Ostrolucká & Bežo, 1994).

2.3.3. Shoot Proliferation

For long-term proliferation of *in vitro* regenerated shoots WPM medium supplemented with 0.5 mg·l⁻¹ BAP and 0.01 mg·l⁻¹ NAA was successfully used. On this medium formation of multiple shoot cultures with good elongation growth was observed, along with different intensity of shoot proliferation in tested species. Increasing of shoot proliferation intensity can be achieved by segmentation of regenerated, elongated microshoots on one-node segments and their further cultivation on the same medium (Figure 1F).

2.3.4. Rooting and Hardening

Spontaneous rooting of microshoots or multiple shoots derived from apical and axillary buds was recorded on media containing low BAP concentration (Figure 1G). In many cases, spontaneous rooting was also observed in embryonic axis cultures.

Microshoots exhibited a good rooting ability on WPM medium with low concentration of auxins IBA 0.3 mg·l⁻¹ and NAA 0.1 mg·l⁻¹ supplemented with activated charcoal 3 g·l⁻¹. The percentage of rooting reached 85–90% depending on species. The addition of activated charcoal to the medium is important for the reduction of light intensity at the base of the microshoots and also for the phenolics absorption (Table 1). After 3–5 weeks of cultivation the root formation occurred on the mentioned medium (Figure 1H).

The rooted plantlets were transplanted into the mixture of peat and perlite (3:1) (Figure 1I) and immediately covered under a plastic tunnel for keeping high air humidity. After 2–3 weeks was air humidity gradually reduced to normal values. The rooted plants were successfully acclimatised under greenhouse conditions without any special light treatment, with regular irrigation, in plastic containers. Plants after 2–3 month of acclimatisation in the greenhouse were subsequently transferred to the soil into the bigger containers and placed outside. Winterisation and survival of plants was successful. After 2 years the oak plants reached the height of 32–45 cm (Figure 1J).

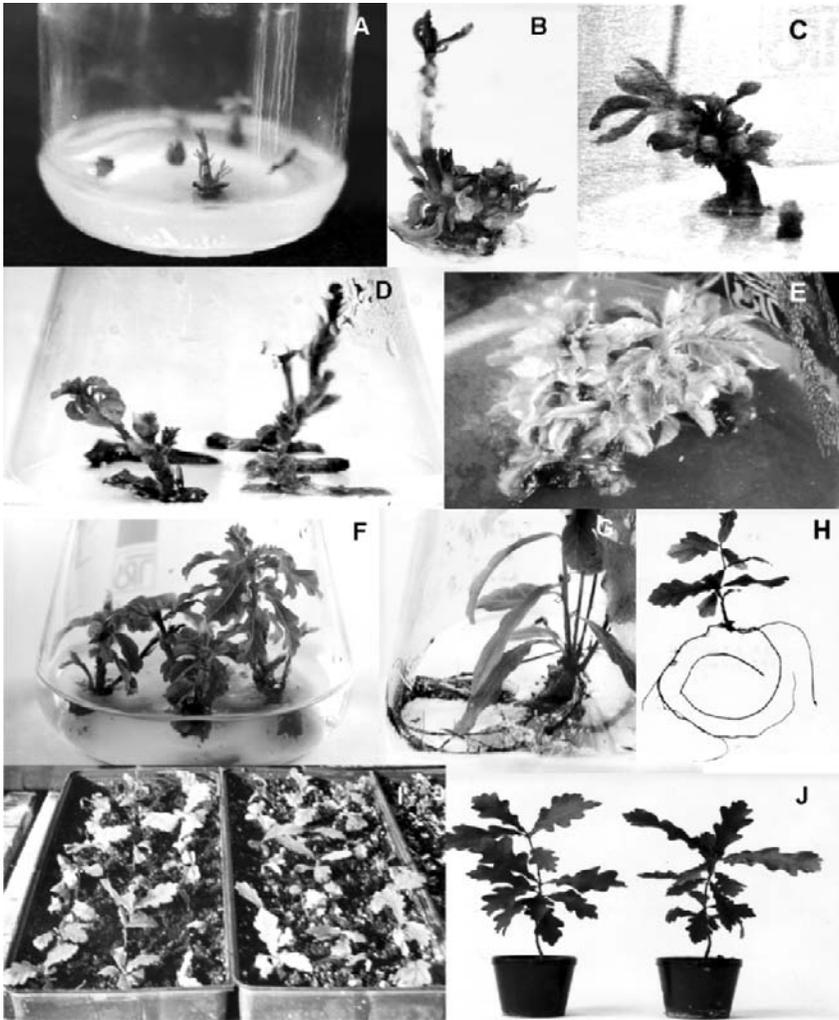


Figure 1. *In vitro* propagation of *Quercus* spp. A) Initial culture of embryonic axis in *Q. cerris* on the medium with BAP $1.0 \text{ mg}\cdot\text{l}^{-1}$ and NAA $0.01 \text{ mg}\cdot\text{l}^{-1}$. B) Multiple shoot regeneration from embryonic axis in *Q. cerris* on the medium with BAP $1.0 \text{ mg}\cdot\text{l}^{-1}$ and NAA $0.01 \text{ mg}\cdot\text{l}^{-1}$. C) Adventitious bud induction in the hypocotylar zone on the shoot regeneration medium in *Q. cerris*. D) Initiation of shoot development from axillary buds on the medium with BAP ($0.5 \text{ mg}\cdot\text{l}^{-1}$) and NAA ($0.5 \text{ mg}\cdot\text{l}^{-1}$) in *Q. virgiliana*. E) Multiple shoot formation from axillary buds on the medium with BAP ($0.1 \text{ mg}\cdot\text{l}^{-1}$) and NAA ($0.1 \text{ mg}\cdot\text{l}^{-1}$) in *Q. cerris*. F) Intensive shoot proliferation on WPM medium with BAP $0.5 \text{ mg}\cdot\text{l}^{-1}$ and NAA $0.01 \text{ mg}\cdot\text{l}^{-1}$ in *Q. robur*. G) Spontaneous rooting of multiple shoots of *Q. robur* derived from axillary bud on WPM medium with low BAP concentration. H) Rooted microshoot of *Q. robur* on the rooting medium WPM with IBA ($0.3 \text{ mg}\cdot\text{l}^{-1}$), NAA ($0.1 \text{ mg}\cdot\text{l}^{-1}$) and activated charcoal ($3 \text{ g}\cdot\text{l}^{-1}$). I) Rooted plantlets transplanted in peat and perlite. J) 2 year-old plants.

2.3.5. Field Testing

In vitro derived *Quercus* spp. plants were after 2 years transferred to experimental fields. From the beginning the plants were characterised by asynchronous growth, what was manifested in differences in plant height. Later no obvious differences were visible between *in vitro* and by cuttings propagated plants. The single plants showed good morphology and physiological health, without morphological anomalies.

3. CONCLUSION

In vitro propagation techniques were proved to be perspective and suitable means of effective propagation and reproduction of selected genotypes of *Quercus* spp., as well as for elimination of problems connected with generative and classical vegetative reproduction. It should be stated that for optimal cultivation and regeneration small protocol modifications may be necessary in different *Quercus* species.

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CHAPTER 9

MICROPROPAGATION OF MEDITERRANEAN CYPRESS (*CUPRESSUS SEMPERVIRENS* L.)

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1. INTRODUCTION

The “Mediterranean” or “common” cypress (*Cupressus sempervirens* L.) is a tall tree (up to 30 m in height), belonging to the genus *Cupressus*, Family Cupressaceae. The Genus includes as much as 25 species (Ducrey et al., 1999), largely diffused in the Mediterranean basin, in Asia and in North America. Of them, the Mediterranean cypress is by far the most important and widespread species in the Mediterranean area. It is native to Northern Persia, as well as Syria, Turkey, Cyprus and several Greek islands. However, during the Roman Empire it was introduced into all the Mediterranean countries, where it can now be considered naturalised. The cypress is monoecious, and bears male and female strobili (cones) separately at the end of short branchlets. Depending on the crown branch habit, the species is divided into two varieties, i.e.:

- *C. sempervirens* var. *horizontalis*, the most common in natural areas, characterised by spreading branches and a broad conical crown;
- *C. sempervirens* var. *pyramidalis* (= var. *fastigiata*), the most popular for ornamental use because of its erect branches, parallel to the trunk, which give the tree its typical columnar shape.

The Mediterranean cypress plays fundamental ecological, economical and ornamental roles in the Mediterranean region. Indeed, the species has important characteristics of marked drought hardiness, and suitability in difficult terrains such as calcareous, clayey or rocky soils. As regards the cypress timber, it has interesting

characteristics of high natural durability and straightness. Due to all these properties, common cypress has been largely utilised in the past for afforestation programs, wood production and the protection against erosion and recovery of degraded areas (Teissier du Cros, 1999). As regards its ornamental use, the characteristic shape of the var. *pyramidalis*, resembling a flame, made the tree from ancient time a typical presence of religious sites (churches and cemeteries), particularly in countries such as Greece, Italy and Spain. In Italy, particularly in Tuscany, it is with the olive the traditional component of the landscape, and it is planted in rows along boulevards and the alleys of approach to ancient villas (Pozzana, 1991). It is also used as wind-breaks for vegetable and fruit tree crops, particularly in France, Spain and Portugal, taking advantage from its columnar and dense crown habit.

Since the 1970s, a serious disease, named the “cypress canker” and caused by the fungus *Seiridium cardinale*, started to spread over large Mediterranean areas and led to extensive damage in forests, nurseries and ornamental plantations, the disease becoming a factor strongly limiting cypress planting. So devastating were the losses incurred that large-scale breeding programmes, founded by the European Union, were initiated in several Mediterranean countries, mainly Italy, Greece and France (Raddi & Panconesi, 1981; Xenopoulos, 1990; Teissier du Cros et al., 1991). As results of these efforts, canker-resistant clones have been recently patented (between the others, “Florentia”, “Etruria”, “Agrimed 1” and “Bolgheri”). These clones have been obtained either by clonal selection in natural stands where the disease was present, or by cross-breeding between selected trees in experimental fields (Raddi et al., 2004).

Cypress is traditionally reproduced by grafting, a technique which allows to propagate individuals selected for their columnar shape and used for ornamental purposes. Although the species can also be propagated by softwood cuttings under mist conditions (Capuana & Lambardi, 1995), this approach is not a common practice of woody plant nurseries. In grafting, scions, collected from selected forms, are side-veneer or cleft grafted on nursery-grown seedling rootstocks during the winter season. In Europe, for instance, over five millions of grafted plants are produced annually (Moraldi et al., 2004). However, this is an extremely labour-intensive and costly practice for the nursery. Hence, the development of effective tissue culture systems could represent a useful tool, not only for mass propagation of selected clones, but also for the gene transfer of important silvicultural traits, as the very long reproductive cycles of conifers makes the conventional breeding techniques very time consuming. In spite of that, up to now *in vitro* culture of common cypress has received little attention; in addition, researches have been focused mainly on the development of *in vitro* regeneration protocols from juvenile material (Fossi et al., 1981; Lambardi et al., 1997; Spanos et al., 1997; Sallandrouze et al., 1999; Lambardi, 2000), but rarely from adult plants (Capuana & Giannini, 1997). Here, protocols for axillary budding, adventitious bud formation and somatic embryogenesis from both juvenile and adult material are reported.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Explants from Juvenile Material

Mature seeds and seedlings are used as source of juvenile plant material. In our experience, seeds are collected from both open and controlled-pollinated trees growing in an experimental orchard, belonging to the Italian CNR, located near to Florence (central Italy, 43° 45' N, 11° 10' E) and established with the aim to select canker-resistant clones. The Mediterranean cypress embryos reach full maturity in late summer of the second year after fertilisation. Hence, the cones have to be harvested at this period and stored in air porous bags and well ventilated rooms. When cone moisture is below 10% (preferably in climatic chamber at an average temperature of 35°C), cones open and the seeds can be easily collected. Seed germination ability of Mediterranean cypress is generally lower than 50%, primarily due to the high percentage of empty seeds (Giannini et al., 1999). The seeds can be stored for up to 10 years at 2–4°C in air-tight containers. An easy way to germinate mature seeds, without stratification and preventing risks of contamination, consists of placing disinfected seeds (see below) over filter paper, moistened with sterile water, inside Petri dishes maintained at a constant temperature of 18–19°C under a 16-h photoperiod (80 $\mu\text{mol m}^{-2} \text{s}^{-1}$). After 5–6 weeks, seedlings 20–30 mm in length, are utilised for *in vitro* establishment of shoot cultures.

2.1.2. Explants from Adult Material

In our work, both very old trees (about 150-year old, from natural growing conditions) and 20- to 40-year old trees (canker-resistant clones from experimental orchards) have been tested for *in vitro* culture during 15 years of experimentation. Apical shoots (not bearing male or female cones) are commonly used as a source of primary explants, collecting them from lateral branches of the lower third of the crown. Indeed, preliminary observations show that *in vitro* morphogenetic competence of shoots collected from basal branches (lower third of the crown) results significantly greater than that of the upper ones. Primary explant collection is performed just before the onset of vegetative growth, around the end of winter growth cessation. However, as cypress growth is potentially a continuous and undetermined process (depending on limiting environmental conditions, such as low temperature and/or drought) and bud burst cannot be taken into account to determine the onset of growth, repeated collections of plant material can be advised during mid winter and early spring. In addition, “re-invigoration” of plants has pursued by side-veneer grafting scions from 30-year old trees onto 1-year old rootstocks. Grafted plants are then grown in pots, under greenhouse conditions, and used as another source of adult explants.

2.1.3. Disinfection of Plant Material

Disinfection of explants is an important step to establish effective shoot cultures of Mediterranean cypress. Recently, effective methods of disinfection have been developed for the various types of explants.

Seeds. Before disinfection, seeds are imbibed for 24 h under running tap water. Filled seeds are treated for 1–2 min with 70% ethanol, followed by 10 min in 0.1% HgCl₂. Then, the seeds are rinsed three times with sterile deionized water (Lambardi et al., 1995).

Shoot tips from seedlings. Five weeks after seed germination, the seedlings (20–30 mm in length) are disinfected by immersion in 70% ethanol for 1 min, followed by soaking in 1% sodium hypochlorite for 15 min. Disinfected explants are then rinsed four times in sterile deionized water. In our experience, following these methods, the rate of contamination is maintained lower than 5%. Moreover, it has been reported that shoot tips collected from older seedlings (18 months) can require a multi-step treatment to achieve good decontamination, e.g., (i) an initial wash of the shoot tips for 10 min in running tap water, (ii) one rinse in distilled water for 15 min, (iii) 10-min disinfection in H₂O₂ (30% v/v), containing 0.025% (v/v) Tween 20, (iv) additional 20 min in 20% (v/v) commercial bleach in tap water, followed by (v) a final rinse of explants (Spanos et al., 1997).

Shoots from adult trees. After collection from adult trees grown in the field, the apical shoots are immediately treated with a 0.1% solution of Benomyl[®] and, after rinsing, stored at 4°C until used. Potted stock plants, maintained under greenhouse conditions, are a better source of explants, as the plant can be periodically treated (even weekly) with a 0.1% solution of Benomyl[®] before the shoot tips collection. The apical shoots (30–50 mm long) from both the provenances are washed under running tap water for at least 60 min, before to be disinfected by immersion in 70% ethanol for 2 min and successive soaking for 20 min in a 1.5% sodium hypochlorite solution, containing drops of Tween 20 as wetting agent. The explants are finally rinsed four times in sterile deionized water. Following this procedure, over 30% fungal and bacterial contamination has still obtained with explants from trees growing in the field, whilst only a 5% of bacterial infection has recorded on shoots collected from grafted plants grown in greenhouse (unpublished data).

2.2. In Vitro Culture

2.2.1. Culture Media

Modifications of SH (Schenk & Hildebrandt, 1972), MS (Murashige & Skoog, 1962), DCR (Gupta & Durzan, 1985), QP (Quoirin & Lepoivre, 1977) and AE (von Arnold & Eriksson, 1981) culture media have been used in different regeneration systems of Mediterranean cypress (see below), according to the formulations reported in Table 1.

Table 1. Basal nutrient media compositions used for mediterranean cypress micropropagation and somatic embryogenesis. PM – proliferation medium, EM – elongation medium, BIM – bud induction medium, EMI – embryogenic induction medium, EMM – embryo maturation medium, S – Sucrose, G – Glucose, F – Fructose, D – Difco Agar, B – Bacto Agar.

Medium	PM	EM	BIM	EIM	EMM
Macro-elements	MS	1/2 SH	1/2 QP	DCR	DCR
Micro-elements	AE	1/2 SH	1/2 QP	DCR	DCR
Myo-inositol (mg l ⁻¹)	100	100	–	200	200
Vitamins	MS	SH	QP	AE	DCR
Amino acids	MS	–	QP	AE	DCR
L-glutamine (mg l ⁻¹)	–	–	–	100	–
Carbon source (g l ⁻¹)	30 S	15 S + 15 S	30 S	30 S	30 S
Activated charcoal (mg l ⁻¹)	–	100	–	–	500
Solidified agent (g l ⁻¹)	7 D	7 D	8 D	7 B	6 B
pH	5.8	5.8	5.7	5.7	5.7

2.2.2. In Vitro Propagation by Axillary Budding

Juvenile material. After excision of the hypocotyl with a scalpel, a portion of the epicotyl with the apical bud is generally used for culture initiation (Capuana & Giannini, 1997). Each primary explant has placed on 75-ml test tube containing the proliferation medium (PM, see Table 1), added of 5 µm BA and 0.1 µm NAA. After 4 weeks, from 4 to 6 axillary shoots develop from pre-existent axillary buds of each healthy explant (Figure 1B). Spanos et al. (1997) used as initial explants shoot tips (50 mm long) excised from 18 month-old seedlings. After the explants were cultured on hormone-free MS medium, an initial proliferation of shoots was observed, although a significant increase in the number of shoots (from 7.2 to 8.2 shoots per explant) was obtained with the addition of BA as growth regulator, at a concentration of 0.001–1.0 mg/l.

Repeated subcultures of explants on a BA-containing medium often results in a poor elongation of the *de novo*-formed axillary shoots. In order to stimulate shoot elongation, the explants are transferred to the elongation medium (EM) containing activated charcoal (Capuana & Giannini, 1997). This way, over 75% of the axillary shoots elongate rapidly and, after 4 weeks shoots longer than 15 mm are excised and transferred to fresh PM. The favourable effect of activated charcoal on axillary shoot elongation has reported for other conifers, such as *Sequoia sempervirens* Lamb. (Boulay, 1978), *Pinus halepensis* Mill. (Lambardi et al., 1993), *Picea abies* L. Karst (Ewald & Suss, 1993). Similarly, the inclusion of fructose in the culture medium alone or in combination with sucrose, showed to stimulate shoot elongation in walnut (Leslie et al., 2005). After five successive proliferation-elongation cycles (i.e., culturing the explants alternatively in the PM and the EM), the average number of elongated shoots longer than 15 mm per explant becomes constant (from 3 to 5), showing that the stabilisation of the culture has been achieved. Following this protocol, shoot cultures of Mediterranean cypress have been maintained for over 3 years in satisfactory conditions.

For root induction, axillary shoots longer than 20 mm are excised from explants at the end of elongation period and placed on root induction medium consisting of half-strength SH with 20 gl^{-1} sucrose and 0.1 mM IBA (Capuana & Giannini, 1997). After 7 days on root induction medium in dark conditions, shoots are transferred to 75 ml jars filled with a solid medium (expression medium) composed of peat, sand and perlite (3:1:1, v:v:v) and moistened with half-strength SH. Each jar with five induced shoots, is incubated at 20°C in a 16-h photoperiod at an irradiance of 80 $\mu\text{E m}^{-2}\text{s}^{-1}$. Under these conditions 82% of induced shoots show 2–3 adventitious roots (longer than 10 mm) after 8 weeks on solid medium (Figure 1C). Axillary shoots, 20–30 mm length, were induced to root on MS ½ with 10 gl^{-1} sucrose and 0.5 mg l^{-1} IBA (Spanos et al., 1997). Adventitious roots differentiated from 95% of shoots after 4 weeks on medium with auxin.

Adult material. Apical portions from the disinfected shoots (20–25 mm in length) are used for *in vitro* establishment of cultures, and axillary bud induction and shoot elongation have been obtained following the same procedure as described for juvenile material. Following the introduction *in vitro*, initial explants from adult stock plants release dark exudations (phenols) which browned the medium surrounding the explants. The detrimental effects of the phenolic exudations are markedly reduced by weekly transfer of explants to a fresh medium, or simply moving the explants in the same test tube on a fresh portion of the medium. This way, after 4 weeks, from 2 to 4 axillary shoots, longer than 10 mm, were developed from the pre-existent buds of each healthy explant (Figure 1A) whilst multiple buds were developed at the base of the primary explants and they formed a cluster. The subsequent transfer of proliferating shoots on EM stimulates the elongation of shoots from axillary buds. At the same time, the adventitious buds at the base of the primary explant remain short and rapidly turn brown.

It is important to note that the new developing axillary shoots display morphological juvenile characters (i.e., juvenile leaf traits with verticillate phyllotaxy). The appearance of juvenile traits can be considered an effect of re-invigoration, due to

exposure of explants to cytokinins (Capuana & Giannini, 1997). Recovery of juvenile traits can be induced through rejuvenating treatments, such as repeated grafting, etiolation and hedging (Fouret et al., 1985; Huang et al., 1992; Giovannelli & Giannini, 2000). However less than 20% of these “re-invigorated” shoots are able to elongate up to 15 mm in 4 weeks on EM. The number of elongated shoots per explant is not improved by maintaining the explants on EM for 6 or 8 weeks; however, the medium is able to increase the length of the longer shoots, i.e., a high number of very long shoots (over 25 mm) has been obtained from each culture vessel. Besides, dividing the elongated shoots into segments (from 5 to 10 mm) has a negative effect on successive growth.

As for juvenile explants, shoots longer than 15 mm are then excised from primary explants and cultured again on PM. Under these conditions, juvenile traits disappear slowly and after five proliferation cycles, the oldest material shows a significant decrease of the proliferation capacity (from 3.5 to 2 mean number of axillary shoot per explant) and an opposite decussate phyllotaxy is newly reached (leaf adult trait). However, significant clonal differences in morphogenetic capacity have been found in *in vitro* competence and persistency of juvenile traits. For instance, primary explants of clones 329 and 771 show a reduced reactivity *in vitro* (lower than 5%) and axillary shoots fail to elongate even after repeated subcultures on EM. In our laboratory, stabilised cultures have been obtained only in the case of five out of 16 clones and it has been maintained for more than five proliferation cycles but *in vitro* growth performances of these clones have remained lower than juvenile explants (unpublished data).

For root induction, shoots longer than 20 mm with juvenile morphological traits and derived from stabilized cultures of five grafted clones and one 150-year-old plant, are rooted following the procedure utilised for juvenile material. After 8 weeks on solid medium only two grafted clones and the 150-year-old plant are able to form adventitious roots but at low rates (from 6 to 15%). Besides, a high percentage (>60%) of shoot tip necrosis has been recorded in all clones. Taken together, these results show that the appearance of juvenile leaf traits did not support the hypothesis that a rejuvenation process will take place during *in vitro* culture.

Culture conditions. During shoot proliferation and elongation, cultures are incubated at $25 \pm 1^\circ\text{C}$ under a 16-h photoperiod at an irradiance of $80 \mu\text{E m}^{-2}\text{s}^{-1}$.

2.2.3. Adventitious Bud Formation

In vitro establishment and adventitious bud induction. Adventitious buds are formed on excised mature embryos cultured on BIM (Bud induction medium) with $10 \mu\text{M}$ BA for 10 days. After induction, embryos with adventitious buds are transferred to the same medium without hormones to induce bud development. After 3–4 weeks, an average of 20 adventitious buds per embryo are recorded and after 24 weeks, it is possible to obtain from 2 to 10 adventitious shoots longer than 10 mm (Lambardi et al., 1995). The adventitious shoots, separated from primary explants, are cultured following the same protocol used for axillary shoot.

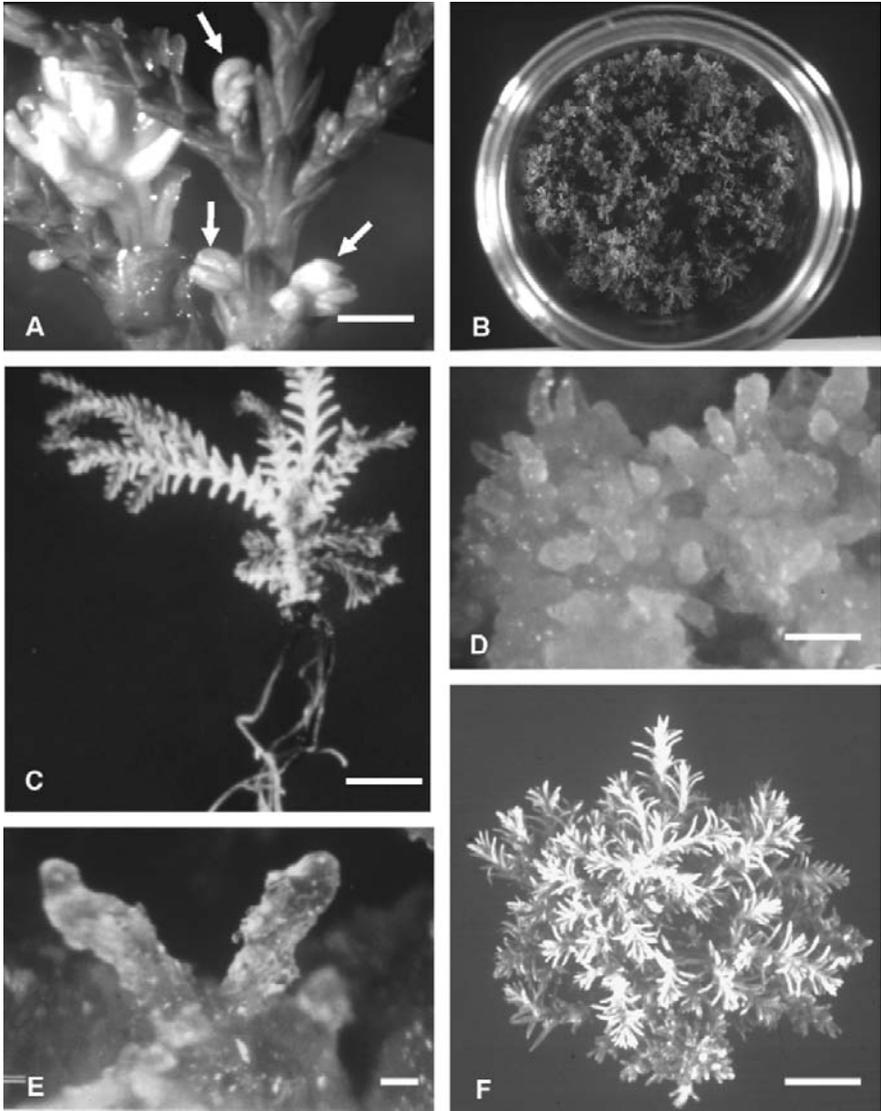


Figure 1. Micropropagation of *C. sempervirens*. A) Axillary budding (arrows) in adult explant (bar = 2 mm). B) 500-ml glass jar with microshoots on proliferation medium (PM) (bar = 17 mm). C) Rooted explant after 8 weeks on solidified expression medium (bar = 10 mm). D) Embryogenic tissue (bar = 1 mm). E) Embryonal and suspensor region (bar = 0.3 mm). F) Cluster with multiple elongating shoots obtained by organogenesis from callus after 6 months in culture (bar = 5 mm).

Rooting. The rooting of adventitious shoots was carried out as described with the axillary shoots. From 15 to 60% of rooted shoots are obtained after 8 months.

2.2.4. Somatic Embryogenesis

Somatic embryogenesis is a powerful system of *in vitro* plant regeneration. When all the steps of a protocol are optimised (i.e., induction and proliferation of embryogenic masses, somatic embryo development and maturation, embryo conversion to plantlets), it is possible to obtain high numbers of plantlets genetically uniform and identical to the tissue (explant) from which the embryogenic line originated. Differently from the broad-leaf woody species in which different tissues (e.g., leaf explants, portions of embryos, flower organs and root tips) have been reported to show embryogenic potential, the elective embryogenic tissue of the conifers is the immature embryo. In time, effective protocols of somatic embryogenesis have been developed for a large number of conifers (Ahuja, 2000) and always using immature embryos as the original explants. In particular, embryogenic masses arise from the suspensor cells, i.e., a small group of elongated cells which are present in basal portion of the embryonic axis.

In cypress, the embryogenic suspensor masses (ESMs) are white, translucent and mucilaginous, with a high percentage of filamentous pro-embryos (Figure 1D). Differently, non-embryogenic callus is white to yellow, never translucent or mucilaginous, and with low organogenic potential. Proliferating ESMs of cypress consist primarily of clusters of somatic pro-embryos, very similar to the late pro-embryo stage of zygotic embryos (Attree & Fowke, 1991). The clusters are polarised structures, initially organised into an embryonic region subtended by multiple, closed and short suspensors. By cleavage polyembryogenesis, these structures continually initiate embryos which generally develop simultaneously to the filamentous stage. We report here a protocol of somatic embryogenesis from immature embryos as developed by Lambardi (2000).

In vitro induction and maintainance of embryogenic suspensor masses (ESMs). The zygotic embryo of *C. sempervirens* reaches the full maturity in the autumn of the second year after fertilisation. In the spring of the second year, the female cone turns brown and the embryo starts to acquire firmness. At this point the seed coat can be removed and the immature embryo safely excised from the megagametophyte.

The first step of the procedure starts with the mechanical isolation of immature seeds in sterile conditions. Thereafter, the isolated seeds will be decontaminated for 1 min with 70% ethanol and 10 min in 0.1% HgCl₂, followed by multiple rinses with sterile distilled water. The disinfected seeds will be stored in the dark at 4°C for 5–7 days in a small amount of sterile distilled water to soften the seed coat before the embryo excision. The immature embryos are then dissected from the megagametophyte working with a stereoscope under the sterile air of a laminar-flow hood.

Culture conditions for the induction and the maintenance of ESMs. To induce EST formation, excised embryos are plated on the EIM (embryogenic induction medium) described in Table 1, supplemented with 500 mg/l casein hydrolysate and 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D). The embryos are then incubated at 23 ± 1°C in the dark, and subcultured every 21 days. In these conditions, ESMs start to appear

at the radicle region of the embryo after 4–5 week of culture. However, it should be underlined that, in the Mediterranean cypress, the ability to initiate ESMs from immature embryos is strongly influenced by the genotype and by the developmental stage of the zygotic embryo. This means that contrasting results can be obtained when collecting the seeds from different trees, or even from the same tree but in different years.

Similarly to other conifers (Becwar et al., 1990; David et al., 1995), the developmental stage of the zygotic embryo is particularly critical for the induction of embryogenic tissue in cypress. As reported by Lambardi (2000), ESMs was mainly found in immature embryos isolated from cones collected in an elapse of time ranging from late-April to late-June. Differently, non-embryogenic calli originated on explants collected throughout almost the entire sampling period. However, it was observed that ESMs always originated from embryos that were morphologically at the same stage of maturity (early-cotyledonary stage), characterised by the two cotyledons just differentiated and still tightly joined. Hence, it is presumable that the time difference among the trees as for the occurrence of the “embryogenic window” (over a 2 week period) reflected a lack of coincidence in the course of their embryo maturation processes.

As for the maintenance and the proliferation of the ESMs, in order to avoid the appearance of extensive browning and necrosis, after the third subculture the embryogenic lines are transferred onto the same medium, but with a lower auxin concentration. In particular, either 2,4-D alone at a 5- μ M concentration, or a combination of α -naphthaleneacetic acid (NAA) and BA (5 μ M each) proved to be effective for the maintenance of the embryogenic lines (Lambardi, 2000). By the regular subculturing every 1–2 weeks in one of the above hormone combination, the embryogenic lines remain prolific (the culture volume doubles approximately every 2 weeks), maintaining high concentrations of filamentous somatic embryos (Figure 1E). Following this procedure, the embryogenic lines can be maintained for several years, although occasionally sudden turning of the lines to a non-embryogenic condition can occur.

Sallandrouze et al. (1999) reported the initiation of a single *C. sempervirens* embryogenic line following a slightly different procedure. The immature embryos were collected in mid-February and cultured on a hormone-free MS (Murashige & Skoog, 1962) medium, supplemented with 15 g/L of both fructose and glucose, 4 g/L charcoal, 10 mL/L coconut water and 7 g/L Bacto-agar.

Embryo development and maturation. For maturation of somatic embryos, the ESMs are transferred on hormone-free DCR medium (embryo maturation medium, EMM), supplemented with 0.5 g/l activated charcoal, and they are cultivated in the darkness. In these conditions, the somatic embryos differentiate until the cotyledonary stage, but the hypocotyls and radicle regions remain poorly developed. To complete the conversion of somatic embryos to plantlets (Figure 1F), the cotyledonary somatic embryos should individually be transferred on filter-paper bridges, soaked with liquid hormone-free DCR medium, within the tissue culture tubes. Differently, Sallandrouze et al. (1999) reported the addition of bovine serum albumin (BSA) into the culture medium to obtain the maturation of cypress somatic embryos to the cotyledonary

stage. When transferred onto a BSA-free medium, the cotyledonary somatic embryos were converted into whole plantlets.

2.3. Hardening and Transfer to the Field

The hardening of plants from micropropagation or from somatic embryogenesis is obtained in 2-liter plastic pots, filled with a peat-sand-perlite substrate (2:1:2, v:v:v) added with osmocote. The potted plantlets are maintained in greenhouse at 20–24°C under misting and daylight conditions for 1–2 months, during which humidity should be gradually reduced from 98 to 65%. The best period to harden-off the plantlets from *in vitro* culture is the early spring. Following this procedure, 100% of successfully hardened plants can be obtained.

Spanos et al. (1997) reported that rooted shoots were weaned into 80-mm pots filled with a loam-peat-sand (7:2:3, v:v:v). Plants were maintained for 14 days under mist conditions at 18–23°C and then placed under glasshouse conditions to complete the hardening for 28 days. This way, high rates of plantlet survival (over 80%) were obtained.

With the above described procedure, also the hardening of the rooted shoots, originally derived from adult material, is easily achieved. However, the plants can show initially typical signs of plagiotrophic growth which, however, starts to disappear after 6 to 8 months.

3. CONCLUSIONS

In the last decades, many breeding programmes have been developed in order to select cypress genotypes of both superior growth habit, and high tolerance to the cypress canker disease. Hence the exploitation of tissue culture systems can offer a valuable alternative to traditional propagation for large-scale clonal reproduction of selected genotypes. *Ex-vitro* plants can be easily obtained from embryos, seedlings or very young plants but it is very difficult with mature selected trees. Preconditioning of adult plant material with re-invigorating treatments as micrografting and repeated grafting onto juvenile rootstock, represents a key step to overcome difficulties of *in vitro* propagation of selected mature trees.

To date, *C. sempervirens* is the only species inside the genus *Cupressus* for which a procedure of somatic embryogenesis has been described and the technique, as well as in many other conifer species, seems to be the most promising among the *in vitro* regeneration systems, taking into consideration its superior multiplication potential. Moreover, embryogenic tissue is an ideal material for genetic transformation, which would be highly advantageous in cypress, considering the time required for the production of new genotypes by sexual crossing.

Data from this report show that two factors – the genotype and the developmental stage of the zygotic embryo – are particularly critical to initiate ESMs from immature embryos. The period during which the embryo responds to inductive treatments can be as short as few days during its course of maturation. In this context, further investigations will be advisable to obtain more information about the detection of the exact

time of the “embryogenic window”. The information could be successfully used to improve the protocols of somatic embryogenesis in *Cupressus sempervirens*.

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CHAPTER 10

IN VITRO SHOOT DEVELOPMENT OF *TAXUS WALLICHIANA* ZUCC., A VALUABLE MEDICINAL PLANT

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1. INTRODUCTION

About 10 genus of *Taxus* spp. are now disposing in temperate zones at the Northern Hemisphere of which the most popular are *Taxus brevifolia* Nutt., *Taxus baccata* L., *Taxus wallichiana* Zucc. and *Taxus cuspidata* Siels et Zucc. For the last three decades, *Taxus* spp. has been concerned after Wani and his colleagues (Triangle Research Institute, NC, USA) discovered a novel anticancer diterpene amide – named “taxol” (paclitaxel) – from the bark of Pacific yew (*Taxus baccata*) extract (Wani et al., 1971; Edgington, 1991). This compound was approved to have clinical treatment of ovarian and breast cancer by the United States Food and Drug Administration (FDA). In addition, taxol also has a significant activity in the treatment of malignant melanoma, lung cancer, and other solid tumors (Wickremesinhe & Arteca, 1993, 1994). Taxol has also been successfully isolated from other species of the genus *Taxus* and from different parts of the plant, including pollen, seed, needles, young stems, woody stems, wood, bark and roots (Wani et al., 1971; Vidensek et al., 1990; Witherup et al., 1990; Fett-Neto et al., 1992; Wickremesinhe & Arteca, 1994). The supply of taxol for clinical use is still limited and depends on extraction from the yew plant, as its bark and needle are the main commercial source. Based on the current bark-extraction procedures, nearly 7,000 kg of bark is needed to produce 1 kg of taxol (Cragg et al., 1993). In addition, *Taxus* species grow very slowly and their seed dormancy is up to 1.5 to 2 years (Steinfeld, 1992). It is very obvious that investigation for alternative sources for the cancer chemotherapeutic agent taxol is urgently needed. Tissue culture of *Taxus* sp. is being considered as a very promising approach towards providing a long-term source of this valuable compound.

Larue (1953) and Tuleke (1959) pioneered the *in vitro* cultures of *Taxus* gametophytes and pollens although taxol was not yet known. From 1970, Lepage-Degivry has published several papers on germination of *Taxus baccata* embryo, focused on breaking seed dormancy (Lepage-Degivry, 1973). Twenty years later, Flores & Sgrignoli (1991) and Chee (1994) reported the embryo culture methods that could overcome the dormancy requirement of *Taxus brevifolia*. Chee (1994) reported that stem-cutting methods could increase the number of available *Taxus brevifolia* trees. Callus culture and suspension culture methods were also studied by Fett-Neto et al. (1992) and Wickremesinha & Arteca (1994), respectively, that could increase the production of taxol.

So far, two *Taxus* species namely *T. chinensis* and *T. wallichiana* have been identified in Viet Nam. *Taxus chinensis* disposes in Northern provinces of Viet Nam, whereas *Taxus wallichiana* grows on 1500-m-high granite mountain of Lam Dong Province. Though this CITES protected species contains high quantity of taxol[®] and 10-deacetyl baccatin III in its bark and needles, only a few studies on regeneration of *T. wallichiana* have been reported. Cutting technique was used during 1994–1996 to preserve *T. wallichiana* at Da Lat Institute of Biology by which the root formation rate was 38% after 90 days (Figure 1A). In general, branches of *Taxus wallichiana* perform vigorous root formation, after 3 months and an average root length of 6–8 cm was recorded.



Figure 1. Cutting technique applied for *Taxus wallichiana*. (A) Rooting of branches. (B) Rooted cutting after 3 months.

For the preservation and mass propagation of this endangered and valuable species, *T. wallichiana*, a protocol for *in vitro* propagation was developed through bud induction, shoot and root regeneration.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Sterilization

Explant collection. *T. wallichiana* explants are selected from young branches bearing many dormant buds (Figure 2). Mark the newly-generated sprouts. After 15 to 25 days, collect the explants by cutting with sharp and clean scissors at the end of the newly-generated buds, leaving the old parts. Only juvenile, green, vigorous sprouts without any infection symptom can be selected.



Figure 2. Plantlets for explant collection. (a) Five-year-old *Taxus wallichiana* yew cultivated in Da Lat Institute of Biology. (b) A young branch bearing dormant buds. (c) Buds. (d) Young shoots.

Explant sterilization. After collection, branches, young stems and shoots are cut into the 5-cm-long segments and washed several times with sterile distilled water. Firstly, dip the segments in washy detergent for 25 to 30 min and rinse under running tap water for 1.5 to 2 h. Surface disinfection is done with 70% ethanol for 30 s, 1%

HgCl₂ added with 2 to 3 drops 0.01% Tween-80 for 10 to 12 min. Then rinse the segments four times with sterile distilled water.

It was demonstrated that explant collection and sterilization can ideally be achieved with 25-day-old young stems and 14-day-old shoots. Table 1 shows the differences in these explants after sterilization and culturing on basal MS medium (Murashige & Skoog, 1962).

Table 1. Contamination was dependent on explant type.

Explant types	Contamination rate (%)	Description
45-day-old branches	100%	Some explants were contaminated, turned brown, and died
25-day-old young stems and 14-day-old shoots	10	Explants were decontaminated, survived, and proliferated

Three kinds of explants, 45-day-old branches, 25-day-old young stems and 14-day-old shoots were examined to investigate the viability and development after sterilization. As demonstrated in Table 1, explants derived from 45-day-old branches are under high risk of contamination (100%). The young explants (young stems and shoots-derived explants) give a lower contamination (10%), with higher survival rate and shoot percentage.

After sterilization, it is recommended to cut the segments into 2 to 2.5-cm-long explants, and place them vertically on the culture media.

2.2. Culture Media

Basal medium containing MS minerals and vitamins (Murashige & Skoog, 1962) supplemented with 20 g.l⁻¹ sucrose is used throughout the whole procedure. Auxins NAA (α -naphthaleneacetic acid), IAA (indole-3-acetic acid) and IBA (indole-3-butyric acid), cytokinins BA (6-benzyladenine) are added at different concentrations to the culture media (Tables 2, 3, and 4). Activated charcoal is also supplemented to reduce the effect of phenolic compounds released by the explants (Pan & Staden, 1998). Media were solidified by adding 9 g.l⁻¹ agar.

Shoot cultures were carried out in 250-ml vessels (40 ml culture medium/vessel). Culture medium pH value was adjusted to 5.8–5.9 by adding 1N NaOH and 1N KCl prior to autoclaving at 121°C, 1 atm (1.02×10^5 Pa) for 30 to 40 min.

It is recommended that an optimal culture medium, supplemented with different plant growth regulators (PGRs), should be tested for addressing specific objectives (see Tables 2, 3, and 4).

2.3. Culture Conditions

Place cultures in the growth chamber at $25 \pm 2^\circ\text{C}$, relative humidity of 75–80%, photosynthetic photon density flux (PPDF) of 45–50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, under 16-h photo-period regime.

Table 2. Concentrations of plant growth regulators α -naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) and activated charcoal supplemented into the half-strength MS basal medium used for shoot growth and elongation.

Medium	NAA (mg.l ⁻¹)	IAA (mg.l ⁻¹)	IBA (mg.l ⁻¹)	Activated charcoal (g.l ⁻¹)
C1	1	–	–	–
C2	3	–	–	–
C3	5	–	–	–
C4	–	1	–	–
C5	–	3	–	–
C6	–	5	–	–
C7	–	–	1	–
C8	–	–	3	–
C9	–	–	5	–
C10	–	–	2.5	2
C11	2.5	–	–	2

Table 3. Concentrations of plant growth regulator (6-benzyladenine i.e. BA) and activated charcoal supplemented into the half-strength MS basal medium used for adventitious bud induction.

Medium	Basal medium	BA (mg.l ⁻¹)	Activated charcoal (g.l ⁻¹)
AB1	½ MS	1	–
AB2	½ MS	3	–
AB3	½ MS	5	–
AB4	½ MS	1	2

Table 4. Concentrations of plant growth regulators α -naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), vitamin B1 and activated charcoal supplemented into the full- or half-strength MS basal medium used for root formation.

Medium	Basal medium	NAA (mg.l ⁻¹)	IAA (mg.l ⁻¹)	IBA (mg.l ⁻¹)	Vitamin B1 (mg.l ⁻¹)	Activated charcoal (g.l ⁻¹)
R1	MS	–	–	5	10	–
R2	MS	5	–	–	10	–
R3	MS	–	5	–	10	–
R4	½ MS	–	–	5	10	–
R5	½ MS	5	–	–	10	–
R6	½ MS	–	5	–	10	–
R7	¼ MS	–	–	5	10	–
R8	¼ MS	5	–	–	10	–
R9	¼ MS	–	5	–	10	–
R10	½ MS	–	–	2.5	–	2
R11	½ MS	2.5	–	–	–	2
R12	½ MS	–	2.5	–	–	2
R13	½ MS	–	–	2.5	–	–

2.4. Establishment of Shoot Cultures

Use young stems 1.5–2 cm long and/or shoots 2–2.5 cm long to establish the shoot cultures.

2.4.1. Shoot Growth and Development

Most of the *T. wallichiana* needles from stem-derived explants were green and alive whereas more than half of needles of the shoot-derived explants became yellowish, turned brown and died after 8 weeks of culture. All shoot-tip cultures produced only one elongated shoot per explant in all the culture media tested, whereas stem explants were characterized by axillary bud elongation (Figures 3 and 4).

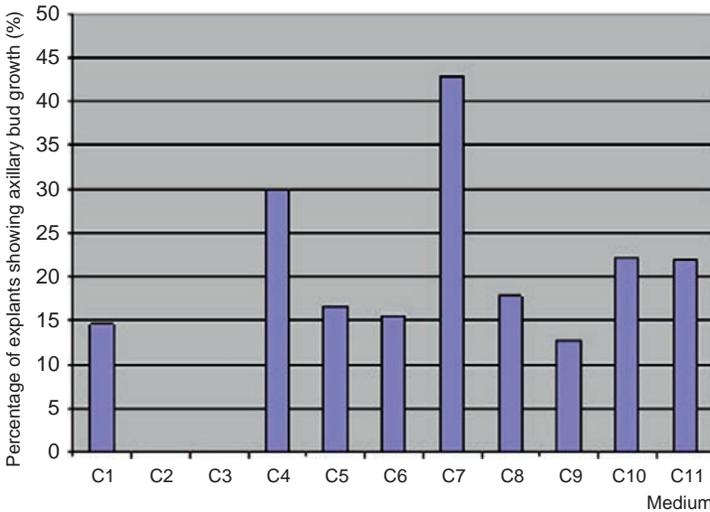


Figure 3. Percentage of *T. wallichiana* explants on different basal MS tissue culture media without activated charcoal showing axillary bud elongation.

For obtaining axillary buds, culture shoots on C7 medium (supplemented with 1 mg·l⁻¹ IBA). For obtaining elongated shoots, place shoots on C4 medium (supplemented with 1 mg·l⁻¹ IBA) (Figures 3 and 4).

At high concentration, auxin inhibits the development of the primordial or axillary buds and induces the formation of callus. Shoots elongated better on media containing lower concentrations of PGRs (Chang et al., 2001). Almost all explants placed on culture medium supplemented with 3 mg·l⁻¹ and 5 mg·l⁻¹ auxin showed lower growth rate, produced phenolics, and formed calli.

To promote shoot elongation and reduce the release of phenolic compounds, add activated charcoal into the culture media. The percentages of explants showing axillary bud growth on culture medium supplemented with 2 g·l⁻¹ activated charcoal can reach to over 80% with an average shoot length of about 3.32 cm, and nearly 90% with an average shoot length of about 3.7 cm on C10 medium and C11 medium, respectively.

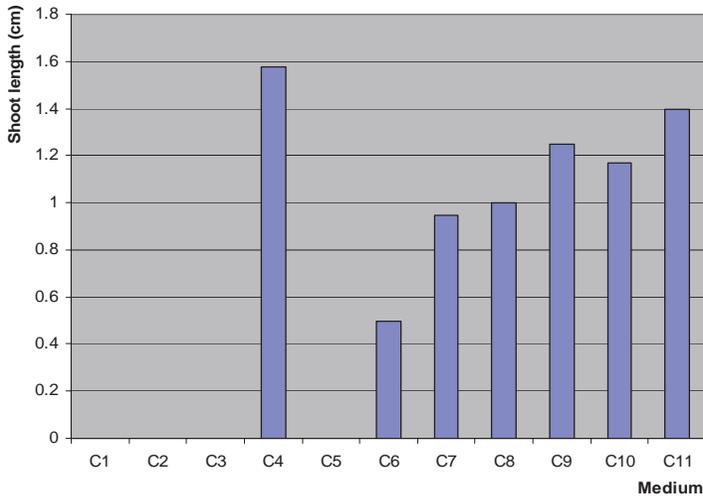


Figure 4. The average shoot length (cm) of *T. wallichiana* explants on different modified MS media without activated charcoal.

2.4.2. Initial Explants for Shoot Growth

Newly-sterilized shoots are preferred in shoot growth in *in vitro* conditions. The newly-sterilized shoots and the *in vitro* 7-week-old shoots and stems were transplanted to C10 and C11 media. After 10 weeks of cultures, all the *ex vitro* explants grew very well, the needles were green; whereas the initial *in vitro* 7-week-old explants showed no growth and had yellowish needles. This is possibly due to their previous physiological states as well as their growth conditions (Table 5).

2.5. Bud Induction

Place young stems 1.5–2 cm in length on culture media. Cytokinins are essential for the induction of adventitious bud primordia in conifers. Among the cytokinins, BA was found to be the most effective for inducing shoots from stem culture of *Taxus mairei* seedlings (Chang et al., 2001).

The induction and multiplication of adventitious buds from young stems were surveyed on AB1 (containing $1 \text{ mg}\cdot\text{l}^{-1}$ BA) and AB4 medium (containing $1 \text{ mg}\cdot\text{l}^{-1}$ BA and $2 \text{ g}\cdot\text{l}^{-1}$ activated charcoal). 22.4% of the explants formed adventitious buds on AB1 medium after 6 weeks in culture. Among them, 0.9% of explants had two buds and 3.45% of the new buds elongated to the average length of about 0.75 cm. Some explants were bloated and formed small calli at their cut ends. 18% of explants induced adventitious buds on AB4 medium; in which 3.33% of the new buds elongated to the average length of about 1.3 cm.

Table 5. Effect of *in vitro* and *ex vitro* derived shoot and stem explants on axillary shoot length (cm) and on percentages of explants with shoots.

Medium	Explants	Axillary shoot length (cm)	Explants with shoots (%)	Note
C10	Stem <i>e</i>	3.00 ± 0.15	100	Green needles
C11	Stem <i>e</i>	6.25 ± 1.25	85.7	Green needles
C10	Stem <i>i</i>	1.93 ± 0.92	52.9	Green needles
C11	Stem <i>i</i>	2.29 ± 1.04	56.3	Green needles, 15.6% explants had 2 shoots
C10	Shoot <i>e</i>	4.24 ± 1.69* 2.21 ± 1.59	100	36.8% had axillary shoot and 52.6% explants showed yellowish needles
C11	Shoot <i>e</i>	3.92 ± 2.06* 3.75 ± 2.25	85.7	The longest shoot were 9.5 cm. 28.6% had yellowish needles, followed by the growth of axillary shoots
C10	Shoot <i>i</i>	0	0	No growth observed. Needles were browning and died
C11	Shoot <i>i</i>	0	0	No growth observed. Needles were browning and died

Stem and shoot *e*: *ex vitro*-derived stem (newly-sterilized stem and shoot).

Stem and shoot *i*: *in vitro* stem and shoot.

*shoots tip length.

After 12 weeks of culture, the number of explants that induced adventitious buds was increased on both AB1 and AB4 media. On AB1 medium, 80.7% of explants were able to produce adventitious buds (Figure 5). Among them, 17.4% of explants formed two buds and 1.8%, 0.9%, and 0.9% of explants formed three, four, and six buds, respectively. On AB4 medium, 41.9% of explants induced adventitious buds. 54.2% of explants forming calli.

According to Ahuja (1985), when activated charcoal was used, shoot elongation and leaf size of *Eucalyptus citriodora* increased but the number of shoots decreased. Webb et al. (1988) found that shoot elongation was promoted by charcoal but this substance inhibited shoot induction when it was included with BA. The *T. wallichiana* explants could be induced on both AB1 and AB4 medium. However, on AB1 medium the explants induced more than one bud per explant, but the shoots were shorter than those ones on the AB4 medium.

2.6. Root Induction

Select and excise shoots (1.5–2.0 cm long) for root induction and place them on culture media supplemented with various auxins (Table 4).

After 10 weeks of culture, all explants placed on R1, R2, and R3 media showed vigorous growth, green needles, and no callus formation was observed at their cut



Figure 5. *In vitro* propagation *T. wallichiana*. (A) Shoots regenerated on culture medium containing $1 \text{ mg}\cdot\text{l}^{-1}$ indole-3-acetic acid (IAA). (B1) Shoot elongation on culture medium containing $1 \text{ mg}\cdot\text{l}^{-1}$ IAA. (B2). *T. wallichiana* plantlet. (C₁, C₂) Bud induction on culture medium supplemented with $1 \text{ mg}\cdot\text{l}^{-1}$ 6-benzyladenine (BA).

ends. Similarly, shoot elongation can be observed in almost all explants placed on R10, R11, and R12 media. The explants formed one to two roots per explant on R7 and R13 medium, respectively. Auxin IBA was more effective than NAA and IAA in promoting root induction.

Although auxin promotes lateral and adventitious root development, our results showed the significant difference, as a special characteristic of *Taxus wallichiana*.

3. CONCLUSION

This chapter describes the procedure for *in vitro* shoot development and bud induction of *Taxus wallichiana* Zucc., an endangered, recalcitrant and valuable medicinal plant. The fact that shoot induction and elongation and root regeneration could be achieved has opened up the novel possibility for *T. wallichiana* large-scale cell

culture to be utilized for taxol and 10-deacetyl baccatin III exploitation. *T. wallichiana* can be propagated by *ex vitro* means. The shoots obtained through *ex vitro* protocol can be applied to produce a significant amount of biomass for conservation and morphological studies of the species. Under *in vitro* conditions the metabolic engineering can be realized for production of taxol and related products. Calli and suspension cultures as well as somatic embryogenesis can also be obtained afterwards from these *in vitro* shoots.

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CHAPTER 11

MICROPROPAGATION OF YEW (*TAXUS BACCATA* L.)

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1. INTRODUCTION

Yews are slow-growing shade tolerant trees which were used over millennia because of their durable and elastic wood, especially for weapons like longbows. This led to an extreme shortage of this material and a dramatic negative selection for phenotypes with straight stem forms over four hundred years ago. After the introduction of firearms, this tree species recovered again. Later on, the accidental poisoning of valuable domestic animals such as horses caused a further clearing of trees (e.g. near roads). A growing interest in yew arose again with investigations on the toxic principle 50 years ago. The mitotic spindle poison paclitaxel was an effective inhibitor of fast growing cancer cells and was mainly concentrated in the bark of the trees. Approximately seven tonnes of yew bark is necessary to obtain one kilogram of paclitaxel. Based on this and on similar calculations it became obvious that the supply of plant material of yew might be limiting factor in the future (Croom, 1995). Therefore, methods for vegetative cutting propagation were developed and improved in different countries (Schneck, 1996; Ewald et al., 2002). Micropropagation methods to multiply selected material were developed at the same time, however, the number of published results concerning adult plant material is limited (*Taxus mairei* – Chee, 1995; Chang et al., 1998, 2001; *Taxus baccata* – Majada et al., 2000). Somatic embryogenesis of *Taxus*, mostly from very juvenile explants such as immature zygotic embryos, was also reported (Wann & Goldner, 1994) but the regeneration of plants was often difficult with regard to embryo formation and synchronous development (*Taxus brevifolia* – Ewald et al., 1995 and *Taxus chinensis* – Qiu et al., 1998). Also, somatic embryogenesis was not satisfactory concerning the percentage of germinating explants (*Taxus brevifolia* – Chee, 1996). Testing these previously described *in vitro* methods based on organogenesis, often led to failure in the laboratory of the author while investigating micropropagation of adult yew material. Insufficient tissue culture

ability of the plant material used as well as other factors might have been responsible for the negative results. Therefore, based on experiences in micropropagation of conifers, a method was worked out in detail to multiply selected yew clones.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Growing Conditions of Mother Plants

Cuttings of adult selected donor trees harvested in September were rooted using rooting paste containing 2 g l^{-1} indole-3-butyric acid (IBA) under high pressure fog in a greenhouse (Figure 1A). These rooted plants were used as donor plants for establishing tissue cultures. Closed buds or shoot tips were harvested as explants after pre-treatment with a fungicide (0.2% Euparen by Bayer, 50% dichlorfluanide) for 24 h.

2.1.2. Disinfection of Plant Material

The surface of the explants was kept dry to allow an effective disinfection. Therefore, no washing was carried out before disinfection and the plants were not watered one day before use. Disinfection was carried out by washing the explants in mercuric chloride solution (0.25%) with one drop of a detergent (e.g. TWEEN 80) for 10–15 min. Afterwards, the explants were rinsed three times with sterile water and placed on nutrient medium in 100 ml-Erlenmeyer flasks.

2.2. Culture Media

Nutrient media used for micropropagation are listed in Table 1. The concentration of basic components (dilution or increase) from well-known plant nutrient media is shown there, whereas growth regulators, which were added, are mentioned in the text separately. In different experiments Woody Plant Medium (WPM, according to Lloyd & McCown, 1981) was found to support the growth and vitality of *Taxus* explants best. Therefore, nutrient media for propagation as well as for elongation was based on this basal medium. The basal medium for rooting was a modified LS medium (L9, based on LS according to Linsmaier & Skoog, 1965).

Table 1. Basal nutrient media compositions used for larch micropropagation (Macroelements, microelements given as dilution or increase of original medium).

Medium	Macroelements	Microelements	Carbon source g l^{-1}	pH
W	1	1	20 sucrose	5.7
Wdouble	2	2	20 sucrose	5.7
L9	1/3	1	5 sucrose	5.7

2.3. Shoot Regeneration and Maintenance

2.3.1. Bud Formation and Propagation

Testing of very efficient cytokinins, like 6-benzylaminopurine (BAP), to stimulate bud and shoot development in combination with nutrient media supplemented with activated charcoal, as it was reported in the literature, led to a rapid necrosis of the material. Tests of different basic media showed that Woody Plant Medium (W) was the most efficient for the growth and vitality of explants. Experiences with other conifers regarding the use of cytokinins (larch, Norway spruce) were also applied to yew. Woody Plant medium with 1.5 mg l^{-1} zeatin (medium called Wz) was used for bud induction. Sometimes, zeatin was replaced by 2iP (N^6 -(Δ^2 -isopentenyl) adenine – 2.84 mg l^{-1}). The explants were kept under continuous red light conditions ($30 \mu\text{E m}^{-2}\text{s}^{-1}$, 650 nm peak emission) at a temperature of 23°C . Although medium Wz led to a reduced elongation of shoots, the formation of axillary buds was forced (Figure 1B). If the concentration of basic medium was doubled (Wdouble) and used with zeatin in identical concentrations as Wz (Wzdouble) a remarkable improvement in shoot elongation, number of lateral buds and needle length was achieved. Nevertheless, a continuous use of Wzdouble reduced the propagation rate. A callus phase – comparable to adventitious bud propagation in other conifers (e.g. Norway spruce) – was not observed. Resulting from these experiments, the nutrient medium Wz was used for continuous propagation of clusters of axillary buds because of its efficiency. Propagation rates of 1.2–1.8 per month were achieved. The addition of 200 mg l^{-1} spermidine boosted these positive results. Using exclusively one medium to get propagation as well as elongation, e.g. as it was elaborated for poplar in our laboratory, was not possible. The conclusion was to establish and to maintain a propagation culture and to transfer buds to an elongation medium to get rootable shoots. Problems during propagation which derived from the occurrence of endophytic bacteria were solved by the addition of 500 mg l^{-1} ticarcilline.

2.3.2. Bud Elongation

Elongation of lateral buds. Tests concerning the serial propagation of bud-bearing stem segments by dissection of elongating shoots comparable to the system used for larch, failed because it was not possible to force the development of axillary buds in stem segments. On the other hand, elongation of shoot tips in this experiment was achieved best using double concentrated W nutrient medium (Wdouble) and a combination of zeatin and kinetin ($0.66 + 0.64 \text{ mg l}^{-1}$) with addition of PVP and arginine (100 mg l^{-1} each). Elongation of lateral buds was not stimulated on this medium, but was stimulated most when a subculture free of phytohormones was included, followed by a subculture with low concentration of thidiazuron (TDZ, 0.01 mg l^{-1}).

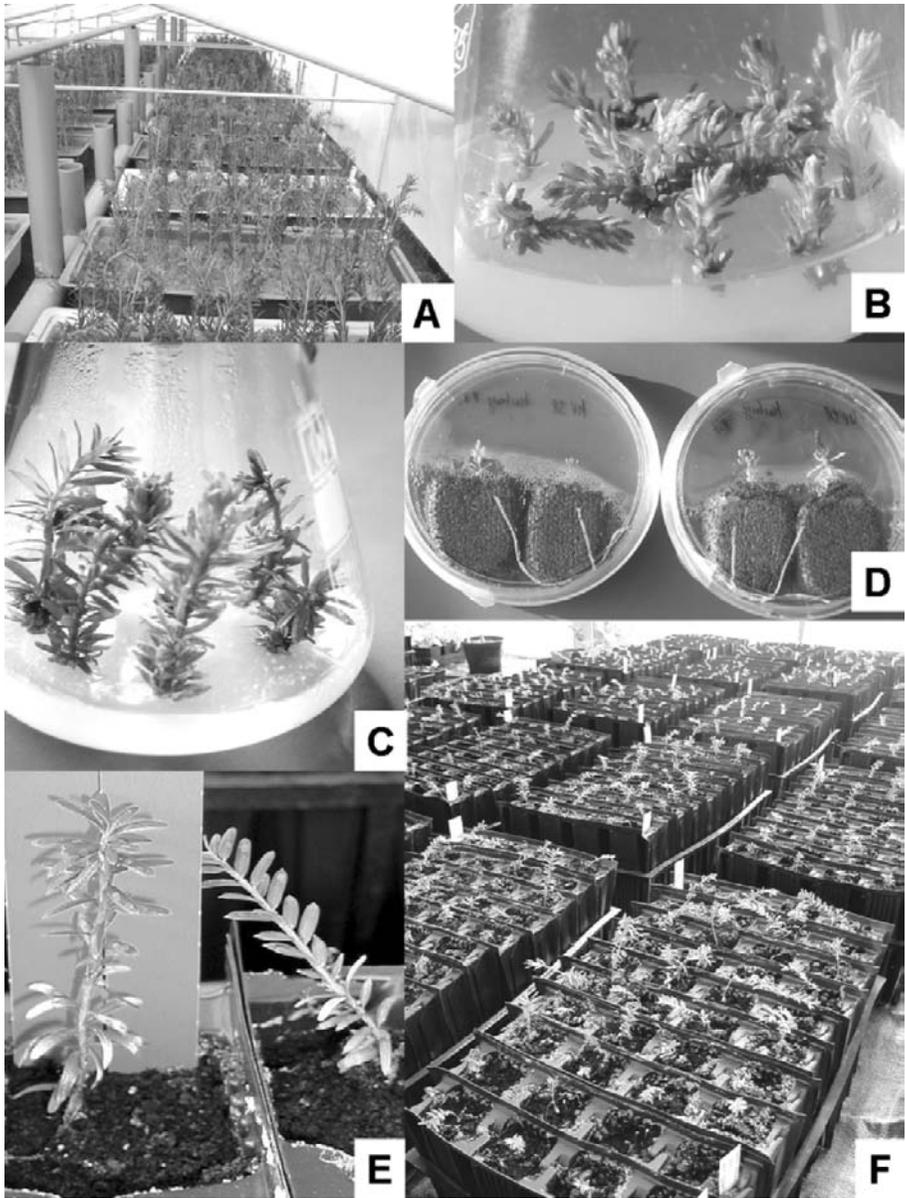


Figure 1. Yew micropropagation: Cuttings from donor trees (A), shoot propagation (B), shoot elongation (C), root formation in peat pellets (D), rooted plants showing radial needle growth (left) or v-shaped needle arrangement (right; E), yew plants after transfer to soil (F).

Shoot elongation. Best elongation of shoots in general was achieved using W medium (Wdouble) supplemented with 0.01 mg l^{-1} TDZ in combination with 200 mg l^{-1} spermidine. In this way it was possible to stimulate the elongation of buds from axillary bud clusters derived from propagation medium (Figure 1C). This combination also led to the best development of needles on the shoots (needle length). Starting with 15 mm long shoots, rootable shoots (35–45 mm) developed after two subcultures (8 weeks). Observing the needle arrangement as a measure for normal shoot development, it became obvious that higher concentrations of TDZ inhibited needle development and elongation of the shoot as well. Rooting experiments led to the conclusion that rooting was improved when only the nutrient medium directly used before rooting contained spermidine. Therefore, shoot elongation after the stimulation of bud development was started on a phytohormone-free medium (1st subculture, 4 weeks) and continued with a TDZ containing medium supplemented with spermidine (2nd subculture, 4 weeks) immediately before root induction.

2.4. Rooting of Shoots

All steps of root induction and development were carried out at 15–17°C and a 16-h light-period (white light-radiation $30 \mu\text{E m}^{-2}\text{s}^{-1}$). There was a remarkable influence of the nutrient medium used before rooting on the rooting percentage later on. Zeatin-containing media used before rooting decreased the rooting success. Spermidine in combination with TDZ (0.01 mg l^{-1}) increased the rooting percentage as well as the vitality of shoots. Compared with other conifers (larch, Norway spruce) the rooting process in yew required a long period of up to 7 months.

Rooting – auxin induction. Rootable shoots (appr. 30–40 mm in length) were induced for root formation on nutrient medium L9 containing 2 mg l^{-1} IBA for 2 weeks. IBA was found to be the best auxin compared with NAA (naphthalene acetic acid). The basal part (2 mm) of the shoots were cut before placing them on induction medium.

Rooting – Agrobacterium rhizogenes induction. Shoots prepared in an identical way as for auxin induction were placed so that the bases of the shoots were in contact, for 24 h with a solution of various *Agrobacterium rhizogenes* strains (OD = 0.6 at 600 nm – nutrient medium 20E; Werner et al. 1975) supplemented with 19.6 mg l^{-1} acetosyringone. Five wildtype strains were tested: A.r. 15834, A.r. DSM 30148, A.r. K599 and A.r. Marburg (kindly provided by Dr. Müller – Phillips University Marburg) and A.r. ZALF (kindly provided by Dr. P. Lentsch/ZALF Müncheberg). Root formation and survival was best using the strain A.r. Marburg compared with IBA treatment. Callus formation as well as decay of shoot bases was reduced during the long-lasting process of root development after use of *Agrobacterium rhizogenes* and especially of the A.r. Marburg strain. This variant of the *Agrobacterium* strain was also the best concerning the general behaviour of shoots during rooting. Therefore, it is worthwhile testing several different *Agrobacterium rhizogenes* strains for root induction, because these natural inducers are capable to support the whole process of root development in some cases much better compared with an auxin induction.

Root development. After the induction period, shoots were placed directly in JIFFY 7 peat pellets and saturated with water. The peat pellets were placed into small plastic greenhouses. Growing peat moss (*Sphagnum spec.*) placed in-between the peat pellets avoided a fungi attack which otherwise occurred very often. The period of root development lasted 5–7 months until roots were visible outside the peat pellets (Figure 1D). For that reason, moisture content of the peat pellets had to be checked weekly (to avoid drying out).

2.5. *Hardening and Transfer to the Field*

After rooting, the plants were transferred into rootainers (RONAASH Ltd. Scotland, 4.5 × 4.5 cm, 20 cm high, each for 40 plantlets) in propagation compost (EINHEITSERDE type VM) and placed under high pressure fog (95% air humidity). Conditioning was carried out by successive reduction of the air humidity over a period of 1 month. Like for other conifers, two main different forms of growth behaviour were visible after transfer to the soil, orthotropic growth and plagiotropic growth. In *Taxus* this growth behaviour was closely related to the position of needles on the elongating shoot. A radial position of needles around the shoot was an indicator for orthotropic growth, similar to normal seedlings. A needle arrangement on two sides only, often v-shaped along shoots, was an indicator for a branch-like or plagiotropic growth (Figure 1E). These two growth characters were observed on shoots within propagation culture *in vitro* for several clones. Small shoots (<20 mm) had mostly radial needles (at least 10:1 relation, number of shoots), whereas longer shoots (>20 mm) showed an almost equal relation (1:1). The amount of shoots forming the v-shaped needle position increased during shoot elongation. There was a close correlation of growth (orthotropic or plagiotropic) and the needle position on the shoot (radial or v-shaped). With increasing shoot length after transfer to the soil (>40 mm), shoots with radial needles on base of shoots often formed at the top of the shoot the v-shaped needle position. Nevertheless, the relation of shoots with different needle positions was approximately 1:1. Observations in the future will give information if some of the trees produced will express an orthotropic growth demonstrating a partial rejuvenation. Examinations of rooted cuttings from adult trees led to the conclusion that orthotropic growth of the plants has not yet been achieved after a period of 6 years in the field. This may indicate that the physiological differentiation of *Taxus* tissues is fixed in material from cutting propagation. The comparison of cutting-propagated versus micropropagated *Taxus* plants (Figure 1F) will allow conclusions about the capacity for the micropropagation cycle to restore a seedling-like (rejuvenated) growth behaviour, which would be very important from the point of view of utilisation of the material in forestry plantations.

3. CONCLUSION

Micropropagation of *Taxus baccata* starting from rooted cuttings was possible. The induction of axillary buds was the first necessary step followed by elongation of the shoots and subsequent root induction. Root elongation of induced microcuttings took place in soil-like substrates. Strains of *Agrobacterium rhizogenes* were able to improve the root formation and survival of plants.

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CHAPTER 12

MICROPROPAGATION OF *LARIX* SPECIES VIA ORGANOGENESIS

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1. INTRODUCTION

Larch is a conifer which is characterised by relatively fast growth. Among conifers, its specialised trait of losing its needles in the winter has some advantages, especially in areas with high levels of air pollution. The breeding history of this tree species in Europe, especially in Germany, goes back more than 40 years. Today several possible parent combinations are known, seed orchards are established, and field trials and natural stands exist, which allow the selection of suitable material for practical purposes as well as for continued breeding and tree improvement. Some of the selected tree stocks are already characterised according to their wood quality and resistance to decay. Larch is known as a wood which normally does not need any chemical protection. This will make larch of increasing interest for forestry in the future. Conifers often flower well only at intervals of several years, in an irregular cycle. As a result, seed material derived from seed orchards and from controlled pollination is not available every year and often is only available in limited amounts. This situation has resulted in the search for vegetative and microvegetative propagation methods for larch. Tissue culture and micropropagation methods were evaluated for these reasons. Seedlings from hybrid larch, characterised by a faster growth rate and an increased tolerance to air pollution, were used to investigate these methods. Larch clones are important for research tasks (e.g. resistance research) and for establishing clonal mixtures suitable for reforestation. Regeneration systems *in vitro* are necessary preconditions for gene transfer as well. Therefore the basic methods were developed and established for juvenile plant material (zygotic embryos, seedlings, saplings). Foresters, however, are generally interested in trees that have proven quality traits such as growth performance and resistance over long periods. This assessment is often

made at half the rotation age. The negative aspect of such an extended assessment is that, by that time, most of the individuals have lost their ability to be propagated vegetatively. Moreover, even when vegetative propagation of selected adult individuals is possible, it is often linked with improper root formation and plagiotropism. For different *Larix* species, there is interest in obtaining propagules with juvenile growth behaviour from selected adult trees. This includes trees from natural stands as well as hybrids derived from breeding experiments. But micropropagation of mature trees is often, although not always, more difficult than *in vitro* propagation of juvenile material such as zygotic embryos or seedlings. This is especially true for some of those conifers used in large scale forestry, including larch (Bonga & von Aderkas, 1988; Chalupa, 1991, 2004; Karnosky et al., 1993). Plant production from shoot formation or shoot development is often lower than from cultures initiated from juvenile plant material. Cultures of adult and juvenile origin also differ in growth behaviour and morphology. Finally, both rooting success and transfer to the soil pose problems because of reduced root growth. Most of the difficulties are due to phase changes during tree development. Nevertheless, it is reportedly possible to overcome these difficulties, at least for those genotypes which showed better responses to tissue culture (Bonga & von Aderkas, 1993). Preconditioning of the plant material (grafting, pruning) is sometimes required and different tissue culture methods must be optimised to gain a degree of reinvigoration and rejuvenation of the plant material. Attention was also focused on possible factors responsible for successful propagation of adult donor trees. This chapter will summarise the work accomplished to date, and will consider possibilities and problems for future work.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Supply of Plant Material

Juvenile plant material. The plant material (seeds) for the development of propagation methods – either from seed orchards or derived from controlled pollination – was provided by different breeders from Brandenburg and Saxony, but also from Russia and China. Various larch species and hybrids have been included in the experiments over the last 20 years: *Larix eurolepis*, *L. decidua*, *L. kaempferii*, *L. sukaczewii*, *L. gmelinii* and others. Especially hybrid larch trees (*L. eurolepis*) characterised by a higher growth performance and frost tolerance were selected for experiments. Elongating shoot tips as well as long-shoot buds of 1- or 2-year-old plants from the nursery were used to establish tissue cultures.

Adult plant material. From adult donor trees, it was possible to use closed winter buds harvested in autumn after needle fall, because this allowed effective disinfection and preparation. Furthermore, good growth of buds and meristems (e.g. for micrografting) was achieved because material was not yet in deep dormancy. Micrografting experiments and attempts to induce adventitious buds demonstrated that this was the best time for establishing cultures from terminal long-shoot buds.

2.1.2. Disinfection of Plant Material

Seeds. Seeds were disinfected for 10 min using mercuric chloride solution (0.25%) containing a drop of detergent (e.g. Tween 80). They were then rinsed three times with sterile deionized water and placed onto a nutrient medium free of phytohormones for germination (BEMB/200). In one case, when seeds of *L. gmelinii* were used, this method failed because of the heavy infection with fungi in the seed coat. Seeds were rinsed for 1 min in 70% ethanol and the seed coat was then removed with a scalpel. The megagametophyte containing the embryo was placed directly on germination medium or was used after a second but shorter disinfection period (3–4 min) with mercuric chloride.

Growing shoot tips/winter buds. Growing shoot tips as well as winter buds from the nursery were sometimes heavily infected, especially during wet weather periods. Thus, if possible, the plants were potted and placed in a greenhouse before taking explants. Treatments with fungicides a few days before harvest of plant material improved the disinfection success. The explants always had a dry surface before disinfection: the method was identical to that used for seeds. Before placing explants on medium, the shoot base was removed with scissors. Used mercuric chloride solution was collected and disposed of as hazardous waste. Mercury could be precipitated after the addition of ammonium disulfide solution and the excess water could then evaporate under an extractor hood. Solid precipitated mercurysulfide was disposed of by specialised enterprises.

2.2. Culture Media

The composition of culture media for larch micropropagation is described in Table 1, modifying the following basal plant nutrient media.

MCM (modified, urea lacking), according to Bornman, C.H. 1983

BEMB (modified,) according to von Arnold, S. & Eriksson, T. 1981
(macroelements), and Boulay, M. 1979 (microelements)

B, according to Boulay, M. 1979

L9 based on L according to Linsmaier, E.M. & Skoog, F. 1965.

Wz based on WPM according to Lloyd, G. & McCown, B. 1981.

SH according to Schenk, R.U. & Hildebrandt, A.C. 1972.

Growth regulators which were supplemented are mentioned separately in the text. All media were solidified with agar or gelrite.

2.3. Shoot Regeneration and Maintenance

2.3.1. Serial Propagation of Juvenile Explants

The organogenic potential of plant parts from larch seedlings can be used for several tissue culture propagation methods. The two possibilities described in the following are serial subcultures without phytohormones and adventitious bud formation. There

Table 1. Basal nutrient media compositions used for larch micropropagation (macroelements, microelements given as dilution of the original medium).

Medium	Macro- elem.	Micro- elem.	Carbon source g l ⁻¹	NH ₄ NO ₃ mg l ⁻¹	PVP mg l ⁻¹	Arg mg l ⁻¹	Gln mg l ⁻¹	pH
MCM	1/2	1/2	15 S		100	100		6.8
Wz gluc	1	1	32.87 G					5.7
BEMB/ 200	1	1 (B-med.)	10 S	200	200			5.8
BEMB/ 600	1	1 (B-med.)	10 S	600	200			5.8
B1	1	1	30 S				146	5.7
L9	1/3	1	5 S					5.7
SH 1/2	1/2	1/2						5.7

PVP – polyvinylpyrrolidone, Arg – arginine, Gln – glutamine, S – sucrose, G – Glucose

are only a few publications which discuss use of phytohormone-free media to induce organ development in larch. Most of the authors tried to stimulate axillary bud development or to induce adventitious buds by phytohormone treatments (see 2.3.3. Adventitious bud formation). By exploiting the capacity for shoot elongation in larch, Douglas fir and Norway spruce, it became obvious that larch had a higher potential for shoot elongation on a phytohormone-free medium compared with Douglas fir or Norway spruce. This was the background used to develop the so-called “serial propagation”, which was carried out according to methods developed for juvenile larch shoots (Hübl & Zoglauer, 1991).

Culture conditions. Serial propagation without phytohormones is based on the continuous growth of elongating larch shoots *in vitro* on B1-medium at a temperature of 23°C. The illumination condition was continuous red light (fluorescent tubes OSRAM L58 W/60, red; 30 µE m⁻²s⁻¹, 650 nm peak emission), which was found to force shoot elongation much better than blue or white light.

Dividing of elongated shoots into segments. Once the shoot axis of the sterile germinated seedling or of a long-shoot bud from a juvenile plant had elongated (Figure 1A), the shoot was cut into a shoot tip and bud bearing stem segments (appr. 15 mm long) which were transferred to fresh B1-medium, where they sprouted and formed long-shoots again.

Subcultures. Subcultures were carried out in 7-week-intervals in tubes or Erlenmeyer flasks. On such an elongated larch shoot, those buds located close to the shoot base are determined to become short-shoots. As in many other tree species, establishment of a propagation culture required a few subculture intervals until optimal propagation factors were reached.

Stimulation of elongation. Shoots showing a larger number of lateral buds with failing bud elongation were treated for 4 weeks with the nutrient medium normally used for adventitious bud induction (MCM + zeatin/kinetin – see 2.3.3.). The medium Wz gluc, containing 1.5 mg l^{-1} zeatin in combination with glucose as a carbon source, was used in the same way to stimulate bud elongation. After this step, the shoots were placed again on the normal elongation medium B1. In many cases, the buds started to sprout shortly after transfer to the phytohormone-free medium. Beyond a certain bud size the inducing effect of the adventitious bud induction medium changed into an elongation-stimulating effect.

Short-shoot stimulation on juvenile explants. With increasing age a larch seedling in the field forms more and more short-shoots. This means that not all buds are able to form an elongating shoot, a long-shoot. Short-shoot buds are characterised by a smaller meristem, which is not as long as the preformed needles, in contrast to a long-shoot meristem. Short-shoot buds form only needle clusters. Terminal buds of larch usually contain long-shoot meristems. The two or three lateral buds below the terminal bud often contain long-shoot meristems as well, whereas the next lower two or three buds can be characterised as intermediate forms of meristems with an increasing tendency to express short-shoot meristems.

In cases where the terminal bud was lost, the next shoot meristem was capable of developing an elongating shoot axis. In juvenile material (4-month-old seedlings) more buds are able to form long-shoots. In older material and especially in old-aged cultures, short-shoot buds refuse to form a long-shoot if the shoot axis is cut into bud bearing stem segments. Therefore an *in vitro* method was developed to stimulate sprouted short-shoot buds to elongate a shoot axis by a combined treatment of cytokinins, light and temperature (Kretzschmar, 1993).

Induction of short-shoots. Non-growing short-shoots were incubated in 100 ml-Erlenmeyer flasks on half strength MCM medium (Table 1). Kinetin (0.5 mg l^{-1}) and 0.05 mg l^{-1} indole-3-acetic acid (IAA) were added as growth regulators. During the induction treatment, the cultures were kept at 17°C under a photoperiod of 16 h in white light (radiation $30 \mu\text{E m}^{-2} \text{ s}^{-1}$) for 4 weeks.

Elongation of short-shoots. After that treatment, the explants were transferred onto the elongation medium free of phytohormones (B1) under continuous red light at 23°C . After 8 weeks, up to 63% of short-shoots elongated and could be used for serial propagation again.

2.3.2. Serial Propagation of Adult Material

The method of serial propagation as already described was also applied to very old material (from over 40 years up to 120 years in age). From 42 clones established that way, it was possible to propagate only 4 lines (Kretzschmar & Ewald, 1994; Ewald & Naujoks, 2000). Lack of elongation capacity in long-shoot buds was still the limiting step. From the established clone lines, three clones were rooted successfully. It was possible to improve the elongation capacity of shoots by a subculture on nutrient

medium containing a cytokinin, based on the experience of short-shoot stimulation in larch described already for juvenile material. The nutrient medium Wz gluc supplemented with 1.5 mg l^{-1} zeatin was used for this subculture. Glucose as a carbon source was sometimes more efficient in other trees as well (G. Naujoks, personal communication 2003; e.g. for oak). For different recalcitrant tree species, this medium was used for induction as well as for organ development. After such an intermediate step the elongation of shoots was forced.

2.3.3. Adventitious Bud Formation – Juvenile Material

Zygotic embryos, germinating seedlings, and shoot tips as well as winter buds were used for the induction of adventitious bud clusters. Zygotic embryos were removed from the endosperm after one day of germination.

Induction of adventitious buds and subculturing. After disinfection, all explants were placed directly on MCM medium (Table 1) supplemented with 1.5 mg l^{-1} zeatin and 0.15 mg l^{-1} kinetin for 4 weeks. Within the following two subcultures (7–8 weeks) clusters of adventitious buds developed on a medium without plant growth regulators (BEMB/200). One propagation cycle consisted of the induction period and two subcultures for the development of induced buds. Afterwards, the bud clusters were divided into single explants. These explants were used for a new cycle. All subcultures were carried out under continuous red light at 23°C .

Elongation of shoots. Shoots with an axis longer than 10 mm after at least three cycles were cultured on a medium without plant growth regulators, but with an enhanced concentration of ammonium nitrate (600 mg l^{-1} ; BEMB/600) in order to support shoot elongation. After shoots had reached a length of 30 mm, they were transferred to standard cultivation medium B1 (Ewald et al., 1997).

2.3.4. Adventitious Bud Formation – Adult Material

Induction of adventitious buds and subculturing. The induction and development of adventitious bud clusters was carried out as described with juvenile material, but using long-shoot buds in October. Physical culture conditions were identical to juvenile material. In some cases the very first induction medium was supplemented additionally with 0.5 mg l^{-1} benzylaminopurine (BAP) to increase the number of buds formed. In the following cycles BAP was omitted to avoid the inhibition of shoot elongation, as had been observed in experiments using BAP for repeated induction steps with different conifers (larch, spruce, yew). The establishment of a well propagating culture of adventitious bud clusters of adult larch continued for a period of at least 2 years (appr. 8 propagation cycles). In the following period, the medium Wz gluc ($+1.5 \text{ mg l}^{-1}$ zeatin) was used to induce buds. In this way the elongation potential could be enhanced.

Elongation. The beginning of the elongation of adventitious buds was achieved, and was visible as a 1–2 mm stem-like region at the base of buds (Figure 11). This occurred after a complete propagation cycle (12 weeks) followed by repeated phytohormone-free subcultures (BEMB/200). Spontaneous rooting was sometimes observed in these

little shoots (Figure 1J – see also 2.4.2.). Such shoots were the basis for ongoing experiments to induce a controlled elongation comparable to the short-shoot stimulation method mentioned before.

2.3.5. Preconditioning of Adult Plant Material (Micrografting)

The micrografting procedure consists of grafting into the top of the sprouting stem axis (epicotyl) after removal of the shoot tip of a very young seedling *in vitro* (Ewald & Kretzschmar, 1996). The method was carried out with disinfected seedlings grown directly in Jiffy-7 peat pellets (\varnothing 38 mm, JIFFY Products Ltd., Norway) in a petri dish. When the epicotyl stem axis of the seedlings reached about 20–30 mm, the upper 10 mm was excised and discarded and the remaining portions of the seedlings were used as rootstocks. It was important to use a sterile or semisterile system to prevent fungal infections of the grafted meristem as well as of the surface of the grafting union. Larch seeds were disinfected as already described. Jiffy peat pellets were fully saturated with deionized water and were autoclaved three times in intervals. Afterwards two peat pellets were transferred into one sterile petri dish (105 mm in diameter). Germinating seeds were placed directly into these peat pellets. Because a minimum length of at least 20–30 mm of the newly formed stem axis is necessary, a longer germination period (appr. 12 weeks starting in June) had to be calculated. Twigs (30 mm) of adult selected donor trees were harvested in October and treated with a fungicide (e.g. 0.2% Euparen by BAYER, 50% dichlorfluanide, w/v) one day before use. Twigs were disinfected as already described. The grafting procedure was carried out under a stereomicroscope in a laminar flow box. To prevent dehydration of the meristem as well as of the cleft of the rootstock, a step-by-step system was developed to make rapid grafting possible. Two kinds of scalpels were used: normal scalpels with replaceable blades and special, extremely sharp small scalpels made of razor blades (handled with a holder) to cut out the meristem and to transfer it. All blades were disinfected during the grafting procedure by wiping them on a wet sponge saturated with 0.4% peracetic acid solution. The petridish with the rootstock was opened briefly and needles along a 15 mm length of the green elongating part (grafting area) of the seedling were removed with a scalpel.

- 1) The twig with the terminal long-shoot bud (Figure 1D) was placed under the microscope. The bud scales were removed with a normal scalpel and discarded. A horizontal cut in the direction of growth near the meristem provided an even surface. A parallel cut behind the meristem counter to the direction of growth provided an area in which the meristem was situated.
- 2) The rootstock was placed under the stereomicroscope again. The shoot axis was cut and a cleft of approximately 3 mm was made in the top with the razor-blade-scalpel.
- 3) The prepared twig with the pre-cut meristem was placed under the microscope again. Two wedge shaped cuts were made in opposite directions to cut out the apical meristem complex (like a piece of cake – about 0.3–0.5 mm in diameter). Dissected tissue included the apical dome and the first ring of needle primordia to prevent desiccation of the meristem after insertion. The first cut was made on

the left and the second on the right side. The meristem was taken out with the left side of scalpel blade to avoid gripping and damaging it with forceps (Figure 1E).

- 4) Afterwards, the meristem was subsequently fixed onto the blade side for transfer and placed in the cleft cut within the rootstock, using the blade to open the cleft and strip off the meristem.

The root system was not disturbed during or after the grafting process because the grafting was carried out within the petridish. All rootstocks and micrografts were cultured at 23°C under continuous red light conditions. After formation of a graft union, the grafts were potted with the peat pellet and transferred to the greenhouse for weaning (Figure 1F). After sprouting of shoots and development of plants, all micrografts should be checked by suitable isozyme analysis. Undetected adventitious bud formation from rootstock material occurred frequently.

Cutting propagation. After 4 years, cuttings taken from micrografts and rooted in June/July with a 2 g l⁻¹ indolyl butyric acid (IBA) containing rooting paste (Figure 1G) resulted in fast growing plants 5 years later. This was impossible directly from the adult donor trees. These plants behaved like seedlings (Figure 1H) and did not show any precocious flower formation.

2.4. Rooting of Shoots

2.4.1. Juvenile Material

Root induction after serial propagation. Newly formed shoot tips, approximately 30–40 mm long and without any visible bud primordia, were subcultured for root induction on L9-medium supplemented with 2 mg l⁻¹ naphthalene acetic acid (NAA) for 2 weeks. The induction by use of NAA was found to be more beneficial than that with IBA (Dembny & Zoglauer, 1992). During root induction and development, the temperature was reduced to 17°C and the photoperiod consisted of 16 h white light (OSRAM L58 W/31-830).

Root development. After 2 weeks, the induced shoots were transferred directly into Jiffy-7 peat pellets (Ø 42 mm) saturated with water. Physical conditions were identical to root induction. The peat pellets were placed into small plastic greenhouses. Growing peat moss (*Sphagnum spec.*) placed in-between the peat pellets avoided a fungi attack which otherwise occurred very often.

Rooting of larch shoots is only possible if the shoots are not vitrified. Rooting rates up to 100% and high quality root systems were obtained. For optimal root development, controlled environmental conditions are very important. Slightly reduced temperature has been described as beneficial for root initiation and development (Hübl & Zoglauer, 1991). Survival of plantlets during rooting and hardening was high, if the temperature was between 17 and 18°C. In a few experiments, not reported here, an increase to 23°C had a detrimental effect on plantlet survival. The root development was undisturbed in JIFFY peat pellets and the roots received an optimal oxygen supply which was better than in agar.

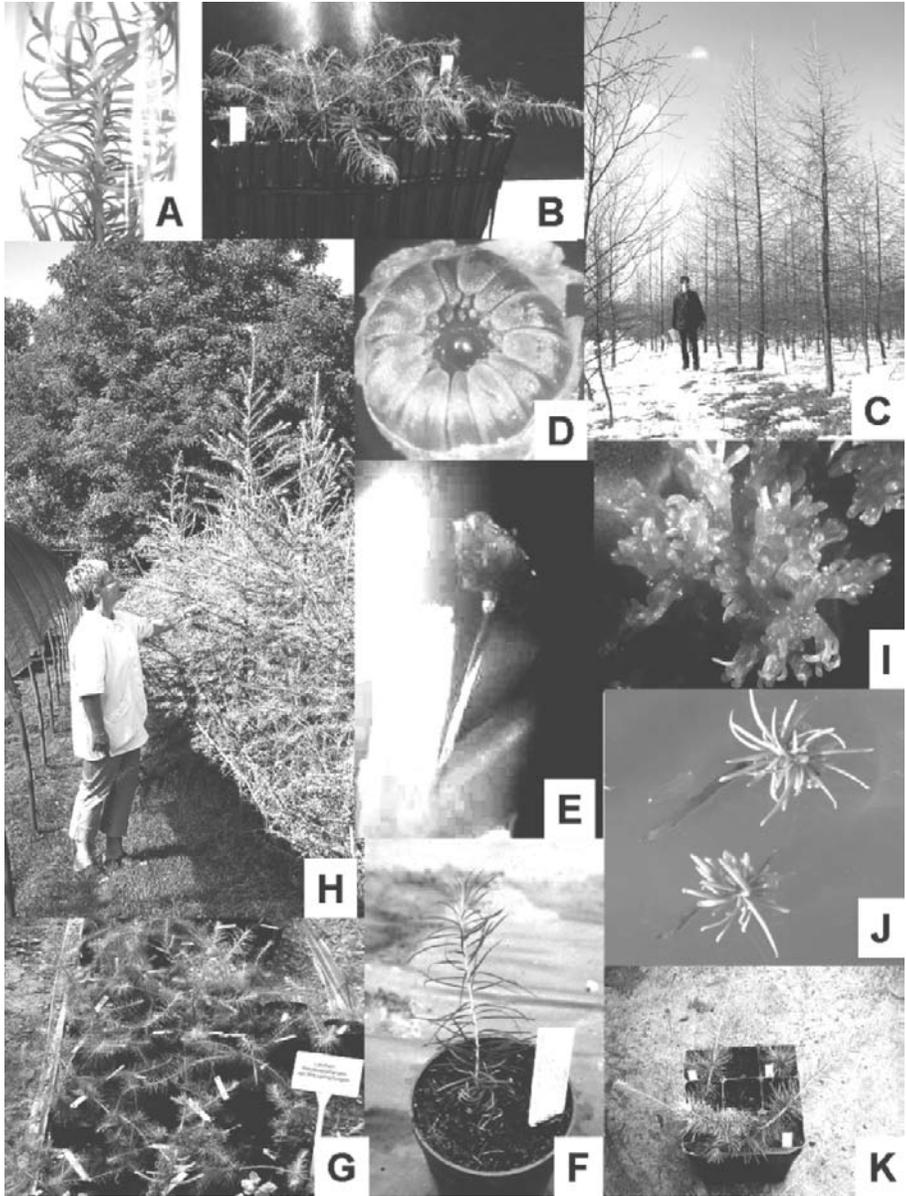


Figure 1. Larch, Serial propagation: bud bearing long-shoot A), plagiotropic growth B), 8-year-old field trial C), Micrografting: long-shoot bud D), cut meristem E), micrografted plant F), micrograft derived cuttings G), 6-year-old cuttings H), Adventitious buds from adult plants: elongating adventitious buds I), spontaneously rooted adventitious buds J), elongating plants from rooted adventitious buds K).

2.4.2. Adult Material

Rooting of elongating shoots from serial propagation. Shoots derived from established propagation cultures were rooted under conditions identical to juvenile shoots, by auxin induction followed by root development in JIFFY-peat pellets.

Rooting of adventitious bud derived shoots. Although the adventitious buds had not developed into long-shoots, single adventitious buds rooted spontaneously in certain conditions. This happened within a propagation cycle during the second subculture free of phytohormones (8th to 12th week) or after additional repeated subcultures free of phytohormones (BEMB/200). It was concluded that at least a partial rejuvenation had occurred. The addition of 200 mg l⁻¹ spermidine to medium BEMB/200 increased the spontaneous root formation up to 11%. Rooted adventitious buds were subcultured on medium BEMB/200 until the root reached a length of 20–30 mm.

Root development and transfer to soil. Rooted buds were transferred to a liquid medium (SH1/2) on a raft in a sterile hydroponic system. There they formed typical short-shoot characteristics. The reduced mineral medium forced root growth. Rooted short-shoots (>100 mm root length) were transferred to the soil and weaned. Some formed a long-shoot after 6–12 months and became upright growing plants (Figure 1K).

2.5. Hardening and Transfer to the Field

2.5.1. Juvenile Plant Material

After 3 months, rooted shoots were transferred together with the Jiffy-7 peat pellet into Rootainers (RONAASH Ltd. Scotland, 4.5 × 4.5 cm, 20 cm high, each for 40 plantlets). The root was not disturbed that way and could continue growing. The containers were placed under a high-pressure-fog system until the plants resumed shoot growth. Air humidity was reduced continuously after 1 month. Plagiotropic growth behaviour became visible after 2–3 months (Figure 1B). Therefore it became necessary to transfer these plants to the nursery or field. The imbalance of fast shoot growth and slower root growth led to the appearance of plagiotropic growth (Ewald, 2000). A larch seedling is characterised by fast root growth in the first year. Based on this root, it starts orthotropic growth in the shoot. *In vitro* larch plants derived from organogenesis showed a relatively fast shoot growth. The root was formed adventitiously and therefore it had to reach an appropriate size to keep the plant growing upright (orthotropic). The faster the transfer to the field the shorter was the period until the plants grew straight. According to our observations, there was no precocious aging due to micropropagation (time until first flower formation), which might have been the case as a result of plagiotropic growth. Plants started to form the first flowers at around the age of 5–7 years like ordinary larch seedlings. The performance of juvenile larch plants propagated *in vitro* was comparable to seedlings, as shown in field trials (Figure 1C, Schneck & Ewald, 2001). Two of the best clones from a field trial were reestablished after successful testing at the age of 10 years. This showed that it was possible to micropropagate juvenile larch, to evaluate these clones in field trials and to re-establish those with the best growth behaviour and stem form after a test period of 10 years.

2.5.2. Adult Plant Material

Plants derived from serial propagation were hardened and transferred to the field like juvenile material. Cutting-derived plants from micrografts 4 years after rooting were comparable to juvenile plants after micropropagation. This was based on all criteria observed (e.g. growth behaviour, time until flower formation). Micrografting restored the rooting capability and is, from our point of view, at present the method of choice for applying an *in vitro* method as a step in improving vegetative propagation of adult larch.

3. CONCLUSION

Larix species can be micropropagated by several methods of organogenesis using juvenile plants. Adult selected trees have been, until now, difficult to propagate due to a lack of knowledge concerning triggering of shoot elongation. Micrografting *in vitro* can support the rejuvenation process of adult donor trees to overcome difficulties in cutting propagation.

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CHAPTER 13

PROPAGATION OF SELECTED *PINUS* GENOTYPES REGARDLESS OF AGE

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1. INTRODUCTION

Most of pine trees do not reproduce naturally from sprouts. Reproduction from artificially rooted propagules of pines has been proved as a successful reproduction method in several countries. This leads us to two different ways to obtain artificial reproduction. Hedging method is used for mass-producing large number of symmetrical and straight cuttings and for maintaining the juvenile nature of propagules (Libby et al., 1972). It has been widely used in breeding programs of plant species for preservation and multiplication of the desired genotypes to establish seed orchards. In this technique we include micrografting which is useful to rejuvenate adult material (Fraga et al., 2002b). Plantlet regeneration from embryos and cotyledons is another approach (Aitken-Christie et al., 1981), it has a great potential for forest tree

multiplication compared to other alternative techniques, such as cutting propagation. In this chapter we describe protocols for *in vitro* micropropagation of selected *Pinus* species like radiata pine (*Pinus radiata* D. Don), austrian pine (*Pinus nigra* Arn.) and caribbean pine (*Pinus caribaea* Mor).

2. *IN VITRO* APPROACHES

Micropropagation is an attractive alternative for the multiplication of selected genotypes. Taking into account the actual barrier that exists in this area for achieving industrial plant production in the following sections we will pay special attention to micropropagation techniques, considering the available information from previous reports and also our own experience with juvenile and mature radiata, austrian and caribbean pines (Rodríguez, 1990; Fraga et al., 2002a,b, 2003; Uribe et al., 2004; Rodríguez et al., 2005b).

A successful micropropagation technique is strongly influenced by the genotype and chronological/physiological age of the donor plant (Rodríguez et al., 2004a,b,c, 2005b). This work is presented in three sections: I) Morphogenesis from embryos and seedlings (Figures 1 and 2a), II) Micropropagation from trees younger than 5 years (Figure 2b), and III) Micropropagation from mature trees (Figure 2c–e). Thereby, the extent of currently available information (Horgan, 1987; Smith, 1997; Fraga et al., 2002a,b; Diego et al., 2004; Rodríguez et al., 2004c) would be of great help to overcome the limitations facing *in vitro* plant propagation.

2.1. *Explants and Sterilization*

Explant selection depends on the final aim of the program. The selection of different explants has shown significant influence on shoot production (Aitken-Christie et al., 1981).

Embryos and cotyledons. To proceed, imbibe seeds in running tap water for 24 h and then stratify in plastic bags for 2 days at 4°C. Seeds are surface sterilized in 5% (v/v) sodium hypochlorite and then washed in sterile water. For embryo culture, dissect embryos from seeds under sterile conditions, or excise cotyledons after aseptically germination (Villalobos & Engelmann, 1995).

There is another similar protocol, place seeds for 30 min in 3% sodium hypochlorite solution containing 0.05% (w/v) of Tween[®] 20 followed by five rinses with sterile water. Once the seed coat is removed the megagametophyte is re-sterilized for 15 min in 15% bleach and for 5 min in hydrogen peroxide (Muriithi et al., 1993). We have proved that mild treatments, 7.5% sodium hypochlorite (v/v) 45 min or ethanol 50% (v/v) 90 min, followed by three rinses in sterile water and after removing seed coats, also work efficiently.

Mature tissues. This type of tissues is difficult to sterilize, and propagation of adult trees can be initiated with different tissues. Apical buds or healthy branch tips are usually collected from vigorous trees, but best responses are obtained when caulinar

terminal portions are taken just prior to active growth manifestation. Any plant material is superficially sterilized dipping into 70% (v/v) ethanol for 15 seconds, washed with sterile water, dipped into a solution of 5% sodium hypochlorite plus 0.05% (w/v) Tween[®] 20 for 15 min, and then washed four times with sterile water in which they were kept until used. On the other hand, mature tissues from field have a higher contamination degree and even endogenous contamination. Protocols based on fungicide–bactericide mixture are used to remove it. Use cuttings 2 cm long from caribbean and radiata selected trees cleaned in laminar flow chamber, stirring continuously in 70% ethanol for 30 seconds and wash with distilled water. Then submerge them in a solution of Benomyl (6 g L⁻¹) plus rifampicin (25 mg L⁻¹) infiltrated with a vacuum pump, wash with distilled water and immerse in sodium hypochlorite 2.5% (v/v) plus Tween[®] 20 0.05% (w/v) solution for 15 min. Finally wash four times with sterile water and put under *in vitro* culture. A mixture of Captan–Benlate in agitation is also employed (Prehn et al., 2003). Another highly effective, but contaminating disinfectant solution is HgCl₂ (0.1–1.0%) for 2–10 min (Gupta & Durzan, 1985).

Micrografting. Apical portion of macroblasts from the selected trees are used as scion source. They are sealed with Parafilm[®] to avoid drying and stored at 4°C for a maximum of 40 days until to be used. Just before sterilization needles are removed and brachyblasts are kept to prevent basal oxidation. Scions composed of basal parts of needles containing an axillary bud (≈40 mm) are sterilized by dipping into 70% ethanol for 30 s in sterile conditions; washed with sterile water, dipped into a 2.5% sodium hypochlorite plus 0.05% (w/v) Tween[®] 20 solution for 15 min, and finally washed four consecutively times with sterile water.

2.2. Media Composition for Organ Culture

There are several culture media used for pine *in vitro* culture, like modified Schenk and Hildebrandt (SH) (Schenk & Hildebrandt, 1972) or Wolter & Skoog (WS) basal medium (Wolter & Skoog, 1966). Most used media have been developed as combination of QL medium (Quoirin & LePoivre, 1977), MS medium (Murahsige & Skoog, 1962) and different growth regulators for each kind of treatment, like benzylaminopurine (BAP), thidiazuron (TDZ), metatopolin (mT), indolebutyric acid (IBA) or naphthaleneacetic acid (NAA) in *P. nigra*, *P. caribaea* and *P. radiata* (Table 1).

In all cases, media are solidified with agar (0.8%) or Gelrite[®] (0.5%), pH is adjusted to 5.8 and media are sterilized for 20 min to pressure conditions of 1 Kg cm⁻² at 120°C. Explants are maintained in sterile conditions at 25 ± 2°C, 70–80 μmol m⁻² s⁻¹ light intensity and 16:8 (day/light) photoperiod.

2.3. *In Vitro* Morphogenesis and Micropropagation from Embryos and Seedlings

Although culture of isolated cotyledons could be an interesting source of new shoots in *P. radiata*, better results are being obtained when whole mature embryos are placed upside down, with cotyledons immersed in the induction medium. QLP has been used as basal medium (Rodríguez et al., 2005b) as shoot induction medium for embryos. QLP supplemented with cytokinins like 22.19 μM BAP, 9.08 μM TDZ or 8.88 μM mT has been usually used.

Liquid medium with same composition is being used for multiplying embryos by temporary immersion systems, with an immersion period of 5 min each 6 h. These systems open the possibility of scaling-up results, achieving 80% of caulogenesis with 10–14 shoot-buds per cotyledon. When embryos or excised cotyledons are first cultured on basal medium, morphogenic capacity declines and reaches to non-responsive situation after few weeks of development on basal media (Figure 1).

2.4. *In Vitro* Vegetative Amplification of Juvenile and Mature Selected Genotypes

The success of *Pinus* micropropagation is highly dependent on original explant genotype and physiological status of donor plant. Therefore, it is very difficult to generalize and to compare shoot production in terms of yield and quality.

Shoots, and adventitious shoots from embryos and seedlings, can be directly included to *in vitro* multiplication culture (Figure 2a). Selected progenies not older than 5 years are able to establish *in vitro* (Figure 2b). Older plant materials remain unresponsive and die shortly before culture establishment. Explants taken from adult trees must be first reinvigorated (Figure 2c–e) before establishment of *in vitro* cultures.

The continuous cultures of shoots, in shoot multiplication QLP₁ and QLP₂ media containing cytokinins, result in limited elongation and unsuccessful rooting. Therefore, in order to establish a highly efficient shoot production chain, it is recommended to use media sequence QLP₂–QLP₁–QLP, associated to a decreasing level of plant growth regulators.

3. MICROGRAFTING, REACTIVATION AND MICROPROPAGATION OF MATURE TREES

Direct *in vitro* establishment of mature explants is not possible in general terms without a previous reinvigoration. Classical *ex vitro* reinvigoration techniques, such as intensive pruning or cascade macrografting, can be used to reinvigorate adult materials (Figure 2c–e).

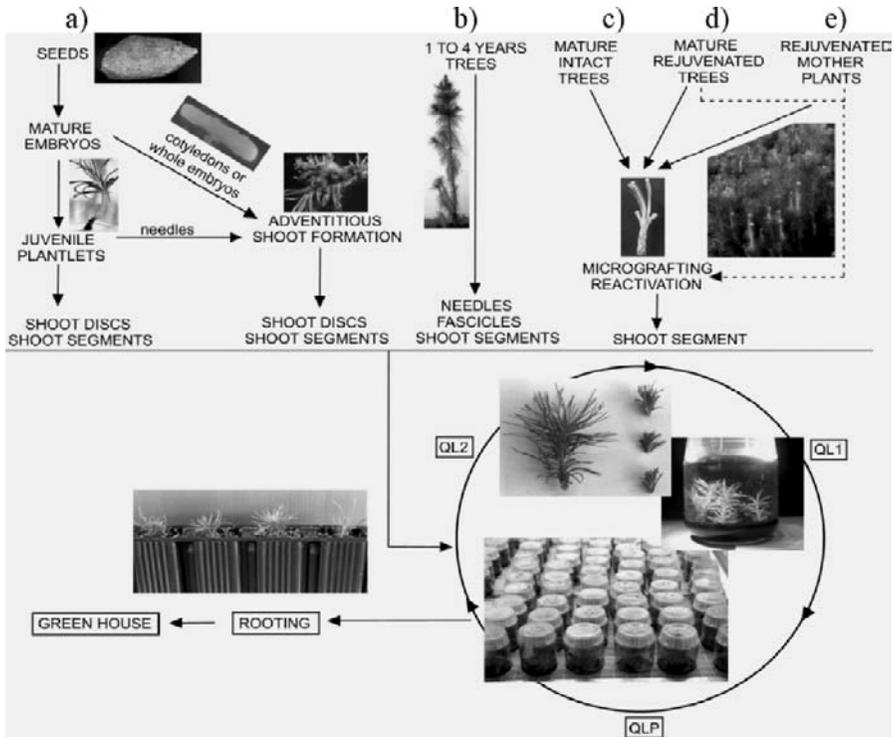


Figure 2. Propagation of *P. radiata*, *P. nigra* and *P. caribaea* genotypes independent of age: (a) morphogenesis from embryos and seedlings, (b) micropropagation from trees younger than 5 years, (c), (d) and (e) micropropagation from mature trees through micrografting, or directly from rejuvenated trees or mother plants.

Scions are obtained from surface sterilizing selected needles (Figure 3a) and cutting its basal part V-shaped (Figure 3b) which allows better scion–rootstock contact. A cut is made underneath cotyledons of 1-month-old seedlings to obtain the rootstock. In its apical part a 3 to 5 mm long incision is made to insert an scion later on (Figure 3c). Rootstock–Scion systems (Figure 3d) are cultured first for 20 days in stimulation medium QLS1. Culture conditions are $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, 16 h photoperiod at $70\text{--}80 \mu\text{mol m}^{-2} \text{s}^{-1}$. After this period, if the scion is established (being green, non-necrotic showing an interphase callus between rootstock and scion), the micrografts are transferred to a QLP culture medium for 40 days. Fully developed scions (Figure 3e) can be directly propagated as juvenile tissue in most of the cases. The proliferation responses develop through consecutive transfers in the following medium sequence: $\text{QLP}_2 \rightarrow \text{QLP}$ or $\text{QLP}_1 \rightarrow \text{QLP}$ being of 25 days each one.

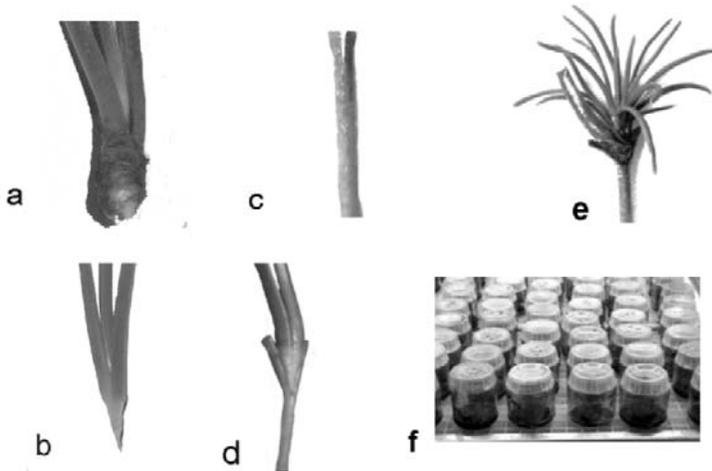


Figure 3. Phases of micrografting in *P. radiata* and *P. nigra*: (a) needles taken from terminal branches were surface sterilized as described before, and then a V cut was made (b) which permits its insertion in a rootstock with a 3–5 mm cleft (c) Rootstock–scion systems (d) were cultured for 60 days till scion was fully developed (e), at this point scions can be used to start proliferative chains (f).

Micrograft development takes place along three phases: after 10 days of culture, an interphase callus between scion–rootstock initiates (establishment). In the following days, vascular tissue between scion and rootstock develops (consolidation) and a subsequent outgrowth of the bud may occur (development). Micrografting has been tested both in juvenile progenies and in different mature trees, obtaining different responses in each case (Figure 4).

Except for explants coming from plant material with limited proliferation ability, the rest of the plant material gradually increased their proliferation capacity. Results of multiplication obtained after 3 consecutive cycles established effective cycloclonal chains and microshoot rooting.

4. ROOTING AND MICROPLANT ESTABLISHMENT

Auxin pulse treatment. There are some protocols in several pine species that use high concentration of IBA. It is possible the use 250 μM IBA for 24 h (Rodríguez et al., 2005a) or 100 μM IBA for 2, 3, 4, 5, 6 h followed by a transfer to a mixture of peat and vermiculite (Muriithi et al., 1993).

Low plant growth regulator concentration. Lower concentrations of IBA and NAA must be used in most cases because they are present for a long time in the plant and may inhibit root development, IAA may be preferred because it is more labile (De Klerk, 2002). In this sense there are some protocols which use low plant growth regulator concentration; use 4.94 μM IBA and 0.54 μM NAA in a medium

containing 1% agar for 10 days and a transfer of the explants for root induction to a mixture of peat: perlite: vermiculite: sand in the ratio of 1:2:2:1 (Bergmann & Stomp, 1994). It is also possible to use 8.2 μM IBA and 5.4 μM NAA for 5 days in 5% agar medium, transferring explants to a $\frac{1}{2}$ LP medium reducing sucrose concentration to 10 g L⁻¹ which facilitates rooting process (Prehn et al., 2003). Also a last protocol uses a 5 day period in agar medium containing 11.42 μM IBA and 2.69 μM NAA prior to shoot establishment into a peat: pumice substrate, which improves rooting to 86%. In all cases *P. radiata*, *P. nigra* and *P. caribaea* rooted plants were observed after 4–6 weeks.

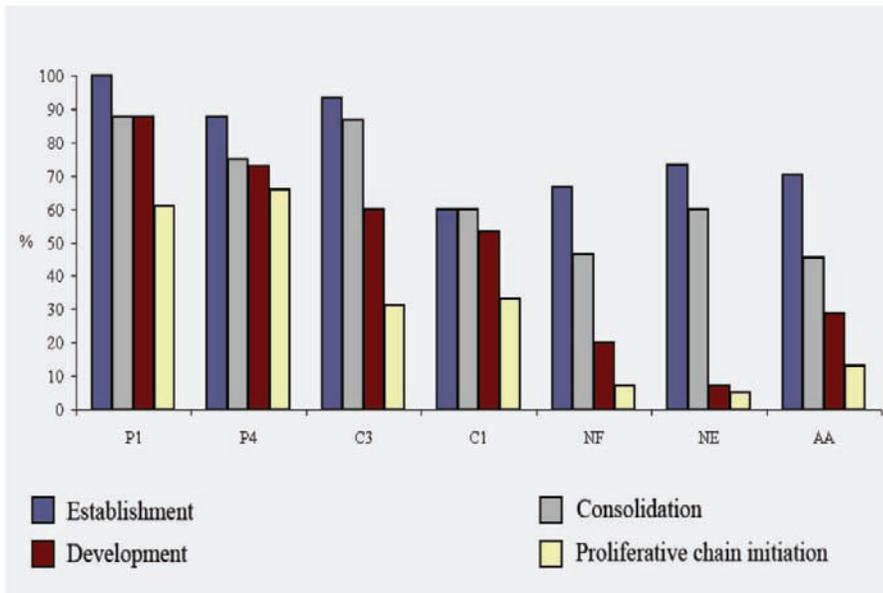


Figure 4. Quantification of micrografting establishment responses and scion outgrowth after homomicrografting in different-origin *P. radiata* materials: Juvenile progenies (J1, J4); mature trees of the same age and origin but subjected to different reinvigoration treatments based in one (C1) or to three (C3) macrograftings; mature trees with different morphogenic potential as limited rooting ability (NE) or not ability to flower (NF); adult field trees older than 30 years (AA).

Bacteria and rooting. Roots can also be induced employing *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens*. This is done either in the presence of IBA, which increases root response, or without it (Bergmann et al., 1997; Li & Leung, 2003). The rooting response depends on the age and origin (direct or indirect caulogenesis) of the plant material, but it is also affected by the proliferation medium. Best rooting induction response was achieved with 1-year-old microshoots (P1). The best culture media for rooting was: (QLP₂-QLP₁-QLP). Several microshoot reinvigoration treatments showed proportional root induction.

5. CONCLUSION

In vitro *Pinus* organogenesis is feasible for several purposes from adventitious organ induction to scaling-up plant multiplication. All desirable responses are restricted by age barrier, which makes difficult reversion to undifferentiated status. Micrografting on juvenile rootstocks is an ideal reinvigoration technique, and it is more effective when donor plant is previously pruned or grafted. Temporary Immersion System facilitates microshoot response, increasing its efficiency. Rooting depends on the quality of microshoots, and basal or low content of plant growth regulators in the culture media greatly enhance microplant production.

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CHAPTER 14

ROOT INDUCTION OF *PINUS SYLVESTRIS* L. HYPOCOTYL CUTTINGS USING SPECIFIC ECTOMYCORRHIZAL FUNGI *IN VITRO*

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1. INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is one of the most widely distributed coniferous species in the world, with a natural range that stretches from Spain to large areas in Siberia (Sarvas, 1964). Genetic improvement of Scots pine by means of conventional breeding is hampered by the long generation time of the species, which is characterized by progressive transition from juvenile to the reproductive mature phase.

Traditionally, the production of clonal material for Scots pine has been based on grafting. However, in seed orchards established using grafting it takes more than 15 years to produce a sufficient number of seeds (Antola, 1990). *In vitro* micropropagation of Scots pine using axillary buds induced on seedlings (Supriyanto & Rohr, 1994) or cotyledons excised from germinated embryos as explants has succeeded at the research level (Häggman et al., 1996). However, in practice the rooting phase has proven problematic particularly because of genotypic variation in the ability to form roots and of increased potential for plagiotrophic growth (Häggman et al., 1996).

Formation of adventitious roots on Scots pine has been studied using hypocotyl cuttings *in vitro*. Depending on the experimental condition, less than 50% of the non-treated cuttings form roots during a 2 months culture period. Treatments with auxin (Grönroos & von Arnold, 1988) and activated charcoal (Grönroos & von Arnold, 1985) can result in both faster rooting and higher rooting frequencies. However, although these methods can induce rooting, results can be variable depending on the culture medium and on timing and concentration of auxin application. Root elongation is also inhibited under some culture conditions, even though the number of cuttings with roots is increased.

In nature, Scots pine lives in symbiosis with specific ectomycorrhizal (ECM) fungi. In this unique interaction, nutrients taken up by the fungus are exchanged for carbohydrates derived from the host plant. The fungi may also release specific plant growth regulators usable to plant roots. The fungal hyphae form a mantle around the short roots of Scots pine and also penetrate between epidermal and cortical cells forming a highly branched structure called a Hartig net (Smith & Read, 1997). The structure of plant roots is strongly modified by ECM symbiosis. Establishment of symbiosis inhibits root hair elongation (Béguiristain & Lapeyrie, 1997; Ditengou et al., 2000) but, conversely, it can stimulate the formation of lateral roots (Karabaghli-Degron et al., 1998; Tranvan et al., 2000; Niemi et al., 2006). In Scots pine, as in other pine species, mycorrhizas are characterized by dichotomous branching of the short roots (Duddridge & Read, 1984; Kaska et al., 1999).

The importance of ECM fungi in the growth and morphology of the roots has resulted in increased interest to use them as promoting agents in adventitious rooting (Niemi et al., 2004). We have developed an in vitro method to induce adventitious root formation in Scots pine hypocotyl cuttings by inoculating them with specific ECM fungi (Niemi et al., 2002a,b). This method has a potential to be used for root induction of micropropagated shoots in vitro. Our method describes the use of specific ECM fungi (*Pisolithus tinctorius* and *Paxillus involutus*) for root induction on Scots pine and may need modifications for use with other host and ECM fungus combinations.

2. EXPERIMENTAL PROTOCOL

2.1. Material

1. Scots pine (*Pinus sylvestris* L.) seeds.
2. Fungal mycelium of the ECM fungi *Pisolithus tinctorius* and *Paxillus involutus*.
3. Sterile water and calcium hypochlorite [$\text{Ca}(\text{OCl})_2$].
4. Sterile filter paper (Schleicher and Schuell 595), glass jars (150 mL), Petri dishes (9 and 14 cm Ø), parafilm.

2.1.1. Sterile Culture Media

Germination of seeds. 0.7% water agar.

Media for fungal cultures. Modified Melin Norkrans (MMN1) medium (Marx, 1969) supplemented with 10 g L⁻¹ glucose and 1.5% (w/v) agar according to Heinonen-Tanski & Holopainen (1991) (Table 1). The pH of the medium is adjusted to 5.8 with 1 N NaOH before autoclaving.

Media for rooting of hypocotyl cuttings. Modified Melin Norkrans medium (MMN2) (Marx, 1969) that differs from MMN1 medium containing 200 mg L⁻¹ glucose and 2% (w/v) agar, and 250 mg L⁻¹ (NH₄)₂HPO₄ instead of NH₄Cl. The pH of the medium is adjusted with 1 M HCl to 5.7 before autoclaving.

Table 1. Composition of Melin Norkrans medium (MMN1) as modified by Heinonen-Tanski and Holopainen (1991).

Component	mg L ⁻¹
KH ₂ PO ₄	500
NH ₄ Cl	250
CaCl ₂ × 2 H ₂ O	66
NaCl	25
MgSO ₄ × 7H ₂ O	150
Thiamine HCl	0.1
FeCl ₃ × 6H ₂ O	0.8

2.2. Method

All the steps are carried out in aseptic conditions in a laminar flow hood. Steps 2.2.1. and 2.2.2. should be started approximately the same date.

2.2.1. Sterilization and Germination of Seeds

1. Surface-sterilize seeds for 20 min in 100 mL of 2% (w/v) [Ca(OCl)₂] solution.
2. Rinse seeds 10 times with sterile water.
3. Transfer seeds with sterile tweezers onto 0.7% water agar in glass jars.
4. Germinate seeds for 3 weeks in a growth chamber at 24 ± 2°C providing a 16-h photoperiod (140–150 μmol m⁻² s⁻¹).

2.2.2. Cultivation of Fungal Mycelium

1. Cut an agar plug of mycelium (5 mm Ø) from the edge of a culture of the ECM fungi *Pisolithus tinctorius* or *Paxillus involutus*.
2. Place mycelial agar plugs onto MMN1 agar in petri dishes (9 cm Ø) and seal dishes with parafilm.
3. Cultivate mycelia for 3 weeks at 21°C in darkness.

2.2.3. Inoculation of Hypocotyl Cuttings

1. Cover the surface of the MMN2 agar with moist sterile filter paper (14 or 9 cm Ø depending on the size of the hypocotyl).
2. Prepare hypocotyl cuttings from 3-week-old seedlings by cutting the stem approximately 5 mm above the root collar.
3. Lay the cutting horizontally on the filter paper covering the surface of the agar (Figure 1).
4. Cut a mycelial agar plug (5 mm Ø) from the edge of the fungal culture and place it beside the base of the cutting (Figure 1).
5. Cover the fungal mycelium and the base of the cutting with a semicircular piece of moist sterile filter paper. This will help prevent desiccation.
6. Seal the petri dishes with parafilm, place them at approximately a 70-degree slant and incubate in the growth chamber under the conditions described for seed germination (2.2.1. above).

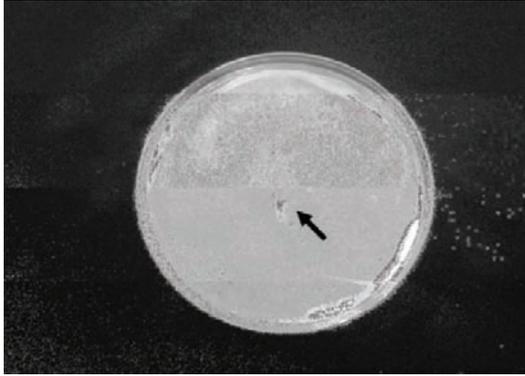


Figure 1. Hypocotyl cutting of Scots pine is laid horizontally on MMN rooting medium covered by filter paper and inoculated with the mycelial agar plug (arrow).



Figure 2. Rooted hypocotyl cuttings of Scots pine after 6 weeks on MMN2 medium. *Pisolithus tinctorius* (bottom left) and *Paxillus involutus* (bottom right) have induced elongation of adventitious roots and primary needles compared to non-inoculated cutting (top center).

Adventitious roots will appear after 2–3 weeks. After 6 weeks, under these incubation conditions, approximately 40% of the non-inoculated cuttings and 80–100% of cuttings inoculated with *Pisolithus tinctorius* or *Paxillus involutus* will have formed roots. The fungi will also enhance elongation of adventitious roots and primary needles (Figure 2). Indications of mycorrhiza formation include the hyphal mantle covering short roots (Figure 3A,B) and penetration of hyphae between root cortical cells (Figure 3B).

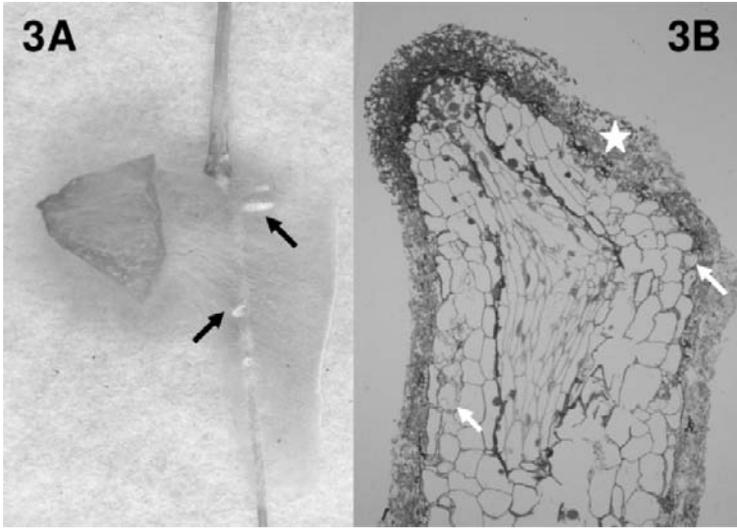


Figure 3. A) The hyphae of *P. tinctorius* cover lateral roots as a mantle (arrows). B) A light micrograph of the lateral root that has started to branch dichotomously due to *P. tinctorius*. The fungal hyphae cover a lateral root (star) and penetrate between epidermal cells (arrows).

3. CONCLUSIONS

Ectomycorrhizal fungi, such as *Pisolithus tinctorius*, can be used to promote adventitious rooting in Scots pine hypocotyl cuttings using the protocol outlined above. This protocol may need modifications for use with other host and ECM fungus combinations. Other ECM as well as arbuscular mycorrhizal fungi and ericoid mycorrhizal fungi have also been shown to improve adventitious root formation. Current and future research on the hormonal interactions between ECM fungi and their host plants will help to elucidate the mechanisms underlying this rooting response to ECM inoculation. Information from this research will be useful for optimizing adventitious root formation during tissue culture and conventional cutting propagation.

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CHAPTER 15

MICROPROPAGATION OF *BETULA PENDULA* ROTH INCLUDING GENETICALLY MODIFIED MATERIAL

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1. INTRODUCTION

Silver birch or European white birch (*Betula pendula* Roth) is medium size deciduous tree, which is naturally widespread in Eurasia and as an escape in North America. According to Organisation for Economic Co-operation and Development (OECD) consensus document on the biology of silver birch (2003), there are some 40 *Betula* species distributed throughout of the northern temperate region. Silver birch is economically the most important deciduous tree species in Nordic countries. In Finland, approximately 15% of growing stock (311 mill. m³) is birch (Finnish Statistical Yearbook of Forestry, 2003), and the birch roundwood is used as a raw material in the chemical pulp industry. Therefore silver birch is the main broad-leaf species of conventional tree breeding in Nordic countries and for instance in Finland and Sweden seed material needed for forest regeneration is derived from seed orchards grown in the polythene greenhouses. These seed orchards have generally been established with grafts but also micropropagated clones are appropriate (Viherä-Aarnio & Rynnänen, 1994). A comprehensive review of the promises and constraints of silver birch breeding, generally and specifically in Finland, is provided by Koski & Rousi (2005).

Molecular breeding of the species is also potential due to the existing genetic transformation techniques (Keinonen-Mettälä et al., 1998; Valjakka et al., 2000) and due to the availability of the extensive expression sequence tag (EST) libraries of the species (Palva, 2000). Vegetative propagation of silver birch can be achieved by several means. Grafts have traditionally been used in establishment of seed orchards. The use of cutting techniques has suffered from a poor rooting success, which has lead to a development of a wide range of *in vitro* techniques. Clonal propagation *in vitro* has succeeded by somatic embryogenesis (Kürten et al., 1990) but it has not

been applied in to a larger scale. Micropropagation has succeeded from leaf callus of young seedlings (Simola, 1985) and also from a mature tree (Iliev & Tomita, 2003). Nevertheless, in most of the micropropagation protocols of the species shoot tips or vegetative buds of adult trees are successfully used as a start material to shoot cultures (Ryynänen & Ryynänen, 1986; Jones et al., 1996). Welander (1993) and Meier-Dinkel (1992) have provided more extensive overviews of micropropagation of *Betula* species including silver birch. Micropropagation has also been used in commercial production of silver birch in Europe. However, both in Finland and Sweden birch micropropagation for forestry, due to regulatory issues, number of clones needed, labour costs, the economical situation etc., has turned out to be unprofitable.

The clonal material of silver birch has also been tested in field trials. Meier-Dinkel (1992), Viherä-Aarnio (1994) and Jones et al. (1996) report the viability of the cloned trees in field experiments. Field tests with 39 clones from 271 selected plus trees planted on 10 different sites have been evaluated up to 10 years of age. Growth traits were under strong genetic influence and showed substantial genetic variation and high potential genetic gain but correlation between growth and growth cessation was weak (Stener & Jansson, 2005). Rousi & Pusenius (2005) studied 8 silver birch clones in two field experiments daily over 6 years. They found that there was large interannual variation in the date of bud burst and especially in the termination of growth, indicating that not only genetic effects but also environmental effects have a strong influence on both bud burst and growth termination. According to Viherä-Aarnio & Velling (2001) the micropropagated plants did not differ from the seed-born plants in growth characteristics or in resistance against pests and herbivores. On the other hand, Laitinen et al. (2004) reported variation in birch bark secondary compounds both between and within clones. Thus, for large scale propagation the importance of pre-testing of the clones in field trials is essential. This has also been taken into account in laws and regulations of many countries considering the use of clonal material in artificial regeneration of forests. The general rule in these regulations being that the better and longer tested the clones are the smaller will be the number of clones (genotypes) which have to be used in the clonal mix intended to be used in clonal plantations.

The main aim of the present article is to describe the *in vitro* propagation protocol options for silver birch leading to well adapted plants to *ex vitro* conditions with good field performance. In addition, the requirements for regeneration of genetically modified material will be considered.

2. EXPERIMENTAL PROTOCOL

2.1. Material

1. Dormant silver birch buds, shoot tips or leaf pieces.
2. Laminar flow hood, growth chamber, stereo microscope, forceps, preparation knives, test tubes, tube caps, baby food jars, magenta caps, magenta jars, parafilm, 70% ethanol.

Table 1. Compositions of culture media for silver birch when shoot tips or vegetative buds are used as explant material. Media: MS – Basal Medium (Murashige & Skoog, 1962), N6 (Welander, 1988), WPM – Woody Plant Medium (Lloyd & McCown, 1980). Plant Growth Regulators (PGRs): BA – 6-benzyladenine, NAA – α -naphthaleneacetic acid, IAA – indole-3-acetic acid, IBA – indole-3-butyric acid.

Medium Composition	Induction			Multiplication		Rooting	
	WPM	MS	N6 ¹	WPM	MS	WPM	MS
Macro-elements	WPM	MS	*	WPM	MS	WPM or 1/5 WPM	1/2 MS
Micro-elements	WPM	MS	MS	WPM	MS or 1/2 MS	WPM	MS
Vitamins	WPM	MS	MS	WPM	MS	WPM	MS
Sucrose (%)	2.0–3.0	3.0	2.0	2.0 or 1.5	2.0 or 1.5	1.0	1.5
PGRs (μ M)							
BA	8.8	4.4	4.4	4.4	4.5		
NAA	0.2		0.005	0.03	1.07		
or				or	or		
BA				2.2	2.22		
IAA				2.85	2.85		
or				or			
BA				4.4			
NAA	4.4						
IBA						0.5	
Agar	0.6–1.0	0.7	0.6	0.6–1.0	1.0	1.0	0.7

Star (*): Macroelements according to Chu et al. (1975). Other components of medium (1): 10 mM ferric-EDTA.

2.2. Sterile Culture Media with Shoot Tips or Vegetative Buds as Explant Material

Induction medium (Table 1). Induction medium to establish shoot cultures: woody plant medium (WPM; Lloyd & McCown, 1980) containing the phytohormones 8.8 μ M 6-benzyladenine (BA) and 0.2 μ M α -naphthaleneacetic acid (NAA) or only 4.4 μ M BA as well as 2.0% or 3.0% sucrose and 0.6–1% agar. Induction on MS-medium (Murashige & Skoog, 1962) with 4.4 μ M BA is also appropriate (Valjakka et al., 2000). In *Betula pendula* var *carelica* shoot cultures were initiated on MS medium with 4.5 μ M BA (Ryynänen & Ryynänen, 1986). The N6 medium supplemented with 4.4 μ M BA and 0.005 μ M NAA has also successfully been used for bud induction by Welander (1988, 1993) and Jansson & Welander (1990). N6 medium

includes the same macronutrients, micronutrients and vitamins as N7 medium described by Simola (1985) and it is used for callus induction as presented below.

Multiplication medium. Multiplication medium applicable for silver birch is WPM with the plant growth regulators 2.2 μM BA and 2.85 μM IAA or 4.4 μM BA together with 0.03 μM NAA or only 4.4 μM BA, 2.0% sucrose and 0.6–1.0% agar. MS medium with 2.2 μM BA and 2.85 μM IAA is also appropriate (Valjakka et al., 2000). In *Betula pendula* var *carelica* bud formation was further induced on MS-medium with 4.5 μM BA and 1.07 μM NAA and shoot development occurred on MS-medium containing half strength of macronutrients, all micronutrients and vitamins, 2.22 μM BA and 2.85 μM indole-3-acetic acid (IAA), 1.5% sucrose and 1.0% agar (Ryynänen & Ryynänen, 1986).

Rooting medium. For silver birch appropriate rooting media are WPM without any growth regulators, containing 1.0% sucrose and 1.0% agar (e.g. Ryynänen, 1999) or WPM with one fifth concentration of the macroelements and indole-3-butyric acid (IBA) at 0.5 μM (Jones et al., 1996), MS without any growth regulators, containing half strength of macro nutrients, all micronutrients and 1.5% sucrose (e.g. Viherä-Aarnio & Ryynänen, 1994).

2.3. Sterile Culture Media with Leaf or Twig Pieces as Explant Material

Callus induction medium (Table 2). As a callus induction medium it is possible to use so called N7-medium (Simola, 1985) which contains the macroelements according to Chu et al. (1975) and microelements and vitamins according to Murashige & Skoog (1962). In addition, the N₇ callus induction medium included 2.3 or 4.6 μM kinetin, 9 or 22.6 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 50 mg l⁻¹ ferric-EDTA, 0.56 mM myoinositol, 0.1% casein hydrolysate, 2.0% sucrose and 0.6% agar.

Callus cultivation medium. Callus cultivation medium (Simola, 1985) included 2.3 μM kinetin, 9 μM 2,4-D, 2.0% sucrose and 0.6% agar. Callus cultivation has also been successful on MS medium including 0.14 mM IAA and 2.3 μM kinetin (Huhtinen & Yahyaoglu, 1973).

Shoot differentiation medium. Shoot differentiation medium (Simola, 1985) included 5 or 10 mg l⁻¹ zeatin or zeatin riboside, 0.5 or 1.1 μM NAA.

Rooting medium. Rooting medium (Simola, 1985) does not necessarily include growth regulators. On the other hand Iliev & Tomita (2003) reported that the highest rooting percentage was achieved when half-strength MS was used together with 2.7 μM NAA and 2.5 μM IBA. Rooting has sometimes also been achieved on half-strength MS medium with 2.9 μM IAA (Lemmetyinen et al., 1998). Our own results indicate that silver birch shoots can be rooted also *ex vitro* without any plant growth regulator treatments. However, *ex vitro* rooting could be improved by submerging the shoots in 2.5 μM IBA for 30 min and then rinsed in water before planting in soil.

Table 2. Compositions of culture media for callus and genetically modified material. Callus and differentiation media: I Callus induction medium, II Callus cultivation medium, III Shoot differentiation medium, IV Rooting medium. Media for genetically modified tissues: I Preculture medium, II Induction medium, III Multiplication medium, IV Rooting medium. Media: MS – Basal medium (Murashige & Skoog, 1962), N7 (Simola, 1985), WPM – Woody Plant Medium (Lloyd & McCown, 1980). Plant growth regulators (PGRs): 2,4-D – dichlorophenoxyacetic acid, BA – 6-benzyladenine, IAA – indole-3-acetic acid, NAA – α -naphthaleneacetic acid, IBA – indole-3-butyric acid, TDZ – thidiazuron.

Medium Compo- sition	For callus				For genetically modified tissues					
	I N7 ¹	II N7	III N7 ²	IV MS	I MS ³	II WPM MS	III WPM MS	IV WPM MS	WPM	
Macro- elements	*	*	*	1/2 MS	MS	WPM MS	WPM MS	WPM MS	WPM	
Micro- elements	MS	MS	MS	MS	MS	WPM MS	WPM MS	WPM MS	WPM	
Vitamins	MS	MS	MS	MS	MS	WPM MS	WPM MS	WPM MS	WPM	
Sucrose (%)	2.0	2.0	2.0	3.0	2.0–3.0	2.0–3.0	2.0–3.0	2.0		
PGRs (μ M)										
2,4-D	9.0	9.0			9.0	1.1				
or	22.6									
BA					4.4		4.4	2.2		
or							2.2			
kinetin	2.3	2.3			or					
or	4.6									
IAA		140.0	0.5	2.9	5.7		2.85	2.85	2.9	
or			1.1	or						
NAA				2.7						
IBA				2.5						
TDZ					0.05	2.3	0.1			
Agar	0.6	0.6	0.6	0.7	0.7–1.0	0.6–1.0	0.6–1.0	0.6–1.0		

Star (*): Macroelements according to Chu et al. (1975). Other components of media (¹): 50 mg l⁻¹ ferric-EDTA, 0.56 mM myo-inositol, 0.1% casein hydrolysate, (²): 5 or 10 mg l⁻¹ zeatin or zeatin riboside (³): 10 mM glutamine.

2.4. Sterile Culture Media for Regeneration of Genetically Modified Tissues

Preculture medium. Preculture medium optimised for *Agrobacterium*-mediated gene transfer of silver birch is (Keinonen, 1999) MSG (Brown & Lawrence, 1968) medium

that is a modified MS medium with glutamine, 9.0 μM 2,4-D and 4.4 μM BA. For biolistic transformation the MS preculture medium with 5.7 μM IAA and 0.05 μM thidiazuron (TDZ) has been appropriate (Valjakka et al., 2000).

Induction medium. Medium appropriate to induce adventitious shoots is WPM with 1.1 μM 2,4-D and 2.3 μM TDZ for material regenerated after *Agrobacterium* mediated gene transfer (Keinonen, 1999) and for material regenerated after biolistic transformation MS with 0.1 μM TDZ (Valjakka et al., 2000).

Multiplication medium. Potential multiplication medium for individual shoots was MS or WPM with 2.85 μM IAA and 2.22 μM BA in the case of shoots derived from biolistic transformation (Valjakka et al., 2000) and WPM with 2.2 or 4.4 μM BA when transformation was done via *Agrobacterium*-mediated gene transfer (Keinonen, 1999).

Rooting medium. Rooting medium used for shoots regenerated after *Agrobacterium*-mediated transformation was WPM with 2.9 μM IAA (Keinonen, 1999) and for shoots from biolistic transformation WPM without growth regulators (Valjakka et al., 2000).

2.5. Method

The regeneration method can be divided into four main steps: explant excision and sterilisation, establishment and proliferation of shoot cultures, rooting, and hardening.

2.5.1. Explant Excision and Sterilisation

1. Collect the dormant silver birch shoot tips, shoot or leaf pieces. Prepare them immediately or let the twigs with dormant buds to vernalise at room temperature (RT) in water containers until the buds start to swell. For genetic transformation experiments use leaf pieces or nodal stem segments derived from *in vitro* shoot cultures (Keinonen-Mettälä et al., 1998; Valjakka et al., 2000).
2. Disinfect the shoot tips or leaves in 70% ethanol for 60, 90 or 120 s (Ryynänen & Ryynänen, 1986; Lemmetyinen et al., 1998). In some cases the explants have needed more severe disinfection. In leaf explants, the ethanol treatment is followed by the immersion of the material in 3% sodium hypochlorite for 2 min (Simola, 1985). For shoot buds or tips 10% sodium hypochlorite (Jones et al., 1996) or 7% calcium hypochlorite (Welandar, 1988) with 0.1% Tween-20 for 15 min. For severely infected material also 0.2% HgCl_2 with Tween-20 for 7 min followed by several rinses with sterile water.

2.5.2. Initiation and Multiplication of Shoot Cultures

1. Remove the outer layers of the apical or axillary bud scales, to expose the shoot apex, and place them on shoot initiation medium. Especially the buds from adult material will secrete high amounts of phenolic compounds into the culture medium quite rapidly and they need to be either replaced in the

medium or transferred to new initiation medium within a few days (Figure 1A). Place the leaf or twig pieces first on callus induction medium and when callus has been formed, generally in 4 weeks, transfer it to shoot initiation medium. Shoot and callus initiation can be achieved in 16-h photoperiod generally between 60 and 114 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 23–25°C, with subculturing every fourth week (Ryynänen, 1999; Keinonen, 1999; Valjakka et al., 2000).

- For shoot multiplication and elongation transfer individual shoots on shoot initiation/multiplication medium. In silver birch it is possible to achieve both adventitious (Figure 1B) or axillary bud induction and shoot multiplication on the same medium (i.e. WPM or MS with 2.2 μM BA and 2.85 μM IAA or WPM with 4.4 μM BA) (Figure 1C). Cultivate in the same photoperiod as during initiation.

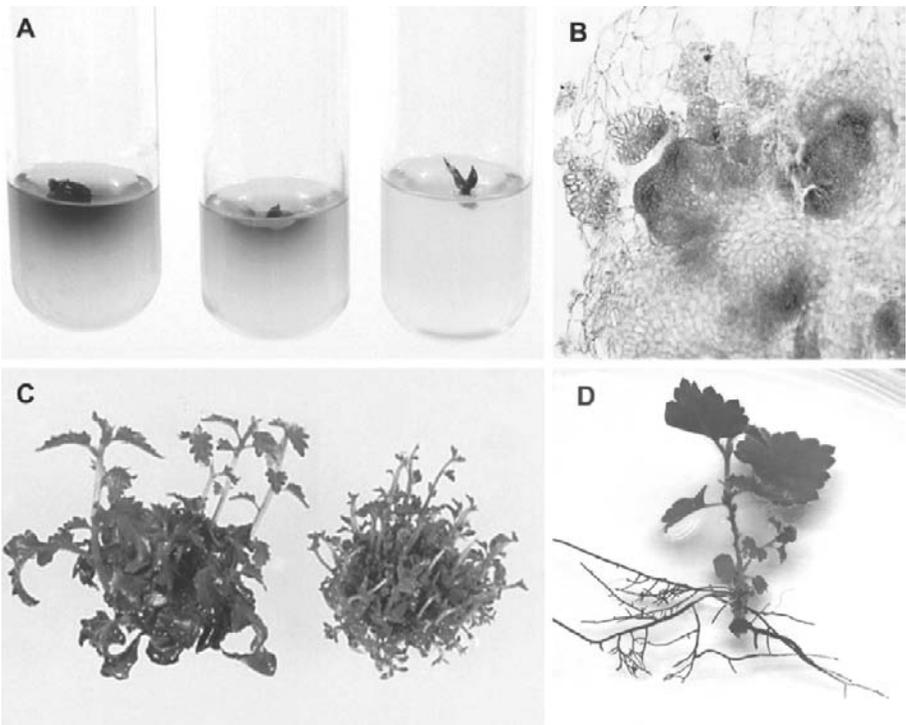


Figure 1. Micropropagation of silver birch (*Betula pendula* Roth) using dormant vegetative buds as explant material. A) Early stages of *in vitro* cultivation of vegetative buds on explants showing severe leakage of phenols into the initiation medium (on the left) and the initiation of new growth (on the right). B) Anatomy of the adventitious buds developing at the base of the *in vitro* shoots. C) Shoot cultures multiplied on N6 medium (on the left) and on the WPM medium (on the right). D) Silver birch shoot rooted on WPM medium. Permission for the use of picture A has been kindly provided by Plenum Publishing Corporation.

3. To improve and succeed in the regeneration of genetically transformed leaf and/or nodal stem pieces of silver birch, the preculture period for 4 to 8 days (Keinonen, 1999; Valjakka et al., 2000) has proved to be appropriate. Although TDZ was necessary for shoot induction, the induced shoots deteriorate if cultivation on TDZ medium is continued. Therefore, the induced shoots have to be transferred into medium containing BA as a cytokinin.

2.5.3. *Acclimatisation and Hardening*

1. Wash the rooted shoots to remove all agar using tap water. It is also possible to transfer non-rooted *in vitro* shoots to *ex vitro* conditions. However, *in vitro* rooted shoots are usually of more uniform quality at least during the early phases of *ex vitro* development than the shoots rooted *ex vitro*. However, if shoots are submerged in IBA before transfer to soil *ex vitro*, rooting is more uniform and faster.
2. Transfer the rooted shoots or shoots for instance into multipots including a wet peat-perlite (1:1 v/v) mixture with (Jones et al., 1996) or without (Ryynänen, 1999) slow-releasing fertilizer or to perlite, unfertilized peat and birch forest soil (2:2:1 v/v) (e.g. Laitinen et al., 2004). Cultivate under decreasing humidity e.g. in propagators for the first 2 weeks before transferring them to the greenhouse conditions.
3. After 4 weeks fertilize the rooted shoots with commercial fertilizers (e.g. 0.2% and followed by 0.3% Superex from Kekkilä, Finland) especially if unfertilized peat has been used before and transplant the plants in individual pots.

3. CONCLUSIONS

Silver birch is an important forest tree species due to wide distribution of the species, economic importance, long conventional breeding practices as well as its potential in molecular breeding and basic research. For many of these approaches the *in vitro* propagation technology available is of utmost importance. For silver birch the micropropagation method is appropriate and applicable for several genotypes although in specific genotypes further optimisation might be needed. Micropropagation of selected genotypes has been used to establish controlled seed orchards in greenhouses and thereby improved the plant material for afforestation. The *in vitro* protocols have enabled the preservation of genetic resources of the species by cryopreservation and it has also been optimised for genetically transformed material. At present, however, silver birch is not directly micropropagated for afforestation purposes in Europe which is more due to high labour costs and legislation regulating the use of clonal material than a technical question. In this paper we are summarising the detailed micropropagation protocols applied for silver birch micropropagation in several tissue culture laboratories.

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CHAPTER 16

PROTOCOL FOR DOUBLED-HAPLOID MICROPROPAGATION IN *QUERCUS SUBER* L. AND ASSISTED VERIFICATION

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1. INTRODUCTION

Cork oak (*Quercus suber* L.) belongs to the family Fagaceae. The tree is robust, up to 20 metres high. The stem may reach 2 metres of diameter. The main raw material produced by this forest species is the corky bark. The cork is a demanded product of economic importance, sustaining an active cork industry. The most valuable manufacture is the cork stopper for wines of high quality.

This forest species has serious drawbacks for the deployment of the classical genetic improvement programmes, mainly based on: 1) its long life and irregular reproductive cycle; 2) low correlation between traits at the juvenile and adult phases; 3) late sexual maturation; 4) the difficulty of seed conservation and of vegetative reproduction; 5) difficulty for the establishment of seed orchards; and 6) the impractical method of repeated backcrossings for the production of pure lines. Somatic embryogenesis has been used to solve the problem of rapid plant propagation of selected trees. A different approach is based on the production of pure lines through doubled-haploid plant regeneration from gametic embryos induced in anther culture.

A protocol for the production of doubled-haploids of cork oak has been developed through anther embryogenesis. By this method, the microspores are subjected to a stress treatment inside the anther cultured *in vitro*. Those microspores leave the gametophytic pathway and react shifting their development to the sporophytic pathway by means of which haploid embryos are obtained. Later on, those embryos develop into haploid plants that can be converted into doubled-haploids. Chromosome duplication either may spontaneously occur or be induced by the application of anti-mitotic chemicals.

2. EXPERIMENTAL PROTOCOL

2.1. Induction of Gametic Embryogenesis

The induction of gametic embryogenesis in *Quercus suber* L. was obtained from anther cultures. During the process, the following relevant factors for the embryogenic response were considered:

1. Correlation between the developmental stages of catkins, anthers and microspores.
2. Influence of a chilling pre-treatment (4°C) on the anther response.
3. Heat shock stress treatments of the anthers.
4. Effect of the addition of activated charcoal to the induction medium for gametic embryogenesis.

2.1.1. Correlation between the Developmental Stages of Catkins, Anthers and Microspores

A key factor for the successful induction of embryogenesis is the adequate developmental stage of the microspore or the pollen grain. Therefore the accurate selection of anther and catkin stages containing a high proportion of embryogenic microspores is crucial. For that purpose, catkins were selected at five different phenologic stages (Figure 1A). Anthers from those stages were dissected and the respective microspores were stained with 4'-6-diamidino-2-phenylindole (DAPI) for the determination of their developmental stage (Figure 1B–E). The anthers were placed on a glass slide in a few drops of 1mg/l DAPI in PBS plus 1% Triton X-100, and tapped softly through the coverglass. Microspores were examined under a Nikon fluorescence microscope and photographed under ultraviolet light ($\lambda=360$ nm) with a digital Coolpix 4500 Nikon camera. We observed a good relationship among the phenologic stage of the cork oak catkin, the anther size and colour, and the microspore stage.

2.1.2. Catkin Selection in *Quercus suber* L.

At the best stage for the induction of gametic embryogenesis in *Quercus suber*, catkins are approximately 20 mm long and 5 mm thick. Flowers of about 2 mm in size start to separate. At this stage, anthers are green-yellowish, of about 1.2 by 1.2 mm size, containing 91% of the microspores in the late uninucleated or vacuolated phase (Figure 1D). The nucleus is moved towards a pole due to the presence of a big central vacuole. These microspores are in the optimal stage for the induction of gametic embryogenesis (Pintos et al., 2005).

2.1.3. Pre-treatment on the Anther Response

Branches bearing catkins (Figure 2B) were collected from selected cork oak trees, transported to the laboratory and preserved in darkness with moist cotton wrapped at the base and enveloped in aluminium foil at 4°C for one, two or three weeks. The highest embryogenic rate was obtained after a pre-treatment at 4°C during one week. Longer cold pre-treatments, e.g., two or three weeks, produce a lower frequency of embryogenesis (0.76% and 0.49% respectively).

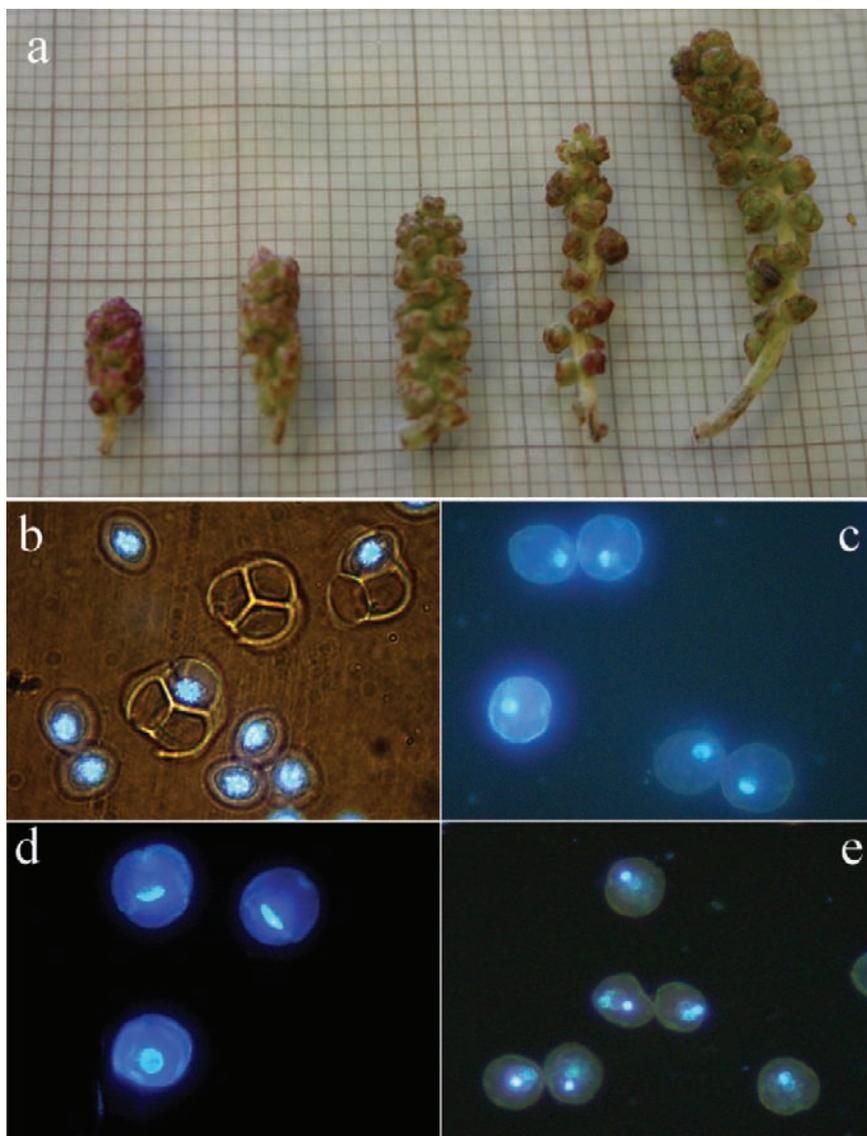


Figure 1. Series of the development of catkins and microspores before pollen release. A) Developmental stages of catkins. B) tetrad stage and microspore release. C) Early uni-nucleated microspore stage. D) Late uni-nucleated or vacuolated stage. E) Early bi-nucleated pollen stage.

2.1.4. *Explant Sterilization*

Catkins were sterilized by immersion in a 2% sodium hypochlorite solution with a few drops of Tween 20 for 20 min, followed by three rinses in distilled sterile water. Anthers were isolated from the catkins in sterile conditions in a laminar flow cabinet and plated in Petri dishes (12 cm diameter, ca 100 anthers per plate) (Figure 2C).

2.1.5. *Heat Shock Stress Treatments of the Anthers*

A temperature shock was applied to the isolated anthers containing vacuolated microspores to induce embryogenesis. No embryogenesis was observed when the anthers were subjected to temperature treatments of 4°C or 25°C between one and ten days, or a heat shock of 37°C. When the anthers were subjected to 35°C for three days, embryogenesis was observed at a very low rate (0.11%). A slightly better rate of embryogenesis was observed with a 33°C treatment between three and seven days, five days being the optimum, with a frequency of embryogenesis of 7.1%.

2.1.6. *Effect of the Activated Charcoal on the Induction of Gametic Embryogenesis*

The induction of embryogenesis from anther cultures of cork oak was obtained on basal medium (abbreviated: SM) (Figure 2C) containing macronutrients (Sommer et al., 1975) microminerals and cofactors (Murashige & Skoog, 1962) and supplemented with 30 g/L sucrose, 10 g/L activated charcoal, and 8 g/L agar. pH: 5.6. (Table 1). Temperature: $25 \pm 1^\circ\text{C}$ in the dark (Bueno et al., 1992a, 2004).

When the medium was not supplemented with activated charcoal, no embryogenesis was induced whatever the heat shock applied. We observed abundant tannin exudates surrounding the anthers, which finally died. The addition of activated charcoal (1%) to the culture medium was necessary to induce anther embryogenesis. Twenty five to thirty days after the heat shock treatment, small globular embryos emerged from inside the anther, breaking through the wall (Bueno et al., 1997) (Figure 2D).

2.2. *Proliferation of Gametic Embryos*

Globular embryos emerged from inside the anther (Figure 2E) and were cultured on SM basal medium supplemented with 500 mg/L glutamine, 30 g/L sucrose and 8 g/L agar (Table 1) and pH 5.6. Temperature: $25 \pm 1^\circ\text{C}$ in the dark (Bueno et al., 1992a). In this proliferation medium, spontaneous secondary embryogenesis occurred, and haploid embryos proliferated (Figure 2F). Every thirty days, these embryos were subcultured on fresh medium of the same composition (Bueno & Manzanera., 1992b). These cultures were maintained along the year, providing embryos for the different experiments of diploidization, maturation and germination.

2.3. *Analysis of the Ploidy Level of Anther-derived Cork Oak Embryo*

2.3.1. *Protocol for Nucleus Release*

For nucleus release, approximately 1 cm²-size of embryogenic mass was chopped with a sharp razor blade in a 55 mm plastic Petri dish containing 400 µl extraction buffer (Partec Cystain UV precise P Kit) and then filtered through a Partec 50 µm

celltrics disposable filter. Small globular embryos, the proliferating tissue of the embryo base and the embryo axis without cotyledons provide a good material for the analysis of the ploidy level. The suspension of released nuclei was stained with 1500 μ l staining solution (Partec Cystain UV precise P Kit) and left for 5 min.

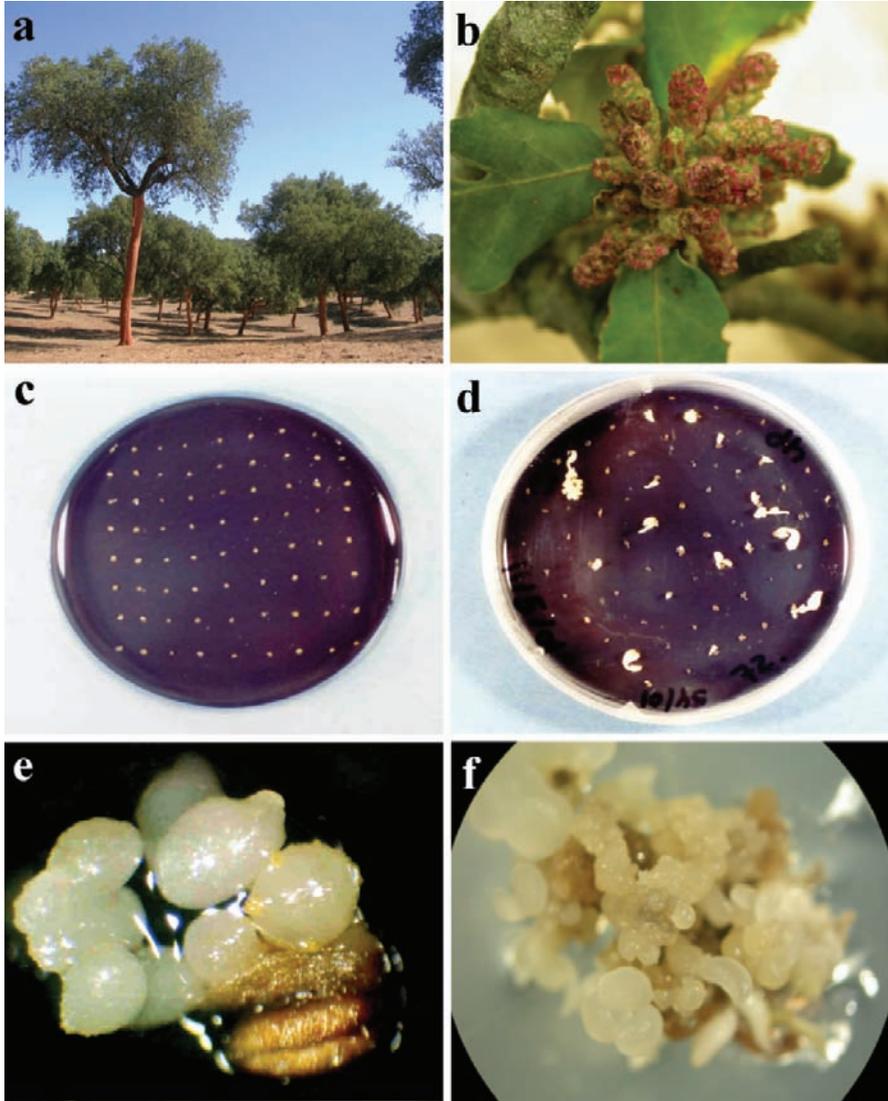


Figure 2. Procedures for induction and proliferation of gametic embryogenesis in cork oak (*Quercus suber* L.). A) *Quercus suber* L. tree. B) Branches with catkins. C) Cork oak anther cultures on induction medium supplemented with activated charcoal. D) Microspore embryogenesis from some cork oak anthers. E) Detail of small globular embryos emerging from inside the anther. F) Proliferation of cork oak haploid embryos.

Table 1. Formulation of culture medium used for doubled haploid micropropagation in *Quercus suber* L. based on modified Sommer et al. (1975) macronutrients and Murashige & Skoog (1962) micronutrients and augmented with culture stage-specific plant growth regulators.

Components	Chemical formula	Stock (g/L)	Medium (mg/L)	
<i>Macronutrients, 100× stock, use 10 ml per L medium</i>				
Potassium chloride	KCl	30	300	
Potassium nitrate	KNO ₃	100	1000	
Calcium chloride-2H ₂ O	CaCl ₂ ·2H ₂ O	15	150	
Magnesium sulfate-7H ₂ O	MgSO ₄ ·7H ₂ O	25	250	
Ammonium sulfate	(NH ₄) ₂ SO ₄	20	200	
Sodium phosphate	NaH ₂ PO ₄ ·2H ₂ O	12.950	129.50	
<i>Micronutrients, 100× stock, use 10 ml per L medium</i>				
Potassium iodide	KI	0.083	0.83	
Boric acid	H ₃ BO ₃	0.62	6.2	
Manganese sulfate-H ₂ O	MnSO ₄ ·H ₂ O	1.69	16.9	
Zinc sulfate-7H ₂ O	ZnSO ₄ ·7H ₂ O	0.86	8.6	
Sodium molybdate-2H ₂ O	Na ₂ MoO ₄ ·2H ₂ O	0.025	0.25	
Cupric sulfate-5H ₂ O	CuSO ₄ ·5H ₂ O	0.0025	0.025	
Cobalt chloride-6H ₂ O	CoCl ₂ ·6H ₂ O	0.0025	0.025	
<i>Iron -EDTA, 100× stock, use 10 ml per L medium</i>				
Iron sulfate-7H ₂ O	FeSO ₄ ·7H ₂ O	2.78	27.8	
Ethylenediamine tetraacetic acid disodium	Na ₂ EDTA	3.72	37.2	
<i>Vitamins, 100× stock, use 10 ml per L medium</i>				
<i>Myo</i> -Inositol		10	100	
Nicotinic acid		0.05	0.5	
Pyridoxine hydrochloride		0.05	0.5	
Thiamine hydrochloride		0.01	0.1	
Glycine		0.2	2	
Ascorbic acid		0.2	2	
<i>Other additives</i>				
Sucrose			30000	
Agar			8000	
<i>Plant growth regulators, glutamine and activated charcoal, add according to culture stage</i>				
Final concentrations (mg/L)				
Culture stage	Glutamine	Charcoal	BAP	IBA
Induction medium	–	10000	–	–
Proliferation medium	500	–	–	–
Maturation medium	–	10000	0.05	–
Germination medium	–	–	–	0.1

2.3.2. Determination by Flow Cytometry

The relative fluorescence of total DNA from isolated nuclei was analysed with a PA Ploidy Analyzer, Partec. Sample size was at least 10,000 nuclei. To determine the standard peak of diploid cells (2C DNA), diploid embryos from zygotic origin were used. The standard peak was adjusted to channel 100 of relative fluorescence intensity. Results were displayed in histograms showing number of nuclei according to relative fluorescence intensity, which is proportional to DNA content. Figure 3 shows examples of histograms of relative DNA content of nuclei released from anther-derived cork oak embryos according to their different ploidy levels. A high percentage of embryos, about 89%, were in fact haploid, confirming their origin from microspores or pollen grains (Bueno et al., 2003). Nevertheless, some exceptions were found, revealing either diploid, triploid genomes or other ploidy levels, 7.9% being diploid, 1.9% haplo-diploid and 1.2% triploid (Figure 3).

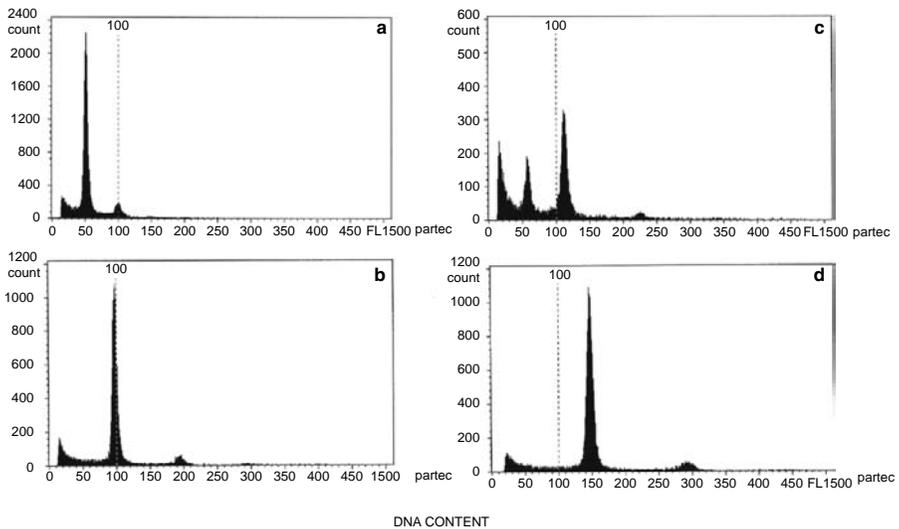


Figure 3. Flow cytometry histograms of relative DNA content of nuclei released from anther derived cork oak embryos stained with DAPI. The frequency (%) of each category of DNA amount is included in the histogram. A) Haploid. B) Diploid. C) Haplo-diploid. D) Triploid.

2.4. Diploidization of Haploid Embryos of *Quercus suber* L.

Haploid embryos were subjected to the antimitotic agent oryzalin for 48 h. Haploid embryogenic masses containing initial translucent globular embryos previously induced in anther culture were immersed in oryzalin (Duchefa®) 0.01 mM in 10% dimethyl sulfoxide (DMSO) and sterilized by ultrafiltration (0.22 μ M). The treatment lasted 48 h in dark at $24 \pm 1^\circ\text{C}$. A treatment with sterile water was used as control. Afterwards, the embryos were rinsed in sterile water, and the excess liquid was removed with filter

paper. Then the explants were subcultured on SM medium supplemented with 3% (w/v) sucrose, 500 mg.l⁻¹ glutamine and solidified with 0.8% (w/v) agar, pH = 5.6.

The explants were viable when survived the treatment and maintained the embryogenic capacity. In the control, survival reached the 96%, while oryzalin 0.01 mM provided at least 78.25% viability. Flow cytometry analysis of haploid embryos treated with oryzalin showed that while control embryos remained haploid, 46.7% oryzalin-treated embryos became diploid (Figure 4) (Pintos, 2005).

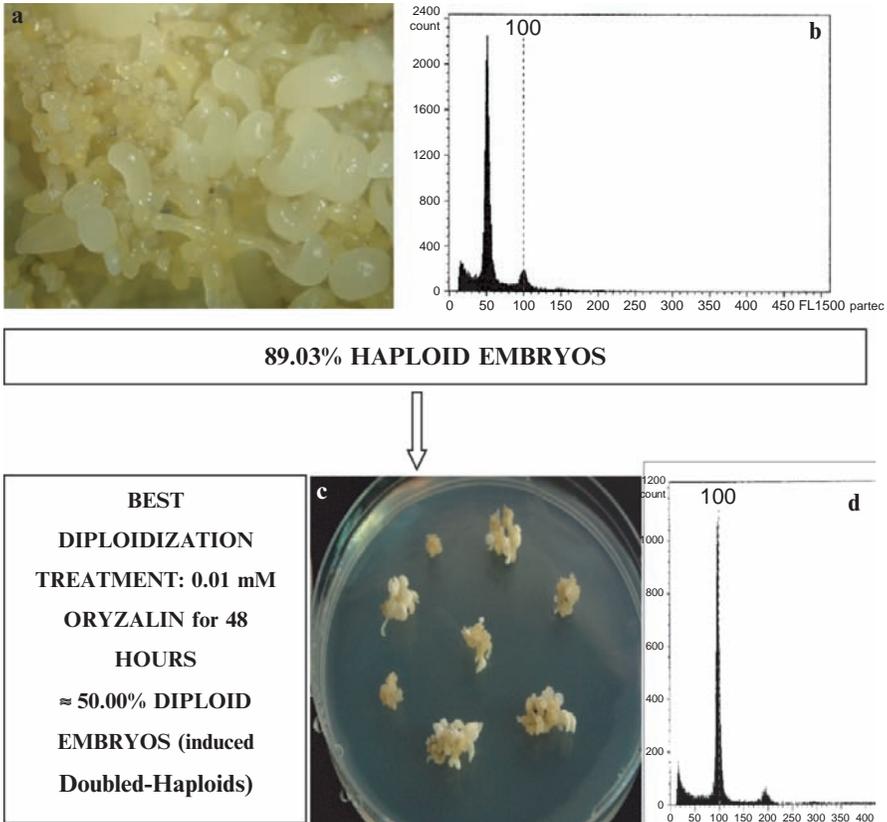


Figure 4. Induction of cork oak doubled-haploids through 0.01 mM oryzalin application. A) Initial haploid embryos. B) Flow cytometry histogram of relative DNA content of nuclei released from anther derived cork oak embryos prior to diploidization treatment. C) oryzalin-treated embryos. D) Flow cytometry histogram of relative DNA content of nuclei released from diploidized cork oak embryos after oryzalin treatment.

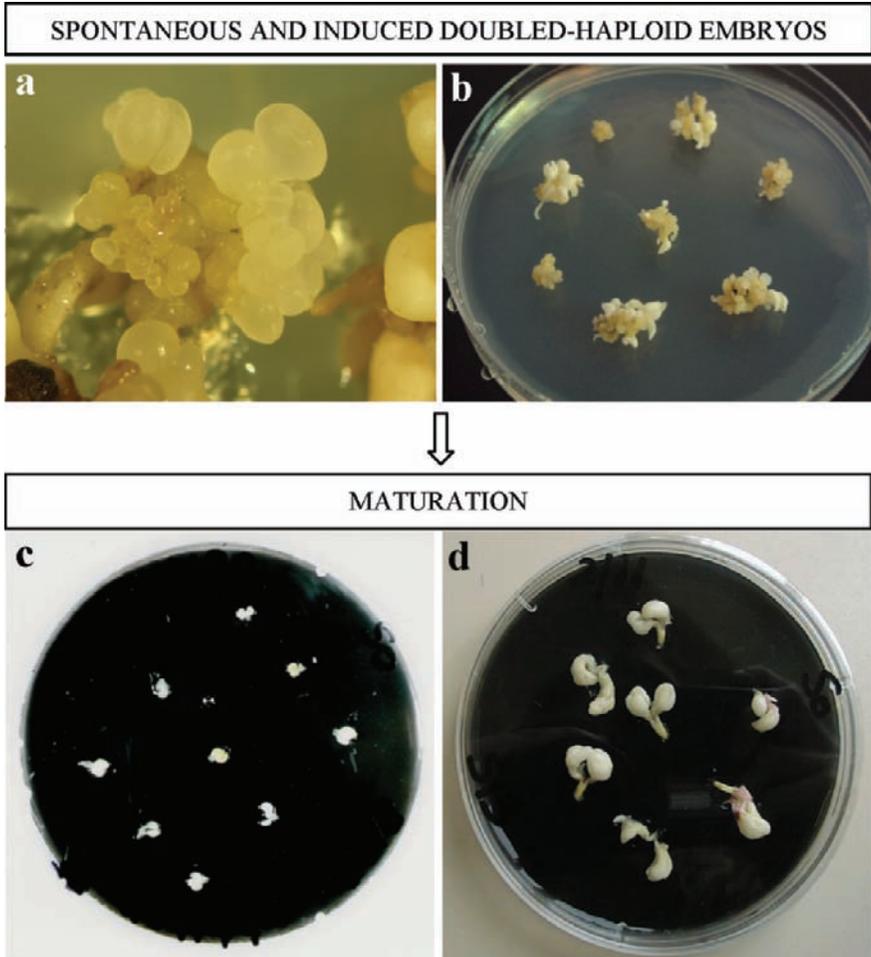


Figure 5. Scheme for the maturation of cork oak doubled-haploid embryos. A) Spontaneously induced doubled-haploid embryos. B) Doubled-haploid embryos induced by anti-mitotic agents. C) Doubled-haploid embryos on maturation medium. D) Cork oak doubled-haploid embryos alter one month on maturation medium.

2.5. Maturation of Doubled-haploid Embryos

Both the anther embryos that spontaneously duplicated their genome and those diploidized by the action of oryzalin were subjected to treatments inducing their maturation (Figure 5A,B). The addition of 1% activated charcoal to the maturation medium provoked a significant increment of both size and weight of the doubled-haploid embryos (Figure 5C,D).

Immature translucent doubled-haploid embryos from either origin were cultured at the cotyledonar stage, about 3–4 mm size and 30 mg fresh weight, on SM basal

medium supplemented with 3% (w/v) sucrose, 1% activated charcoal and solidified with 0.8% (w/v) agar, pH = 5.6. Then the cultures were subjected to a temperature of $25\pm 1^\circ\text{C}$ in darkness for one month and next at 4°C during two months (Pintos, 2005).

2.6. Germination of Doubled-haploid Embryos

Mature cork oak doubled-haploid embryos (Figure 6A) were prepared for the germination treatments by immersion in sterile distilled water for 24 h at 4°C .

The best germination rates (18.7%) were obtained when the mature embryos were cultured on basal SM medium supplemented with 10 g/L agar, 15 g/L sucrose, 0.1 mg/L IBA and 0.05 mg/L BAP. (Figure 6B). The pH of the medium was adjusted to 5.7 and the embryos were cultured under a photoperiod of 16 h light and 8 h darkness. Temperature: $25 \pm 1^\circ\text{C}$.

2.7. Acclimation of Doubled-haploid Embryos

Plantlets germinated in the previous medium were carefully taken out of the test tube and agar was washed from the roots with tap water. Those plantlets were then transferred to nursery pots containing a mixture of peat:perlite:vermiculite 1:1:1 (Figure 7). Afterwards, the plantlets were subjected to a preventive fungicide treatment with 1.8 g/L PREVICUR[®] (propamocarb). The first days, plantlets were acclimated in a chamber with high relative moisture, close to 100% (Figure 8). After acclimation, those plantlets were transferred to bigger pots to permit further growth. Three growth seasons later, they were transferred to soil (Bueno & Manzanera, 2003). A total of fourteen doubled-haploid cork oak plantlets from anther culture were acclimated.

2.8. Marker-assisted Verification

A problem found in the induction of embryogenesis through anther culture was the actual origin of those embryos. Totipotency of plant cells would permit the regeneration either from haploid cells of the anther cavity (microspores) or from diploid cells of the anther wall. The genetic composition of both types of regenerants would differ. Thanks to flow cytometry, we observed in cork oak that the main composition of the regenerated embryos was haploid (89.03%), which clearly indicated their haploid origin, in this case microspores. Nevertheless a small percentage were either diploid (7.87%) or triploid (1.24%) (section 2.3). In order to elucidate if those embryos were originally haploids which spontaneously duplicated or triplicated their genome, or alternatively those embryos were regenerated from the diploid tissue of the anther wall, a genetic test was designed. Analyses were performed through micro-satellite markers.

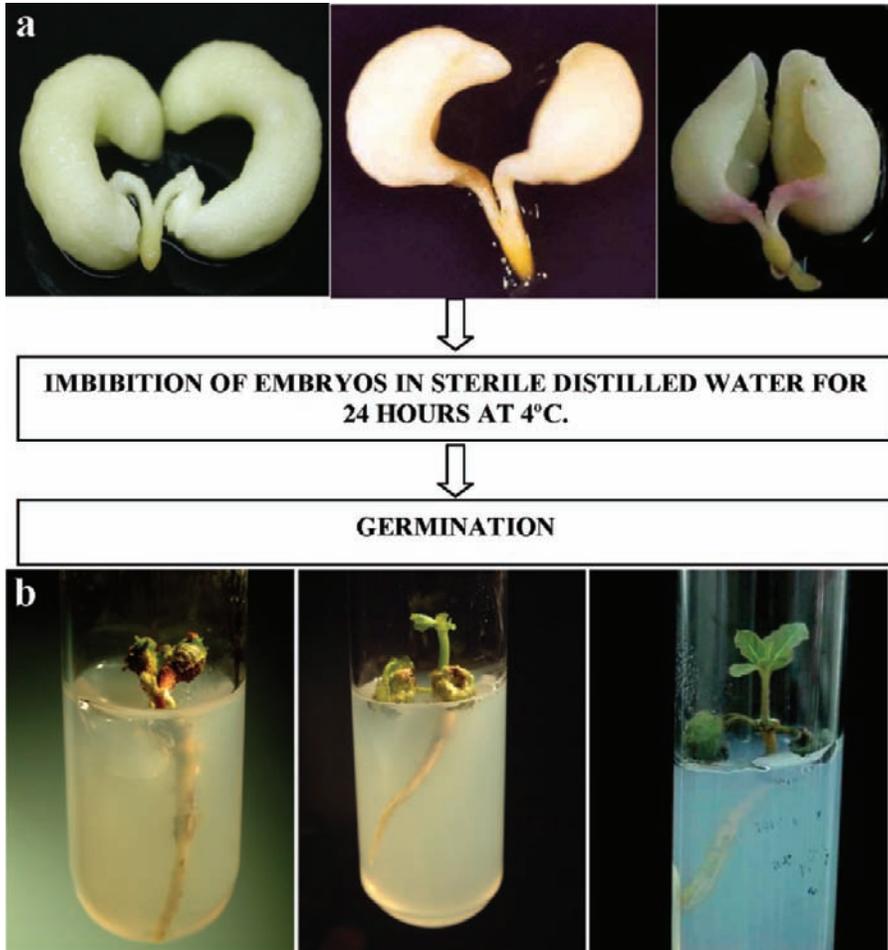


Figure 6. Scheme for the germination of cork oak doubled-haploid embryos. A) Mature doubled-haploid embryos. B) Germinated doubled-haploid embryos.

2.8.1. Verification through Microsatellite Markers

Extraction of leaf DNA. First, DNA is extracted from leaves of the parent trees, following the protocol described by Ziegenhagen et al. (1993). DNA samples were taken from 12 trees.

1. About 200 mg of leaf samples are placed in a sterile Eppendorf tube and 1 ml extraction buffer is added (100 mM sodium acetate pH 4.8, 50 mM EDTA pH 8, 500 mM NaCl and 2% polyvinyl pyrrolidone, PVP). The buffer pH is adjusted at 5.5 and 1.4% sodium dodecyl sulfate (SDS) is added. The mixture is grinded with a sterile stick.

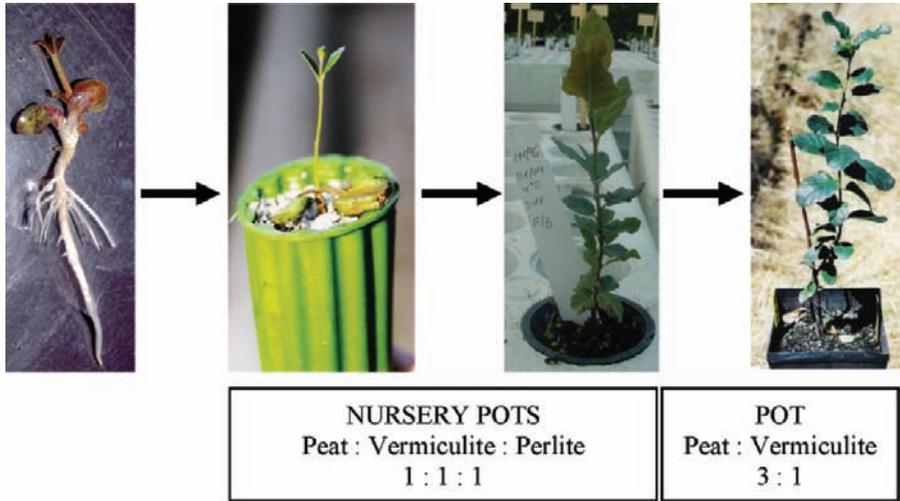


Figure 7. Scheme for the process of acclimation of cork oak doubled-haploid plantlets obtained by anther culture.

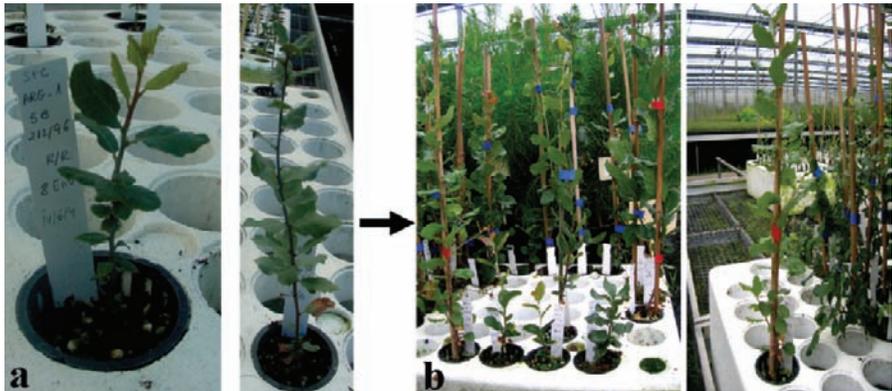


Figure 8. Cork oak doubled-haploid plantlets obtained from anther culture. A) One-year-old plantlets. B) Two-year-old plantlets.

2. The closed Eppendorf tubes are incubated in a bath at 65°C for 20 min and then centrifuged at 10.000 rpm for 10 min. The supernatant is pipetted to a new tube where 1/3 volume potassium acetate 5M, pH 5.2, is added. The tube is gently shaken and incubated for 30 min at 0°C.
3. The samples are centrifuged at 10.000 rpm for 10 min. Then the supernatant is discarded and a 60% volume of isopropanol is added. The tube is gently

shaken and incubated for 30 min at -20°C . The samples are centrifuged again at 10.000 rpm for 10 min. Then the supernatant is carefully discarded and the pellet is dried at room temperature for 10 min. The pellet is resuspended in 200 μl TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8) and incubated at room temperature for 30 min. Alternatively, the suspension may be incubated at 4°C overnight.

4. Afterwards, 200 μl phenol in 1M Tris-HCl pH 8 and hydroxyquinoline are added and the mixture is vigorously shaken. Centrifuge at 10.000 rpm for 10 min. The supernatant is discarded and 200 μl of chloroform are added. Again, the sample is centrifuged at 10.000 rpm for 5 min. The supernatant is discarded and 300 mM sodium acetate, pH 5.2, and 2.5 volume of 96% ethanol are added. The mixture is incubated overnight at -20°C or for 1 h at -80°C .
5. After a new centrifugation at 10.000 rpm for 10 min, the supernatant is carefully discarded and the pellet is dried at room temperature as long as necessary. The pellet is resuspended in 20 μl TE buffer. Finally, the DNA is purified with the cleaning kit GENE CLEAN® (BIO 101).

Extraction of embryo DNA. Also, the DNA of the embryos regenerated from anther cultures from tree 3M was extracted following the protocol of Doyle and Doyle (1990). A total of 24 embryos from different anthers were analysed: Six embryos were taken from an anther (allocated A) in which previous flow cytometry analysis had determined that all embryos from that anther were haplo-diploid. Twelve embryos were taken from two anthers (allocated B and C) in which previous flow cytometry analysis had determined that all embryos from those anthers were haploid. Six embryos were taken from an anther (allocated D) in which previous flow cytometry analysis had determined that all embryos from that anther were diploid.

1. Each embryo is placed in a sterile Eppendorf tube and 100 μl sodium bisulphite 3,8 g/L are added. In each tube, 300 μl extraction buffer (0.35 M Sorbitol, 0.1 M Tris and 5 mM EDTA, pH 8.2) are added. The embryos are grinded with a sterile stick. Then, 300 μl lysis buffer are added (0.2 M Tris, 0.05 M EDTA, 2 M NaCl and 20 g/L CTAB, pH 7.5). Then, 120 μl Sarkosil 5% are added and the mixture is vigorously shaken in a vortex. The tubes are closed and incubated in a bath at 65°C for at least 15 min.
2. Then, the tubes are removed from the bath, 600 μl of chloroform is added and the mixture is vigorously shaken to obtain an emulsion. The sample is centrifuged at 12.000 rpm and 4°C for 10 min. The supernatant is pipetted in a new tube, 400 μl isopropanol are added and the mixture is gently shaken. The formation of DNA bundles may be visible. Then, centrifuge at 12.000 rpm and 4°C for 5 min. The supernatant is discarded and the pellet is dried at room temperature for about 10 min. The pellet is washed with 10 μl ethanol 70% and again let to evaporate. The pellet is resuspended in 50 μl TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8) and warmed with a bath at 65°C for 15 min. Finally, the DNA extraction is preserved at -20°C .

Amplification of cork oak DNA. DNA amplification from leaf extracts of adult trees of *Quercus suber* L. was successfully obtained for three microsatellites assayed (SsrQpZAG15, SsrQpZAG46 y SsrQpZAG110) (Gómez et al., 2001a,b). DNA was amplified with a Perkin-Elmer 9600 thermocycler. The amplification mixture was prepared for a final volume of 25 µl per tube. Tube size was 0.2 ml. The 25 µl reaction mixture contained 20 ng genomic DNA, 200 µM of each primer (the direct primer was fluorescently labelled, Progenetic®), 100 µM of each dNTP (dATP, dCTP, dTTP, dGTP), 50 mM KCl, 10 mM TRIS-HCl (pH 9), 2.5 mM MgCl₂ and 0.5 Units EcoTaq-DNA polymerase (ECOGEN®).

Fluorescently labelled amplification products were separated and analysed in an automated DNA sequencer (ABI PRISM™ 310 Genetic Analyzer, Perkin-Elmer). Homologous fragments of the same microsatellite amplification differing in size, i.e., at least one base pair, were considered “alleles” of the same “gene” or *locus*.

The microsatellite marker-assisted analysis of anther diploid embryos provided evidence of the presence of a single allele per *locus* (Figure 9), that is, those individuals were homozygous for each of the *loci*, while the parent tree was heterozygous for all three *loci*. Similarly, all the anther-derived embryos with haplo-diploid DNA content had a single set of alleles, a part of which was doubled (Figure 9). These results prove that the diploid anther embryos of *Quercus suber* L. are not from somatic but from gametic origin, and subsequently these embryos experienced spontaneous duplication of their haploid genome. These embryos are actually doubled-haploids.

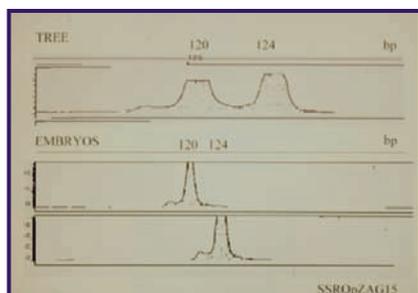
3. CONCLUSION

We have presented a new protocol to obtain doubled-haploids of cork oak (*Quercus suber* L.), from induction to plantlet regeneration. A correlation between the phenologic traits of catkins, the size and colour of anthers and the developmental stage of the microspores has been established. Gametic development was diverted to the sporophytic pathway by temperature stress.

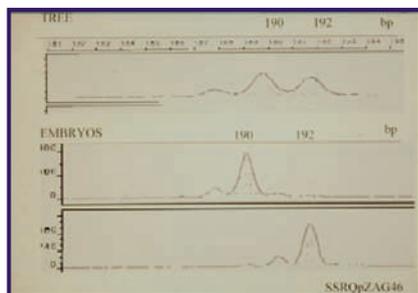
Ploidy level of the cork oak embryos induced from anther cultures was verified by flow cytometry. The haploid genotype was the most frequently observed, although a small percentage of diploid embryos was also found. Microsatellite markers permitted the verification of the gametic origin of those anther embryos which had diploid genome. Spontaneous diploidization of anther embryos was rare. As a consequence, the regeneration of DH plants had to be obtained through treatments with oryzalin 0.01 mM.

Maturation in medium supplemented with 1% activated charcoal provided a significant increase in embryo size and weight. Stratification at 4°C for two months in the same maturation medium favoured the germination on medium supplemented with 6-benzyl-adenine and indole-3-butyric acid, and the subsequent acclimation to soil. Doubled-haploid cork oak plantlets have been established in a field trial.

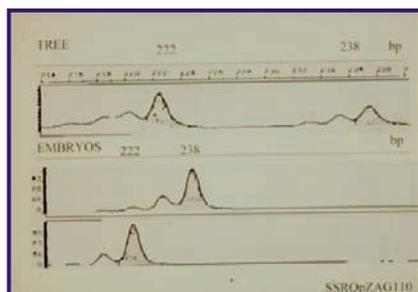
Acknowledgements. This research was supported by project RTA 2005-0018-C02-02 and AGL 2000-0029-P4-03 from the Spanish Ministry of Education and Science. Dr. Pintos was recipient of a PhD grant of the National Institute of Agronomic Research (INIA).



Tree allele (bp)	ANTHER				Total No. embryos
	A	B	C	D	
120	---	6	---	1	7
124	6	---	6	5	17



Tree allele (bp)	ANTHER				Total No. embryos
	A	B	C	D	
190	---	4	---	6	10*
192	6	2	6	---	14*



Tree allele (bp)	ANTHER				Total No. embryos
	A	B	C	D	
222	6	---	6	6	18
238	---	6	---	---	6

Figure 9. Microsatellite (SSR) composition of the parent tree showing heterozygous genotype and of embryo samples, showing only one allele per locus. Allele sizes in bp. Number of anther-induced embryos bearing each allele (*SsrOpZAG15*, *SsrOpZAG46*, *SsrOpZAG110*) detected in cork oak tree. * 1:1 segregation at the 0.05 significance level (chi-squared test).

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CHAPTER 17

IN VITRO PROPAGATION OF *FRAXINUS* SPECIES

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1. INTRODUCTION

The genus *Fraxinus*, a member of the Oleaceae family, includes over 65 ash species native to the temperate regions of the northern hemisphere (Miller, 1955). Several of the ash species are important forest trees noted for their tough, highly resistant to shock, straight grained wood as well as being excellent shade trees for parks and residential areas (Dirr, 1998). Economically, the most important species include white ash (*F. americana* L.) and green or red ash (*F. pennsylvanica* Marsh.) in the United States and Europe or common ash (*F. excelsior* L.), flowering ash (*F. ornus* L.), and narrow leaf ash (*F. augustifolia* Vahl.) in Europe and Asia Minor.

Most ashes are deciduous trees that produce inconspicuous apetalous flowers in terminal or axillary clusters in the spring just before or with the leaves (Dirr, 1998). Fruits are bore in open panicles of elongated, winged, mostly single seeded samaras that mature in late summer or fall. Mature samaras can be dried to 7 to 10% moisture and stored under refrigeration in sealed containers for more than 5 years with little loss in viability (Bonner, 1974). Most species of ash exhibit some form of seed dormancy due to immature embryos, internal growth inhibitors, and/or to impermeable seed coats. The standard treatments to overcome seed dormancy involve various combinations of after-ripening at 20 to 30°C for 30 to 90 days to mature embryos and/or stratification at 1 to 5°C for up to 150 days to overcome internal factors (Bonner, 1974).

Propagation is usually by seed collected in the fall and sown immediately or artificially stratified for 90 to 120 days before sowing in the spring. Reliance on seed propagation for conventional breeding is problematic as it may take 10 to 25 years for trees to attain reproductive maturity and then abundant seed crops may only be produced every 3 to 5 years (Bonner, 1974). Although there are no reliable methods

for rooting softwood cuttings, ash cultivars can be propagated by budding, grafting, and possibly layering (Hartmann et al., 1997). *In vitro* propagation through axillary shoot micropropagation, adventitious shoot organogenesis, or somatic embryogenesis is promising for several of the ash species. The objective of this chapter is to describe the procedures we have used and to compare them to some of the most promising *in vitro* approaches used by other researchers for the different ash species.

2. EXPLANT SOURCES AND DISINFESTATION

2.1. Stored Seed

We have found that *in vitro* establishment of *Fraxinus* species using vegetative buds from non-stratified embryos as explants is easier than using shoot tips or apical buds from seedlings or adult trees (Preece et al., 1987). Seed is easily peeled from the samara and harbors few microorganisms so it is easily disinfested. Seed of most ash species contains a single embryo fully differentiated into hypocotyl, cotyledons, and epicotyl. The embryo is surrounded by endosperm and may extend from half to the full length of the seed with cotyledons pointing away from the wing on the samara (Miller, 1955; Bonner, 1974). The major problem associated with *in vitro* germination of ash seed is overcoming dormancy due to inhibitors within the endosperm or an impermeable seed coat (Preece et al., 1995). Dormancy can be overcome by excising the embryo from the endosperm and testa (Arrillaga et al. 1992b); however, this technique is labor intensive and frequently results in damaged embryos unable to survive surface disinfested with dilute solutions of sodium hypochlorite (NaOCl) or other less commonly used disinfectants.

Excellent *in vitro* germination of non-stratified, surface-disinfested seed of ash is achieved by excising 1 to 2 mm from the end of the seed that contains the tips of the cotyledons (Preece et al., 1989, 1995). To use this approach, each seed has to be marked with indelible ink, surface disinfestations in 1% NaOCl and 0.01% Tween-20 solution for 20 to 30 minutes followed by three rinses with sterile distilled water, then cut under sterile conditions. We have also found removal of approximately 1 mm from both the apical and basal ends of the seed coat was equally effective for white and green ash (Van Sambeek et al., 2001). Germination rates of sound, surface-disinfested seed typically exceed 95% with fewer than 10% of germinants showing microbial contamination during the first month in culture. With either cutting technique, the cotyledons start emerging from seed coat within a week of placement *in vitro* followed within a week or two by an elongating epicotyl. The cut seed technique can also be used with immature seed collected at the liquid-endosperm or seed-filling stages with germination exceeding 80% and more than 60% of these germinates producing elongating epicotyls within 4 weeks (Preece et al., 1995).

2.2. Shoot Tip and Nodal Segments

Many of the early trials on *in vitro* propagation of ash started with the culture of defoliated shoot tips taken from seedlings or from branches cut from adult trees and forced in the laboratory (Browne & Hicks, 1983; Chalupa, 1984; Preece et al., 1987;

Arrillaga et al., 1992a; Perez-Parron et al., 1994). Excised shoot tips or apical buds are most commonly surface disinfested by immersion in 70% ethanol, then in 0.3 to 1.6% NaOCl mixed with a non-toxic surfactant like Tween 20 for 5 to 20 minutes, and, finally, in multiple rinses with sterile deionized water. Preece et al. (1987) found forcing new shoot growth on branches from adult trees was more effective with green ash than it was white ash. Browne and Hicks (1983) reported that more than 60% of white ash shoots excised from branches forced in the laboratory were still free of contamination after 2 to 4 weeks *in vitro*. Perez-Parron et al. (1994) reported less than 20% contamination after 4 weeks for narrow-leaf ash shoots excised from branches forced in the laboratory.

No reports were found that described the grafting of dormant branch tips of adult trees to seedling rootstocks to force new shoot growth as a source of explants for any of the ash species. This approach has been successfully used on black walnut, a species more recalcitrant to *in vitro* culture than ash (Van Sambeek et al., 1997). Laboratory observations indicated that tissues originating from adult ash trees may produce phytotoxic exudates *in vitro* and, like black walnut, may initially require more frequent transfers to new medium than do explants from germinating seeds (Compton & Preece, 1988).

2.3. *Epicormic Sprouts*

We have also experimented with forcing epicormic sprouts in the laboratory or greenhouse on branch segments cut from basal branches or stems of adult trees. Dormant buds on basal branches exhibit many of the traits that the tree possessed when the buds were first formed and are a promising source of juvenile explants for *in vitro* culture. The forcing of epicormic sprouts in the laboratory or greenhouse on stem or branch segments cut from adult trees has been reported for both white and green ash trees (Van Sambeek et al., 2002; Aftab et al., 2005). Explants taken from epicormic sprouts collected in the field or forced under mist are very difficult to surface disinfest (Preece, et al., 1987). However, explants from epicormic sprouts forced in the laboratory or greenhouse with hand watering or drip irrigation are relatively easy to surface disinfest with dilute NaOCl solutions (Van Sambeek et al., 1997; Van Sambeek & Preece, 1999; Aftab et al., 2005).

No published reports were found that described greenhouse or laboratory forcing of epicormic sprouts on branch pieces as an explant source for any of the ash species; however, this technique has been successfully used with silver maple, black walnut, and eucalyptus (Ikemori 1987; Bailey et al., 1998; Van Sambeek et al., 1998a; Aftab et al., 2005). Softwood cuttings of white ash are easily rooted when excised from epicormic sprouts forced in the greenhouse on branch segments cut from basal branches of adult trees (Van Sambeek et al., 1998b; Van Sambeek & Preece, 1999). Rapid rooting of the softwood cuttings with or without auxin treatments is evidence that epicormic sprouts forced on basal branches from adult trees retain more juvenile traits than shoot tips forced on terminal branch tips that are cut from the same adult trees that traditionally are difficult to root or used as explant sources for establishing *in vitro* cultures.

3. MICROPROPAGATION

3.1. Laboratory Procedures

3.1.1. Basal Media

Much of the early research on *in vitro* culture of ash consisted primarily of testing various media and plant growth regulators to identify conditions leading to successful establishment and rapid axillary shoot proliferation. High-salt basal media normally produce the best results based on screenings done with various combinations of nine different basal media and three ash species (Chalupa, 1984; Navarrete et al., 1989; Perez-Parron et al., 1994). For *in vitro* propagation of white and green ash, we use slightly modified versions of MS (Murashige & Skoog, 1962) and DKW (Driver & Kuniyuki, 1984) using 10 or 20 ml of six stock solutions (Table 1). As shown in Table 2, the basal medium is supplemented with various combinations and concentrations of the plant growth regulators thidiazuron (TDZ), benzylaminopurine (BAP), isopentenyladenine (2iP), indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) for the different *in vitro* propagation stages. The pH of the medium is routinely adjusted to 5.6 to 5.8 before addition of plant growth regulators and heating to melt agar when used. No published reports were found comparing effects of different gelling agents; however, 7 to 8% Difco Bacto agar is most often used for agar-solidified medium. Approximately 20 ml of basal medium is added to 25 × 150 ml glass culture tubes capped with semi-transparent, autoclavable Magenta closures or 30 ml of basal medium is added to 120 ml glass jars or Magenta GA7 vessels capped with autoclavable Magenta lids. Basal media are routinely autoclaved at 121°C (1.2 Kg cm⁻²) for 20 to 30 minutes depending on size of culture vessels.

3.1.2. In Vitro Environment

Established cultures are routinely transferred or subcultured to new medium monthly inside a laminar flow hood disinfested with 70% ethyl alcohol. Cultures are normally maintained on open shelves in climate-controlled laboratories (26 ± 3°C). Shelves are lighted with 40-watt cool white fluorescent lamps providing 35 to 40 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ of photosynthetically active radiation with a 16-h photoperiod.

3.2. Micropropagation by Axillary Shoot Proliferation

3.2.1. In Vitro Establishment from Seed Explants

For *in vitro* germination of white or green ash seeds, we typically place surface disinfested, cut seeds on agar-solidified medium (Figure 1). We have published on several techniques that can be used to accelerate the establishment phase when using cut seeds for both white and green ash (Navarrete et al., 1989; Preece et al., 1995; Van Sambeek et al., 2001). Changing the concentration of the cytokinin analog TDZ in EEM (Table 2) affects *in vitro* establishment and growth to a greater extent than does changing concentrations of 2iP or BAP (Figure 2). We found epicotyl elongation and axillary shoot initiation from the cotyledonary node of germinating ash embryos can be accelerated by adding a liquid overlay of the establishment medium

midway between the monthly transfers to new agar-solidified medium. Inserting the radicle end of the emerging germinate into the solidified EEM before applying the liquid overlay will strongly inhibit radicle elongation and promote axillary shoots from the cotyledonary node. Typically over half the white and green ash germinants possess visible epicotyls ranging from 4 to 10 mm in length and cotyledons ranging from 25 to 40 mm in length after the first month of culture.

Table 1. Composition of stock solutions for preparation of MS and DKW basal media.

<i>Stock solution and components</i>	<i>for MS medium</i>	<i>for DKW medium</i>
	<i>g/L</i>	<i>g/L</i>
<i>Stock solution A (nitrogen):</i>		
Ammonia nitrate	82.5	98.0
Potassium nitrate	95.0	—
Calcium nitrate	—	98.0
<i>Stock solution B (sulfates):</i>		
Magnesium sulfate heptahydrate	18.5	37.0
Potassium sulfate	—	78.0
<i>Stock solution C:</i>		
Calcium chloride dihydrate	22.0	7.35
Potassium phosphate	8.5	13.0
<i>Stock solution D (chelated iron):</i>		
Ferric sulfate heptahydrate	1.39	1.65
Sodium ethylene dinitrotetraacetic acid	1.88	2.25
<i>Stock solution E (micronutrients):</i>		
Manganese sulfate monohydrate	1.110	1.700
Zinc sulfate heptahydrate	0.430	—
Zinc nitrate hexahydrate	—	0.850
Boric acid	0.310	0.250
Potassium iodide	0.042	—
Sodium molybdate dehydrate	0.013	0.020
Cupric sulfate pentahydrate	0.0013	0.0125
<i>Stock solution F (organics):</i>		
Myo-inositol	5.00	5.00
Glycine	0.10	0.10
Pyridoxine hydrochloride	0.025	—
Nicotinic acid	0.025	0.05
Thiamine	0.005	0.10

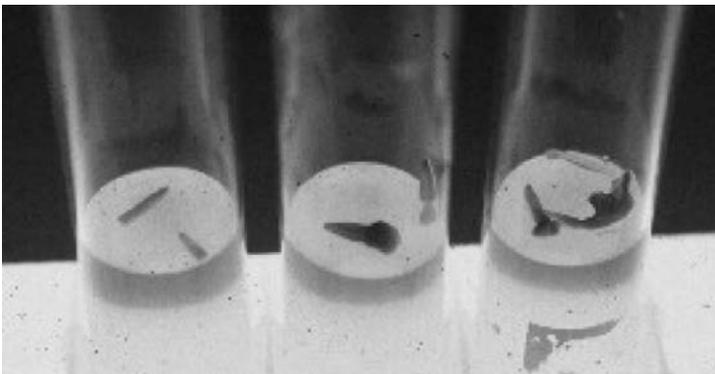
Add 20 ml of each stock to 800 ml of distilled water before bringing final volume to 1 L. Add sucrose and plant growth regulators, adjust pH to 5.6 to 5.7 before autoclaving, add and melt agar, then dispense into culture vessels before autoclaving.

Table 2. Plant growth regulator concentrations within different media for *in vitro* ash propagation.

PGR and Other additives elongation	Explant establishment (EEM)	Axillary proliferation (APM)	Adventitious root induction (RIM)	Adventitious root (REM)
Basal medium ¹	1X	1X	0.5X	0.5X
Sucrose (g/L)	30.0	30.0	15.0	15.0
TDZ (μM)	10.0	3.0	—	—
BAP (μM)	1.0	1.0	—	—
IBA (μM)	1.0	1.0	5.0	—
NAA (μM)	—	—	5.0	—

¹Basal medium at 1X uses 20 ml of each stock and 0.5X uses 10 ml of each stock.

As part of the establishment phase, we transfer germinates of both white and green ash after 4 weeks to new agar-solidified axillary proliferation medium (APM) in which the TDZ concentration has been reduced from 10 to 3 μM (Table 2). Germinants are trimmed to remove half to two-thirds of each cotyledon and all but 1 cm of the hypocotyl before inserting into new APM to the depth of the cotyledonary node. TDZ at 3 μM represents a compromise between maximizing proliferation rates and minimizing unwanted organogenic callus production from tissues touching the medium (Navarrete et al., 1989). The retention of IBA in the proliferation medium aids in keeping the unwanted callus healthy which otherwise can decline, become necrotic, and release toxic exudates with subsequent loss of established cultures. Typically over half the white ash cultures will possess two axillary shoots from the cotyledonary node in addition to the epicotyl while most green ash cultures will consist primarily of the elongating epicotyl after 8 weeks in culture (Van Sambeek et al., 2001).

**Figure 1.** Disinfested cut-seed explants of white ash on EEM for 0, 1, and 2 weeks.

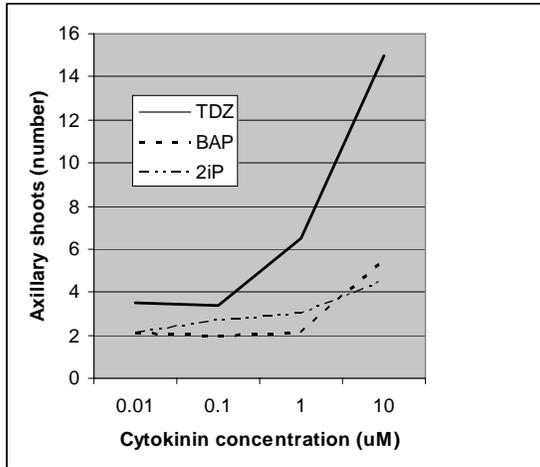


Figure 2. Influence of cytokinins on number of axillary shoots on germinants from cut, non-stratified seeds of white ash after 12 weeks *in vitro*.

Kim et al. (1997) have also reported procedures for *in vitro* culture from embryo establishment to microshoot rooting for three green ash clones. They germinated their non-stratified, cut seed of green ash on MS without plant growth regulators and after 3 weeks transferred germinants to MS supplemented with 10 µM TDZ and 10 µM BAP to induce axillary shoot proliferation. When they subcultured these culture, they obtained the highest rates of axillary shoot proliferation (4 to 8 axillary shoots per culture) with a cytokinin mix of 5 µM TDZ and 5 µM BA. They also reported the production of organogenic callus on tissues touching the medium such that axillary shoots from the cotyledonary node could not be distinguished from the regenerating adventitious shoots. In contrast to our results with white and green ash, Hammatt and Ridout (1992) reported *in vitro* germination and axillary shoot proliferation of common ash was better on DKW medium than on MS medium.

3.1.2. *In Vitro* Establishment from Shoot Tip Explants

In our early research, using shoot tips taken from seedlings, we found shoot tips from white ash, but not from green ash, could be established and initiate axillary shoot proliferation with the best proliferation occurring on liquid WPM supplemented with 44 µM BAP (Preece et al., 1987). Chalupa (1990) reported modest axillary shoot production from seedling shoot tips of European ash when cultured on MS or DKW supplemented with either 0.04 µM TDZ or 9 to 12 µM BAP and 0.5 µM IBA. With the ease that mature seed could be established as a juvenile source of ash explants, it appears few researchers have continued to pursue using explants from seedlings grown in the greenhouse to obtain juvenile explants.

We have also tried to force shoot tips on branches excised from adult white and green ash trees as a source of explants. When placed on WPM supplemented with 4.4 µM BAP and 5 µM IBA, shoots tips would elongate and form callus, but failed

to produce axillary shoots after 5 months in culture (Preece et al., 1987). Likewise, Browne and Hicks (1983) found shoots forced on branch tips from mature white trees showed little *in vitro* development on LS medium supplemented with BAP. Perez-Parron et al. (1994) successfully established shoot tips forced on branches from adult narrow-leaf ash and achieved axillary shoot proliferation when subcultured on DKW supplemented with 4.4 μM BAP and 1 μM IBA, especially if nodal segments were placed horizontally on a new culture medium when subculturing. Hammatt (1994) reported the successful establishment and axillary shoot proliferation from adult European ash when cultured on DKW supplemented with 22 μM BAP.

3.1.3. Initiation of *In Vitro* Axillary Shoot Proliferation

We achieve relatively high rates of *in vitro* axillary shoot proliferation for both white and green ash using nodal segments with monthly transfers and liquid overlays of ASP medium (Van Sambeek et al., 2001). To initiate the axillary shoot proliferation stage, 2-node explants are harvested from the elongating epicotyl and axillary shoots from 2 month old or older cultures, leaf blades excised, and then stems are placed horizontally on agar-solidified medium with the basal node slightly buried (Figure 3A). Two weeks later, a 0.5 cm deep liquid overlay of the same proliferation medium is added. Raising the TDZ concentration of the proliferation medium will increase the number of axillary shoots from the nodes; however, it will also increase the amount of unwanted organogenic callus produced on tissues in contact with the medium and can lead to abnormally thickened shoots (Figure 3B). Typically between 40 and 70% of white and green ash subcultures will produce between 0.3 and 2.5 cm^3 of callus between monthly transfers to new medium. The amount of callus and whether it is organogenic varies depending on the tree from which the original seed explant originated (Preece & Bates, 1995). There is a trend for organogenic callus to gradually change from producing adventitious roots initially to adventitious shoots with later subcultures.

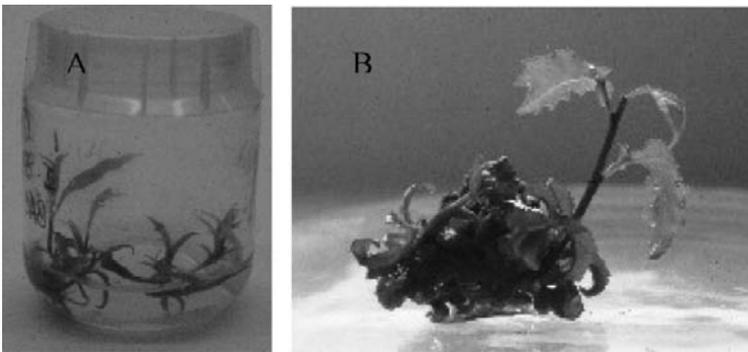


Figure 3. A) White ash subculture with proliferating axillary shoots at the stage when liquid APM overlays are added to improve axillary shoot growth. B) White ash culture from a germinating embryo on EEM with 10 μM TDZ showing extensive callus formation and indistinguishable axillary and adventitious microshoots.

After 1 month on proliferation medium, white ash 2-node segments typically produce an average of 5.8 new axillary shoots which is more than double the 2.3 shoots from green ash nodal segments (Van Sambeek et al., 2001). In addition, the longest axillary shoot after 1 month in subculture tend to be slightly longer on white ash than on green ash nodal explants although there can be substantial variation among clones within an open-pollinated family or species. Genotypic differences in axillary shoot proliferation rates among cultures arising from open-pollinated seed of different trees also have been reported by others for green and European ash (Tabrett & Hammatt, 1992; Kim et al., 1997).

3.2. *Micropropagation by Regeneration of Adventitious Buds and Shoots*

For adventitious shoot regeneration, cut seeds (either immature or mature) are prepared and best placed on agar-solidified MS medium supplemented with 10 μM TDZ and 0.1 or 1 μM 2,4-D. Organogenesis occurs in the callus formed where the cut ends of the cotyledons and hypocotyls touch the medium (Bates et al., 1992; Preece & Bates, 1995). If cotyledons are detached from the embryonic axis, organogenesis is reduced and shoot development will be slower than if cotyledons remain attached to the embryonic axis. If organogenic cultures are transferred to the ASP medium, buds are more likely to elongate into shoots that can be excised, rooted under mist, and acclimatized to a normal greenhouse environment. We have also achieved adventitious shoot regeneration on the unwanted callus that forms on tissues touching the medium during the *in vitro* establishment and axillary shoot proliferation stages of both white and green ash (Navarrete et al., 1989; Van Sambeek et al., 2001). Adventitious shoots typically have a thinner more transparent stem and narrower more succulent unifoliate leaves than the stem and leaves on the developing axillary shoots in these cultures. Kim et al. (1997) also reported formation of organogenic callus around the nodes on their green ash subcultures in contact with the culture medium.

Tabrett and Hammatt (1992) reported high rates of adventitious shoot regeneration on excised hypocotyls from immature and mature seed of European ash when cultured on MS supplemented with 20 μM TDZ and 0.5 μM IBA. Hypocotyls from immature seed tended to have higher rates of regeneration with fewer necrotic cultures. When using seed that had been dried and stored, the best regeneration rates from excised hypocotyls occurred on MS supplemented with 0.5 μM TDZ and 0.5 μM IBA. Higher levels of TDZ tended to result in more cultures that produced vitrified (hyperhydrous) adventitious shoots. Most excised hypocotyls exposed to a primary medium with TDZ for 2 to 5 weeks and then transferred to DKW supplemented with 20 μM BAP produced adventitious shoots that could be rooted.

We have also achieved embryogenesis on white ash by placing cut seeds on MS medium containing a high auxin to low cytokinin ratio (Preece et al., 1987; Bates et al., 1992; Preece & Bates, 1995). The highest rates of embryogenesis have been observed when cut white ash seed were cultured either on MS containing 10 μM 2,4-D with 0.1 or 1 μM TDZ or on DKW medium supplemented with 5 μM BAP and either 1 or 5 μM 2,4-D. The number of germinants showing embryogenesis on the DKW medium could be increased to 20% by transferring germinants with callus

to DKW medium without plant growth regulators. When embryogenic callus was subcultured, new embryos were produced from either the callus or on the surface of the first-formed embryos. Only callus still attached to the original germinant remained embryogenic. A high percentage of the embryos showed abnormal development and only a few could be germinated and developed into normal plantlets when the epicotyls were excised and rooted *ex vitro* (Bates et al., 1993).

4. ROOTING

4.1. *In Vitro* Rooting

We developed a two step procedure under which both white and green ash microshoots can be synchronously rooted *in vitro* (Navarrete et al., 1989; Van Sambeek et al., 2001). For step one, 2 to 6 cm long microshoots are pulsed in the dark for 4 to 8 days with the basal end set 1 cm deep into agar-solidified RIM (Table 2). We have observed that minor changes in the NAA concentration can markedly alter the number of adventitious roots produced both within and among clones. For step two, pulsed microshoots are transferred to individual culture tubes with 7 to 10 cm deep REM (Table 2). Adventitious roots typically emerge in 10 to 14 days after initiating auxin pulse for green ash and 12 to 15 days for white ash (Figure 4A). Green ash microshoots generally have slightly higher rooting percentages and produce more adventitious roots (greater than 80% with 3 or more adventitious roots) than white ash microshoots. There is some evidence that the number of adventitious roots initiated during the auxin pulse does not increase during greenhouse acclimation and following planting into field studies (Van Sambeek et al., 1999). Preece et al. (1991) did find that reducing the MS macrosalt and sucrose concentrations in REM would increase the number and length of secondary roots but did not substantially alter the number or length of the primary adventitious roots. Perez-Parron et al. (1994) reported rooting percentages in excess of 90% for microshoots originating from both juvenile and adult narrow-leaf ash when rooted on WPM supplemented with 1 μM IBA as the auxin.

Several studies have shown that microshoots of some ashes can be rooted *in vitro* without an auxin treatment (Preece et al., 1995, Kim et al., 1998); however, more synchronous rooting is achieved using auxins. Kim et al. (1998) showed that both the culture medium and auxin concentrations could dramatically alter the number and elongation rate of adventitious roots on green ash microshoots. Microshoots continuously exposed to 5 μM IBA in liquid MS averaged between 4 and 6 adventitious roots while microshoots in agar-solidified MS averaged fewer than 2 adventitious roots after 5 weeks. The addition of NAA to the culture medium doubled or tripled the number of adventitious roots although most roots were thick and did not elongate normally when they remain on the root induction medium. Preece et al. (1987) also reported that roots of white ash microshoots when left in agar-solidified WPM supplemented with either 0.5 or 5 μM IBA for 1 month were abnormally thickened and brittle. Stunting could be minimized by the addition of 10 g l^{-1} activated charcoal to the rooting medium.

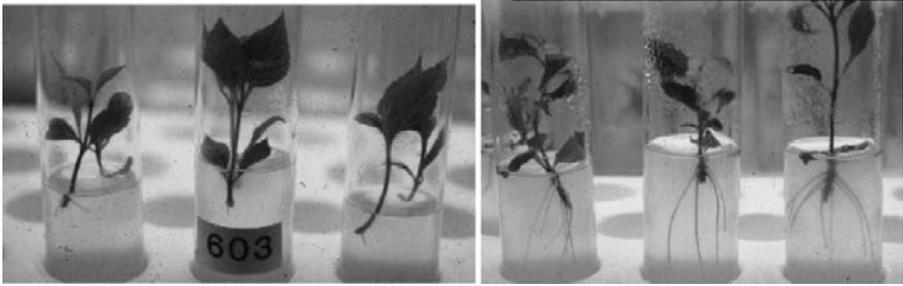


Figure 4. A) White ash microshoots a few weeks after being transferred to REM after having been in RIM for 1 week. B) One-month-old rooted microshoots of white ash ready to be transferred to potting medium and set under mist in a greenhouse.

4.2. *Ex Vitro* Rooting

We have also successfully rooted white ash microshoots under mist in the greenhouse both with and without the use of auxins. For *ex vitro* rooting microshoots are normally placed in peat plugs or a soil-less peat medium. Bates et al. (1992) reported excellent rooting on microshoots excised from adventitious shoots using a 15 second quick dip in 1,000 μM IBA dissolved in 10% ethanol before setting in trays of sterile vermiculite kept under mist in a greenhouse for 3 to 4 weeks.

5. ACCLIMATIZATION AND FIELD PERFORMANCE

5.1. *Acclimatization*

White and green ash microplants rooted *in vitro* can be easily acclimatized to a greenhouse environment if left on the REM until adventitious roots start to curl at the bottom of the culture tube or until shoots start to produce new leaves (Figure 4B). Microplants are then removed from the REM, rinsed free of agar, and transferred to an autoclaved soil-less medium. Plantlets are placed in a high humidity environment until they develop secondary roots along the adventitious roots and additional new leaves with normal functioning stomata (Preece & Sutter, 1991). Initially, plantlets produce simple leaves with a gradual transition to compound leaves with increasing numbers of leaflets (Figure 5). After 2 to 4 weeks under decreasing humidity, plantlets can be moved to a greenhouse bench for additional shoot and root growth and then forced to set a terminal bud.

Dormant plantlets can be transferred to refrigerated coolers for three or more months to meet normal chilling requirements before field planting. Occasionally, we have had difficulty in acclimatizing microplants from somatic embryos because the roots would not elongate or develop secondary roots. These plantlets can be successfully acclimatized by excising the shoot and treating it as a softwood cutting. Following the gradual exposure to reduced relative humidity over a 2 to 4 week period, survival of over 65% has been reported for microplants of white, European, flowering, and narrowleaf ash (Preece et al., 1987; Chalupa, 1990; Arrillaga et al., 1992a; Perez-Parron et al., 1994).



Figure 5. White ash microshoots acclimatized to a greenhouse environment beginning to transition from simple to compound leaves.

5.2. Field Performance

We have successfully spring-planted, in-leaf white ash plantlets in several field studies with little transplant mortality. In one study we are following the growth of white ash microplants from 12 clones (Van Sambeek et al., 1999). Six years after planting, survival averaged between 70 and 100% except for a single clone where all the microplants had died. No relationship has been found between numbers of adventitious roots and fifth-year stem height and diameter for the eleven surviving clones. Few clones changed their relative ranking when ranked by average height from the second through the sixth year. Initially more variation in stem height and diameter existed within clones than among clones; however, after 6 years variation among clones was twice that within clones. All clones except the one that did not survive exhibited normal growth and morphology including the clone originating from organogenic callus. Chalupa (1984) reported successful establishment after one winter of European ash microplants in the field. To date, Bates et al. (1992) are the only researchers to report the occurrence of abnormal development and that was on one white ash microplant from a somatic embryo that exhibit atypical phyllotaxy on the main stem.

6. CONCLUSIONS

Successful *in vitro* propagation from explant establishment through field planting of microplants has been reported for white, green, European, flowering, and narrowleaf ash. In most cases, explants from adult trees have not been established *in vitro*. Embryo dissection or the cut seed technique works well to overcome embryo dormancy in mature, non-stratified seed for all five species. Axillary shoot proliferation is commonly reported on shoots harvested from *in vitro* germinants when subcultured monthly on a high-salt, agar-solidified medium supplemented with thidiazuron (TDZ). The use of liquid overlays of the same medium has been shown to increase

proliferation and axillary elongation rates. Organogenic callus producing adventitious shoots is frequently produced on cut surfaces of germinants and axillary shoots when exposed to TDZ with or without 2,4-D. Pulsing microshoots for a week on a low salt medium supplemented with both IBA and NAA and transfer to medium without plant growth regulator leads to synchronous adventitious rooting. With gradually declining humidity, ash microplants are easily acclimatized to a greenhouse environment where they can put on additional height and growth. Field plantings show most microplants develop normally and the amount of phenotypic variation among clones is much greater than the variation within a clone after a few years.

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CHAPTER 18

MICROPROPAGATION OF BLACK LOCUST (*ROBINIA PSEUDOACACIA* L.)

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1. INTRODUCTION

Robinia genus has about 10 species and 4 hybrids that are native to eastern North America and Mexico. They are *Robinia boyntonii**, *R. elliottii**, *R. hartwegii** (*R. viscosa* var. *hartwegii*), *R. hispida*, *R. kelseyi**, *R. luxurians**, *R. nana**, *R. neomexicana*, *R. pseudoacacia*, *R. viscosa* (*: not accepted as distinct by all authorities), *R. × ambigua* (*R. pseudoacacia* × *R. viscosa*), *R. × holdtii* (*R. neomexicana* × *R. pseudoacacia*), *R. × longiloba* (*R. hispida* × *R. viscosa*), and *R. × margarettiae* (*R. hispida* × *R. pseudoacacia*) (<http://en.wikipedia.org/wiki/Robinia>).

Robinia pseudoacacia (black locust) is a commercial species. It is natively distributed in south eastern United States; on the lower slopes of the Appalachian Mountains, with separate outliers north along the slopes and forest edges of southern Illinois, Indiana, and Missouri (<http://www.nps.gov/plants/alien/fact/rops1.htm>). Black locust is the first forest tree species introduced in Europe from North America. The Parisian Botanical Garden introduced this species already in 1601 (as reviewed by Davis & Keathley, 1987). The species was introduced to Qingdao City of China in 1897 (Pan & You, 1994).

Black locust is able to fix atmospheric nitrogen and enrich the soil fertility. The wood is valued for its durability and high fuel value. The tree provides good forage for bees and generally planted on reclaimed drought and barren lands to control erosion. It is also used for ornamental purposes. Moreover, its fast growth rate and great potential for environmental restoration have drawn attention to woody biomass plantation, establishment of energy plantation that would transfer existing traditional forests into energy forests (Rédei, 1999).

The research is being carried out to improve wood properties of the species for construction, furniture, and other industrial uses (Molnár & Bariska, 2006). The wider

uses of wood have led to the nursery propagation and plantation forestry. However, the conventional plant propagation by grafting, cutting or root sucker is too slow to meet the needs of large-scale plantations.

Micropropagation is an effective route to produce plants rapidly (Shu & Liu, 2003) and a strategy to minimize somaclonal variation among regenerated plants (Scowcroft, 1985). The explants collected from young black locust trees or twigs have high multiplication rate *in vitro* and the micropropagated shoots have high rooting efficiency (Barghchi & Chi, 1998). In this paper, we will describe the experimental protocol to micropropagate black locust efficiently. The protocol can also be utilized in germplasm conservation and genetic studies.

2. EXPERIMENTAL PROTOCOL

2.1. Preparation of Explants

2.1.1. Acquiring of Explants

Well suited mother plants (Figure 1A) showing vigorous growth rates in the field are most appropriate for micropropagation of black locust. The young shoots sprouted from the lower trunk or from underground roots were collected in spring before budding (Kamlesh et al. 1995). About 30 to 40 cm long segments excised from the shoots were cultured in water in a phytotron. The water was changed every day. The temperature in the phytotron was between 24 and 26°C, the relative humidity (RH) 70–80% during shoot cultivation, 16-h photoperiod and with light illumination at $80 \mu\text{mol m}^{-2} \text{s}^{-1}$. In these conditions, the axillary buds sprouted after 14 days. Within 28 days, the new green shoots reached 3–4 cm in length. The new shoots were excised in 1.0–2.0 cm long segments with at least one axillary bud on each segment. Thereafter, they were used as explants (Figure 1B–C).

2.1.2. Surface Sterilization

Shoot segments were cleaned by brushing with suds and rinsed by running tap water for 20–30 min. They were sterilized by dipping into 70% ethanol for 30 s, and then in 0.4% active chlorine of sodium hypochlorite solution (bleach) to which Tween-20 was added at the rate of one drop per 50 ml and shaken gently for about 20 min. The explants were rinsed by distilled sterile water three times (each for 3 min) and kept in the sterilized distilled water for 5 min in order to wash out residual bleach sticking to the plant material. All of these steps were carried out in a laminar flow hood. The shoot segments were individually dried on a sterilized filter paper in a Petri dish (10-cm diameter). Some necrotic ends, which were probably caused by sterilization, were cut off. The explants were cultured on initiation medium (one explant per vessel) (for medium formula, see Table 2).

2.2. Culture Media and Conditions

2.2.1. Culture Media

The black locust culture media were modified from the basic Murashige & Skoog medium (1962). The changes were mainly made in the concentrations of minerals, plant growth regulators and sucrose. The substances supplemented are listed in Table 2. Different compositions were used in initiation, proliferation and induction of roots.



Figure 1. Micropropagation of black locust (*Robinia pseudoacacia* L.). (A) Mother plants with 35 year age. (B) Explant on initiation medium. (C) Sprouting of axillary bud. (D) Shoot elongation and multiplication. (E) Rooting culture. (F) Plant in nutritious plate plug, five weeks after transplantation. Courtesy of Professor Xiaofang Luo.

The pH of the medium was adjusted to 5.8 using 1M NaOH or HCl. Agar gel strength was 1200 g/cm². The agar was dissolved by heating in a microwave oven (2 min for each 250 ml media in a 1000-W microwave oven) and 10 ml was dispensed in each glass tube (25 × 12.5 mm) for initiation culture and 40 ml into each culture vessel (250 cm³ in size) for subcultures, respectively. The glass tubes or vessels were sealed with whorl plastic cover, marked and autoclaved at 121°C (1.05 kg/cm²) for 20 min.

2.2.2. Culture Conditions

Initiation, multiplication and root induction on black locust cultures were achieved at $26 \pm 1^\circ\text{C}$ under a 16-h photoperiod with fluorescent illumination (TLD 36W/33, OSRAM, Germany) at a light intensity of $36 \mu\text{mol m}^{-2} \text{s}^{-1}$. The growth of cultures declined at a temperature below 23°C , and hyperhydricity occurred at 30°C . The leaves of the explants crumpled and the caudexes became transparent and curved. In some of the explants, the hyperhydricity could be recovered to normal growth by transferring them onto the fresh culture medium, and maintained at the room temperature for more than 60 days.

Table 1. Composition of culture media for black locust.

Compounds	Initiation medium	Proliferation medium	Rooting medium
MS medium	Full strength	Full strength	Half strength ^a
Sucrose (g/L)	30	20–30	20
Agar (g/L)	5	5	5
NAA (mg/L)	0.1	1	0.2
6-BA (mg/L)	0.5	1	—
IBA (mg/L)	—	—	0.1
pH	5.8	5.8	5.8

^aHalf strength means the concentration of major element salts is halved.

NAA: naphthalene acetic acid; 6-BA: 6-benzylamino-purine; IBA: 3-indolebutyric acid.

2.3. Initiation of Shoot Cultures

The sterilized explants were grown on the initiation culture medium for about 5 to 10 days until sprouting. The contaminated cultures were discarded after autoclaving at 121°C (1.05 kg/cm^2) for 20 min. It was important to take care of the contaminated cultures.

In general, the number of contaminated explants was low, indicating the selected sterilizing agent was effective. Too high concentrations of the sterilizing agent were injurious to both contaminating microorganisms and plant material. At lower concentrations, the contamination rate of plant material was very high. Generally, 50% of decontamination could be achieved by optimizing the sterilization procedure without damaging plant material seriously.

2.4. Elongation and Multiplication of Shoots

Shoots derived from the initial shoot cultures were cut into 1.5 cm long segments which were cultured on the proliferation culture medium (Table 1, Figure 1D). Usually 4–5 segments were placed in each culture vessel. After 4–5 days, shoots from the adventitious buds grew fast throughout the following 35 days. To keep the cultures in vigorous growth, the subcultures were carried out on the fresh culture medium, at a every 28-day interval.

2.5. Rooting and Acclimatization

The shoots approximately 2-cm long were transferred on the rooting medium (Table 1, Figure 1E). Four shoots were placed in each vessel (250 cm³ in size) and usually the roots protruded after 21 days of culture on the rooting medium. At this stage, plantlets had dark green leaflets and 2 to 3 white roots.

Since *in vitro* plantlets are grown under highly controlled culture conditions, they would desiccate and die by transferring them directly to the field conditions. Therefore, the plantlets were acclimatized at first in the greenhouse conditions for 21 days. The culture vessels were placed facing direct sunlight in order to expose them to the scattered light and daily temperature fluctuations.

Acclimatization of the plantlets to the field conditions took more than 10 days. An adaptation period of less than 10 days was not sufficient and a long adaptation period (30 days) might cause nutrient deprivation in the medium. Moreover, roots were still growing and extending for a long period of time in the rooting medium. These roots were easily damaged while cleaning them with water for removing agar sticking to them and consequently decreased in plantlet survival rate.

In order to avoid dehydration and burning, the tender plantlets were kept away from the strong light conditions. Adaptation to sunshine was performed in two steps: Firstly, the plantlets were placed under a gentle and scattered sunlight environment to mimic the *in vitro* condition. Secondly, the plantlets were moved to a scattered sunlight gradually so that they could adapt to natural growing conditions.

2.6. Transplantation

Acclimated plantlets, rooted in the culture vessels, were transplanted in a rooting mixture (sand : peat : perlite or vermiculite) for field transplantation (Figure 1E). At first, the roots of *in vitro* plantlets, sticking with agar, were submerged in hot water (at 40°C) for about 30 min to wash it off. Thereafter, plantlets were transferred either directly in sand [sand, peat and perlite (1:1:1 v/v/v)] and vermiculite mixture (1:1 v/v) or firstly in vermiculite for 21 days followed by planting in sand (see above) and vermiculite mixture. The vermiculite was sterilized by spraying 0.3% KMnO₄ solution, and it was ready to use after completely draining out KMnO₄ solution. After being adequately watered, the plants were kept in the greenhouse for about 21 days before moving them to the fields.

The selection of the rooting substrate for transplanting the plantlets should be critically made. Vermiculite would be a better choice. The plantlets growing in it showed vigorous and strong growth potential. Leaves were green and glossy. New shoots developed and grew 1–2 cm long within 21 days after transplantation in the field. On the contrary, the plantlets in perlite were stagnant and weak.

The young plants had to be transplanted to bigger containers or fields after 21 days. After that, the plants became much stronger. Their stems lignified and their resistance to environmental stresses increased. The final survival rate of the plants was over 98%. If the plantlets did not form roots or their roots were damaged, new roots could be induced by 500 mg L⁻¹ indole-3-butyric acid (IBA) solution treatment for 5 s, before transferring in sterilized vermiculite.

Moisture of rooting substrate was a critical factor on survival of the plantlets. High moisture results in low aeration and furthermore the roots might be infected by mildew and rot. On the contrary, lower moisture of the substrate dehydrates leaves and plantlets die due to drought stress.

Humidity and air temperature were also important factors on the survival of the plantlets after transplantation. Ventilation system in the greenhouse was required to maintain stable conditions and to reduce fungal diseases and death of the plantlets. In general, the plantlets were kept in the mist house or greenhouse with a black plastic shield meshwork after transplantation. Some time the plantlets were grown under arch cotes covered with plastic film. In this case, the plastic shield meshwork was covered above the plastic film. Anyway, water could be sprayed onto the plastic film to low the temperature if necessary. In the morning and evening, both sides of the plastic films of the cote were uncovered to let in the fresh air in cote space and to reduce fungi inside. Temperature in the cote was kept at $25 \pm 1^\circ\text{C}$. Relative humidity was at about 70–80%. The plastic film could be uncovered gradually 7 days later if necessary.

After transplantation, plant leaves were sprayed with the modified Hoagland's nutrient solution (1/5 dilution) at a regular interval of 3–5 days (Table 2).

Table 2. Composition of a modified Hoagland's nutrient solution for growth of plants^a.

Compounds	Nutrient elements	Final concentrations		
		μm	mg/L	
Macronutrients	N	16 000	224	
	K	6 000	235	
	KNO ₃ , Ca(NO ₃) ₂ ·4H ₂ O, NH ₄ H ₂ PO ₄ , MgSO ₄ ·7H ₂ O	Ca	4 000	160
		P	2 000	62
	S	1 000	32	
	Mg	1 000	24	
Micronutrients	Cl	50	1.77	
	KCl, H ₃ BO ₄ , MnSO ₄ ·H ₂ O,	B	25	0.27
	ZnSO ₄ ·7H ₂ O, CuSO ₄ ·5H ₂ O,	Mn	2.0	0.11
	H ₂ MoO ₄ , Fe-EDTA	Zn	2.0	0.131
		Cu	0.5	0.032
	Mo	0.5	0.05	
	Fe	20	1.12	

Adapted from Epstein, 1972.

^aThe compounds are added separately from stock solutions to prevent nutrient precipitation during the preparation of the nutrient solution (see Epstein, 1972 for details)

3. CONCLUSIONS

Woody plants are typically perennial and complex organisms. Their micropropagation systems are quite different and usually recalcitrant for *in vitro* propagation. Techniques of *in vitro* culture of black locust are relatively well established. Further

research is, however, needed to obtain high quality micropropagated plants for afforestation and forest tree breeding purposes. Micropropagation technique is also important when utilizing somaclonal variation and/or gene engineering techniques. Micropropagation may also be used to preserve gene resources of black locust and combined with tree breeding, it may improve industrial utilization of the black locust wood.

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CHAPTER 19

ALBIZIA ODORATISSIMA L.F. (BENTH) MICROPROPAGATION

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1. INTRODUCTION

Albizia odoratissima commonly known as black siris is a multipurpose nitrogen fixing tree species belonging to the Leguminosae family. The plant is native to the humid tropical parts such as India, Bangladesh, China, Myanmar and Sri Lanka. It is a large erect tree and attains a height of 22–26 m and diameter of 120–150 cm. It occurs from sea level to 1500 m (Troup, 1921), and grows sporadically in both dry and moist deciduous forest zones.

Albizia odoratissima is hermaphroditic and deciduous with a short leafless period from December to February. Flowers are corymbs, pale yellowish white, fragrant and generally appear from March to June. Fruits appear in early August and start ripening at the end of October. In Northeast India and Bangladesh, it has been extensively planted as a shade tree in tea and coffee plantations (Sana, 1989). The shade extends the productive life of crop plants and increases annual yields. Sana (1989) reported that *Albizia odoratissima* contributed 16 kg/ha of nitrogen from 655 kg dry weight of leaf litter and through this way it provides organic matter and soil nutrients to the rhizosphere of understory plants. Tree canopies decrease soil desiccation, and suppress weed growth and protect plants from hail and rainstorms. *Albizia odoratissima*'s presence in the tea monoculture reduces incidence of tea pests particularly red spider mites and scarlet mites.

The timber of *Albizia odoratissima* is hard, heavy and strong. Its heartwood is brown with lighter and darker streaks. The wood could be used for making cabinet, high-class heavy furniture, agricultural implements, carts and paneling. *Albizia odoratissima* is one of the preferred native species for plantation programs because of its greater adaptability to sites. Although native species are often slower growing

than exotic species, they are well adapted to local climatic and edaphic condition and enhance the naturalness of landscapes, and many produce premium quality timber. In Sri Lanka, this species is popularly known as Ceylon rosewood and has been identified as one of the important indigenous species for *ex situ* conservation. This tree species has encountered genetic degeneration due to extensive felling of phenotypically superior trees for commercial purposes. It is confined to a few natural habitats and its natural regeneration is threatened. Hence, there is an important need to protect the genetic resource of this species by establishing *ex situ* conservation stands (Dominique, 1997).

Albizia odoratissima is commonly propagated through seeds. However, the seeds are frequently infested by bruchids. The larvae of *Bruchidius bilineatopygus* cause heavy damage to developing pods and seeds. Besides, wild stand seedlings are highly variable in terms of growth and biomass production. More uniform, healthy and vigorously growing planting stock of *A. odoratissima* that can benefit agroforestry systems can be obtained from vegetatively propagated superior trees. For the majority of tree species, propagation by rooted cuttings is often characterized by a rapid loss of rooting capacity with increasing age of the mother plant (Biondi & Thorpe, 1981). In many species, rooted branch cuttings tend to continue to grow with a horizontal orientation and bilateral symmetry (plagiotropism) until the terminal meristem changes to radial symmetry and vertical growth (orthotropism). This reversal to normal growth displays frequent intra and inter clonal variation, and thus erratic in selection program. Thus the evaluation of genotypes in a selection experiment becomes almost impossible (Libby, 1974). Micropropagation technique could be used as a complimentary approach for rapidly establishing a clonal orchard from selected trees or control pollinated seeds from which further propagules will be produced by rooted cuttings. In addition, tissue culture technique could be a viable alternative to conventional vegetative propagation methods for mass multiplication, tree improvement programs on superior canopy characteristics and resistance to pests and diseases (Hossain, 1997).

In vitro regeneration has already been reported in *Albizia odoratissima* (Phukan & Mitra, 1983). There are no other reports on *in vitro* propagation of this tree species to the best of our knowledge. With an ultimate objective to establish a method for clonal propagation of mature black siris tree, in the present investigation initial studies were conducted with the seedling explants. This is primarily to generate information on the nature of responses, exhibited *in vitro* by the tissues of this plant, which can be extended to studies with mature explants. The plants raised from tissue culture of seedling-derived explants will also be useful to confirm the previous observations (Mascarenhas et al., 1987) regarding reduction of the juvenile phase of seedling-raised tree.

2. EXPERIMENTAL PROTOCOL

The method developed for micropropagation of *Albizia odoratissima* using seedling explants as well as two-year-old saplings is presented below.

2.1. Growing Conditions of Donor Plants

2.1.1. Two-year-old Saplings

The potted plants were grown outdoors at 32–38°C, a 16-h photoperiod of natural light with photosynthetic photon flux density of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a relative humidity of 60%.

2.1.2. In Vitro Germinated Seedlings

The donor plants of 14-day-old seedling explants were grown at $25 \pm 2^\circ\text{C}$, 16-h photoperiod, photosynthetic photon flux (PPF), 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$, at the culture medium level provided by cool white fluorescent light (Philips, India).

2.1.3. Pretreatment and Surface Sterilization of Seeds

Select fresh stock of healthy uniform seeds. Treat with 1N sulfuric acid for 15 min (Figure 1A,B). Wash thoroughly under running tap water to remove H_2SO_4 . Surface sterilize with 0.05% (w/v) aqueous solution of mercuric chloride for 5 min (Figure 1C). Under aseptic conditions, wash thoroughly with sterile distilled water.

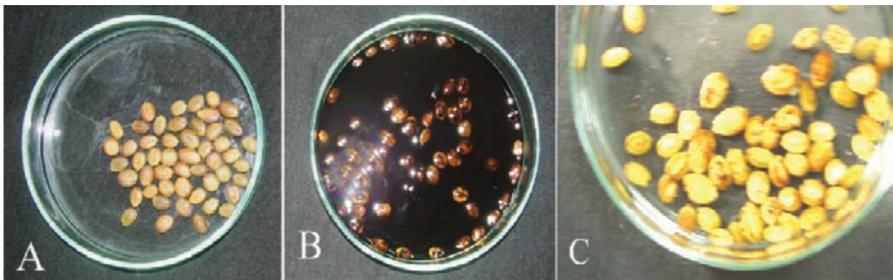


Figure 1. Procedure for *in vitro* germination of seeds of *Albizia odoratissima*. A) Freshly collected good, healthy seeds. B) Treatment with 1N Sulfuric acid. C) Surface sterilization of acid treated seeds with 0.05% (W/V) aqueous solution of mercuric chloride.

2.1.4. Inoculation and In Vitro Germination of Seeds

Transfer seeds to a sterile petri plate and dry for 2 min using autoclaved filter paper strips. Inoculate 10 seeds per conical flask (250 ml) on MS-1 medium.

Incubate the seeds at $25 \pm 2^\circ\text{C}$ at 16-h photoperiod with a photosynthetic photon flux (PPF) density of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the culture medium level provided by cool-white fluorescent light (Philips, India). Seeds will germinate within 2 weeks (the percentage of germination is 70 to 80%) (Figure 2A).

2.2. Explants Dissection

2.2.1. Explants Excised from In Vitro Germinated Seedlings

Three types of explants can be effectively isolated from seedlings including the cotyledonary nodes, leaf nodes and hypocotyls.

Make a transverse cut in the epicotyl region of the seedling, which is located in between the leaf nodes and cotyledonary nodes (Figure 2B). Remove the shoot tip and leaves attached to the nodal segment in order to obtain leaf nodal explants (0.5 cm long) with two axillary buds (Figure 2C). Cut at the hypocotyl region of the

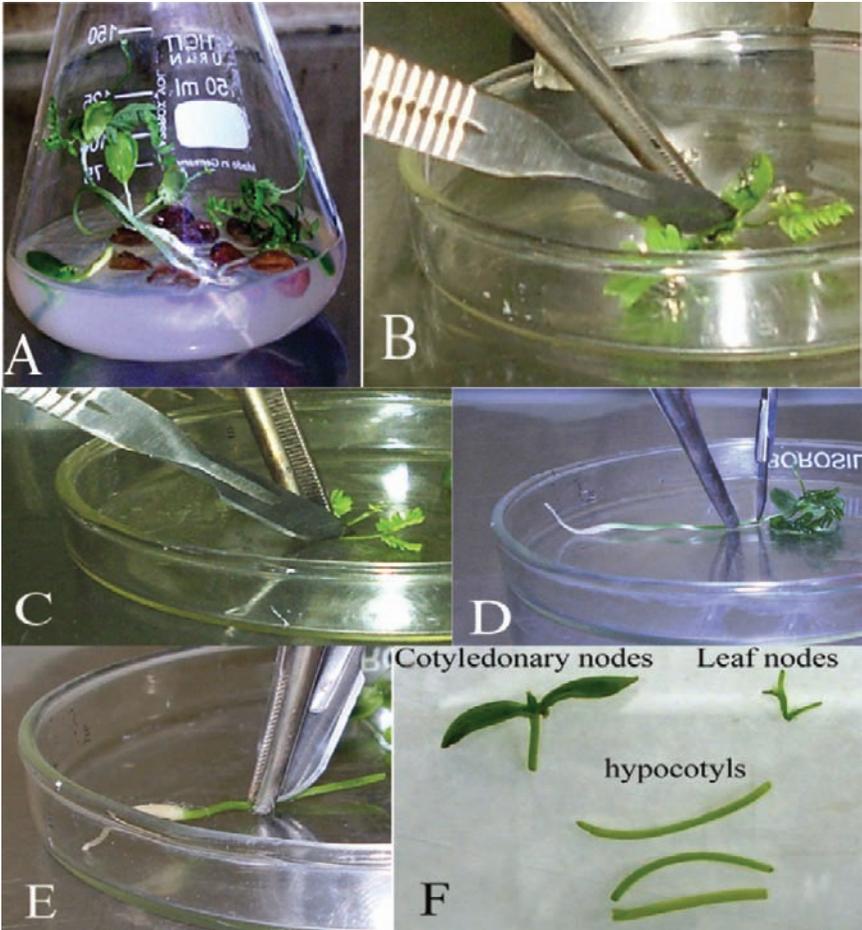


Figure 2. Stepwise procedure used to excise leaf nodes, cotyledonary nodes and hypocotyls from in vitro germinated seedlings. A) In vitro germinated seedlings of *Albizia odoratissima* 14 days after inoculation of seeds. B) Make a transverse cut in the epicotyl portion which is located in between the leaf nodes and cotyledonary nodes. As cotyledonary nodes and leaf nodes are present in close proximity careful manipulation is necessary to avoid damaging the axillary buds found at the base of leaf. Note that the basal cut end of leaf nodes does not contain any epicotyl region in figure F. C) Cut and remove the leaves and shoot tip attached to the leaf nodal segment. D) Cut at the hypocotyl region 0.5 cm below the cotyledonary nodes. E) Cut the remaining hypocotyl region into small segments (1.5 cm long). F) Cotyledonary nodes (1 cm long), leaf nodes (0.5 cm long) and hypocotyls (1.5 cm long) excised from in vitro germinated seedlings.

seedling 0.5 cm below the cotyledonary nodes to obtain cotyledonary nodes (1 cm long) (Figure 2D). These two types of explants are suitable for developing protocols for axillary shoot proliferation.

Cut the remaining hypocotyl region into small segments (1.5 cm long) (Figure 2E). This explant can be used for the development of protocol for adventitious shoot organogenesis. Leaf nodes, cotyledonary nodes and hypocotyls obtained by using the above procedure are shown in Figure 2F.

2.2.2. Collection and Surface Sterilization of Nodes from Two-year-old Saplings

Collect fresh, green and healthy top three branches of two-year-old saplings (Figure 3A). Using surgical blade cut and remove the leaves attached to the nodes (Figure 3B,C). Cut the stem portion of the branch 0.5 cm above and 0.5 cm below the nodes in order to obtain leaf nodes (Figure 3D–F). Put the nodal explants in a non-sterile conical flask, wash thoroughly under running tap water for 30 min. Immerse the explants in a detergent solution (2 or 3 drops of tween-20 per 100 ml water) for 3 min and thoroughly wash with non-sterile distilled water. Transfer the leaf nodes to a sterile conical flask. Surface sterilize with 0.10% (w/v) mercuric chloride for 10 min. Under aseptic condition wash thoroughly with sterile distilled water and inoculate immediately.

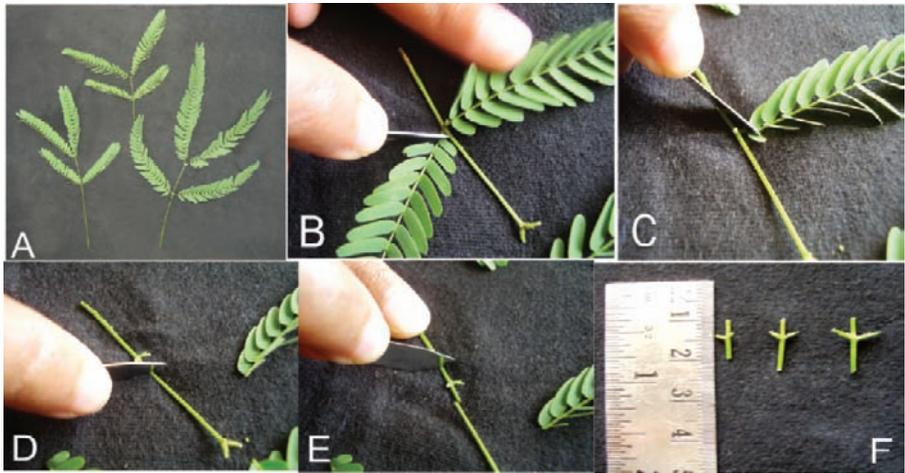


Figure 3. Procedure for the excision of leaf nodes from freshly collected branches of two-year-old saplings. A) Freshly collected young branches of two-year-old saplings. B–C) Removal of leaves attached to the leaf nodes. D–E) Cutting the stem segment, separation of leaf nodes. F) Leaf nodes excised from branches of two-year-old saplings.

2.3. Culture Medium

2.3.1. Basal Medium Components

The basal medium commonly used for *Albizia* tissue culture is MS (Murashige & Skoog, 1962) with modifications to achieve stage dependent optimizations by particularly manipulating the hormonal content. Table 1 lists the components of MS medium.

2.3.2. Medium Preparation

Prepare the basal medium including all additives except growth regulators as shown in Table 1. Adjust medium to pH 5.7 with 1M NaOH and HCl and dispense in 150×25 mm culture tubes (15 ml per tube), 250 ml conical flasks (25 ml per flask). These vessels are autoclaved for 1 min at 121°C and 1×105 Pa (1.1 Kg cm^{-2}) and kept in a cool place ready for culturing.

Table 1. Formulation of culture medium used for *Albizia odoratissima* micropropagation based on MS salt augmented with culture stage-specific plant growth regulators.

Components	Chemical formula	Stock (g/L)	Medium (mg/L)
<i>Macronutrients, 10 × stock, use 100 ml per L medium</i>			
Ammonium nitrate	NH_4NO_3	16.5	1650
Potassium nitrate	KNO_3	19	1900
Calcium chloride	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	4.4	440
Magnesium sulfate	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	3.7	370
Potassium orthophosphate	KH_2PO_4	1.7	170
<i>Micronutrients, 1000 × stock use 1 ml per L medium</i>			
Boric acid	H_3BO_3	6.2	6.2
Cobalt chloride	$\text{COCl}_2 \cdot 6\text{H}_2\text{O}$	0.025	0.025
Cupric sulfate	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	0.025
Manganese sulphate	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	16.9	16.9
Potassium iodide	KI	0.83	0.83
Sodium molybdate	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	0.25
Zinc sulphate	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6	8.6
<i>Iron-EDTA, 100 ×, use 10 ml per L medium</i>			
Iron sulfate	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	2.78	27.8
Ethylenediamine tetra acetic acid di-sodium	Na_2EDTA	3.73	37.3
<i>Vitamins, 100 ×, use 10ml per L medium</i>			
Myo-inositol		10.0	100
Nicotinic acid		0.05	0.5
Pyridoxine hydrochloride		0.05	0.5
Thiamine hydrochloride		0.01	0.1
Glycine		0.2	2
<i>Other additives</i>			
Sucrose			30000
Agar			8000
<i>Plant growth regulators add according to the type of medium</i>			
Type of medium			
MS-1	Half strength MS basal medium without plant growth regulators		
MS-2	MS + 10 μM BAP and 1.5 μM NAA		
MS-3	MS + 5 μM BAP and 1.5 μM NAA		
MS-4	MS + 10 μM BAP and 10 μM 2-iP		
MS-5	MS + 5 μM BAP and 10 μM kinetin		
MS-6	MS + 7.5 μM BAP and 0.5 μM NAA		
MS-7	MS + 25 μM IBA		
MS-8	MS basal medium without plant growth regulators		

2.4. Axillary Shoot Proliferation

2.4.1. Nodes from Two-year-old Saplings

With the help of sterile forceps and a sterile surgical blade (No. 10), trim the nodal explants (with two axillary buds) by removing the tissues at the basal end of the explants which turned brown during the process of sterilization. This would substantially help improve the responsiveness of the explants as the deteriorated dead cells were removed and thereby exposing the fresh live tissues to the culture medium. Inoculate nodal segments vertically on MS-2 medium in such a way that the axillary bud is in contact with the medium. Buds sprout and turn green in 10–15 days and callus develops at the basal cut end of the nodes. After 8 weeks 2–3 shoots with the average height of 1.5 cm develop from nodes (Figure 4A).

2.4.2. Multiplication in Subculture

Take elongated shoots with 2 or 3 internodes. Cut the shoot into small segments so that each segment includes nodal region with single axillary bud. Inoculate on MS-3 medium. After about 3–4 weeks each segment gives rise to 2 to 3 shoots which can be further subcultured (Figure 4B).

2.4.3. In Vitro Germinated Seedling Explants

Inoculate cotyledonary nodes and leaf nodes on MS-4 medium and MS-5 medium, respectively. Place the explants vertically with the basal end slightly inserted, 1–2 mm deep, into the medium taking care that the axillary buds are in contact with the medium. Incubate cultures at $25 \pm 2^\circ\text{C}$ in the light (16-h photoperiod with a photosynthetic photon flux (PPF) density of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$). Within 20–25 days about 6–7 shoots with the average height of 2–2.5 cm develop from cotyledonary nodes (Figure 4C) whereas about 3–4 shoots with the mean height of 1–1.5 cm develop from leaf nodes (Figure 4D). These shoots can either be subcultured for further multiplication or kept for rooting.

2.5. Adventitious Shoot Organogenesis from Hypocotyls

Inoculate hypocotyl segments vertically with the basal cut end inserted slightly 0.5 cm deep into the MS-6 medium. After incubation for about a week, callus develops at the basal cut end of hypocotyls. However, shoot buds start emerging after 3 weeks of incubation at both the cut ends. After about 3–4 weeks each segment gives rise to 14–15 shoots with the average height of 1.5–2 cm (Figure 4E).

2.6. Cytological Changes during Adventitious Shoot Organogenesis

The *in vitro* organization and morphogenesis of multicellular plants depends upon the integration and mutual interaction of various organs, tissues and cells. There is a need for much more intensive anatomical examination by both light and electron microscopy to trace the emergence of organs within the initially relatively uniform cell mass of callus or suspension culture aggregates. This part of the work was carried out to analyze the ontogenesis of adventitious buds from hypocotyls which would contribute to the understanding of the mechanisms controlling morphogenesis.

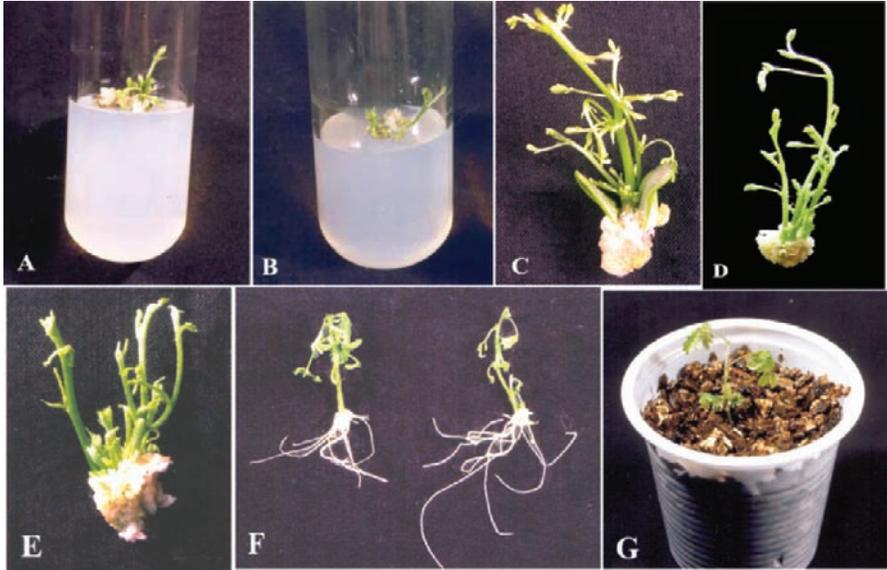


Figure 4. *In vitro* plant regeneration of *Albizia odoratissima* from seedling explants. A) Leaf nodes of two-year-old saplings cultured on MS-2 medium. B) Subculture of nodes with single bud derived from microshoots of leaf nodes of two-year-old saplings on MS-3 medium. C) Cotyledonary nodes cultured on MS-4 medium showed multiple shoots. D) Leaf nodes showing multiple shoots 4 weeks after culturing on MS-5 medium. E) Adventitious shoot regeneration from hypocotyls cultured on MS-6 medium. F) Rooting of *in vitro* regenerated shoots 4 weeks after culturing on MS-8 medium. G) Hardened *in vitro* raised plants of *A. odoratissima* in vermiculite medium.

Take hypocotyl segments cultured on MS-6 medium at weekly intervals up to 4 weeks (0 day (control), 7, 14, 21 and 28th day) for cytological analysis. Take free hand sections of these cultured hypocotyls using razor blade and take photographs using Nikon automatic camera Model FX-35A attached to Nikon Labophot (Japan) binocular microscope. On 0 day (control) hypocotyls show a single layered outermost epidermis, cortex and a continuous vascular cylinder with three layers of cambial cells, the primary phloem external to the cambium and the primary xylem external to the cambium (Figure 5A). On 7th day the number of cell layers in the cambium increases due to periclinal divisions of the original cambial cells (Figure 5B). On 14th day meristematic regions composed of small tightly packed cells develop in the cambium (Figure 5D). These meristematic regions differentiate into vascular elements. Thus nodules have a cambial origin and arise without significant callus formation (Figure 5E).

2.7. Rooting

Select elongated shoots (3–4 cm long) for rooting. Inoculate the shoots on MS-7 medium and culture for 24 h and thereafter transfer these shoots on MS-8 medium.

Note the emergence of roots and the development of complete plantlets after 4 weeks (Figure 4F).

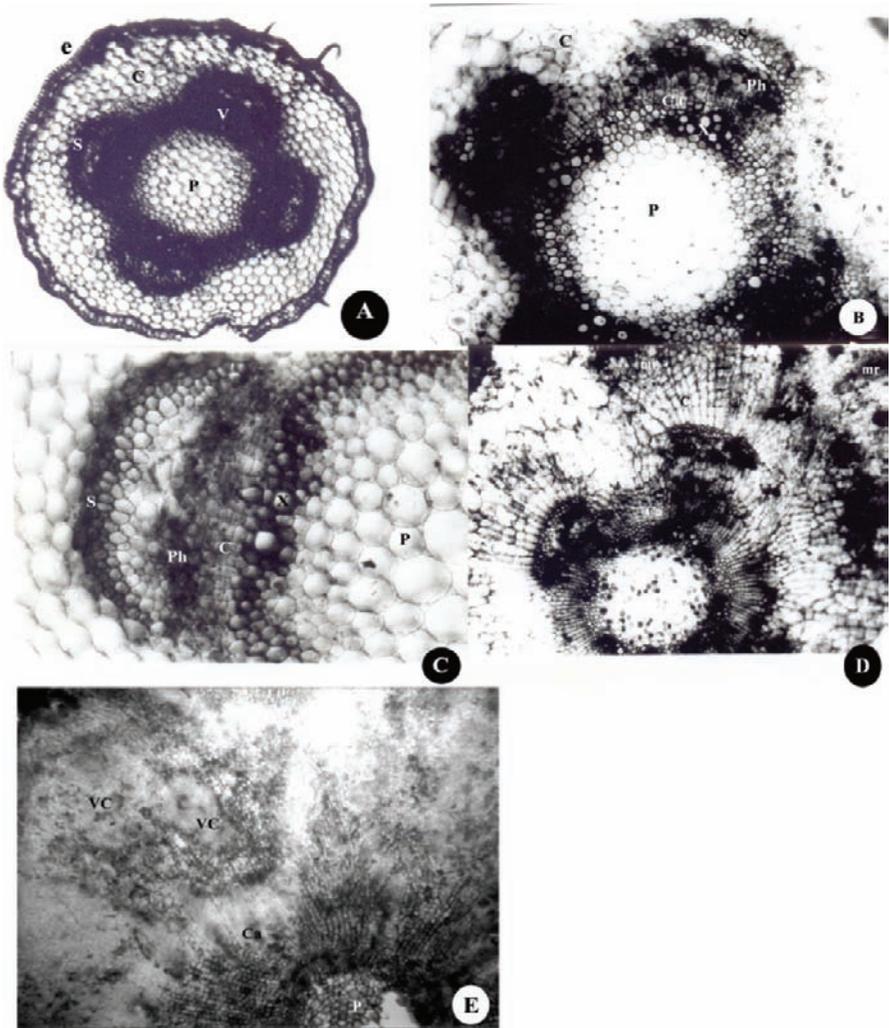


Figure 5. Histological analysis of adventitious shoot organogenesis from hypocotyls of *A. odoratissima*. A) Transverse section of hypocotyls on day 0. B–C) Transverse sections of hypocotyls on day 7. Note that the cambial cells just started to divide in figure C. D) Cross section of hypocotyls on day 14 shows that the number of cell layers in cambium increased due to periclinal divisions. E) Some of the meristematic centers differentiate into vascular elements of the differentiated shoots on day 14. C – Cortex; Ca – Cambium; e – epidermis; mr – meristematic center; P – Pith; Ph – Phloem; S – sclerenchymatous cap; V – Vascular bundle; VC – Vascular center; X – Xylem.

2.8. Hardening

Remove plantlets, which have attained the height of about 4–5 cm from culture vessels. Wash the root system thoroughly in running tap water to remove traces of medium adhering to the plantlet. Transfer the individual plantlets into small plastic pots (10 cm diameter) containing sterilized vermiculite moistened with half strength MS medium without sucrose (Figure 4G). To retain high humidity cover the potted plantlets with clear plastic sheet and keep under culture room condition. After 1-month transfer plantlets to polybags (15 × 25 cm) containing soil, sand and farmyard manure in the ratio of 3:1:1 and keep in a shade house.

3. CONCLUSION

This chapter described a reliable and reproducible micropropagation protocol for *Albizia odoratissima* based on axillary shoot proliferation as well as adventitious shoot organogenesis from seedling explants. The results have shown that the efficiency of micropropagation system is influenced by both the physiological state of the donor plants and also by the type of explants. This is evident in the present study that the number of shoots produced by the explants of more juvenile source (14-day-old seedling) was more than that of the older explant source (two-year-old sapling). Even though the clonal propagation system developed for the matured tree is much more valuable than the juvenile seedlings, this study is conducted primarily to generate information on the nature of responses exhibited *in vitro* by the tissues of this plant which can be extended to studies with mature explants. As most of the woody species segregate for phenotypic traits when propagated by seeds, the plants regenerated *in vitro* from seedling explants would display inherent genetic variation. This could, however, be controlled by using an elite seed population as was selected by Pradhan *et al.* (1998) for their study on propagation of *Dalbergia latifolia*. The method developed for adventitious shoot organogenesis permits the rapid rate of plant production and histological analysis showed that the shoot buds originated from cambial cells thus minimizing the risk of producing genetically off types.

Cloning of mature trees through tissue culture is generally preferred to that of juvenile tissue as it is not always possible to determine if the juvenile tissue will have the desired qualities at maturity. However, explants derived from mature trees show inherent recalcitrance to *in vitro* condition. Therefore, improvements and refinements in the technique will be necessary. The cost per plant would be high particularly if one considers application of this method for mass propagation. Development of somatic embryos and encapsulation with enriched polymers and production of synthetic seeds could be a solution to this problem of economics of micropropagation.

New developments in genetic engineering procedures, including the recent somaclonal, gametoclonal and embryo rescue technologies show promise for their inclusion and applications in tree breeding programs for the genetic improvement of forest tree species. These technologies when combined with the methods of micropropagation for raising seed orchards could greatly reduce the time normally taken in conventional tree breeding programs.

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CHAPTER 20

MICROPROPAGATION OF *SALIX CAPREA* L.

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1. INTRODUCTION

The genus of willows (*Salicaceae*) comprises approximately 500 tree and shrub species, mainly growing in the northern cool and temperate zones. Because of numerous varieties and crossing progenies, it is difficult to give an exact species number. Willows prefer humid or wet sites in floodplain forests, along flowing water and on marshland. Under favourable conditions they represent a modest pioneer tree species with good vegetative and generative propagation capacity in most cases. Willows play an important role for the development of landscape structures and for maintaining the ecological balance. Several projects on *Salix* tree improvement were initiated because willows are used as raw material for bioenergy and pharmaceutical products, stream bank stabilization, nutrient filters, and phytoremediation. Especially the more tree-like growing willow species are of increasing interest for use in short rotation coppice plantations because of their high growth potential.

Although most of the *Salix* species show quite good regeneration properties, there are a few species among them, e.g. sallow (*Salix caprea* L.), which are difficult-to-root by wood cuttings. Micropropagation is a suitable tool to facilitate the multiplication of selected willow genotypes which were characterised beforehand for their outstanding growth performance, disease resistance traits or wood components.

Several authors have reported successful tissue culture methods for different willow species. Looking at the literature, it is obvious that the more willow species are studied, the broader is the range of media variants with different basic nutrient compositions and phytohormones. Grönroos et al. (1989) described their first results on induction of embryogenic callus using isolated pistils of *Salix viminalis*. On nutrient medium with a combination of the plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA) three different callus types were formed and

somatic embryos developed after transfer to hormone-free medium. Bergman et al. (1985) tested the influence of BA on the micropropagation potential of five different willow clones, including one of *S. caprea*. Neuner and Beiderbeck (1993) analysed nine clones of *S. caprea* for their tissue culture ability. Both publications emphasised the difficulties in cutting and microcutting propagation of willow and the strong influence of genotypes on rooting success in this species. Callus proliferation in *Salix exigua* with 2,4-D was induced by Stoehr et al. (1989), followed by shoot regeneration with BA and rooting without hormones. A recent publication by Santos et al. (2005) also described possibilities for callus induction in *Salix humboldtiana* Willd with BA, 2,4-D and 1-naphthaleneacetic acid (NAA). Chung and Carrasco (2000) presented experiments with 13 different *Salix* species (e.g. *Salix stipularis*, *S. triandra*, *S. purpurea*, *S. dasyclados*, *S. burjatica*, *S. calodendron*, *S. sericans*, *S. eriocephala* and others). They tested the influence of basic nutrient elements and the concentration and effect of BA and gibberellic acid (GA₃) on micropropagation behaviour. As a result they reported that the responses obtained were dependent on the type and concentration of growth-regulators used and were strongly dependent on species and genotype.

Gebhardt (1992) reported that cytokinin-containing media resulted in shoot tip browning of *Salix viminalis*, *S. fragilis*, *S. x lispoclados*, *S. x rubens* and *S. petandra*. He recommended a shoot-division-method on medium without cytokinins. The suitability of this micropropagation mode could be partly confirmed by Liesebach and Naujoks (2004) during their investigations on vegetative propagation of *Salix caprea*. Furthermore, there are reports on application of tissue culture methods for special purposes. Crossing products from artificial pollination of *Salix fragilis* x *S. lispoclados* are unable to survive under conventional conditions. Agrawal and Gebhardt (1994) established and propagated these hybrid seedlings via embryo rescue *in vitro* on BA containing medium. In a project dealing with alpine willow species as pioneer wood for revegetation of areas endangered by erosion, Büttner et al. (2006) presented their first results on tissue culture establishment for 11 difficult-to-propagate *Salix* species selected in alpine regions. On media with cytokinin/auxin combinations, the first step of establishment was successful for the majority of these clones.

In the following, a protocol which was developed for micropropagation of *Salix caprea* clones is presented. This method may also be suitable for many other willow species.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Growing Conditions of Mother Plants.

Accelerated sprouting of twigs in the greenhouse. One-year-old twigs, 40–50 cm long, were harvested from adult donor trees at the end of January, put into vessels filled with tap water and placed on tables in the greenhouse. To reduce surface contamination, the twigs were sprayed with 0.2% Euparen (fungicide by BAYER, 50% dichlorfluanide, w/v). The temperature was in the range from 18 to 26°C. Additional artificial lighting was installed during winter and early spring for prolonging the illumination period to about 12 hours a day aiming to force sprouting.

Nursery plants. Rooted cuttings from adult donor trees were grown in plastic pots in the nursery during spring and summer under irrigation. One year after rooting, the new shoots were used for tissue culture establishment.

Greenhouse plants. The most favourable preconditions for the establishment of plant material *in vitro* were given after growing potted willow plants (e.g. from cutting propagation attempts) for a period of 2–6 months in the greenhouse. In these conditions the shoots were elongated quickly and the surface of leaves and stems was relatively clean.

2.1.2. Explant Excision and Sterilization

Explants from sprouting twigs in the greenhouse. Four to six weeks after transferring the twigs to the greenhouse, 2–2.5 cm long apical shoot tips and nodal segments with leaves were excised from the newly growing shoots and were used as explants. The area of the biggest leaves was reduced to 1/3 their full size and the explants were shaken in 0.2% Euparen solution for 2 min. After drying with a paper towel, the disinfection procedure was immersion for 9 min in 0.25% mercuric chloride with two drops of the detergent Tween80, followed by rinsing three to four times in sterile deionised water. Before transfer to culture vessels, the base of the explants was cut again for better nutrient uptake (final length of the explants: 1.5–2 cm).

Nursery plants. From the middle of August to the beginning of September, healthy apical shoot tips and nodal segments, each 2–2.5 cm long, were taken from container-grown plants in the nursery when the weather was warm and dry. Big leaves were cut to 1/3 their size, afterwards the explants were rinsed for 2 min in 0.2% Euparen. After drying between paper towel sheets, the disinfection with 0.25% mercuric chloride and a few drops of Tween80 was extended to 20 min because of the high contamination risk from the explants grown outdoors.

Greenhouse plants. When mother plants were growing for several months in the greenhouse before establishment *in vitro*, shaking of the explants in a fungicide could be omitted, but all other steps of surface disinfection had to be done as described above. Since the lignification level of the shoot tips and nodal segments was different, mercuric chloride for surface disinfection had to be applied for 8 min with explants taken in April, but 10 min with explants taken in October followed by the rinsing in sterile deionised water.

2.2. Culture Medium

2.2.1. Media Composition

Two different basic nutrient media were modified for the successful establishment of *Salix caprea* clones (see Table 1): MCM according to Bornman (1983) and WPM by Lloyd and McCown (1981). The propagation media MCM_{AK}, WPM_{AK} and WPM1/2_{AK} were prepared without any plant growth-regulator, but with 0.1% activated charcoal (Darco G60 by SERVA). The medium WPM1/2₅₀ contained 50 mg l⁻¹ indole-3-butyric

acid (IBA) for root induction. SERVA agar, 10 g l^{-1} (gel strength ~ 800), was used as gelling agent.

2.2.2. Medium Preparation

For preparing WPM based media variants, powdered media supplied for example by DUCHEFA Biochemie B.V. were used. MCM_{AK} had to be produced with stock solutions of the different components. The high amount of auxin (50 mg l^{-1} IBA) was dissolved separately with a few ml of undenaturated ethanol (70%) before it was added to the total quantity. After adjusting the pH, the media were heated till all compounds were completely dissolved. While stirring carefully, the medium was distributed with a dispenser pump to suitable culture vessels. Twenty five ml medium were filled into 100 ml Erlenmeyer flasks and 10 ml medium into small glass tubes, 95 mm long with 28 mm diameter. After closing with aluminium foil the medium was autoclaved at 121°C (pressure: 105 kPa) for 20 min.

2.3. Shoot Regeneration and Maintenance

For culture initiation after surface disinfection, the shoot tips and nodal segments were put into the glass tubes with medium MCM_{AK} or WPM_{AK} . The cultures were kept at $20\text{--}22^\circ\text{C}$ with a light intensity of 1600 to 1700 lux (27.5 to $28 \mu\text{mol m}^{-2} \text{ s}^{-1}$) supplied by warm-white fluorescent tubes (e.g. Phillips TLD 58W/93, OSRAM L58W/31-830 Warmton Lumilux warm white) and a 16-hours photoperiod. If enough explants are available for each clone, both nutrient media variants should be tested, because different sallow genotypes may have different media preferences for establishment of viable cultures.

During the first month, explants from donor trees with the best tissue culture ability showed spontaneous rooting. Subsequently shoots started elongating and the shoot tip could be cut if it was about 1.5–2 cm long (Figure 1A). At least one or two axillary buds were left at the remaining rooted part of the plantlet. Within the next weeks, new shoots began sprouting from these buds. After transfer to fresh nutrient medium, the shoot tips rooted again spontaneously and could also be used as mother plants for microcuttings which could be harvested periodically every 3–4 weeks. Rooted plants had to be transferred to fresh medium not later than after 3 months if they were set aside as further stock plants.

Explants from some recalcitrant donor trees were not able to form roots spontaneously. In these cases, shoot tips had to be put on a half concentrated Woody Plant Medium ($\text{WPM1}/2_{50}$) supplemented with 50 mg l^{-1} IBA for 1 week for root induction and afterwards on the growth-regulator-free medium ($\text{WPM1}/2_{\text{AK}}$) for root development. During the following 3 weeks, after the appearance of roots shoot elongation started. Further propagation *in vitro* could be done as described above *via* repeated cutting of the newly formed shoots and application of a pulse treatment with a high amount of auxin. After several microcutting cycles executed in this manner, it became obvious that the difficult-to-root sallow clones were capable of forming roots spontaneously.

Table 1. Media composition for tissue culture of *Salix caprea* L. (all amounts given in mg l⁻¹).

Component	Medium designation			
	MCM _{AK}	WPM _{AK}	WPM1/2 _{AK}	WPM1/2 ₅₀
KNO ₃	2000			
NH ₄ NO ₃		400	200	200
CaCl ₂ × 2 H ₂ O		96	48	48
Ca(NO ₃) ₂ × 4 H ₂ O	500	556	278	278
(NH ₄) ₂ SO ₄	400			
K ₂ SO ₄	150	990	495	495
MgSO ₄ × 7 H ₂ O	250	370	185	185
KH ₂ PO ₄	270	170	85	85
Na ₂ EDTA × 2 H ₂ O	37.3	37.3	18.65	18.65
FeSO ₄ × 7 H ₂ O	27.8	27.8	13.9	13.9
H ₃ BO ₃	1.5	6.2	3.1	3.1
MnSO ₄ × H ₂ O	0.17	22.3	11.15	11.15
ZnSO ₄ × 7 H ₂ O	3.0	8.6	4.3	4.3
KI	0.25			
Na ₂ MoO ₄ × 2 H ₂ O	0.25	0.25	0.125	0.125
CuSO ₄ × 5 H ₂ O	0.025	0.25	0.125	0.125
CoCl ₂ × 6 H ₂ O	0.025			
Myo-inositol	90.0	100	50	50
Thiamine-HCl	1.7	1.0	0.5	0.5
Nicotinic acid	0.6	0.5	0.25	0.25
Pyridoxine-HCl	1.2	0.5	0.25	0.25
Pantothenat	0.5			
Biotin	0.125			
Folic acid	1.1			
Glycine	2.0	2.0	1.0	1.0
IBA				50
Activated charcoal	1 000	1 000	1 000	
Sucrose	20 000	20 000	10 000	10 000
pH	6.4	5.7	5.7	5.7

2.4. Rooting and Hardening

Spontaneous rooting of micropropagated willow shoots was observed on medium MCM_{AK}, WPM_{AK} or WPM1/2_{AK}. Another possibility was the root induction with a high dose of IBA like already presented (see 2.3, Shoot regeneration and maintenance and Figure 1B).

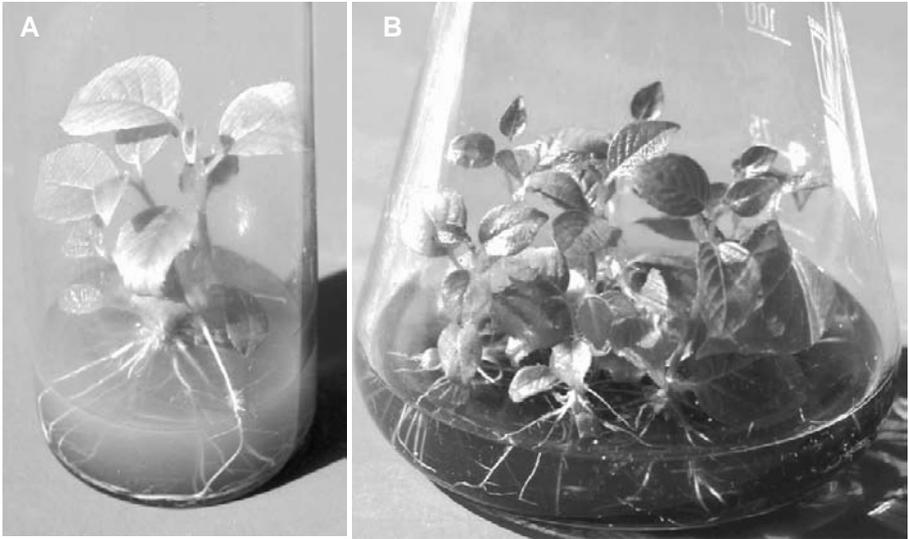


Figure 1. A) Shoot development from *Salix caprea* plantlets after repeated cutting. B) Shoots of *Salix caprea*, 3 weeks after pulse treatment with 50 mg l^{-1} IBA and subsequent transfer to phytohormone-free medium for root development.

Well rooted, vigorous plantlets were transferred to a standard seedling soil:sand mixture (3:1) and kept in the greenhouse under high air humidity (80–85%) maintained by fog irrigation and plastic foil cover. Containers of the type “Rootrainer” (RONAASH Ltd. Scotland, $4.5 \times 4.5 \text{ cm}$, 20 cm high, each for 40 plantlets) were very useful for the transfer of micropropagated plants to soil. The temperature in the greenhouse was in the range of 18–28°C and during winter time, additional light was required to extend the illumination period to about 12 hours.

About 3 weeks later, the air humidity had to be reduced successively and sallow plantlets were grown for several weeks until they reached a height of about 20 cm (Figure 2A).

After hardening, if they showed a sufficient level of lignification, the micropropagated plants were transplanted to the nursery. Growing in containers or planted in rows in the open ground, the plants were kept in the nursery (Figure 2B) for later use in establishing field trials, planting clonal archives or founding short rotation coppice plantations. The plants showed a normal growth habit and they were comparable to trees derived from hardwood cutting propagation.



Figure 2. A) Micropropagated plantlets of *Salix caprea*, 4 weeks after transfer to the soil. B) Sallow plantlets at the end of the first growing period in the nursery.

3. CONCLUSION

Salix caprea L. (sallow) belongs to the rapidly growing willow species, but is difficult to propagate *via* conventional vegetative methods like hardwood cuttings. Micropropagation can facilitate the supply of plant material from juvenile or adult trees selected for outstanding growth or resistance characteristics. For tissue culture establishment, plant material harvested in the field, grown in the greenhouse or in the nursery was used. After surface disinfection, explants were put on hormone-free basic nutrient medium enriched with activated charcoal. In cases of spontaneous rooting, subsequently the plants were used several times for microcutting propagation. To improve the rooting success, a pulse treatment with high IBA concentration was applied. After transfer to soil under greenhouse conditions, acclimatized *Salix* plants from micropropagation could be cultivated for one or two years in the nursery until use for field plantations.

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CHAPTER 21

MICROPROPAGATION OF *CEDRELA FISSILIS* VELL. (MELIACEAE)

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1. INTRODUCTION

Cedrela fissilis Vellozo (Meliaceae) is a valuable fast growing timber tree species native of the endangered Atlantic Forest (South Brasil). Its wood has excellent multiple properties, which is used for the furniture, navy and construction industries. This species has antimalarial, antibacterial and diuretic properties and is also used in folk medicine in diarrhoea control and as a cicatrization agent (Carvalho, 1994). Lago et al. (2004) studied the chemistry of the genus *Cedrela* that focused on the isolation of limonoids chemical composition and antibacterial activities of volatile oils from leaves and stem barks of *C. fissilis*. Aromatic oil with medicinal properties is obtained from its distilled wood (Reitz et al., 1979).

Viana et al. (1999) reported that selective logging has depleted the most valuable genotypes leading to the genetic erosion of *Cedrela* species in Brazil and Central America. Like several members of the Meliaceae it is highly valuable plantation species. The damage caused by shoot borers is the main constraint to its large-scale utilization in reforestation programs and secondary metabolism studies (Haines, 1994). It is attacked by *Hypsipyla grandella* Zeller (Lepidoptera, Pyralidae) and *Oncideres* sp. (Cerambycidae) and a suitable method to control it is yet to be found. Therefore alternative methods including silviculture control and tree improvement programs have been considered by Maruyama et al. (1989).

The development of *in vitro* culture systems for *C. fissilis* is therefore the basis to ensure the introduction of genes for insect resistance, clonal propagation of selected resistant clones, germplasm conservation, synthetic seed technology and secondary metabolite production. The few studies available on *in vitro* culture of the genus *Cedrela* are on micropropagation by shoot tip culture, *in vitro* conservation and organogenesis by hypocotyl segment culture of *C. odorata* (Maruyama et al., 1989, 1997 a,b; Cerdas et al., 1998). Micropropagation procedures have been established for *Cedrela odorata* L. and *Swietenia macrophylla* (Lee & Rao, 1988; Maruyama et al., 1989; Cerdas et al., 1998) and a somatic embryogenesis system was developed for *Swietenia macrophylla* (Maruyama & Ishii, 1999). An efficient micropropagation protocol was developed for *C. fissilis* by Nunes et al. (2002) using nodal segments from juvenile origin for axillary shoot proliferation. Shoot proliferation was significantly affected by medium salt content, explant origin and 6-benzyladenine concentration. Rooting was achieved on half strength Murashige and Skoog medium either with or without growth regulators. Regenerated plants were successfully acclimatized on sterilized sand and for further plant development the sand:soil (1:1) mixture was the best substrate. The survival rate of plantlets under *ex vitro* conditions was 100% after 3 months.

Storage of calcium-alginate encapsulated shoot tips of the Amazonian species *Cedrela odorata* L., *Guazuma crinita* Mart. (Sterculiaceae) and *Jacaranda mimosaeifolia* D. Don. (Bignoniaceae) at above freezing temperatures (Maruyama et al., 1997a) has been reported. Cryopreservation, using a vitrification procedure has also been undertaken for *Guazuma crinita* Mart. bud clusters (Maruyama et al., 1997b). The development of germplasm conservation programs for *Cedrela spp.* is of increasing importance and these should include biotechnological techniques (Nunes et al., 2003) including tissue culture (artificial seeds comprising alginate-encapsulated vegetative propagules) and cryopreservation.

Rapid screening at the callus level is required to select the most promising culture conditions for the establishment of ideal plant cell suspension cultures to produce secondary metabolites. The solid phase micro extraction technique (SPME) was successfully used to extract volatile compounds released by small samples of embryogenic cultures and somatic embryos of sweet oranges (Alonzo et al., 2001). A sensitive headspace solid phase micro extraction method was developed to analyze the volatiles produced by callus culture of *C. fissilis*.

The protocols described herein are efficient and reproducible for shoot initiation, rooting, acclimatization, artificial seeds using alginate encapsulated shoot tips and nodal segments of *C. fissilis*. The chapter also deals with protocols on callus culture initiation from juvenile seedling stock explants to study organogenesis and secondary metabolism and a rapid and sensitive method to analyze the volatiles produced by small samples of calli.

2. EXPERIMENTAL PROTOCOL

2.1. Establishment of Shoot Cultures

The culture media for different steps of *in vitro* propagation are shown in Table 1.

2.1.1. Seed Surface Sterilization and Germination

1. Surface sterilize seeds of *C. fissilis* in commercial bleach (2.5% active chlorine) added with 2–3 drops of Tween 20 for 75 min.
2. Rinse three times for 10 min in sterile distilled water and culture on MS basal medium (MS, Sigma Co., USA) (Murashige & Skoog, 1962) supplemented with 2% (w/v) sucrose and 0.2% (w/v) Phytigel (Phytigel, Sigma Co., USA). Adjust the pH to 5.8 before adding Phytigel and dispense the medium into 25 × 150 mm glass culture tubes (10 ml/tube) before autoclaving at 121°C for 18 min.
3. Seal the vessels with transparent polypropylene film (76 × 76 mm) and place the cultures 25°C, 70% RH under a 16-h photoperiod and photosynthetic photon flux of 20–25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by fluorescent light tubes. Use these culture conditions in all the protocols mentioned below unless otherwise stated.

After 4 weeks of *in vitro* growth the seedlings are about 8.2 ± 1.0 cm long, root length of 8.6 ± 1.2 cm, and 0.505 ± 0.1 g fresh weight. They have one cotyledonary node, two epicotyl nodes, one apical bud and 3.1 ± 0.5 leaves.

2.1.2. Shoot Initiation and Multiplication

1. Remove the cotyledonary (0.8–1.0 cm long comprising two axillary buds) and epicotyl node cuttings (comprising two axillary buds inserted at the base of the first pair of leaves above the cotyledons) from 30-day-old seedlings aseptically grown in the light.
2. Place the explants horizontally and on MS medium supplemented with 2% (w/v) sucrose, 0.2% (w/v) Phytigel and 2.5 μM 6-benzyladenine (BA).
3. Incubate the cultures at 25°C, 70% RH under a 16-h photoperiod and photosynthetic photon flux of 20–25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by fluorescent light tubes.

Shoot initiation starts by releasing axillary buds from apical dominance on the culture medium containing 2.5 μM BA. The highest number of nodes per explant could then be excised for further multiplication and production of new microplants. Originally there are only two axillary buds per explant. Calli proliferate and adventitious buds are produced at the base of the axillary buds (Figure 1A). The multiplication rate increases to 6–7-fold after 60–90 days in the second subculture cycle. The subculture of each of the single node cuttings produces a new plant (Figure 1B). After 60–90 days shoot cultures from cotyledonary nodes produce ca. 6–7 nodes per

explant while the epicotyl node cuttings produce on an average 4.0 nodes per explant, which can then be excised for further multiplication and production of new microplants. The number of nodes/explant is low when epicotyl-derived explants are used and is affected by the slow growth and senescence after 30 days (Nunes et al., 2002). The multiplication rates for hardwood species are low, 5 to 10 propagules per culture cycle. However, even a multiplication factor of this order can account to million propagules per year for many species (Haines, 1994).

2.1.3. *Rooting*

1. Remove the single node cuttings from 60-day-old shoots produced in the multiplication medium.
2. Inoculate the explants on either half or full strength MS medium supplemented with 2% (w/v) sucrose, 0.2% (w/v) Phytigel and 2.5 μM indole-3-butyric acid (IBA).
3. Incubate the cultures at 25°C, 70% RH under a 16-h photoperiod and photosynthetic photon flux of 20–25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by fluorescent light tubes.
4. Evaluate adventitious roots regeneration after 10 days of culture.

On half strength MS supplemented with 2.5 μM IBA 100% rooting ratio is achieved at 10th day of culture and 87% at the 12th day without plant growth regulators (Figure 1C). On full strength MS either without plant growth regulators or supplemented with 1.25 μM IBA the maximum rooting percentages (93%) are only achieved within 18–35th day of culture. Successful micropropagation of many woody species is frequently limited by their recalcitrance to form adventitious roots.

2.1.4. *Acclimatization*

1. Remove the 30-day-old rooted regenerated plants from the test tubes, wash roots with running water to remove Phytigel. Care should be taken to prevent damage to roots.
2. Transfer the rooted plants to trays containing steam-sterilized medium grade river sand covered with a polyvinyl chloride (PVC) transparent film.
3. After the first week gradually remove the PVC film from the seed trays and expose them to the relative humidity (average 70%) of the culture room.
4. After 21 days transfer plants to 250 ml plastic pots containing a 1:1 mixture of sand and soil. At all stages of acclimatization keep them in the culture room, under identical culture conditions to those used for the tissue culture studies. After 90 days acclimatization transfer the plants to the greenhouse.

After 90 days acclimatization in 1:1 mixture of sand and soil the surviving rate is 100%, the plants are in average 19.8 cm long and have 12.7 leaves (Figure 1D) (Nunes et al., 2002).

Table 1. Composition of culture media used for micropropagation and *in vitro* conservation of *Cedrela fissilis*. Basic MS medium containing appropriate concentrations of gelling agents, sucrose and plant growth regulators was used for different objectives. The photosynthetic photon flux at culture level was 20–25 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Objective	Explant	Medium strength	Sucrose ($\text{g}\cdot\text{l}^{-1}$)	*PGR (μM)	Gelling agent ($\text{g}\cdot\text{l}^{-1}$)
Seed germination and establishment of axenic seedlings	Zygotic seeds	full	20	—	Phytigel (2)
Shoot initiation and multiplication	Cotyledonary and epicotyl nodes	full	20	BA (2.5)	Phytigel (2)
Rooting	Single node cuttings	half	20	IBA (2.5)	Phytigel (2)
		full	20	IBA (1.25)	Phytigel (2)
Propagule encapsulation	Cotyledonary and epicotyl nodes, shoot tips	full	20	—	Sodium Alginate (40)
Storage of alginate-encapsulated propagules	Cotyledonary and epicotyl nodes, shoot tips	full	—	—	Agar Type A (4)
Post-storage recovery of alginate-encapsulated propagules	Cotyledonary and epicotyl nodes, shoot tips	full	20	BA (2.5)	Phytigel (2)
Rooting of alginate-encapsulated propagules	Microshoots from cotyledonary and epicotyl nodes	full	20	IBA (1-2.5)	Phytigel (2)
	Microshoots from shoot tips	full	20	IBA (5)	Phytigel (2)

*PGR = Plant Growth Regulator

2.2. *In vitro* Conservation of Vegetative Propagules

The different culture media used for different steps of *in vitro* conservation of vegetative propagules are shown in Table 2.

2.2.1. Dehydration

1. Remove shoot tips (4 mm long comprising one apical bud) and cotyledonary nodal segments (4–5 mm long) from 30-day-old sterile seedlings.
2. Dehydrate the shoot tips and cotyledonary nodal segments in open Petri dishes, in the flow laminar cabinet for 1–4 h.
3. Transfer explants to the recovery medium containing MS medium supplemented with 2% (w/v) sucrose, 0.2% (w/v) Phytigel and 2.5 μM BA.
4. Incubate cultures at 25°C, 70% RH under a 16-h photoperiod and photosynthetic photon flux of 20–25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by fluorescent light light tubes.
5. Evaluate shoot regeneration after 14 days of culture.

Nunes et al. (2003) observed that non-encapsulated shoot tips and cotyledonary nodes tolerates 1–4 h of air dehydration in a sterile laminar flow. Shoot regeneration rate is (90–100%) on the 7th day of culture when the propagules are dehydrated for 1–2h (water losses of 61.1–72.8%, for shoot tips, and 44.6–68.4%, for cotyledonary nodes) and reduced to 40% when the water loss is >82.2% (3–4 h air dehydration) on a fresh weight basis. Air dehydration is suited for conservation of vegetative propagules although complementary studies on viability after longer storage periods must be undertaken.

2.2.2. Encapsulation

1. Excise shoot tips, cotyledonary and epicotyl nodal segments (4–5 mm long) from 30-day-old sterile seedlings.
2. Immerse the explants in autoclaved MS medium supplemented with 2% sucrose (w/v) and 4% (w/v) sodium alginate (Sigma Co., USA).
3. Collect individually the explants with a sterile pipette and drop them in a sterile of 1.4% (w/v) calcium chloride solution.
4. After 30 min decant the calcium chloride solution and rinse the alginate beads three times with sterile medium. Each bead contains one vegetative propagule.
5. Transfer the alginate beads to the storage medium.

2.2.3. Storage of Alginate-encapsulated Explants

1. Collect the alginate-encapsulated explants and transfer to baby jars containing 25 ml mixture of water and agar (Agar Type A, Sigma Co., USA) at 0.4, 0.7 or 1.0% (w/v).
2. Incubate the cultures at 25°C under 16-h photoperiod and photon flux of 20–25 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at culture level, supplied by fluorescent light tubes.

2.2.4. Post-storage Recovery

1. After 3, 6 and 9 months of storage transfer the alginate-encapsulated explants to recovery medium consisting of MS medium supplemented with 2% (w/v) sucrose, 0.2% (w/v) Phytigel and 2.5 μM BA.
2. Evaluate shoot regeneration after 14 days incubation in the recovery medium.
3. Transfer the microshoots to MS medium containing 2% (w/v) sucrose, 0.2% (w/v) Phytigel and 2.5 μM IBA (microshoots from cotyledonary nodes) and 5.0 μM IBA (microshoots from encapsulated shoot tips).
4. After 45 days the microplants are ready for acclimatization.

Nunes et al. (2003) observed high regeneration rates (90–100%) for propagules exposed to all treatments. They survive storage at 25°C for 3 months, however, after 6 months storage, survival of encapsulated shoot tips on 0.4% (w/v) agar decreases to 40–44%. This value is significantly greater than those obtained for cotyledonary nodes, at all agar concentrations tested, and when compared to shoot tips on 1% (w/v) agar. After 9 months storage the viability of the explants declines drastically (6–8%). Plants regenerated from encapsulated cotyledonary nodes and apical shoot tips did not show morphological variation after 15 days in the recovery medium (Figure 1E, F). Maximum rooting (58%) occurs in plantlets derived from encapsulated cotyledonary nodes, in 2.5 μM IBA, and from shoot tips, in 5.0 μM IBA. Vegetative propagules for synthetic seed production and germplasm storage at above freezing temperatures have been reported in *Cedrela odorata*, *Guazuma crinita* and *Jacaranda mimosaeifolia* (Maruyama et al., 1997a).

2.3. Callus Induction

The culture media for different protocols of callus induction are shown in Table 2.

2.3.1. Callus Induction from Cotyledonary Nodes and Shoot Tips

1. Inoculate either cotyledonary node cuttings or epicotyl node cuttings (0.8–1.0 cm long) obtained from 30-day-old aseptically grown seedlings on MS medium supplemented with 2% (w/v) sucrose, 0.2% (w/v) Phytigel and combinations of 2.5 μM BA and 5 μM α -naphthalene acetic acid (NAA). Place explants horizontally into the medium.
2. Incubate cultures at 25°C in either light (under 16-h photoperiod and photon flux of 20–25 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at culture level, supplied by fluorescent light tubes) or in the dark.
3. After 8 weeks evaluate the callus fresh and dry mass.

For both cotyledonary and epicotyl node explants, high callus fresh weight (ca. 1.3 g, for cotyledonary nodes and 0.7–0.9 g, for epicotyl nodes) is obtained on the culture medium containing 2.5 μM BA and 2.5 or 5 μM NAA (Nunes et al., 2002). When BA and NAA are used separately, the fresh weight of calli is decreased. Differences in dry weight are detected when cotyledonary node-derived calli grow in

the light (ca. 60 mg) or in the dark (50 mg), however no differences are observed in the fresh weight. Depending on the treatment the calli were either friable or showed globular structures and/or rhizogenesis (Figure 2A,B).

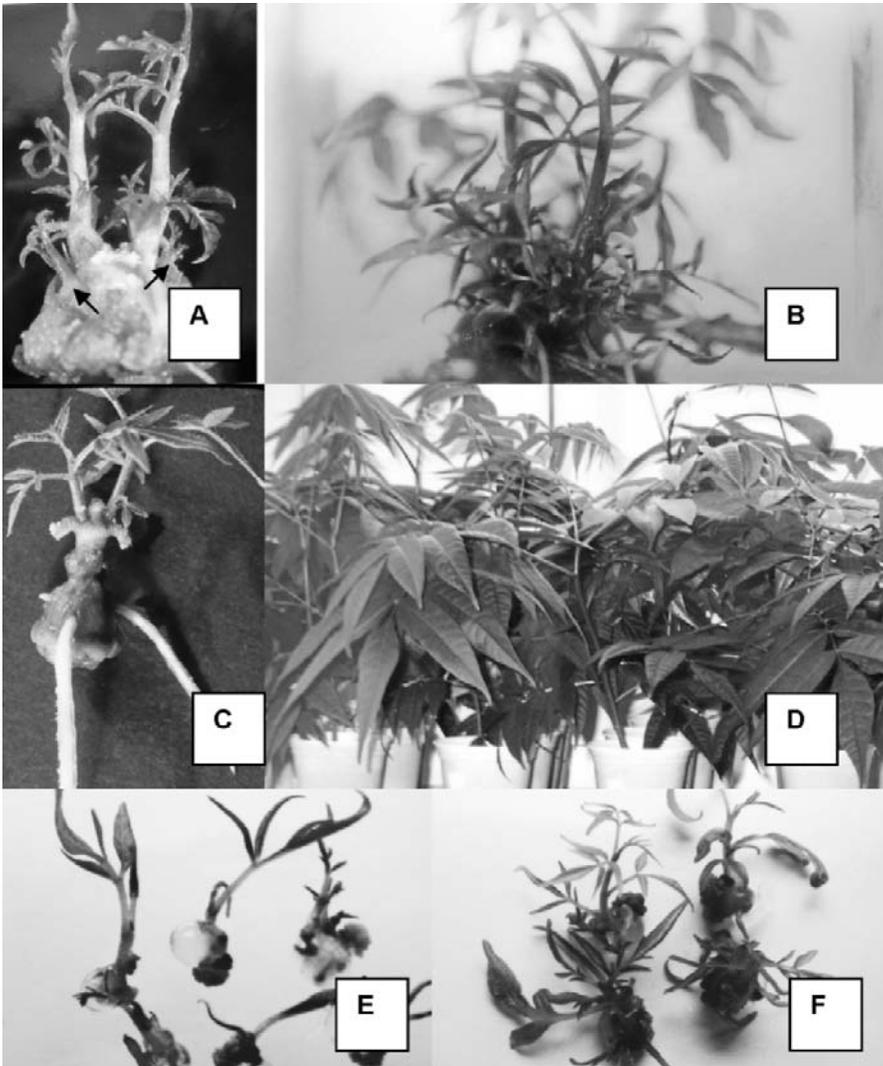


Figure 1. Shoot cultures of *Cedrela fissilis* derived from cotyledonary node cuttings. A) Callus and new buds are formed at the base of both axillary buds after 45 days and B) shoot proliferation at 90 days. C) A rooted shoot on MS medium 15 days after transfer. D) Acclimatized plants after 90 days. E) Shoot development from alginate encapsulated shoot tips and F) cotyledonary nodes after 15 days on the recovery medium.

Table 2. Composition of culture media used for callus induction and direct organogenesis of *Cedrela fissilis*. Basic MS medium containing 20 g·l⁻¹ sucrose, 2 g·l⁻¹ phytigel, and plant growth regulators in appropriate concentrations was used for different objectives. The photosynthetic photon flux at culture level was 20–25 μmol m⁻² s⁻¹.

Objective	Explant	*PGR (μM)	Culture condition
Callus induction	Cotyledonary and epicotyl nodes	BA (2.5)+NAA (2.5–5)	light
	Epicotyl nodes	BA (2.5)+NAA (2.5–5)	light
		NAA (2.7)	light or dark
		2,4-D (1.15–4.6)	light
		2,4-D (4.6)	dark
	Root segments	2,4-D (2.3–4.6)	dark
	Cotyledon basal end segments	2,4-D (2.3–4.6)	dark
Cotyledon middle portion segments	BA (2.5–15)+NAA (10–15)	light	
Direct organogenesis (shoots)	Intact cotyledon	BA (0.6)	light
		TDZ (0.3)	light
		TDZ (0.6)	dark
Direct organogenesis (roots)	Intact cotyledon	—	light or dark
	Seedlings	NAA (10.74–21.48)	dark

*PGR = Plant Growth Regulator

2.3.2. Headspace Solid Phase Micro Extraction (SPME) and Mass Spectrometer Analysis Procedure for Rapid Screening of Calli for Production of Volatiles

1. SPME device and fiber of polydimethylsiloxane (PDMS) (100 μm) were obtained from Supelco (Bellefonte, PA, USA). Condition the fiber thermally (300°C) prior to their first absorption in the hot port of the gas chromatograph instrument according to the supplier's instructions.
2. Collect a portion of 0.5 g (wet weight) of fresh callus produced after 8 weeks culture on MS basal medium supplemented with 2% sucrose, 2.5 μM BA, 5 μM NAA and 0.2 % (w/v) Phytigel under either light or dark and smash it gently.
3. Add a portion of 10-mL tissue sample in a 16-mL vial containing a magnetic spin bar (PTFE) and seal the vial immediately with a septum to prevent sample evaporation.
4. Pierce the septum vial with the protecting needle and expose the PDMS fiber to the sample headspace. After 1 h of extraction of the organic compounds from the headspace onto the fiber, with the maximum agitation of the sample, withdraw the fiber into the needle, remove from the vial and immediately insert into the heated GC-MS injection port.

5. Use a Hewlett Packard 5890 II capillary gas chromatograph equipped with a mass spectrometer and a split-splitless injector to carry out the HS-SPME-GC experiments.
6. Use a HP-5 fused silica capillary column of 30 m \times 0.25 mm ID and a phase thickness of 0.25 μ m for GC separations of the pre-concentrated compounds.
7. Set the temperature program for the analyses as follows: hold the initial temperature of 35°C for 3 min and then increase to 280 at 10°C min⁻¹. Set the injector and detector temperatures at 250 and 280°C, respectively. The run time is 28.6 min. Use helium as a carrier gas at a flow rate of 1.0 mL min⁻¹.
8. Inject the samples in the splitless mode. Operate the spectrometer in electron impact mode (EI) with 70 eV detection volts and scan range of 35–450 m/z.

Although the CG-MS profiles do not correspond to the actual composition of the sample due to the nature of the equilibrium in the SPME method, this method is suitable for extracting and pre-concentrating the volatile compounds. It helps in comparing chromatographic profiles of different samples extracted under similar experimental conditions. Comparison of retention indexes and mass spectra allows the identification of 13 compounds (eight monoterpenes, three sesquiterpenes and two alkanes), of the 21 volatiles detected by the headspace SPME technique in callus grown in the light (Table 3). Ten compounds were identified in callus cultured in the dark. Tridecane, germacrene *D* and α -alaskene were detected only in callus produced in the light. Only germacrene *D* was previously reported as a constituent of volatile oils from leaves and stem bark of *C. fissilis*.

2.3.3. *In Vitro* Low Temperature Storage of Epicotyl Nodes and Shoot Tips for Callus Production

1. Inoculate either epicotyl node cuttings or shoot tips (0.8–1.0 cm long) obtained from 30-day-old aseptically grown seedlings on MS medium supplemented with 2% (w/v) sucrose and 0.2% (w/v) Phytigel.
2. Incubate the cultures at 5°C in the dark.
3. After storage transfer the explants to MS medium supplemented with 2% (w/v) sucrose, 0.2% (w/v) Phytigel, 2.5 μ M BA and 5.0 μ M NAA.
4. Incubate the cultures at 25°C in the light.
5. After 8 weeks collect callus to evaluate the fresh and dry mass and for the secondary metabolism studies.

The explants withstand storage at 5°C in the dark up to 8 weeks. No differences in callus dry weight are detected when the explants are stored at low temperature when compared with the explants stored at 25°C. However, callus from stored epicotyl nodes shows higher dry weight (ca. 56 mg) than the shoot tips (ca. 38 mg). Low temperature storage of vegetative propagules is very simple and effective method for short-term germplasm storage of callus for secondary metabolism studies.

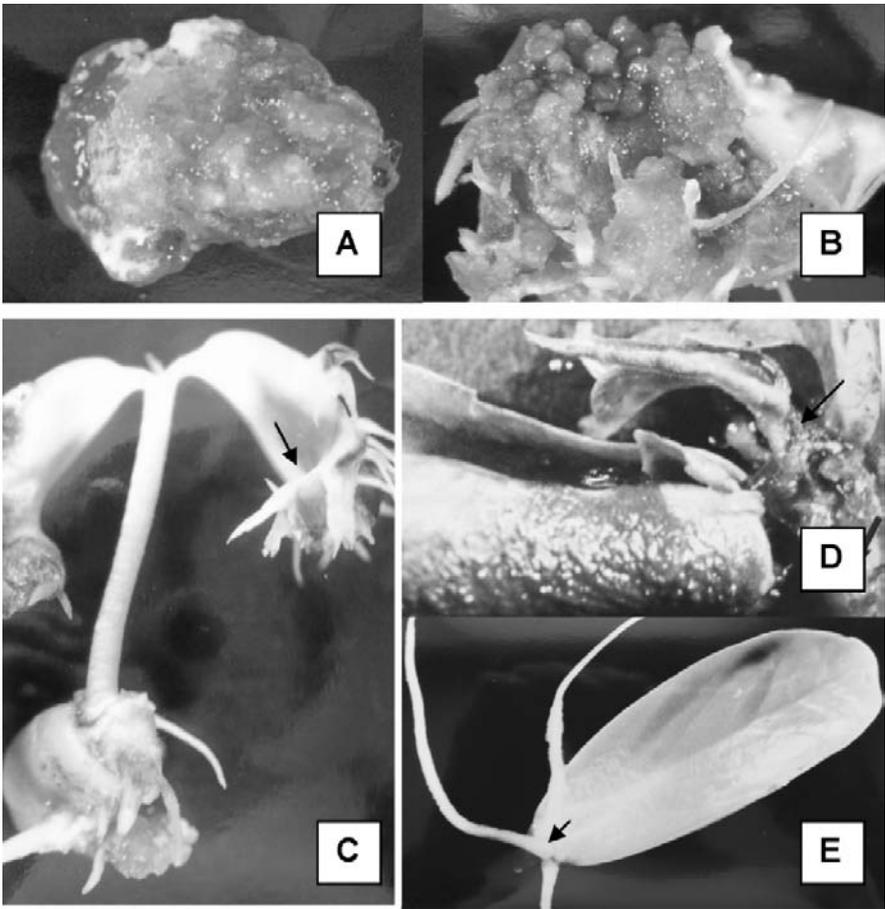


Figure 2. Calli from cotyledonary node of *Cedrela fissilis* cuttings produced on MS medium supplemented with $2.5 \mu\text{M}$ BA and $5 \mu\text{M}$ NAA (A,B). Note the globular aspect and regeneration of adventitious roots (B). (C) Direct rhizogenesis in cotyledons in 30-day-old intact seedling produced from seed germination on MS medium supplemented with $21.48 \mu\text{M}$ NAA. Adventitious shoots (D) and roots (E) produced at the basal end of isolated cotyledons cultured on plain MS medium respectively, under light and in the dark, after 20 days.

2.3.4. Callus Induction from Root and Epicotyl Node Segments

1. Inoculate either root segments (1.0–1.2 cm long) or epicotyl node segments (1.0–1.2 cm long) excised from 30-day-old aseptically grown seedlings in the light on MS medium supplemented with 2% (w/v) sucrose, 0.2 % (w/v) Phytigel and with different concentrations of either 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 1.15, 2.3 and $4.6 \mu\text{M}$) or NAA (0, 1.35, 2.7 and $5.4 \mu\text{M}$). Place explants horizontally into the medium.

2. Incubate the root cultures in the dark and the epicotyl cultures either in the light or in the dark.
3. After 45 days the globular calli are collected to determine the fresh and dry mass.

High callus fresh weight is obtained from root segments cultured on 1.15–2.3 μM 2,4-D. The epicotyl segments cultured in the light produce superior values of callus fresh weight at all concentrations of 2,4-D or at 2.7 μM NAA; in the dark the best concentration for callus induction is either 4.6 μM 2,4-D or 2.7 μM NAA.

Table 3. Peak area percentage of the volatile constituents collected in the head space SPME of callus of *Cedrela fissilis* cultured either in the light or in the dark during 8 weeks on MS basal medium supplemented with 2.5 μM BAP and 5 μM NAA.

Compound	R.I. ^a	Callus cultured in the light	Callus cultured in the dark
Unknown	974	0.73	0.62
Limonene ^b	1031	0.67	1.33
1,8-Cineol ^b	1033	9.40	10.19
Terpinolene ^b	1088	0.72	0.99
Camphor ^b	1143	2.10	2.41
Borneol ^b	1165	1.10	1.12
α -Terpineol ^b	1189	3.95	4.50
Bornyl acetate ^b	1285	44.01	54.03
Tridecane ^d	1300	0.75	—
α -Terpenyl acetate ^b	1350	9.87	13.10
Tetradecane ^d	1400	1.72	0.79
Unknown	1410	1.18	1.37
Unknown	1482	0.65	—
Germacrene D ^c	1491	2.02	—
Unknown	1499	0.61	1.90
α -Alaskene ^c	1513	1.12	—
6,11-oxo-Acor-4-ene ^c	1531	4.01	3.92
Unknown	1615	2.65	0.8
Unknown	1717	10.32	1.19
Unknown	1893	1.57	—
Unknown	1900	0.84	—

^aRetention Index, ^bmonoterpenes, ^csesquiterpenes, ^dalkanes

2.3.5. Callus Induction from Cotyledon Segments

1. Excise segments (0.8–1.0 cm) either from the basal end or from the middle of cotyledons from 30-day-old seedlings aseptically grown in the light.
2. Inoculate the segments from the middle portion of the cotyledon on MS medium supplemented with 2% (w/v) sucrose, 0.2% (w/v) Phytigel and with different combinations of BA (0, 1.25, 2.5, 5, 10 and 15 μM) and NAA (0, 1.25, 2.5, 5, 10 and 15 μM).
3. Inoculate the basal end segments on MS medium supplemented with 2% (w/v) sucrose, 0.2% (w/v) Phytigel and either 2.3 or 4.6 μM 2,4-D. Place explants horizontally into the medium.
4. Incubate the middle portion cotyledon cultures in the light and the basal end cotyledon cultures in the dark.
5. After 45 days globular calli can be collected for fresh and dry mass assessments.

High callus fresh weight (ca. 300 mg) from the middle portion of the cotyledons is produced in 80–100% of the explants when 2.5–15 μM BA is combined with either 10 or 15 μM NAA. Rhizogenesis occurs in all treatments except in the absence of NAA or when 15 μM BA is combined with 1.25–15 μM NAA. These explants produce calli with lower fresh weight (<100 mg) when cultured with either 2.3 or 4.6 μM 2,4-D. The basal end segments, on the contrary produce calli with higher fresh weight when cultured at these 2,4-D concentrations.

2.4. Direct Organogenesis

The culture media for protocols of direct organogenesis are shown in Table 2.

2.4.1. In Isolated Cotyledons

1. Excise cotyledons from 20-day-old seedlings aseptically grown in the dark and inoculate them on MS medium supplemented with 2% (w/v) sucrose, 0.2% (w/v) Phytigel and with different concentrations of either BA (0, 0.3, 0.6 and 1.25 μM) or thidiazuron (TDZ). Place explants horizontally into the medium.
2. Incubate the cultures either in the light or in the dark.
3. After 30 days evaluate the cultures for shoot and/or root production.

Shoots are directly produced at the basal end of 20–30% of the explants cultured in the light, in the presence of either 0.6 μM BA or 0.3 μM TDZ (Figure 2D). In the dark shoots are formed in 20% of the explants only when cultured with 0.6 μM TDZ. Direct rhizogenesis is observed in 40–60% at the basal end of the explants cultured in the absence of BA or TDZ, either under light or dark (Figure 2E). Therefore, isolated cotyledons are promising explants to be used to develop systems for genetic transformation to produce transgenic shoots or roots for further studies on secondary metabolism.

2.4.2. In Intact Seedlings

1. Surface sterilize seeds in commercial bleach (2.5% active chlorine) with 2–3 drops of Tween 20 for 75 min.
2. Rinse three times for 10 min in sterile distilled water and culture on MS medium supplemented with 2% (w/v) sucrose, 0.2% (w/v) Phytagel and 0, 10.74, 21.48 and 42.96 μM NAA.
3. Incubate the cultures in the dark.
4. After 60 days evaluate the cultures for morphogenic events.

Direct organogenesis (rhizogenesis) is observed in the distal portion of cotyledons of seedlings (20–30%) grown at either at 10.74 or at 21.48 μM NAA (Figure 2C). The competence of cotyledons to produce roots without callus formation is a clear indication that they may also have the ability to originate shoots directly when properly manipulated with cytokinins. These are important achievements to foster studies on the development of consistent direct shoot/root regeneration for genetic transformation.

3. CONCLUSION

This chapter highlights *C. fissilis* tissue culture and a reproducible micropropagation protocol based shoot multiplication. The results showed that BA is indispensable for the sprouting and multiplication of axillary buds of cotyledonary node cuttings. They can then be used as starting plant material for further rapid multiplication of selected genotypes and in alginate-encapsulation of *in vitro* derived vegetative propagules for synthetic seed production. We established a reliable micropropagation and callus culture protocols from juvenile seedlings of *C. fissilis*. This technology may provide a valuable tool in a tree improvement program, although the study on micropropagation from adult trees will be essential.

In vitro germplasm conservation of *C. fissilis* requires 6–8 subcultures per year in fresh culture media. We have achieved in reducing maintenance cost, risk of contamination by preserving ca. 44% viability of encapsulated shoot tips. However, more studies are necessary to improve the efficiency of the system. Another advantage of the described protocol is that it does not require liquid nitrogen and reduce the cost of storage. Therefore storage of alginate-encapsulated germplasm at room temperature is a practical alternative for the conservation of tropical forest trees.

The callus culture protocols will foster the studies on secondary metabolites and genetic transformation systems. Lago et al. (2004) identified volatile oils from leaves and stem barks of *C. fissilis* by direct analysis with GC-MS performed by hydrodistillation in a Clevenger-type apparatus. This is a laborious method and requires relatively large amount of fresh plant material. It is unsuitable to perform rapid screening for volatile production of newly initiated cell cultures. Therefore, the headspace SPME technique would be an ideal tool to probe calli for the production of volatiles of *C. fissilis*. The method was validated preliminary on characterization of callus grown in different culture conditions and it is suitable for screening of *in vitro* cultures for volatile oil production.

These protocols are suitable for vegetative propagation, cryopreservation and secondary metabolism studies. *Ex situ* storage procedures are now available for the medium- to long-term conservation of *C. fissilis*. They offer new opportunities for the conservation, sustainable management and utilization of this valuable fast growing timber tree.

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CHAPTER 22

MICROPROPAGATION OF MATURE TREES OF *ULMUS GLABRA*, *ULMUS MINOR* AND *ULMUS LAEVIS*

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1. INTRODUCTION

About 40 species of elms (*Ulmus* spp.) occur throughout the temperate regions of the Northern Hemisphere. Elm trees are highly valued for their cold tolerance, their landscape value as well as for their timber. Unfortunately, in recent decades the fungal vascular wilt disease caused by the vascular wilt fungus *Ophiostoma ulmi* and more recently by highly aggressive *O. novo-ulmi* (Et-Touil et al., 1999), has devastated the elm populations of Europe, North America and central Asia. This has led, in both Europe and North America, to the beginning of breeding programmes in an attempt to produce resistant individuals. However, production of disease resistant elm trees via conventional breeding has met with limited success owing to the long time scales involved in breeding programmes and difficulties in obtaining locally climatically adapted trees. Some resistant material has recently been released in North America and the Netherlands for trial plantings (Gartland et al., 2001).

The attention is now being focused on biotechnology with the goal of genetic improvement through the transfer of foreign genes into the genome of elm cells (Fenning et al., 1996). However, this strategy can only be initiated after the establishment of efficient protocols for plant regeneration from cells and tissues of elm trees. Various micropropagation systems have been reported for a range of elm species and hybrids (Chalupa, 1994; Gartland et al., 2000; Mala, 2000; Biroscikova et al., 2004). Protocols of this type have been already developed from differentiated explants (Fenning et al., 1993), from callus and suspension cultures (Karnosky et al., 1982). Another way that could play an important role in current elm improvement

programmes is somatic embryogenesis (Conde et al., 2004). The presented protocol describes an efficient method for *in vitro* micropropagation of wych elm (*Ulmus glabra* Huds.), smooth-leaved elm (*U. minor* Gled.) and European white elm (*U. laevis* Pall.) by the axillary bud culture method (see Figure 1).



Figure 1. Stand of *Ulmus glabra* donor plus trees in Ore Mountains.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Growing Condition of Mother Plants

For the establishment of primary elm cultures the plant material from 40 to 80 years old donor plus trees of *Ulmus glabra*, *U. laevis*, and *U. minor* (15 clones from each elm species) has been used. The origins of the different elm species are summarized in Table 1.

2.1.2. Explant Excision and Sterilization

Dormant axillary buds collected in February were used for tissue culture establishment. Thirty buds have been taken from each clone for primary cultivation. The buds were first rinsed under tap water to remove surface dirt, and sterilized by immersion in 0.1% NaClO₃ for 10 min followed by immersion in 0.01% HgCl₂ for 15 min. The buds were further rinsed three times with autoclaved distilled water for 15 min. Finally, the apical meristems were excised from buds by means of sterile incisors and scalpel.

Table 1. *The origin of elm species.*

<i>Species</i>	<i>Natural area</i>	<i>Number of clones</i>	<i>Altitude (m)</i>	<i>Average annual rainfall (mm)</i>
<i>U. glabra</i>	Ore Mountains	10	800–900	680
	Giant Mountains	5	750–800	720
<i>U. minor</i>	Lowland of Labe river	3	170–200	450
	Lowland of Labe river	6	250–300	450
<i>U. laevis</i>	South Moravia	6	150–160	350
	South Moravia	6	150–160	350
	Lowland of Labe river	2	190–200	450
	Lowland of Labe river	7	180–200	450

2.2. Induction of Organogenesis

The excised apical meristems from dormant buds were placed onto agar-solidified (6 g l⁻¹) Murashige & Skoog (1962; MS) basal medium supplemented with benzylaminopurine (BAP), β -indolebutyric acid (IBA), glutamine and sucrose (Table 2).

Table 2. *Growth substances, organics and sucrose concentrations supplemented to basal MS medium used for various morphogenetic programs in micropropagation of elm.*

<i>Program</i>	<i>Time of culture</i>	<i>Growth substances (mg l⁻¹)</i>	<i>Organics (mg l⁻¹)</i>	<i>Sucrose (g l⁻¹)</i>	<i>Strength of MS</i>
Induction of organogenesis	4–6 w	BAP (0.2) IBA (0.1)	Glutamine (10.0)	30.0	Full
Shoot multiplication	3–4 w	BAP (0.5) IBA (0.1)	Glutamine (200.0) Casein-hydrolysate (200.0)	30.0	Full
Rooting of shoots	1 w	NAA (1.0)	0	10.0	One-third
	3–4 w	0	0	10.0	One-third

The pH of media was adjusted to 5.8 with 1 M KOH. The buds were cultured at 24°C under 16-h photoperiod with white fluorescent light (36W/33 Philips tubes, Eindhoven, The Netherlands; irradiance of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 4–6 weeks. Organogenic activity of apical meristems appeared approximately after 2 weeks of cultivation. The differentiation of new adventitious shoots was initiated from the meristemoids formed in calli on the basis of primary explants and/or from axillary buds (Figure 2A). The average number of newly regenerated shoots was 1–2 in *U. glabra* and *U. laevis* and 3–4 in *U. minor* where the additive shoot were also formed from axillary buds. The average loss of primary explants, due to contamination and/or overgrowth of callus, was detected in all clones between 10% and 40%.

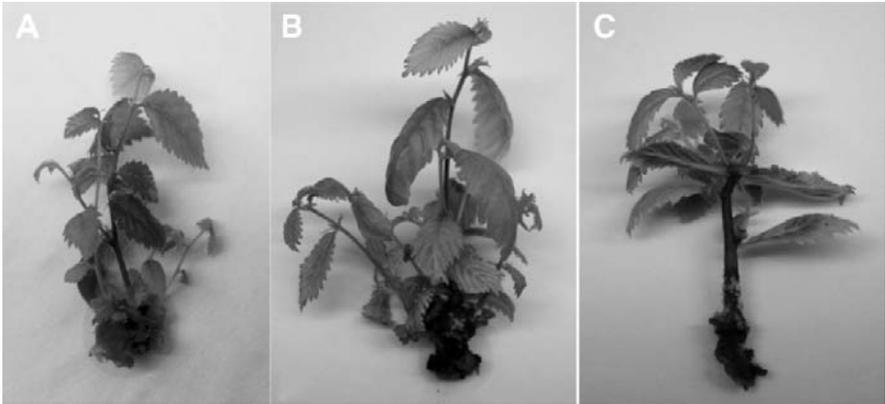


Figure 2. A) Induction of organogenic activity in primary explant. B)–C) The shoot forming capacity of apical and basal parts of elm multiplied shoots. B) The new adventive shoots arose from meristemoids on the bases of apical segments. C) The new shoots in basal parts originated from axillary buds.

2.3. Shoot Multiplication

The shoots growing from the buds of 1–2 cm long were excised and placed onto the fresh medium. For subsequent transfer we used MS medium modified by an increased concentration of BAP, IBA, glutamine, and casein-hydrolysate. Explants were grown under the same growth conditions as during the organogenesis induction stage and the cultures were transferred onto the fresh media every 3–4 week interval. After the first transfer to the multiplication medium, the numbers of growing shoots in elm species increased differently. Both the numbers and the lengths of newly formed shoots varied among elm species (Table 3). No changes in the shoot numbers were observed during the following transfers to the fresh media.

Table 3. The differences in average numbers and length of newly developed shoots between elm species after 4 weeks of cultivation.

Species	Number per explant \pm SD	Shoot length mm \pm SD
<i>U. glabra</i>	3.8 \pm 1.6	50.8 \pm 11.2
<i>U. minor</i>	4.6 \pm 1.8	30.2 \pm 16.3
<i>U. laevis</i>	4.2 \pm 1.5	48.4 \pm 15.1

2.3.1. Multiplication from Shoot Apical and Basal Segments

We focused on studying the possibility to increase the multiplication rate. Regenerated shoots of *U. glabra* were cut into two parts, apical and basal, and both parts (each about 2 cm long) were cultivated separately on the shoot multiplication medium for 4 weeks. The efficiency of cut shoots in terms of shoot multiplication and root initiation was compared in 50 microcuttings each of both parts, respectively. The

shoot forming capacity was higher in the apical part where the mean numbers of shoots per apical segment explant were 5.5 ± 1.6 . The basal parts responded to culture on multiplication medium with the mean number 3.8 ± 1.8 of shoots per explant. In the apical parts the new adventitious shoots arose from the meristemoids that were formed in calluses on the bases of apical segments (Figure 2B). The removal of the apex released the axillary buds from apical dominance and the adventitious shoots formed on the elm shoot basal parts originated from axillary buds (Figure 2C).

2.4. Rooting of Shoots

Mixed samples of 50 microcuttings about 3–4 cm long from 15 clones of each elm species were used for rooting and acclimatization. The rooting was performed in two steps. Transferred shoots were cultured in black boxes in air-conditioned room at 25°C for 1 week on the agar medium with one-third strength MS enriched with α -naphthalenacetic acid (NAA) as the sole growth regulator (Table 2). After 7 days the shoots were transferred onto the hormone-free medium (one-third strength MS) and exposed to 16 h photoperiod (white fluorescent light, $30 \mu\text{mol m}^{-2} \text{s}^{-1}$). Roots appeared in all species after 7–8 days. The percentages of rooted *U. minor* and *U. laevis* microcuttings were high, $95.8\% \pm 2.1\%$ and $92.8\% \pm 3.6\%$, respectively, while in *U. glabra* it reached only $85.6\% \pm 2.7\%$. After 3–4 weeks rooted shoots were transferred into non-sterile substrate for acclimatization (Figure 3A).

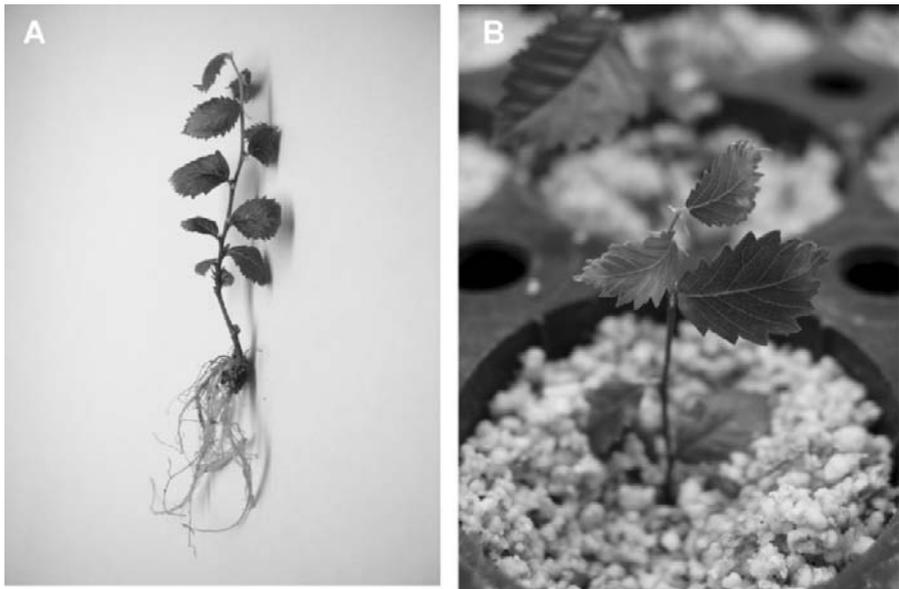


Figure 3. A) Rooted elm plantlet after 4 weeks cultivation in agar medium. B) An elm plantlet growing in perlite for 15 days.

2.4.1. Rooting of Apical and Basal Shoot Segments

The differences in root development of apical and basal segments of *U. glabra* were scored as mean rooting time. The apical and basal parts of 50 shoots (about 2 cm long) from multiplying explant cultures were used for rooting. The timing of root formation was significantly delayed in the apical part (13 days) in comparison with the basal part where the first roots appeared after 7 days. However, the percentage of rooted segments was similar $84.8\% \pm 2.1\%$ and $87.8\% \pm 2.1\%$ in the apical and basal segments, respectively.

2.5. Acclimatization and Hardening

2.5.1. Growth in Perlite (Beads of Expanded Basic Silicates)

Rooted plantlets were transferred into side slit cells with perlite (BCC Growing trays, Sweden; size of side slit cell: the cones without bottom, height 100 mm, upper diameter 50 mm, inferior 40 mm) and watered by one-tenth strength MS medium (Figure 3B). Plantlets were cultivated for 30 days under continuous white fluorescent light (36W/33 Philips Tubes, The Netherlands; $30 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 25°C. The relative humidity in air-conditioned room was during the first 15 days of culture 90% and then decreased for the next 15 days to 70% relative humidity.

2.5.2. Cultivation in Peat–Soil Mixture

Plantlets growing for 1 month in perlite were transferred into peat–soil–perlite mixture (2:1:1, w/w/w) and cultured in BCC Growing trays (size of cell with eight vertical interior projections: height 190 mm, upper diameter 67 mm, inferior one 45 mm) for 15 days under continuous white fluorescent light (36W/33 Philips Tubes, The Netherlands; $30 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 25°C and 70% relative humidity in air-conditioned greenhouse. During acclimatization period (approximately 8 weeks) only 5% mortality of plantlets was recorded. Average heights of plantlets measured at the end of acclimatization period were: 28.4 ± 12.1 cm in *U. minor*, 32.8 ± 14.2 cm in *U. glabra*, and 37.5 ± 13.6 cm in *U. laevis*.

2.5.3. Hardening

After 6 to 8 weeks in greenhouse the plantlets were transplanted to the outdoor protected nursery beds during May and June (Figure 4A). During the spring of the next year were the plantlets planted on the trial plots. The minimal percentage of plantlet mortality (up to 7%) was recorded after transfer them onto outdoor beds.

2.6. Field Testing

The field trials were established for micropropagated trees from *U. glabra* in April, 1998. The main aim of the field trials was to compare the growth rate, morphology and vitality of plants regenerated from mature mother tree via organogenesis with 2-year-old seedlings derived from the same provenance. The trial plots were established at two localities (altitude 520 and 560 m above sea), the spacing of trees in the field trials was 2×2 m and the tree height, vitality, stem and treetop

morphology were assessed each year. There were no significant differences in the growth, vitality and morphology of *in vitro*- and generative-propagated trees. The growth rate of micropropagated trees is slightly higher than that of seedlings in the course of the followed 7 years (Table 4, Figure 4B). Vigorous 7-year-old regenerates showing normal growth characteristics are presented in Figure 4B.

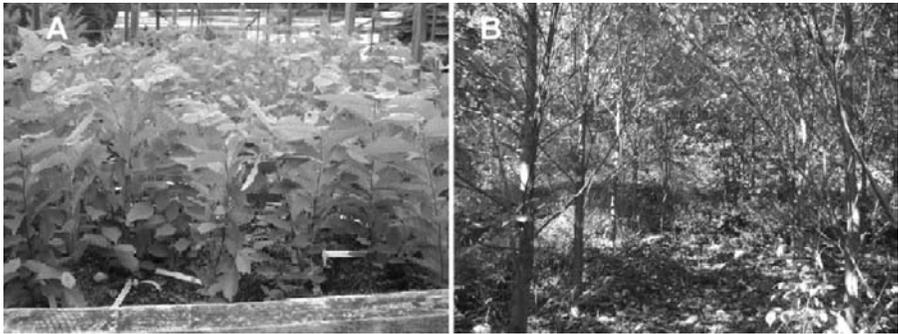


Figure 4. A) Elm plantlets after 1 - year growth on the outdoor protected nursery beds. B) *In vitro*-propagated 7-year old *U. glabra* trees growing on the field trial.

Table 4. Comparison of the growth and mortality of *in vitro*-propagated *U. glabra* trees and generative- propagated trees from the same provenance growing at the stand Polná (560 m above sea level).

Year	<i>In vitro</i> -propagated			Generative-propagated		
	No. of trees	Average height \pm SD [cm]	Average increment \pm SD [cm]	No. of trees	Average height \pm SD [cm]	Average increment \pm SD [cm]
1998	92	106.8 \pm 32.2	–	56	59.3 \pm 18.3	–
1999	92	135.6 \pm 35.5	38.7 \pm 15.9	55	70.8 \pm 15.6	14.2 \pm 8.2
2000	90	151.9 \pm 37.2	16.4 \pm 10.6	55	85.3 \pm 22.5	23.2 \pm 23.6
2001	90	191.9 \pm 48.1	40.0 \pm 26.9	55	108.2 \pm 59.8	45.0 \pm 30.7
2002	90	241.5 \pm 62.6	49.6 \pm 30.2	55	174.4 \pm 64.4	66.2 \pm 36.2
2003	90	292.2 \pm 84.4	50.7 \pm 31.3	55	197.0 \pm 87.2	29.6 \pm 20.1
2004	90	323.6 \pm 98.5	31.4 \pm 25.5	55	239.4 \pm 95.1	39.5 \pm 30.9
2005	90	374.8 \pm 51.2	51.1 \pm 35.1	55	286.0 \pm 92.9	37.8 \pm 27.3

2.7. Genetic Stability

Genetic stability of *U. glabra*, *U. minor* and *U. laevis* during *in vitro* cultivation was studied by the method of isoenzyme analysis. Polymorphism of following enzymes was studied: malate dehydrogenase (MDH), phosphoglucomutase (PGM) and phosphogluconate dehydrogenase (6-PGDH). No genetic changes were found for selected *Ulmus* sp. clones within investigated enzymatic systems (6-PGDH, MDH and PGM) that would be caused by the induction of organogenesis on dormant buds

of donor individuals and/or by their subsequent, long-term *in vitro* cultivation (Ivanek et al., 2005).

2.7.1. Enzyme Extraction and Isoenzyme Analyses

Sprouting elms leaves were sampled and stored at -15 to -25°C . Samples were homogenized with extraction buffer according to Wendel & Weeden (1989). Extraction buffer contained 1% polyvinylpyrrolidone (PVP), avg. mol. wt. 360,000, 7% PVP (avg. mol. wt. 40,000), 10% sucrose, 200 mg l^{-1} ethylenediaminetetraacetic acid (EDTA), 150 mg l^{-1} dithiotreitol, 175 mg l^{-1} ascorbic acid, 1 g l^{-1} bovine serum albumine, 275 mg l^{-1} β -nicotinamide adenine dinucleotide (NAD), 225 mg l^{-1} β -nicotinamide adenine dinucleotide phosphate (NADP), 50 mg l^{-1} pyridoxal 5-phosphate and 6.6 ml l^{-1} mercaptoethanol in 1 M Tris (hydroxymethyl)-aminomethane citrate buffer, pH 6.7). The isoenzymes were separated by horizontal one-dimensional electrophoresis on starch gel at 3 – 5°C using histidine citrate buffer (pH 5.7). Isoenzyme patterns of MDH, PGM and 6-PGDH were stained according to Pasteur et al. (1988). MDH was stained using mixture of 20 ml 0.2 M Tris (hydroxymethyl)-aminomethane hydrochloride buffer (Tris HCl, pH 8.0) and 20 ml 0.5 M DL-malic acid (pH 7.0), containing 500 mg l^{-1} NAD, 500 mg l^{-1} nitrotetrazolium blue chloride (NBT) and 125 mg l^{-1} phenazine methosulphate (PMS). For PGM staining, 40 ml of 0.2M Tris HCl (pH 8.0) was used, containing 12.5 g l^{-1} disodium salt of glucose-1-phosphate; 1.25 mg l^{-1} glucose 1,6- diphosphate, 40 units glucose-6-phosphate dehydrogenase; 5 g l^{-1} MgCl_2 hexahydrate, 500 mg l^{-1} NAD, 500 mg l^{-1} NBT and 250 mg l^{-1} PMS. The 6-PGDH was stained using 40 ml of 0,2 M Tris HCl (pH 8.0), containing 1.25 g l^{-1} trisodium salt of 6-phosphogluconic acid, 2.5 g l^{-1} MgCl_2 hexahydrate, 500 mg l^{-1} NAD, 500 mg l^{-1} NBT and 125 mg l^{-1} PMS.

Based on the increasing mobility in electrical field the isoenzyme alleles were marked by capital letters in alphabetical order, reflecting mobility of isoenzyme patterns. Zymograms were evaluated both by visual interpretation and in digital manner using Pharmacia Biotech software ImageMaster.

2.8. Cold Storage of *in Vitro* Cultures

The elm shoot cultures (10 cultures of each 15 clones of *U. glabra*, *U. minor* and *U. laevis*) were stored at 5°C without marked influence on the survival and proliferation capacity of *in vitro* cultures for up to 12 months without subculture. As already mentioned above, the shoot cultures were transferred onto the fresh media every 3–4 week interval. However, for the cold storage elm multishoot cultures were used 7 days after the last subculture. The cultures were transferred from standard culture conditions to the cold room where they were kept at 5°C for up to 12 months, 16 h photoperiod with lower light intensity ($15 \mu\text{mol m}^{-2} \text{s}^{-1}$). After 12 months, cultures were subcultured to the fresh medium and further maintained at standard condition. The regeneration capacity and growth of the cold stored shoots increased after the second subculture, however, this effect was not a permanent one and the followed parameters returned to the control levels (non-stored cultures) in

subsequent subcultures. Approximately 75% of *U. glabra*, *U. minor* and *U. laevis* cultures regenerated after 12 months of cold storage without subculture.

2.9. Statistical Analyses

One-way analysis of variance (ANOVA) was used for the statistical evaluation of the results.

3. CONCLUSION

This protocol describes an efficient method for *in vitro* micropropagation of elms, important European native trees. To date, 8-year-old *in vitro*-propagated trees (Figure 4B) do not show abnormal morphological variation or growth characteristics in comparison with generative-propagated trees. We focused also on studying the possibility to increase the elm explant multiplication rate by cutting the regenerated shoots to apical and basal parts. Our attempt was to establish a relationship between endogenous hormonal content and *in vitro* morphogenic responses of elm explants. The shoot forming capacity was higher in the apical part but the timing of root formation was in this type of explant significantly delayed (compared with the organogenic potential of basal part). We have shown that the efficiency of our elm multiplication system in terms of shoot multiplication and rate of root initiation was significantly influenced by the endogenous levels of auxin, cytokinins (predominately zeatin and dihydrozeatin), polyamines and phenolic acids in the explants (Mala et al., 2006). Our results demonstrate that this protocol can be used routinely for multiplication of elms.

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Section B

CHAPTER 23

MICROGRAFTING IN GRAPEVINE (*VITIS* spp.)

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1. INTRODUCTION

Grapes are sought after fruit grown for its high nutritional quality and taste and its use in the wine industry. Grapes are mainly cultivated in the temperate regions but some cultivars that are tolerant to high temperatures have been introduced to tropical and subtropical countries. Commercial production of grapes is approximately 60.9 metric tonnes (FAOSTAT, 2004). Over the past decade substantial increases in grapevine plantings have been driven by increasing wine exports (Pathirana & McKenzie, 2005). Amongst the genus *Vitis*, *V. vinifera* and its selections are the commercially popular table varieties as well as used in wine making.

Most commercial plantations utilize direct planting of selected varieties. However, cultivated varieties of grape are susceptible to microbes, mites, insects, nematodes and more importantly to *Phylloxera* leading to decline in total crop productivity. Therefore it is necessary that the planting material obtained for the establishment of new vineyards must be free from diseases, especially viral. Since once a vineyard is established, minimizing losses caused by viral infection is difficult to achieve (Martelli, 1993) numerous attempts have been made at generating resistant varieties but have met with limited success. To overcome this, several methods have been developed to index the disease status of vines such as, (a) serological (Monis & Bestwick, 1996), (b) molecular (Goszczyński & Jooste, 2003), (c) woody grafting (Martelli et al., 1993) and (d) immunosorbent electron microscopy, sequential poly-acrylamide gel electrophoresis for viroids, dsRNA analysis and nucleic acid hybridization (Macquaire et al., 1993; Martelli et al., 1993). During this period, the idea has emerged 'to graft fruit-bearing scions onto resistant rootstocks to get infection free vines'.

Conventionally, grapes are mainly propagated by cuttings, layering and grafting. Sprouts that arise during vegetative propagation serve as the base material for further clonal multiplication and propagation. In some grape varieties and some rootstocks,

cuttings have however proved difficult to root. Insufficient disease-free planting material led to alternate avenues being sought to meet the growers' demand. In breeding programs aimed at grape improvement, the major constraint has been the vines' long generation cycle and highly heterozygous nature.

Interestingly the demand for seedless progeny is high, but the proportion that can be generated from crosses between seeded and seedless varieties, is low. In this area, application of *in vitro* techniques for the rapid propagation of grape, holds immense potential. The explant used in grape micropropagation has mostly been shoot tips and axillary buds (Salunkhe et al., 1999) and the success was found to be highly genotype dependent. Herbaceous grafting under green house conditions has been practiced with cucurbits and solanaceous plants for many years (Oda et al., 1994; Hartmann et al., 1997). This process involves placing a shoot tip or meristem on the freshly cut surface of a seedling epicotyl, where it is held in place while it heals under high humidity conditions (Walker & Golino, 1999).

A similar but relatively new technique 'green-grafting', has been developed for the vegetative propagation of grapevines and has generally been promoted as a method to rapidly produce grafted grapevine plants (Carlson, 1963; Walter et al., 1990; Kaserer et al., 2003). Grapevines have been green grafted not only under green house but also under *in vitro* conditions (Bouquet & Hevin, 1978; Walker & Meredith, 1990). This tissue culture based grafting system referred to as 'micro-grafting', was introduced by Tanne et al. (1993). Micrografting of grapevine was aimed mainly to eliminate viruses in certification programmes (Kim et al., 2005). However, this also finds application in the rapid propagation of scarce or hard-to-propagate varieties (Gray & Fisher, 1986; Lee & Wetzstein, 1990; Lewandowski, 1991).

Greenhouse-grown mother vines of rootstocks and scions are ideal and can supply the needed cuttings for year-round grafting after virus-free certification. The woody cuttings with one bud each are selected and planted, with the bud facing upwards in standard potting mix on a mist bed. Success with green grafting is dependent upon high-quality cuttings under optimized conditions of light, humidity, fertilization and insect/pest control. Avanzato and Tamponi (1988) have described improved grafting success with the application of heat to the walnut graft union. The scion cuttings should have a full sized leaf, which is trimmed by about half, and has an active lateral bud. The rootstock cuttings need to be at least 25 cm long to accommodate adequate spacing between the roots and graft union which is essential for field planting (Walker & Golino, 1999).

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Supply of Plant Material

Green shoots from green house grown rootstocks and scions have to be selected for culture initiation (tissue culture) and micrografting. The material could be virus-free rootstocks (*Cabernet franc* and LN 33) and virus-infected scions (*Cabernet franc*

and *Cabernet sauvignon*) as reported by Pathirana and McKenzie (2005) or any cultivated variety and any rootstock of choice.

2.1.2. Disinfection of Plant Material and Culture Initiation

Surface sterilization of rootstock cuttings and scion cuttings is carried out for 30s in 70% ethyl alcohol followed by 1% sodium hypochlorite solution containing 0.2% Tween 80 for 20 min with shaking, on a rotary shaker, 50 rpm (Pathirana & McKenzie, 2005). Surface sterilization can also be done by thoroughly washing cuttings with soap and water and subsequently immersing in 0.1% HgCl_2 for 10 min followed by rinsing (4–5 times) with sterile tap water (Mhatre et al., 2000). The surface sterilized shoots are then cut into single node pieces and cultured on defined media (Figure 1A). To achieve sufficient multiplication, the shoots can be split into half lengthwise at the node and cultured horizontally with the cut surface in contact with the medium and the bud facing upwards (Pathirana & McKenzie, 2005). For micrografting, shoots from the second subculture are used.

2.2. Serial Multiplication of Plant Material

2.2.1. Culture Media

The composition of culture media for micropropagation of grapevine has been described in Table 1. Protocols for efficient *in vitro* multiplication in cultivated varieties of grapes are available (Mhatre et al., 2000; Mezzetti et al., 2002; Pathirana & McKenzie, 2005) which can be implemented perhaps with minor variation to suit each variety, for mass propagation of rootstock or scion prior to micrografting. Mhatre et al. (2000) have described a step wise protocol for the efficient and cyclic recovery of shoots of *V. vinifera* cvs. Thompson, Sonaka and Tas-e-Ganesh. They have defined specific media that can be used at four distinct stages in the recovery of complete plants using nodal culture namely, *initiation*, *multiplication*, *shoot elongation* and *rooting* (Table 1b,c,d,e). Pathirana and McKenzie (2005) have described a medium for cvs. *Cabernet franc* and *C. sauvignon* containing BAP alone to multiply shoots with roots or BAP and IAA which supports shoot multiplication as well as rooting (Table 1a).

2.2.2. Culture Procedure

Stem segments containing a single node should be cultured per culture vessel and allowed to grow (Figure 1A). In a span of 3 to 4 weeks on initiation medium (Table 1b) three shoots can be produced (Mhatre et al., 2000). One of the shoots can be rooted on rooting medium (Table 1e) and the other two can be subcultured on multiplication medium (Table 1c) for further proliferation (Figure 1B). Periodically, explants from this can be subcultured or rooted (Figure 1C,D) as per requirement leading to a cyclic and abundant supply of shoots or plantlets (Mhatre et al., 2000).

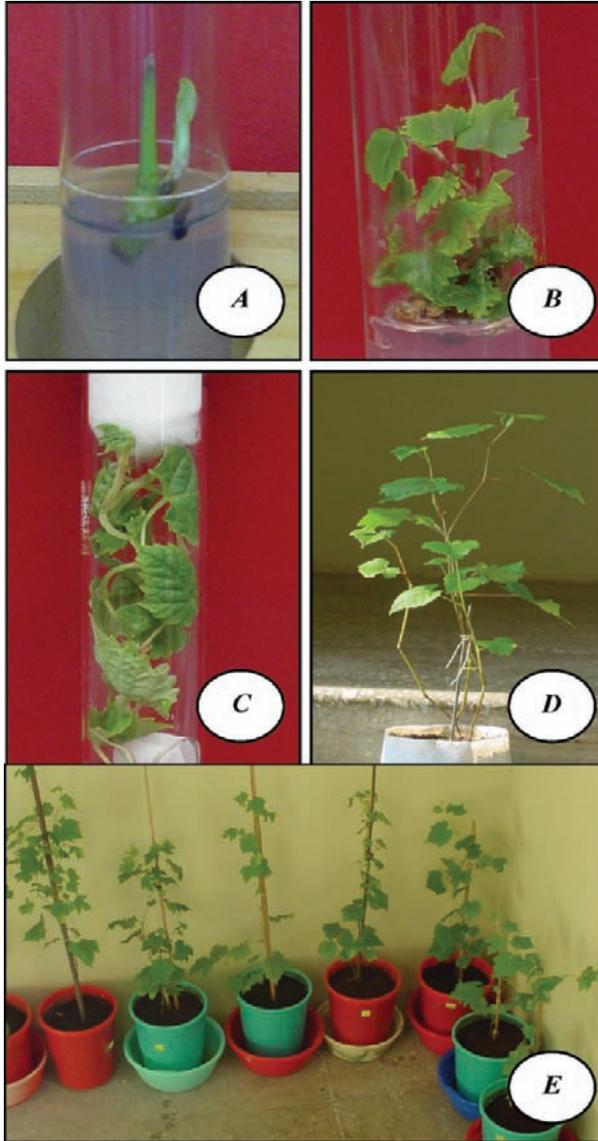


Figure 1. Micropropagation of grapevine cv. Anab-e-Shahi via nodal culture. A) Cultured axillary bud, B) Axillary bud producing multiple shoots, C) Rooting of shoot in liquid rooting medium, D) Acclimatized plant in polybag, E) Tissue culture derived potted plants.

Table 1. Commonly used nutrient media for multiplication of grapevine shoots.

Sl #	Medium	Macro elements	Micro elements	Vitamins	Growth adjuvant mg l ⁻¹	Carbon source g l ⁻¹	Reference
a.	¹ MS	1/2	1	² LM-1 + inositol-2	BAP 0.5+IAA 0.08	30	Pathirana & McKenzie, 2005
b.	³ NN	1	1	⁴ LS-1	Thiamine 10 + Ad. sul. 40.53 + monobasic NaH ₂ PO ₄ 218.4 + BAP 2.25 + NAA 0.09	20	Mhatre et al., 2000
c.	⁵ WPM	1	1	⁶ B ₅ -1	Cal. Pan. 2 + monobasic NaH ₂ PO ₄ 168 + BAP 2.25+ IBA 0.5	30	
d.	MS	1	1	1	BAP 0.5 + IAA 0.2	20	
e.	MS	1/2	1/2	1	IAA 0.1	10	

¹MS, Murashige & Skoog (1962) medium; ²LM, Lloyd & McCown (1980) organics; ³NN, Nitsch & Nitsch (1969) medium; ⁴LS, Linsmaier & Skoog (1965) medium; ⁵WPM, woody plant medium (Sigma catalogue, 1994); ⁶B₅, Gamborg et al. (1968) medium; BAP, benzyl amino purine; IAA, indole-3-acetic acid; Ad. sul., adenine sulphate; NAA, naphthalene acetic acid; IBA, indole butyric acid.

2.3. In Vitro Grafting

2.3.1. Using Shoot Apices

In vitro micrografting can be attempted using shoot apices (0.3 mm) as explants, of the chosen rootstock and the scion to generate shoots on a large scale (Cantos et al., 1995). Once the rootstock culture is established as a rooted shoot, its top portion can be cut so that there is no nodal region left and a small cleft is made in the cut end. The shoot portion (bearing 3–4 buds) of the scion is sliced from the rest and inserted in the cleft of the rootstock. The entire set is again cultured *in vitro* on a medium that supports growth (Table 1). Bass et al. (1988) have used animal serum in the culture medium to facilitate the development of grapevine meristems (0.1 mm, 1–2 leaf primordial) and report an increase in the number of meristems developing into plantlets as well as in the rate of their *in vitro* differentiation. The healing of the callus occurs during the first 6 days and the establishment of vascular junction – between the 8 and 12th day after grafting. This is the critical period for successful micrografting and results in 60% survival of the micrografted plants (Cantos et al., 1995).

2.3.2. Using *in Vitro* Produced Shoots

Tissue culture generated rootstock rooted plantlets, grown until several leaves are produced (6–8 weeks) can be divided into 3–4 explants, each containing 1–2 nodes with leaves. The apex of the rootstock nodal explant is cut longitudinally with a sharp sterilized blade, producing a small (2–4 mm) longitudinal cleft leaving the rootstock decapitated. An appropriate scion with a single node and a leaf can be selected to match the size of the rootstock. The basal part of the stem of the scion should be cut in such a way that the wedge formed, matches the cleft of the rootstock. It is then fitted on to the cleft of the rootstock grown in the medium (Figure 2). The graft tissue is allowed to develop around the union during subsequent subculture. In this way, two grafts can be established per tissue culture vessel (Pathirana & McKenzie, 2005). After insertion of the scion into the rootstock cleft, wrapping in sterile aluminium foil to hold the graft can also be attempted to strengthen and support the graft (Tanne et al., 1993). However, to accomplish this is laborious and time consuming under aseptic conditions. In case the scion does not fit properly on the rootstock, dipping the lower end of the scion in the medium before fitting it helps to establish the graft. This is because of two reasons (i) the cut surfaces are kept moist until relative humidity of the tub/vessel is established and (ii) dipping ensures that the nutrients are directly supplied to the graft site (Pathirana & McKenzie, 2005).

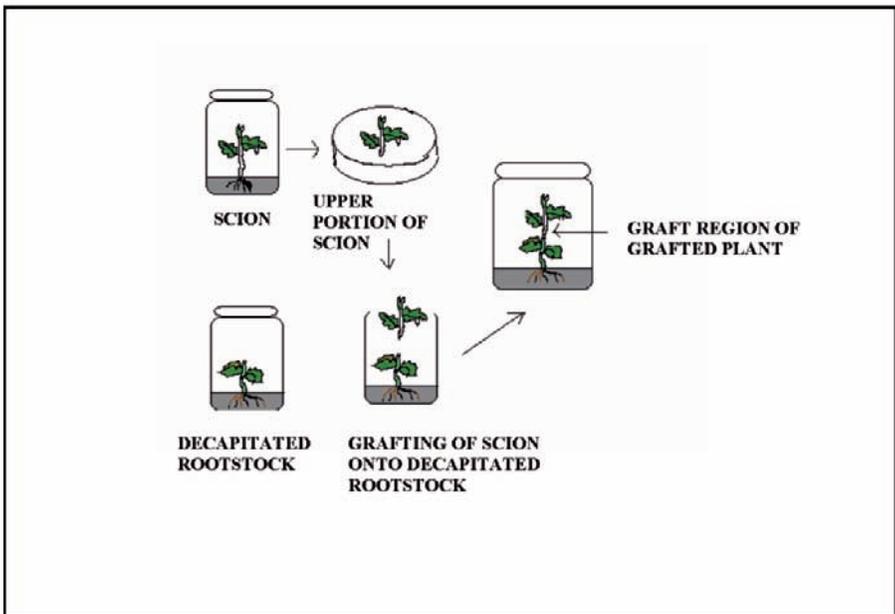


Figure 2. Diagrammatic representation of *in vitro* micrografting.

2.4. Culture Conditions

All media should be solidified with gelrite (Sigma, 3 g l⁻¹) and pH should be adjusted to 5.8 prior to autoclaving at 121°C, 103.4 kPa pressure, for 20 min. Preferably 290 ml clear wide-mouth disposable tissue culture tubs with snap-on lids containing 50 ml medium are the culture vessels ideal and desirable for such work. The wide mouth allows easy access during the *in vitro* grafting of scion on to the rootstock and this type of vessel generates good air circulation needed for the stability of the graft. Cultures should be placed under 16 h light, 8 h dark photoperiod at 30 μmol m⁻² s⁻¹ light intensity at 24°C (Pathirana & McKenzie, 2005).

2.5. Rooting and Transfer to Soil

2.5.1. Rooting of Shoots

Rooting in *in vitro* grafted plants is achieved on defined medium (Table 1a) for a month, after which the grafted plants are ready for transplanting. Some rootstocks do not respond to this medium. In such cases it is necessary to achieve rooting of the rootstock prior to the micrografting step, on medium 'e' (Table 1) for 6 weeks. The roots are trimmed and the young plants are transferred to sterile soil mix. Air circulation is gradually increased and the plants are ready for the greenhouse transplantation. Rooting of grafted plants can also be done as a two step procedure in which roots are only first initiated *in vitro* and subsequently grown *in vivo* on root growing media such as peat, perlite or vermiculite (or a mixture of all three), which are commercially available (Sigma-Aldrich or at local nurseries).

2.5.2. Acclimatization of Micrografted Plants

The successful grafts require appropriate acclimatization to external environmental conditions in order to obtain a high percentage of survival. It is necessary to have minimum 2–3 leaves developed on the grafted plant before transfer to soil. The time required to reach this stage varies between 5 and 6 weeks after the graft is stabilized. Steam sterilized soilmix suitable to grow grape is always recommended. The plants should be covered with polythene bags with perforations, to retain appropriate moisture and humidity and should be kept in rooms equipped with controlled conditions of temperature, light and humidity.

2.5.3. Graft Incompatibility and Histology

The term 'incompatibility' with respect to grafted fruit trees is defined as a phenomenon of premature senescence caused by physiological and biochemical factors (Feucht, 1988). When two plants of different genotype are grafted, the degree of adaptation or ageing depends on the plasticity of both partners to perform in the enforced symbiosis. Hence, diseases (causative agents), genotypic response of the symbiont to the changed environment or severe environmental stress may shorten the life span of the sensitized graft combination. Histological observations in *Opuntia* spp., micrografts have revealed that there are five developmental stages of graft union formation (a) development of a necrotic layer, (b) proliferation of a

callus bridge at the graft interface, (c) differentiation of new vascular cambium, (d) restoration of new vascular tissue, and (e) restoration of the continuity of external epidermal tissue at the union zone (Estrada-Luna et al., 2002).

3. CONCLUSION

Several methods are available for *in vitro* micrografting in various plants such as, *Citrus* (Navarro, 1992), *Larix* (Ewald & Kretschmar, 1996) and *Persea* (Raharjo & Litz, 2005). In grape, micrografting is one of the methods to recover disease-free plants of *Vitis* spp., for a large scale plantation, for sanitation programs and quarantine procedures and for effective indexing of viruses (Tanne et al., 1993; Valat et al., 2003; Pathirana & McKenzie, 2005). The success of micrografting is cultivar dependent and greatly influenced by the shoot tip source although no marked effect is observed on the growth of the micrografted plants (Baydar & Celik, 1999). The major constraint in this work, however, is to obtain contamination-free cultures once the grafts have been stabilized. Skillful manipulation of acclimatization conditions of micrografted plants holds the key to retain the *ex vitro* viability of *in vitro* generated plants. Besides this, micrografting also finds use in the basic study of graft incompatibility, physiology of the graft union and plant aging (Navarro, 1992). This technique also finds application in cultivated grapevine varieties which are difficult-to-root. The major cost in plant propagation by tissue culture is manual labor. Automation of tissue culture manipulations has been attempted before which can limit *in vitro* manipulations to only one inoculation of plant material on a fresh multiplication medium (Vanderschaeghe & Debergh, 1988). Once the bottleneck of obtaining aseptic plants is overcome using automation, the protocol can be extended to micrografted plants (*in vitro*), leading to mass propagation of disease-free micrografted plants ready for field transfer.

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CHAPTER 24

MICROGRAFTING GRAPEVINE FOR VIRUS INDEXING

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1. INTRODUCTION

Grapevine (*Vitis vinifera* L.) is a widespread and highly valuable horticultural crop. Around 2.25 million ha are grown worldwide, mainly in the southern temperate zone and in the subtropics. Italy (8.6 million Mt from 0.84 million ha), France (6.8 million Mt from 0.85 million ha), the United States (7.1 million Mt from 0.38 million ha), Spain (6.1 million Mt from 1.17 million ha) and China (6.6 million Mt from 0.45 million ha) are the main producers, accounting for half the total world production of 66 million Mt (FAOSTAT data, 2007). More recently, substantial increases in the plantings in the southern hemisphere (Australia, South Africa, Argentina, Brazil and Chile) have been driven by the increasing wine exports from these countries.

Expansion of grape cultivation in many countries has been based on imported grapevine stocks of established cultivars and this trend is continuing. Importation of grape varieties to establish new vineyards increases the risk of previously unknown viruses being introduced. Importantly, planting materials obtained locally to establish new vineyards must also be free from viruses.

It is estimated that global losses due to viral infection of grapevines are over 1 billion US dollars due to effects such as yield reduction, maturity delay, reduced sugar content in the fruit and decreased plant vigour. However, the means to reduce losses caused by viral infection once a vineyard is established are extremely limited. Therefore, it is important to have robust, sensitive, rapid and reliable techniques for assessing grape clones for any viral infections they may carry.

Woody-grafting of vines on to red-berried indicators such as *Vitis riparia*, Gloire de Montpellier, Baco 22A, *Vitis rupestris* St George, LN33, Kober 5BB, 110R, *Cabernet franc*, *Pinot noir*, etc., is the established method of indexing. However, this method is time consuming and labour-intensive, with a 2–3 year incubation period of the grafted indicators in a nursery. A relatively new technique,

green-grafting, developed for the vegetative propagation of grapevines, has been successfully adopted in several grape-growing countries to rapidly diagnose viral disease (Walter et al., 1990; Lahogue et al., 1995; Kaserer et al., 2003; Pathirana & McKenzie, 2005a).

In 1993 Tanne et al. reported micrografting grapevine for the purpose of viral indexing, specifically for the detection of corky-bark virus. This closterovirus causes retardation of leaf growth, irregular wood maturity and soft, rubbery canes with longitudinal cracks at their base. It can be latent in many grapevine cultivars, making its detection difficult. However, when susceptible cultivars are exposed, the symptoms significantly reduce overall plant vigour. Micrografting allowed symptoms of corky-bark virus to be detected 8–12 weeks after grafting, compared with 2 years using the standard indexing procedure.

Pathirana & McKenzie (2005b) have further refined the method of Tanne et al. (1993) and used it for the rapid detection of grapevine leafroll-associated closterovirus III (GLRaVIII). The grapevine leafroll-associated closteroviruses are phloem-restricted and non-mechanically transmissible. *Vitis* species are the only known plant hosts for these viruses. Certainly they have not been identified in any other wild or cultivated plant species (Martelli et al., 1993). The virus has easily recognizable symptoms on grapevines – downward rolling of older leaves, reddish-purple discoloration of leaves in red-berried cultivars, and yellowing of leaves in white-berried cultivars. Necrotic areas can develop in the interveinal tissue when the leaf is heavily infected (Martelli et al., 1993; Emmett & Hamilton, 1994). The spread of the virus has been associated with mealybug species, *Pseudococcus longispinus*, *P. viburni*, *P. maritimus*, *P. calceolariae*, *Planococcus citri* and *P. ficus* (Homoptera: Pseudococcidae) (Martelli et al., 1993; Petersen & Charles, 1997; Golino et al., 2002). Grapevines infected with grapevine leafroll-associated closteroviruses are subject to yield losses of 20–40% due to delayed maturity and decreased sugar content of the fruit.

The successful application of micrografting in grapevine for the detection of GLRaVIII, which has substantially different symptoms to corky-bark, indicates that there is scope to apply the micrografting procedure for routine virus indexing following its successful application to other viruses. It may, however, be used already to detect leafroll and corky-bark viruses, based on the success of the established methods (Tanne et al., 1993; Pathirana & McKenzie, 2005b).

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Growing Conditions of the Mother Plants

To establish tissue cultures, mother plants should be preferentially grown under greenhouse conditions. Plants can be established in the greenhouse from single bud woody cuttings from field-grown plants. The cuttings should be placed bud-side up in standard potting mix in a mist bed. The woody cuttings need to be from plants that have not gone into dormancy. Material from actively growing plants produces shoots within a few weeks, unlike dormant buds which take a few months to shoot.

Once the shoots are induced, the pots can be transferred to the greenhouse and allowed to grow under optimal conditions. Green shoots for tissue culture establishment can be selected from the resulting plants (Figure 1). Field-grown plants may also be used to establish tissue cultures but they usually carry more contamination, therefore surface sterilization must be more thorough when using them as source material.

The indicator plants used for viral indexing must be maintained separately from plants that have unknown virus status. A regular spray programme to control the insect vectors of viruses, such as mealy bugs, is also important.



Figure 1. *Vitis vinifera* growing in the greenhouse ready for initiating tissue cultures.

2.1.2. Explant Excision and Sterilization

Green shoots harvested from the greenhouse-grown plants (0.5 cm diameter) should be cut into pieces, each having a single bud. They can be surface sterilized by dipping in 70% ethyl alcohol for 30 s followed by 1% sodium hypochlorite solution containing 0.2% Tween® 80 for 20 min. For better sterilization, the container with the shoots immersed in the sterilant should be shaken on a rotary shaker (50 rpm). The explants are rinsed three times for 1 min in sterile water. Aseptically cut the bleached ends of stem pieces before they are placed into tissue culture. Usually whole shoot pieces do not result in active shoot production as readily as when young shoots are split in half aseptically and placed horizontally on the media with the cut surface touching the medium and the bud facing up.

2.2. Culture Medium

A wide variety of tissue culture media has been successfully used to grow and maintain grapevine plants in tissue, culture. One such medium consists of half strength MS (Murashige & Skoog, 1962) macro salts and full strength MS micro salts, Lloyd & McCown (1980) organics with double the concentration of inositol, 3% (w/v) sucrose and solidified with Gelrite® (3 g l⁻¹) (Table 1). The medium should be adjusted to pH 5.8 before autoclaving for 20 min at 121°C and 103.4 kPa pressure. Clear wide-mouth disposable tissue culture tubs (about 300 ml capacity) with snap-on lids or glass bottles with a wide mouth may be used. Each tub or bottle should be dispensed with 50 ml media and the shoot initiation and plant growth will be optimal in culture rooms maintained at 23–27°C with a 16 h light, 8 h dark photoperiod at 25–40 µmol m⁻² s⁻¹ light intensity.

Table 1. Chemical composition of grapevine tissue culture medium used in micrografting for indexing GLRaVIII (Pathirana & McKenzie, 2005b).

Component	mg/l ⁻¹
CaCl ₂ ·2H ₂ O	220
CoCl ₂ ·6H ₂ O	0.025
CuSO ₄ ·5H ₂ O	0.025
FeSO ₄ ·7H ₂ O	27.8
H ₃ BO ₃	6.2
KH ₂ PO ₄	85
KI	0.83
KNO ₃	950
MgSO ₄ ·7H ₂ O	185
MnSO ₄ ·4H ₂ O	15.1
Na ₂ MoO ₄ ·2H ₂ O	0.25
Na ₂ EDTA·2H ₂ O	37.3
NH ₄ NO ₃	825
ZnSO ₄ ·7H ₂ O	8.6
Nicotinic Acid	0.5
Thiamine HCl	1.0
Pyridoxine HCl	0.5
Glycine	2.0
Inositol (Myo)	200
Sucrose	30,000
Gelrite®	3000
pH	5.8

2.2.1. Shoot Regeneration and Maintenance

It is easier to produce the quantity of shoots required for micrografting through shoot multiplication *in vitro* rather than establish new tissue cultures from greenhouse-grown plants. Rapid multiplication of shoot material can be achieved by supplementing the basal medium described above with 0.5 mg l⁻¹ 6-benzylaminopurine. On average, 2.8 shoots per explant can be harvested within 1 month of subculture in this medium. Shoots with 2–3 nodes can be aseptically cut and subcultured on to fresh shoot multiplication media for further multiplication or placed on rooting media in preparation for micrografting. In general, the virus-infected material will grow slower and produce symptoms of viral infection in culture, e.g. the symptoms of GLRaVIII are dark red leaves, particularly in the variety *Cabernet franc* (Figure 2A).

2.2.2. Rooting

Generally hormones are not required for root induction, therefore excised shoots placed on the basic culture medium outlined in Table 1 would be expected to produce roots within 2–3 weeks. As there are cultivar differences in growth and rooting of grapevine in tissue culture, all cultivars may not root easily. Thus, if root production is delayed, the incorporation of 0.08 mg l⁻¹ 3-indoleacetic acid is beneficial. Very often the shoots excised from rooted plants grown *in vitro* produce roots easily. Thus the need for exogenous auxin for rooting is experienced mainly at the initial establishment stage.

2.2.3. Micrografting Technique

The rootstocks and scions for micrografting should be selected from established, actively growing plants in tissue culture. Rootstock plantlets obtained *in vitro* are allowed to grow until several leaves are produced (6–8 weeks) and then divided into 3–4 explants, each containing a single node with a leaf. The apex of the rootstock nodal explant can then be cut longitudinally with a new, sharp scalpel blade, producing a small (2–4 mm) longitudinal cleft, and placed on the basic medium. A scion with a single node and a leaf is then selected to match the stem width of the rootstock. The basal part of the stem of the scion should be cut into a wedge to match the cleft of the rootstock and carefully fitted on to the cleft of the rootstock on the medium (Figure 2B). In contrast to the technique described by Tanne et al. (1993), wrapping the graft is not necessary if the scion and rootstock are carefully selected and because the humidity in the tissue culture tub is inherently high. However, if the scion does not fit properly on the rootstock, dipping the lower end of scion in the medium before fitting it helps to establish the graft, probably by keeping the cut surfaces moist until the relative humidity of the tub is established after closure and by supplying nutrients directly to the graft site.

Successful grafts form callus at the graft union within a few days (Figure 2C) and continue to grow, with new shoots emerging from the rootstock and scion leaf axils. We find that about 80% of the micrografts are successful; there were no significant differences between the combinations of rootstock and scion we tried.

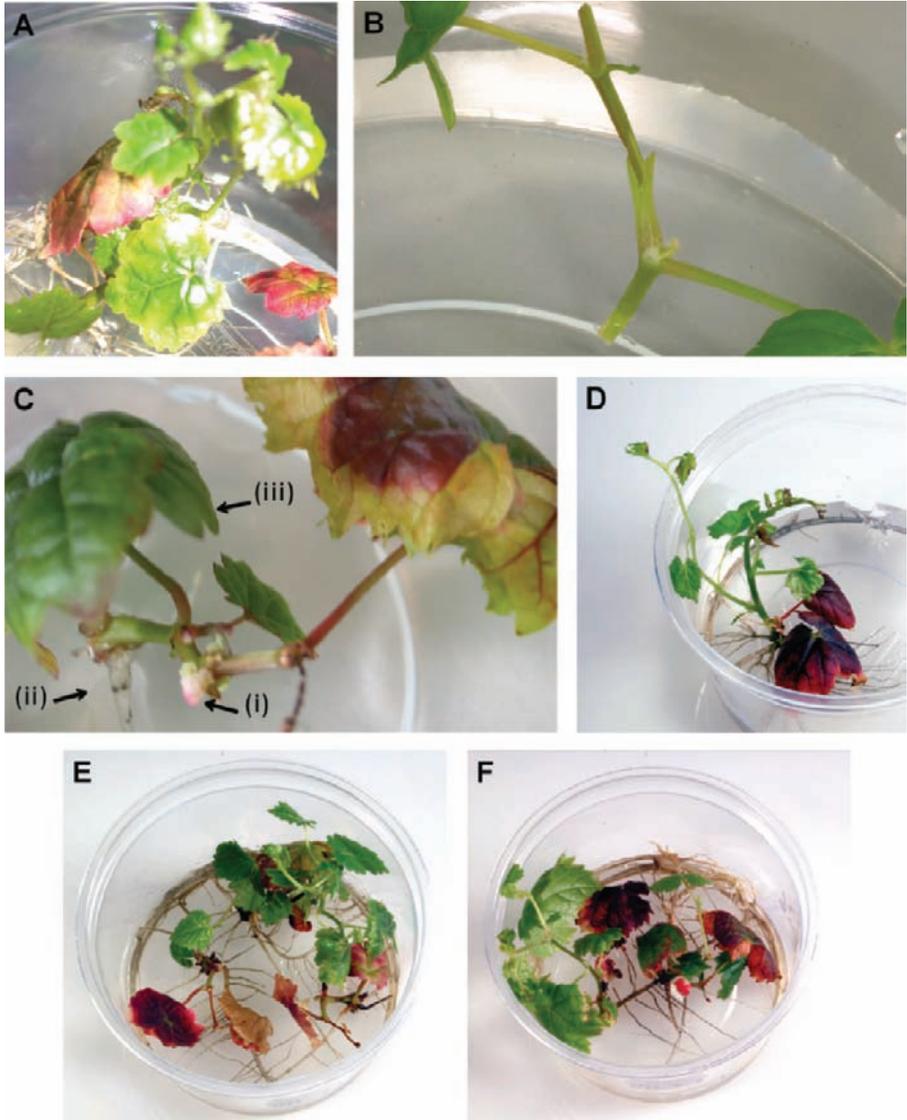


Figure 2. Micrografting of GLRaVIII infected grapevine scions to virus-free rootstock *in vitro*. A) Leafroll-infected Cabernet franc. B) Newly made micrograft. C) Callusing of the graft union (i), root initiation (ii) and leaf rolling symptoms on rootstock leaf (iii) of Cabernet sauvignon indicator after 3 weeks of micrografting. D) Poor leaf development of scion and reddening of rootstock leaf after 12 weeks of micrografting. E) and F) Typical symptoms of leafroll virus in grafted plants after 12 weeks. Reproduced from pathirana & Mckenzie (2005b) with kind permission of springer science and business media.

Root initiation on the rootstock can be expected 2–3 weeks after grafting (Figure 2C). Successful grafts first produce callus at the point of grafting before the vascular connections are established.

2.2.4. Detection of Viral Symptoms in Grafted Rootstock

Tissue culture is an ideal environment for monitoring the presence of GRLaVIII because symptoms of this virus are ideally expressed at temperatures between 22 and 25°C. When an infected scion is micrografted on to an uninfected rootstock of the indicator variety (e.g. *Cabernet sauvignon*), the basal leaf of the rootstock turns red, at first at the edges, within 2–3 weeks (Figure 2D). There appears to be no difference in the rate of symptom appearance in the rootstock when different scions are used. However, the degree of expression of the symptoms may vary depending on the indicator rootstock. Other serotypes of leafroll virus may also produce symptoms easily when micrografted, but experimental evidence is lacking to date.

Three months following micrografting, shoots can be expected to produce 4–8 leaves and further general symptoms of leafroll may be observed. These include rolling of lower leaves, stunted growth and small, underdeveloped leaves (Figure 2E, F). Seventy to ninety percent of micrografts can be expected to display at least one visual symptom of leafrolling disease by this time if the scions used are all infected.

3. CONCLUSION

The described micrografting technique for grapevine is a robust and reliable method, providing actively growing plant material of a uniform nature established in tissue culture. Micrografting success appears to be most reliant on the good fit of scion to root stock and conditions of high humidity in the tissue culture system. Wrapping of the graft union is not necessary.

The technique is particularly useful as a rapid method for detecting viral contamination in grapevine and has been applied successfully to detect corky bark and GRLaVIII viruses within 8–12 weeks (Tanne et al., 1993; Pathirana and McKenzie, 2005b). Within the limited amount of experimental evidence available to date, it appears that the incompatibility seen in other methods of grafting applies less to micrografting. However, selection of the proper indicator rootstock is important to maximize the chances of detecting the viruses because not all the rootstocks used in woody grafting produce similar results. Validation of this method for other viruses that infect grapevine could support the grapevine industry by increasing the rate and ease of detection of contaminating viruses that have a serious economic impact on both imported and domestic grapevine material.

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CHAPTER 25

APRICOT MICROPROPAGATION

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1. INTRODUCTION

Apricot (*Prunus armeniaca* L.) is the second most important species of the stone fruits, although far behind peach and very much geographically located by its peculiar ecological needs. During the last years the world production of apricot fruit has oscillated around 2.5 million tons, and more than half of world production concentrated in the Mediterranean basin countries.

Micropropagation has been applied to the commercial production of fruit and nut crops since the late 1970s. Initially, it was used for small (soft) fruit crops such as strawberry and raspberry and for the rootstocks of several tree fruit species, especially peach (Zimmerman & Debergh, 1991). Apricot is well suited for *in vitro* propagation because cuttings are difficult to root and scions are currently budded onto apricot seedlings (Reighard et al., 1990). Therefore, growing apricots on their own roots appears to be a logical undertaking. Very little, however, has been reported on the tissue culture of this species and most of it is relatively recent. Most commercial production of micropropagated fruit trees has been focused on the production of rootstocks. Although grafting of scions onto seedling rootstocks is a common practice, the production of scions on their own roots has been limited. To our knowledge, apricot cultivars have never been commercially micropropagated, and also apricot rootstocks. Only one small Spanish company has been producing small quantities of selected apricot cultivars on their own roots, for the last 2 years. Here we describe a protocol containing different steps necessary to micropropagate apricot cultivars. It has proven useful for most cultivars tested although often some parameters have to be optimized for each single cultivar. When parameters depended on the genotype, it is indicated in the text and a range is provided.

2. EXPERIMENTAL PROTOCOLS

2.1. Introduction and Establishment of Apricot *in Vitro*

Contamination is a serious problem in *in vitro* cultures. The first and foremost step in establishing *in vitro* cultures of any plant is to eliminate microorganisms. Shoot tips and axillary shoots are easier to propagate compared with other types of explants such as meristems. Meristem culture is often used to produce pathogen-free plants from a systemically infected individual (Cassells, 1991) and is also recommended for establishing aseptic explants for micropropagation. In woody species, and particularly in *Prunus*, the available reports on meristem tip culture are rather limited (Pérez-Tornero et al., 1999a; Manganaris et al., 2003).

2.1.1. Meristems

Plant material. Plant material used to introduce apricot cultivars through meristems is obtained between January and March, when buds are starting to swell, from shoots in dormancy. Shoots are removed from adult apricot field-grown trees.

Disinfection and bud dissection. Dormant shoots are cut in two- or three-node sections. These stem sections are washed carefully with water and detergent (Mistol®, Henkel Ibérica, S.A.). They are shaken for 5 min in 70% ethanol, followed by 20 min treatment with a 20% solution of Domestos® (0.8% NaClO). Finally, stem sections are rinsed three times with sterile distilled water.

Bud dissection and meristem isolation. Meristem tips are dissected from lateral and apical buds of disinfected shoots. Hold the basal end of the stem with gloved fingers while dissection. The instruments should be changed frequently so that only a sterile surface touches the bud. First, bark surrounding the bud is removed by means of a sequence of short longitudinal cuts (Figure 1A), and then the outer bud scales are removed by short longitudinal and transverse cuts (Figure 1B, C). This process is continued until the meristematic dome and a few leaf primordia are exposed. The meristem is then removed by cutting at the base leaving an explant averaging 0.5 to 1 mm long and which has a wood portion that allows further manipulations and culture in the nutrient culture medium (Figure 1D).

Culture medium. Macro- and micronutrients, organics and vitamins (Quoirin & Lepoivre (QL), 1977; Table 1) are used for the culture medium of meristems, supplemented with 2% sorbitol and solidified with 0.6% agar (Hispanlab, S.A.). A two-step culture medium is employed (Pérez-Tornero et al., 1999a), first the medium contains 2.2 to 8.8 μM benzyladenine (BA), depending on the cultivar used. These media ensure a high meristem survival rate and the development of a rosette of leaves (Figure 2A).

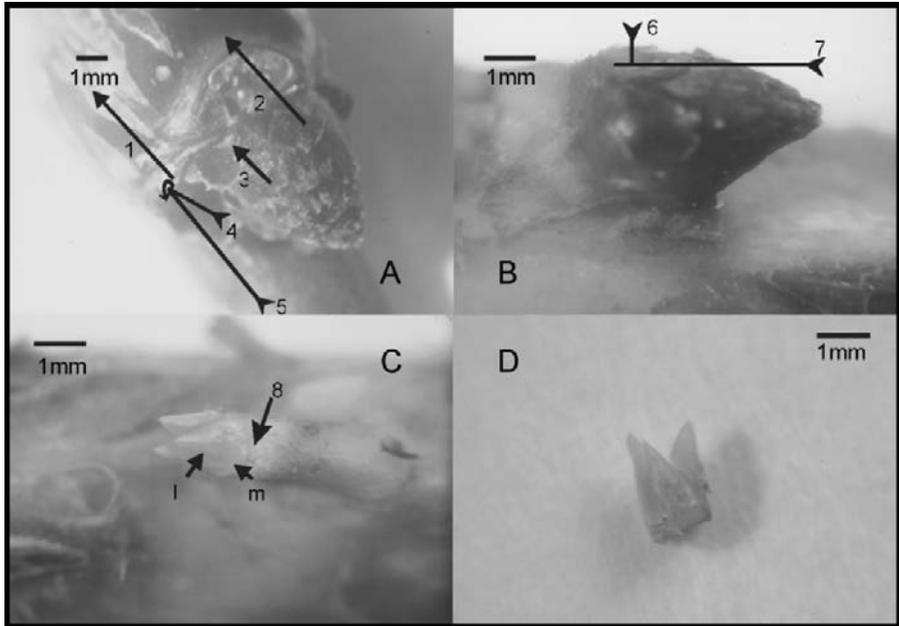


Figure 1. Apricot meristem tip dissection. (A) Intact dormant bud on a stem piece. Arrows indicate the location and sequence of cuts 1 to 5 to remove bark surrounding the bud. (B) Appearance of the bud after cuts 1 to 5. Arrows 6 and 7 show the cuts necessary to remove outer bud scales. (C) Meristem tip explant containing a meristematic dome (m) and a few leaf primordia (l). Arrow 8 indicates the point to cut and excise the explant. (D) Aspect of the newly-explanted meristem tip.

The addition of gibberellic acid (GA) at 5.8 to 11.6 μM and reduction of BA at between 2.2 and 4.4 μM , depending on the cultivar, promotes explant elongation (Figure 2B) when a rosette of leaves has developed. The medium is dispensed in 25 \times 150 mm culture tubes covered with permeable membrane caps and sterilized in the autoclave at 121°C for 21 min after plant growth regulators (PGRs) are added and the pH is adjusted to 5.7 with 1N NaOH. Meristems are subcultured to the fresh culture medium every 2 weeks and maintained at 23°C under cool white fluorescent light (55 $\mu\text{mol m}^{-2} \text{s}^{-1}$) with a 16 h photoperiod. Elongated shoots are transferred to jars containing proliferation culture medium (Figure 4).

2.1.2. Axillary Shoots

Plant material. Axillary shoots are introduced *in vitro* from actively growing plants. Preferably, apricot cultivars are grafted onto the apricot rootstocks and maintained in large pots under the greenhouse conditions, to improve sanitary conditions.

Table 1. *Macronutrients, micronutrients, vitamins and organics compounds of the culture media used in apricot micropropagation (concentrations in mg/l).*

	Introduction and establishment of meristems	Shoot proliferation	Rooting
MACRONUTRIENTS			
KNO ₃	1800	1800	900
NH ₄ NO ₃	400	400	200
Ca(NO ₃) ₂ ·4H ₂ O	1199.46	1199.46	599.73
CaCl ₂ ·2H ₂ O	–	–	–
MgSO ₄ ·7H ₂ O	359.65	359.65	179.825
KH ₂ PO ₄	270	270	135
K ₂ SO ₄	–	–	–
MICRONUTRIENTS			
H ₃ BO ₃	6.20	4.80	4.80
CoCl ₂ ·6H ₂ O	0.025	–	–
CuSO ₄ ·5H ₂ O	0.025	0.25	0.25
MnSO ₄ ·H ₂ O	0.76	33.50	33.50
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.39	0.39
KI	0.08	–	–
ZnSO ₄ ·7H ₂ O	8.60	17.00	17.00
FeSO ₄ ·7H ₂ O	27.80	33.80	33.80
Na-EDTA·2H ₂ O	37.30	45.40	45.40
VITAMINS			
Thiamine	1	2	2
Nicotinic acid	1	1	1
Biotin	0.1	–	–
Folic acid	0.01	–	–
p-amino benzoic acid	1	–	–
Riboflavin	0.1	–	–
Ca-pantothenate	0.5	–	–
ORGANICS			
Myo-Inositol	50	100	100
Glycine	–	2	2

Disinfection and node dissection. Shoots are taken from rapidly growing new branches during spring (Figure 3A). Expanded leaves are removed. Shoots are washed with water and detergent (Mistol®, Henkel Ibérica, S.A.). The protocol for disinfection with ethanol and bleach is followed accordingly as described earlier. Node explants, 2 cm long, are cut and cultured upright with the basal end of the node embedded a few mm into the culture medium (Figure 3B).

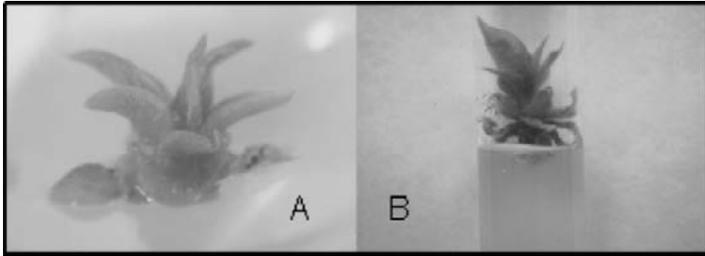


Figure 2. (A) Leaf rosette after 2 weeks in culture ready for the first subculture. (B) Elongated shoot.

Culture medium. The shoot proliferation culture medium, supplemented with 3% sucrose and solidified with 0.6% agar, is also used for the *in vitro* introduction and establishment of axillary shoots (Hispanlab, S.A.). The PGRs used in the introduction medium are 1.76 μM BA and 0.2 μM indole-3-butyric acid (IBA). The medium is dispensed in 25 \times 150 mm culture tubes covered with permeable membrane caps. Sterilization of media and culture conditions are described above. Axillary buds sprout in this medium (Figure 3C, D) and, after removing contaminated nodes, elongated shoots are transferred to jars containing proliferation medium (Figure 4).

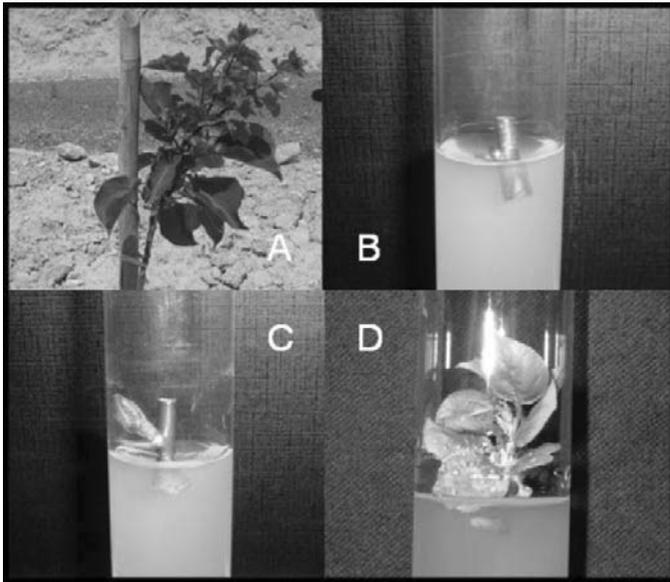


Figure 3. *In vitro* introduction of apricot by axillary shoots. (A) New branches of apricot growing during spring (B) Node explant introduced in the culture medium. (C and D) Sprouting of axillary buds.

2.2. Shoot Multiplication and Maintenance

2.2.1. Culture Medium and Conditions

QL macronutrients and Driver & Kuniyuki (DKW) (1984) micronutrients, organics and vitamins (Table 1), are used for the propagation of apricot shoot tips. The medium is supplemented with 3% sucrose and 0.2 μM IBA, and solidified with 0.6% agar (Hispanlab, S.A.). BA at 1.78 to 3.11 μM is added to the medium, depending on the genotype (Pérez-Tornero & Burgos, 2000). Higher concentrations could produce rosette leaves, hyperhydricity and cytokinin habituation. Medium is dispensed in jars and sterilized as described above. Culture conditions are described above. Proliferating shoots (Figure 4) are transferred to the fresh medium every 21 days. When sub culturing, explants are divided into new shoots approximately 1 cm long, leaving smaller buds attached to the main shoot. Some researchers have successfully used sorbitol instead of sucrose in the proliferation medium; this improved the number of shoots and the rooting efficiency of shoots in a sucrose-containing medium (Marino et al., 1993).

Some reports indicated that the ability to produce axillary shoots is poor or almost absent as in cv. Canino (Snir, 1984); however conditions described here have allowed up to 4 shoots per explant in this cultivar (Pérez-Tornero et al., 2000). On an average 1.5 to 4 shoots (1 to 1.5 cm long) per explant were obtained on different culture media depending on the cultivar. A recent report has shown that combining 4 mg/L GA_3 with a high concentration of BA (1 or 2 mg/L) improved shoot length and productivity and reduced hyperhydricity in cv. Bebecou (Koubouris & Vasilakakis, 2006). Since apricot has a tendency to form rosettes, this may also be useful for other cultivars.

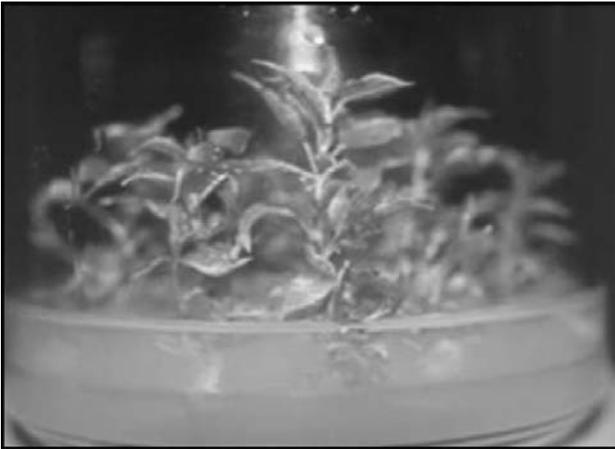


Figure 4. Apricot shoots after 21 days in a proliferation medium.

2.2.2. Hyperhydricity

Hyperhydricity is a physiological disorder frequently related to the *in vitro* environment during micropropagation of woody plants. Apricot shoot tips at the proliferation stage are very sensitive to hyperhydration (Figure 5), although the degree of symptoms is genotype-dependent.

Treatments that decrease hyperhydricity, without affecting micropropagation rates in apricot, are the application of a bottom cooling system for 1 or 2 weeks in each culture cycle; and an increase in the agar concentration of the culture medium (0.6–0.8%) or the use of Agargel™ (0.5%) as gelling agent (Pérez-Tornero et al., 2001). However, the effectiveness of each method depends on the genotype and only bottom cooling have consistently prevented hyperhydricity in all apricot cultivars tested.



Figure 5. Hyperhydric apricot explants of two different cultivars.

Bottom cooling system. High relative humidity (RH) is probably the most important environmental factor that induces hyperhydricity. The bottom cooling system (Figure 6) decreases the RH inside the *in vitro* culture jar by condensing water on the cooled culture medium (Vanderschaeghe & Debergh, 1987). Vessels are placed on an iron plate cooled by a system of copper tubes. The water, cooled by a cryostat (Selecta, Frigiterm-10), circulates constantly through the tubes. The temperature of the water is adjusted to 13°C in the cryostat to obtain a gradient of approximately 4°C between the bottom and the top of the jar. RH within the jar is then decreased to approximately 75% (Vanderschaeghe & Debergh, 1987). Hyperhydricity can be avoided by placing proliferating shoots in the bottom cooling system in the first week of the culturing cycle. More than 64% of hyperhydric shoots could be returned to normal by keeping them for 3 weeks in the bottom cooling system.

2.3. Rooting

2.3.1. Culture Medium and Conditions

QL macronutrients diluted to half strength, DKW micronutrients, organics and vitamins (Table 1), plus 40 mg/l phloroglucinol, are used for the rooting of apricot shoots. Sucrose is used at 2%, agar at 0.6% (Hispanlab, S.A.) and IBA at 1 to 3 μM , depending on genotype (Pérez-Tornero & Burgos, 2000).



Figure 6. Bottom cooling system.

Medium is prepared, dispensed and sterilized as described previously. Culture conditions are as described above. Proliferating shoots should be at least 2 cm long. The basal callus is eliminated and shoots are cultured for 21 days in the rooting medium, where induced adventitious roots grow (Figure 7).

Although rooting of micropropagated apricot shoots is not a problem, a high percentage of shoots shows symptoms of apical necrosis on the rooting media, sometimes exceeding 80% and also affecting axillary buds. Plantlets with necrotic apices frequently are unable to grow and cannot be acclimatized. This effect is highly genotype-dependent.

2.3.2. Apical Necrosis during Rooting

Apical necrosis can be related to lower endogenous cytokine concentration in the shoot apex (Kataeva et al., 1991) in apricot (Pérez-Tornero & Burgos, 2000). Apical necrosis during rooting can be solved by immersing shoot tips in concentrated BA solution (22.19 to 44.38 μM) just before explanting shoots on the rooting media. It is necessary to dry the excessive BA solution to avoid any interference with rooting.

2.4. Acclimatization

Rooted explants are transferred to a 200-cc alveolus containing a mixture of peat and perlite (2:1). Acclimatization of plants occurs within a tunnel (Figure 8) with >85% relative humidity, achieved by means of intermittent mist. To harden plants, the plastic covering the tunnel is opened gradually from a few minutes a day until normal greenhouse conditions can be maintained without desiccation of the plants. The acclimatization period is 15 days, and then acclimated plants are transferred to a 2.5 litre pot and maintained in the greenhouse. In optimal conditions high frequency acclimatization (70–80%) can be obtained.



Figure 7. Apricot explant rooted and ready to be acclimated.

2.5. Behaviour of Plants on Their Own Roots

Behaviour of micropropagated plants on their roots was compared with grafted plants of the same cultivars. The trunk diameter 25 cm above the ground indicates that more vigorous trees are obtained from *in vitro* culture. The young self-rooted trees also produce greater yields than grafted trees, which may indicate a faster entry into production (Pérez-Tornero et al., 2006). Differences are more or less significant depending on the genotype.

The improved behaviour of self-rooted apricot trees may be related to the elimination of the grafting point in self-rooted trees. Also, self-rooted apricots show a fibrous root system with many different main roots coming from the base of the stem in a “star” pattern, this it is probably more efficient in colonizing the soil compared with tap roots from apricot seedlings.

2.6. Storage of *in Vitro* Cultures

In vitro repositories of valuable plant genotypes offer a number of advantages over conventional methods. Greenhouse space and maintenance are reduced, and the stored material is protected from insect pests and pathogens and can be micropropagated rapidly when desired. Minimal growth storage is a very simple technique that allows storage of plants *in vitro* for long period of time.



Figure 8. Acclimatization tunnel.

The main objective of the minimal growth procedure is the extension of the normal subculture interval from 2–6 weeks to a much longer period (up to 12 months). It also increases flexibility in micropropagation and overcomes temporary difficulties in production. The common procedure requires incubation of *in vitro* plantlets at reduced temperature and low light intensity (or in darkness).

In vitro apricot shoots can be stored for at least 6 months without subculture (Pérez-Tornero et al., 1999b) when they are stored at 3°C on the proliferation medium in darkness (Figure 9). Normal proliferation rates are obtained after storage and shoots can be rooted and acclimatized.

3. CONCLUSIONS

Apricot cultivars can be efficiently micropropagated with the methodology described here. *In vitro* introduction and establishment are better achieved if axillary shoots are used as original explants. However, when internal contaminants are a serious problem, the culture of meristem tips overcomes this problem. A modified QL medium is ideal for proliferation of apricot shoots, whilst hyperhydricity problems, often presents in apricot cultures, can be controlled by using a bottom cooling system. Rooting of apricot shoots can be obtained readily but apical necrosis

occurs frequently and needs to be controlled by immersing the shoot tips in concentrated BA solution. High percentages of apricot *in vitro* plantlet acclimatization can be achieved by using standard procedures. Micro propagated plants, on their own roots, are an alternative to grafted plants when the chosen rootstocks are apricot seedlings. A better root system and the elimination of problems related to the grafting point are characteristics of self-rooted plants obtained by micro propagation.



Figure 9. Apricot shoots stored for 6 months without subculture at 3°C on a proliferation medium in darkness.

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CHAPTER 26

IN VITRO CONSERVATION AND MICROPROPAGATION OF BREADFRUIT (*ARTOCARPUS ALTILIS*, MORACEA)

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1. INTRODUCTION

Breadfruit (*Artocarpus altilis*, Moraceae) has been used for more than 3,000 years by Pacific islanders as a traditional food crop (Ragone, 1997). Breadfruit is rich in carbohydrates (76.7%) and nutritional energy (Adebowale et al., 2005) and readily consumed at all stages of maturity. The range of traditional uses of breadfruit includes roasted, baked, boiled, dried, pickled, and fermented fruits, as well as processed flour (Ragone, 2003). Prepared breadfruit has a moderate glycaemic index and there are multiple nutritional benefits to including breadfruit as a dietary staple (Ramdath et al., 2004). Breadfruit trees are also a good source of medicine, insecticides, adhesives, timber, and shelter and highly valued as a primary component of traditional agro-forestry systems in Oceania (Morton, 1987; Ragone, 1997; Zerega et al., 2004). Breadfruit varieties exist in two ploidy levels. Triploid accessions ($2n = 3x = 84$) lack the ability to produce seeds while diploid accessions ($2n = 2x = 56$) differ in the ability to produce seed (Ragone, 2001; Zerega et al., 2004, 2005, 2006).

The conservation of breadfruit germplasm is of global significance, and this important tree species is one of 35 crops covered by the International Treaty for Plant Genetic Resources for Food and Agriculture (Fowler et al., 2003). A number of breadfruit cultivars have already disappeared or are on the edge of becoming rare and endangered (Ragone, 1997). Hurricanes, devastating tropical storms, cultural and

environmental changes in the Pacific Islands are potential threats to indigenous breadfruit germplasm. Breadfruit varieties are traditionally propagated vegetatively, vegetatively, via root cuttings for transport, sharing of genetic resources and to maintain genetic uniformity but potential problems with these traditional propagation methods include low survival rates, microbial infections, and difficulties in modern transport arising from international plant quarantine restrictions.

In vitro culture and micropropagation using meristem proliferation have proven to be an effective method for conservation, propagation and distribution of a wide variety of plant species. In recent years, tissue culture techniques have been applied to the propagation of tree species related to breadfruit such as jackfruit (Amin & Jaiswal, 1993), but the success with breadfruit cultivars has been slow and less than desirable. Recently, we have developed efficient protocols for mass propagation from axillary buds and controlled environment production of axenic plants for germplasm distribution (Murch et al., 2007). This chapter provides a detailed account of various steps involved in the culture, maintenance, and sustained production of breadfruit germplasm.

2. PROPAGATION PROTOCOLS

2.1. Donor Breadfruit Materials

Two types of donor plants, mature and juvenile trees, from three breadfruit (*Artocarpus altilis*) varieties (Maafala, Puou and Puupuu) were used in micropropagation experiments. Plant material from mature trees was collected from the germplasm collection at the Kahanu and McBryde gardens of the National Tropical Botanical Garden, USA. Greenhouse-grown juvenile trees were obtained from the first cycle of *in vitro* propagation of breadfruit accessions at the University of Guelph (Guelph, ON, Canada).

2.2. Explant Preparation from Donor Trees for the Initiation of *In Vitro* Cultures

1. Collect the axillary shoots tips (terminal bud and 0.5–1.0 cm of wood tissue) (Figure 1A) from donor trees.
2. Place the shoot tip explants in a 1L beaker and wash thoroughly with running tap water.
3. Surface sterilization of the explants is carried out in a flow laminar flow bench.
4. Place the explants in 70% ethanol for 1 min.
5. Replace ethanol with 10% bleach (5.25% sodium hypochlorite) and incubate for 15 min with occasional swirling of the explants for uniform exposure to the sterilant.
6. Wash the explants 3–5 times with sterile distilled water.

7. The tissue of the explant at the point of severance from the mother plant turns brownish during surface sterilization. Remove browning basal part of the shoot buds with a sharp scalpel and also peel off the stipule and unopened leaves surrounding the shoot meristems using a pair of forceps and scalpel.

2.3. Tissue Culture Media

The tissue culture media used in micropropagation of breadfruit are given in Table 1. Media were prepared by dissolving all media components in a flask of distilled water placed on stirring/hot plate and pH adjusted to 5.75. For solidification, 2.2 g l⁻¹ gelrite is added into the medium and dissolved by heating the medium on the stirring/hot plate. The medium is dispensed into tissue culture vessels using a dispenser and sterilized for 20 min (121°C, 1.4 × 10⁴ kg/m²).

2.4. Initiation of Aseptic Stock Cultures

1. Place surface sterilized shoot tip explants in tubes containing 22 ml of MS medium with 1 ml l⁻¹ plant preservative mixture (PPM). Use of PPM is necessary to reduce the risk of contamination especially when starting explants are collected from mature tress in tropics.
2. In case of contamination of cultures, surface sterilize the shoot tips from contaminated cultures by dipping the tips in 70% alcohol for 30 s, then immerse in 10% bleach for 3–5 min, rinse 5 times with sterilized distilled water. Culture the re-sterilized tips individually in culture tubes, each containing 22 ml of MSO medium and examine the cultures regularly to ensure that they are free of contamination.
3. Once aseptic cultures are established in 2–3 weeks period, transfer clean cultures with emerging shoots on *Artocarpus altilis* Shoot (AS) medium which contains a combination of 2 µM BA (0.450 mg l⁻¹) and 3 µM kinetin (0.645 mg l⁻¹).
4. Grow cultures for about 4 weeks in a culture growth room adjusted to 28°C and 16 h photoperiod (25–40 µmol m⁻²s⁻¹) provided by cool white fluorescent tubes (Figure 1B).
5. Excise the shoots developing from the shoot tip explants with the help of sterile scalpels and place them in Magenta boxes each containing 65 ml AS culture medium. The buds should be pushed 0.5–1.0 cm deep into the medium with shoot tip well above the surface of the medium.
6. Maintain stock cultures by subculturing shoots with two nodes to fresh medium every 3 weeks. Up to five shoots can be cultured per Magenta box (Figure 1C).

Table 1. Media composition for micropropagation of breadfruit (*Artocarpus altilis*) (all amounts given in mg l⁻¹).

Components	Medium ^a			
	MSO ^b	AS	SAR	LAR ^c
Major elements				
NH ₄ NO ₃	1,650	1,650	1,650	1,650
KNO ₃	1,900	1,900	1,900	1,900
CaCl ₂ × 2H ₂ O	440	440	440	440
MgSO ₄ × 7H ₂ O	370	370	370	370
Na ₂ EDTA	37.3	37.3	37.3	37.3
KH ₂ PO ₄	170	170	170	170
FeSO ₄ × 7H ₂ O	27.8	27.8	27.8	27.8
Minor elements				
H ₃ BO ₃	6.2	6.2	6.2	6.2
MnSO ₄ × 4H ₂ O	22.3	22.3	22.3	22.3
ZnSO ₄ × 4H ₂ O	8.6	8.6	8.6	8.6
KI	0.83	0.83	0.83	0.83
Na ₂ MoO ₄ × 2H ₂ O	0.25	0.25	0.25	0.25
CoCl ₂ × 6H ₂ O	0.025	0.025	0.025	0.025
CuSO ₄ × 5H ₂ O	0.025	0.025	0.025	0.025
Organic constituents				
Sucrose	30,000	30,000	30,000	30,000
Glycine	2	2	2	2
Myo-inositol	100	100	100	100
Nicotinic acid	1	1	1	1
Pyridoxine-HCl	1	1	1	1
Thiamine-HCl	10	10	10	10
Growth regulators				
BA	–	0.450	–	–
Kinetin	–	0.645	–	–
IAA	–	–	0.175	0.875
Solidifying agent				
Gelrite	2,200	2,200	2,200	–

AS, *Artocarpus altilis* Shoot (AS) medium; SAR, solid *Artocarpus altilis* rooting medium; LAR, liquid *Artocarpus altilis* rooting.

^apH of all media was adjusted to 5.75 before the addition of solidifying agent and sterilization in the autoclave.

^bMSO medium used in the first shoot tip culture contain 1 ml l⁻¹ PPM.

^cLAR medium contains 5 times more IAA than SAR in the first step of mass propagation in liquid system.

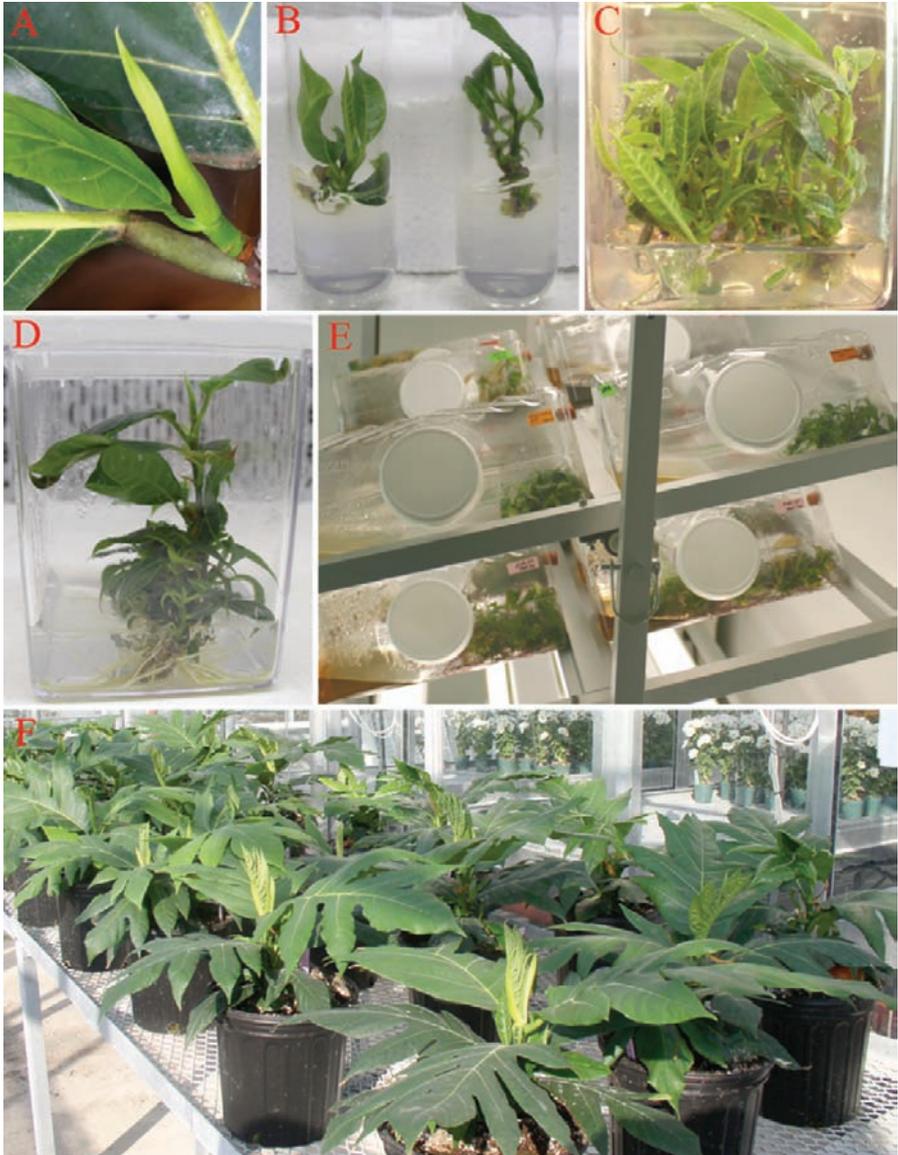


Figure 1. Micropropagation of breadfruit. A) Breadfruit axillary shoot bud on a mature tree, B) Established axenic cultures of shoot buds, C) Aseptic stock cultures, D) Shoot proliferation and rooting in solid culture system, E) Mass production of plants in a liquid lab temporary immersion system, F) Established juvenile breadfruit trees in the greenhouse.

2.5. Micropropagation

Mass production of rooted breadfruit plants can be achieved by placing differentiated shoots from aseptic stock cultures in solid or liquid culture systems. The solid and liquid systems can also be utilized in combination to maximize the plantlet proliferation and growth rates.

2.5.1. Solid Culture System for Rooted Plant Production

1. Excise 2 cm-long shoot explants with 2 nodes from the aseptic stocks and remove the leaves of the lower node.
2. Place up to five shoot explants in Magenta boxes containing 65 ml of solid *Artocarpus* rooting (SAR) medium supplemented with 1 μM IAA (0.175 mg l^{-1}). Ensure that the shoot explants are in contact with the culture medium by pushing the bottom portions (0.5–1.0 cm) of the buds gently into the medium. Place the cultures in the same growth room as stock cultures.
3. Examine the cultures for rooting response after 2 weeks. Normally, the roots differentiate within 2 weeks of culture from nodal section in contact with the medium.
4. Whole plantlets with about 6 cm long shoots and well established root systems develop after 6 weeks of culture. Nearly 100% of the shoot cultures form plantlets suited for potting and transfer to a growth chamber (Figure 1D).

2.5.2. Liquid Culture System for Plant Production

1. Excise 0.5–1 cm long shoot explants with a single intact node.
2. Place 15 explants in a Liquid Lab™ vessel with 100 ml of liquid *Artocarpus* rooting (LAR) medium with 5 μM IAA (0.875 mg l^{-1}).
3. The vessels are placed on the Liquid Lab™ Rocker system (Southern Sun Biosystems Inc., Hodges, SC, USA) to provide a gentle side to side rotation with a 30 s cycle at 1.2 min interval between cycles. Growth conditions in the culture room are set to provide a 16 h photoperiod with an intensity of 25–40 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at a temperature of 20–24°C.
4. Replace the culture medium after 13 weeks with 100 ml of liquid MSO and every 4 weeks thereafter.
5. After 6 months of culture, all explants turn to clumps of shoots with well developed roots (Figure 1E). Separate the rooted shoots from clumps and transfer them to a growth chamber for further development.

2.6. Transfer of Rooted Plants to Greenhouse

1. Plantlets with well-formed roots obtained from both solid and liquid culture systems are suitable for transplantation. *In vitro*-rooted plants are acclimatized before transfer to greenhouse.
2. Wash the plants with tap water to eliminate nutrient media and transplant in 4-inch plastic pots containing a soil-less mix (Promix BX, Premier Horticulture Ltd, Quebec, Canada).

3. After potting, plants are watered with 20-8-20 fertilizer (Plant Products Co. Ltd, Brampton, On, Canada) daily.
4. Place the pots in a growth chamber set for 16 h light at 26°C, 8 h dark at 24°C, and 95% relative humidity. Reduce the relative humidity by 5% every week for 5 weeks and keep it constant at 70%.
5. Five weeks after transplantation, transfer the transplants into 6-inch pots and grow them for an additional 12 weeks in the same chamber.
6. At the end of 12 week, transfer the plants to 1-gallon pots and place the potted plants in the greenhouse. Almost 100% of the transplants survive and grow to the size of juvenile trees, ready for planting outdoors, within 6 months after transplantation (Figure 1F).
7. Greenhouse-grown juvenile trees are continuous sources of axillary shoot tips for micropropagation that are free from most of the biotic contaminants encountered with the materials grown in tropics.

2.7. Determination of Nuclear DNA Content and Ploidy Stability by Flow Cytometry Analysis

The flow cytometry protocol presented here has been optimized to determine the nuclear DNA content, ploidy level, and genetic stability in breadfruit. Carrot (*Daucus carota* cv. Red Cored Chantenay) is used as internal reference since co-processing of carrot and breadfruit tissues does not change the relative position of breadfruit peaks. Carrot cells at the G0/G1 phase (2C) contain 0.98 pg DNA/nucleus (Arumuganathan and Earle, 1991a). DNA analysis is performed using nuclei isolated from young leaf tissues.

1. Place leaf tissues of breadfruit and carrot (about 50 mg tissue from each) into 65 × 15 mm Petri dishes kept on ice.
2. Add 1.5 ml of ice cold nuclei isolation buffer (NIB) (Table 2) into the Petri dishes on ice and slice the tissues finely with the help of a sharp razor blade.
3. Filter the homogenate through a 37 µm nylon filter to eliminate the tissue debris and collect the filtrate in a 1.5 ml eppendorf tube.
4. Centrifuge the tube at 8000 g for 5 s to pellet the nuclei.
5. Decant the supernatant and place the tubes on a rack placed on ice.
6. Re-suspend the nuclei in 300 µl of NIB with 25 µg ml⁻¹ RNase.
7. To stain the nuclei, add 10 µl of propidium iodide (PI) from a 1 mg PI ml⁻¹ stock solution prepared by dissolving 1 mg PI in 1 ml milliQ water.
8. Analyze 10–15,000 nuclei/sample with a flow cytometer (e.g., a Coulter EPICS Elite ESP Flow cytometer, Coulter Corp., Miami, Florida, USA).

The nuclear DNA content is calculated using the linear relationship between the ratio of the 2C peak positions of breadfruit/carrot on the histogram of fluorescence intensities (Arumuganathan & Earle, 1991b) using the equation described below:

$$2C \text{ nuclear DNA content of breadfruit (pg DNA/nucleus)} = \frac{(\text{mean position of breadfruit nuclei})}{(\text{mean position of carrot nuclei})} \times 0.98$$

9. The nuclear DNA contents of accessions Puou and Maafala are almost identical (1.80 ± 0.1 and 1.87 ± 0.1 pg/2C), but both differ from accession Puupuu (2.84 ± 0.04 pg/2C). Puupuu contains about 50% more nuclear DNA than the other two accessions. Therefore, the nuclear DNA contents of accessions Puou and Maafala correspond to diploid level whereas nuclear DNA content of accession Puupuu corresponds to a triploid level (Figure 2). Analysed micropropagated plants did not show significant differences from donor plants in nuclear DNA content and ploidy levels indicating that mass propagation systems established for breadfruit are likely to produce genetically stable plants.

Table 2. Composition of the Nuclei Isolation Buffer (NIB) (for 100 ml solution) (modified from Bino et al., 1992).

Chemical	Amount of chemicals	Final concentrations of chemicals ^a
HEPES	360 mg	15 mM
Na ₂ EDTA	37.22 mg	1 mM
KCl	597 mg	80 mM
NaCl	116.9 mg	20 mM
Triton X-100	200 µl	0.2% (v/v)
Sucrose	10.3 g	300 mM
Spermine	17.4 mg	0.5 mM
PVP-40	1 g	1%

^aAdjust pH to 7.5 and store NIB at -20°C until use.

3. CONCLUSION

Breadfruit is a versatile and nutritious food crop which can provide multiple nutritional benefits and is considered a primary component of traditional agro-forestry systems in Oceania. Global distribution of this important species is currently limited due to poor propagation efficiency with conventional methods. The protocols described above allow for efficient establishment of *in vitro* cultures which can be perpetually used for replenishment, conservation and distribution of elite breadfruit cultivars without environmental, agricultural and geographical constraints. This technology has potential to promote sustainable agriculture and food security in the tropics where breadfruit is recognized as a multipurpose life-supporting tree.

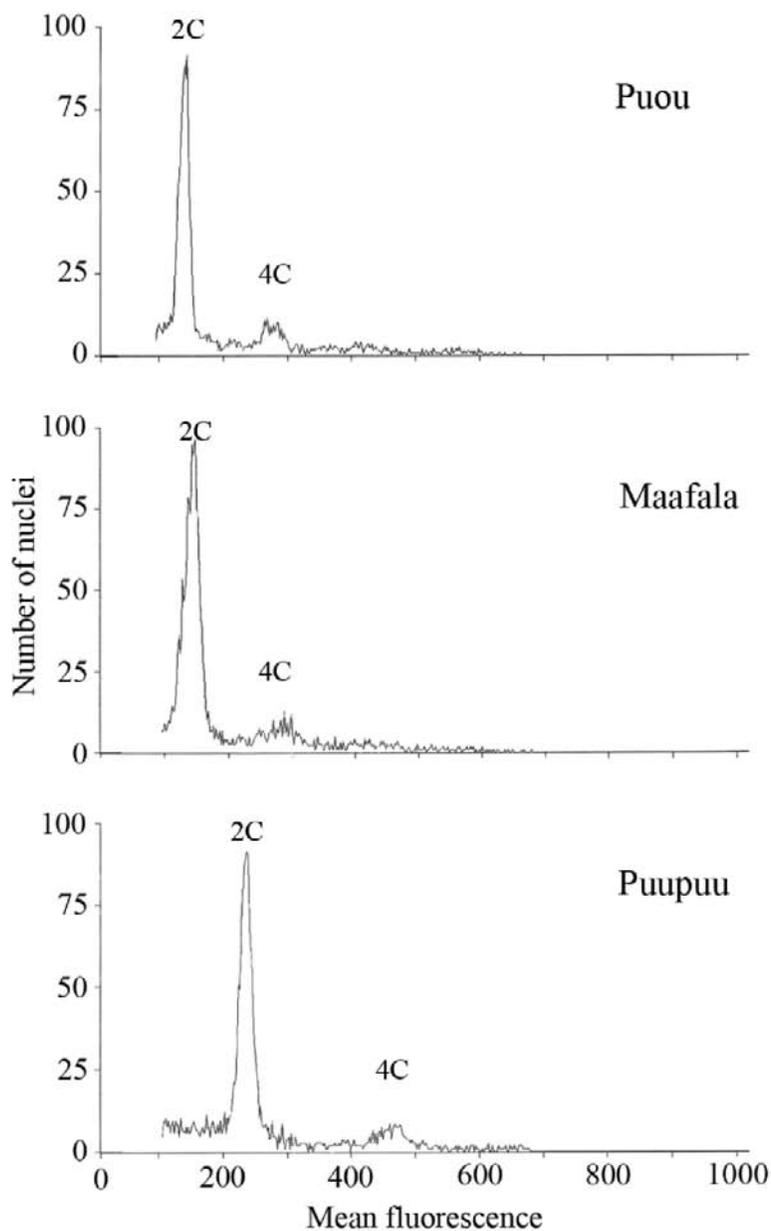


Figure 2. Flow cytometry analysis of nuclei of leaf tissue from three breadfruit accessions (Puou, Maafala, and Puupuu). Internal standard control was not used in these samples. The 2C and 4C peaks reflect G₀/G₁ and G₂/M stages. Puou and Maafala are diploids whereas Puupuu is a triploid.

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CHAPTER 27

MICROGRAFTING OF PISTACHIO (*PISTACIA VERA* L. CV. SIIRT)

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1. INTRODUCTION

Pistacia vera L. is a dioecious tree species cultivated widely in the Mediterranean regions of Europe and North Africa, the Middle East, China and California, USA. Development of pistachio plantations is limited by the absence of adequate nursery stock due to the difficulty of propagating *Pistacia* plants by conventional methods, such as cutting and grafting. Consequently, many efforts have focused on establishing *in vitro* propagation procedures for *Pistacia vera* and several other *Pistacia* species (Hansman & Owens, 1986; Barghchi & Alderson, 1989; Onay & Jeffree, 2000; Onay, 2003; Onay et al., 2004a,b,c; Onay, 2005).

The initiation of cultures from mature material usually involves pruning, grafting as well as BA and GA₃ spray treatments that stimulate new growth of shoots (Barghchi, 1986; Gonzales & Frutos, 1990). There has been some success in establishment of mature pistachios by direct organogenesis (Yang & Lüdders, 1993; Dolcet-Sanjuan & Claveria, 1995; Onay, 2000) and somatic embryogenesis (Onay et al., 1995, 1996, 1997, 2000; Onay et al., 2004a). Onay (2000) harvested 3–4 cm long terminal lignified stem sections from 30-year-old pistachio trees and immersed the cut ends in plant growth regulator solutions, before placing them in a greenhouse medium. New, softwood shoots were forced in the greenhouse under a 24 h photoperiod until they were sufficiently large to excise, surface disinfest and place *in vitro*. However, despite this progress, establishment and multiplication of field-grown mature *P. vera* clones remain problematic. *In vitro* micrografting can overcome these limitations. The first attempts to rejuvenate mature materials by micrografting *in vitro* mature scion shoot tips onto juvenile *P. vera* rootstocks were reported by Barghchi (1986). However, the growth of scion was very slow and elongation did not occur. Micrografting was investigated *in vitro* as well as *in vivo* by Abousalim & Mantell (1992), but rejuvenation was not reported when mature

trees were used as scions. Recently, pistachio has been successfully micrografted both *in vivo* and *in vitro* (Onay et al., 2003a,b, 2004b). This chapter contains a detailed protocol for *in vitro* micrografting of pistachio. The vigor of the proliferated shoots was restored, and complete plants were established in soil and grown in the greenhouse. This method can also be suitable for many other recalcitrant species.

2. EXPERIMENTAL PROTOCOL

2.1. Materials

1. Sodium hypochlorite (NaOCl) (10–14% available chlorine), absolute ethanol, sterile distilled water (SDW).
2. Slivers of stainless steel, razor blades, flow hood, sterile Petri dishes (9 cm), Erlenmeyer flasks, pipettes, forceps, scalpels, aluminum foil, Magenta GA 7 vessels.
3. Mature seeds, decapitated seedlings, mature apical tips and the regenerated apical or axillary shoots.
4. Tissue culture chamber and shaking incubators.
5. N⁶-benzyladenine (BA), kinetin, (kin), indole-3-butyric acid (IBA), ascorbic acid, casein hydrolysate.
6. Media (see Basal Culture Medium (BCM) (Table 1) and formulations of pistachio micrografting media (Table 2).

2.2. Methods

Application of *in vitro* micrografting would require the establishment of *in vitro* shoots derived from mature trees for the improvement of pistachio (1), as a source of scion, *in vitro*-germinated seedling root stock (2), a technique *in vitro* grafting, and (3) acclimatization of micrografts (4).

2.2.1. Scion Preparation

Slow growth due to the browning and blackening is a major problem in establishing axenic cultures from field-grown pistachio explants. The mature pistachio explants release large quantities of polyphenols into the medium within a few hours of culturing, which results in the death of cultures. We have found that a series of washes with sterile distilled water followed by bleach treatment were highly effective in overcoming this problem.

Explants. The genotype used for this study was *Pistacia vera* ‘Siirt’ from an orchard in Diyarbakır Province of Southeast Turkey. An experiment was carried out to investigate whether the degree of the juvenility or maturity of an apical meristem depends on its distance (along trunk and branches) from the root-shoot junction. Three types of explants (apical tips) were excised from the (1) far end of the orthotropic trunk; (2) far end of the plagiotropic trunk, 3) and from a pruned trunk.

Table 1. Pistachio basic culture media (BCM).

<i>Constituent</i>	<i>mg/L</i>	<i>Constituent</i>	<i>mg/L</i>
<i>Basal Salts</i>		<i>Organic Additives</i>	
Ammonium nitrate	1650.0	Sucrose	Varies
Boric acid	6.20	Casein hydrolysate	Varies
Calcium chloride anhydrous	332.20	L-ascorbic acid	Varies
Cobalt chloride hexahydrate	0.0250	Myo-Inositol	100.0
Cupric sulphate pentahydrate	0.0250	Thiamine hydrochloride	10.0
Disodium EDTA dehydrate	37.260	Nicotinic acid (Free acid)	1.0
Ferrous sulphate heptahydrate	27.80	Pyridoxine hydrochloride	1.0
Magnesium sulphate anhydrous	180.70	Agar	Varies
Manganese sulphate monohydrate	16.90	pH	5.7
Potassium iodide	0.830		
Potassium nitrate	1900.0		
Potassium phosphate monobasic	170.0		
Sodium molybdate dehydrate	0.250		
Zinc sulphate heptahydrate	8.60		

Table 2. Formulations of pistachio micrografting media¹.

<i>Organic Additives</i>	<i>RPM</i>	<i>SPM</i>	<i>CMM</i>	<i>MDM</i>
	<i>Rootstock Production Medium</i>	<i>Scion Production Medium</i>	<i>Culture Maintenance Medium</i>	<i>Micrograft Development Medium</i>
Sucrose	20.000	30.000	30.000	30.000
N ⁶ -Benzyladenine	0.5	1.0	0.5	0.5
Casein hydrolysate	–	200	200	–
l-ascorbic acid	–	200	200	200
Agar	5700	5700	5700	7000

¹All units are in mg/L (or ppm). All chemical used were obtained from Sigma Ltd.

The pH of all media is adjusted to 5.7.

Levels of survival, the average shoot length and the average shoot number were assessed after 5 weeks of culture. The position of the explants cultured did not influence the survival rate but the highest length of shoots was obtained with the apical tips explanted from the pruned shoot trunks. Explants from the pruned shoot trunk produced most shoots and means were greater than the explants excised from other positions.

1. Collect the actively growing shoot tips (3–5 cm long) from a pruned trunk of mature pistachio by cutting with a sharp scalpel and cover immediately the cut edge with a moistened cotton material.
2. Bring the shoot tips to the laboratory, chop off the leaves (with petioles) and surface sterilize or dip the defoliated shoot tips into the sterile distilled water (SDW) if they were not used for initiation of cultures.

Explant sterilization and initiation of cultures. In fact, it is difficult to establish *in vitro* cultures from lignified stem sections from mature pistachio trees during the dormant season. We have been conducting research on forcing shoots for years. Shoots were forced in the greenhouse under a 24 h photoperiod until they were large enough to excise, disinfest surface and place *in vitro*.

1. Remove the stem section of the explants (use only shoot tips, 15–20 mm long) and sterilize them.
2. Treat with 20% commercial bleach with 14% available chlorine for 35 min on a shaker at 150 rpm.
3. Gently wash with SDW at least 3 times for 5 min.
4. Cut the basal end of the rinsed explants.
5. Shake the explants with SDW at least twice for 1 h on horizontal type shaker at 150 rpm with SDW.
6. Inoculate 3–5 mm apical tips on scion production medium (SPM) (Table 2).
7. Incubate cultures in a growth room under the light (at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density; fluorescent lamps, 75W) and a photoperiod of 16 h at $25 \pm 2^\circ\text{C}$ for 4 weeks.
8. Excise the sprouting apical tips with minimal mother tissue attached. Note that shoot tip region consist of apical meristematic bud surrounded by a number of leaf primordia encased by fleshy green-white tissue of a leaf sheath.
9. Transfer the regenerated shoots to the shoot culture maintenance medium (CMM) (Table 2).
10. Incubate until the explants are at 3–4 leaf stage. An early subculture is recommended if the apical tips start releasing the polyphenols in the culture medium.
11. Use freshly sprouted apical tip or healthy growing shoot tips (4–6 mm long) as scions (Figure 1A).

2.2.2. Culture Maintenance

A regular pattern of subculture of the regenerated shoot cultures is necessary at every 3 or 4 week interval on the fresh MS medium (full strength) supplemented with 0.5 or 1.0 mg l⁻¹ BA and 0.2 mg l⁻¹ GA₃ for long-term maintenance of mature regenerated shoot cultures.

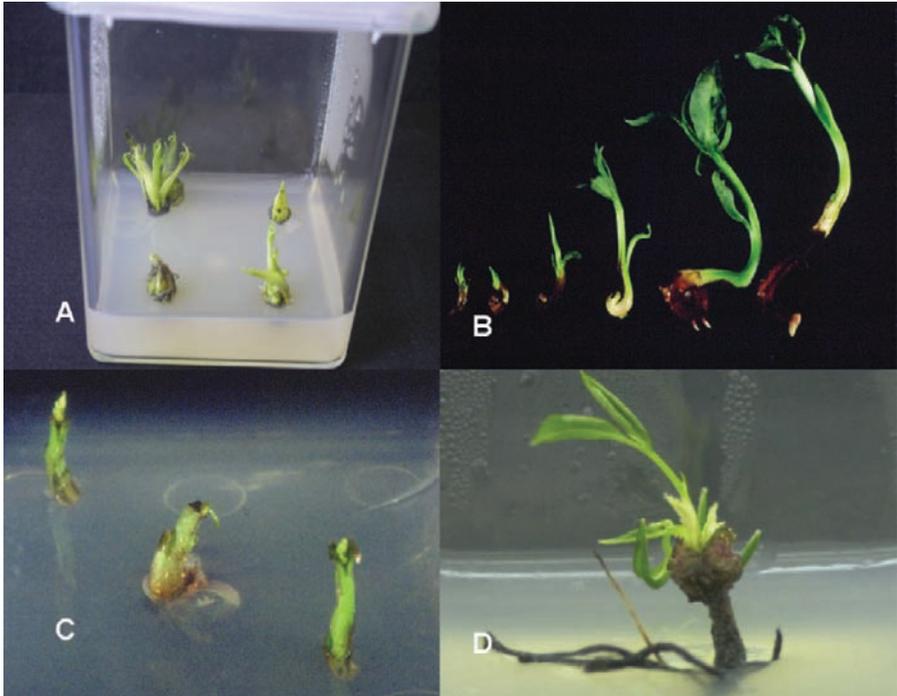


Figure 1. A) *In vitro* forced apical tips of 20-year-old *Pistacia vera* L. B) Rootstock development from germinating seed culture in RDM + 1 mg l⁻¹ BA. C) A close up photo of slit micrografted rootstocks. The excised shoot meristems (4–6 mm) were inserted into the stem axis. D) Shoot tips of *P. vera* micrografted onto seedling rootstocks *in vitro* after 6 weeks in culture.

In order to examine the effect of medium strength on shoot growth and multiplication, the concentration of nutrients in BCM varied (quarter, half to full and double strength). The medium strength used affected the mean shoot number and mean node number. Explants from plantlets cultured on quarter and half strength medium regenerated less shoots and nodes than those from explants cultured in full and double strength media (Onay et al., 2003b). Therefore, we have used half or full strength medium for culture initiation and maintenance. In failing to do so, the cultures can rapidly turn dark brown and lose their organogenesis potential regardless of state of medium.

2.2.3. Rootstock Production

Pistacia vera, with reduced vigor, is commonly used rootstock worldwide. Most pistachio orchards in Iran, Turkey, Syria and Tunisia are planted by using this rootstock. As seed sources, in this study, the following open pollinated rootstocks were used: *Pistacia vera* L., *P. terebinthus* L., and *P. khinjuk* Stocks. At the end of 14th day *in vitro* culture period, observations were taken from the total of 50 seedlings on: stem diameter (mm), stem height (cm), and length of the roots (cm).

Stem diameter. *In vitro* micrografting, stem diameter is very important. Onay et al. (2003b) observed stem diameter differences between rootstocks. *P. vera* L. had the biggest stem diameter which is important to accommodate the microscions while *P. terebinthus* L. and *P. khinjuk* stocks showed a reduced stem diameter. As expected, an effect was found between stem height records of the seedlings. In relation with the number of roots, *P. vera* L. had less roots with a couple of adventitious roots.

In vitro seed germination type. This type was also tested for the best rootstock production on RPM with or without 0.5 mg l⁻¹ BA. In this experiment, intact kernels, half kernels with embryos were isolated and cultured on RPM. After 14 day culture period, records on a total number of 40 explants were taken on stem diameter (mm), stem height (cm), and length of roots (cm).

The cultured explants exhibited various responses according to with and without BA treatments. In the presence of BA, the length of root was reduced, and the isolated embryos gave shorter root length than the intact and half kernels. Regarding stem diameter, important differences between rootstocks were observed (Onay et al., 2003b). The intact kernels gave the biggest stem diameter among the explants tested. Considering observations made, *Pistacia vera* L. is promising for *in vitro* micrografting.

Explant

1. Use mature dry nuts of *Pistacia vera* 'Siirt' to raise *in vitro* seedlings for rootstocks.
2. Bring the seeds to the laboratory and remove outer pericarp and shells of seeds before sterilization.

Surface sterilization of mature seed

1. Sterilize kernels, from which the outer pericarp and shells are removed by immersion in a 20% (v/v) commercial bleach solution for 30 min on a shaker at 150 rpm.
2. Gently wash with SDW at least 3 times for 5 min.
3. Shake with SDW at least 30 min on a shaker at 100 rpm.
4. Remove the seed coats and then cut the half of the cotyledons.
5. Incubate the cultures in Magenta GA 7 vessels (Chicago Corp.) containing 50 ml RPM supplemented with 1.0 mg l⁻¹ BA in a growth room.

6. Incubate the cultures in a growth room at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux intensity (fluorescent lamps, 75W) and a photoperiod of 16 h at $25 \pm 2^\circ\text{C}$ for 10–14 days. Use the seedlings (Figure 1B) as rootstocks for grafting.

2.2.4. Development of Grafting Method

Various grafting procedures were tried within the culture vessels without removing the rootstocks from the medium (Figure 2):

1. *Slit micrografting*: The rootstock was decapitated to remove all leaves and a vertical slit was made on the stump, and the scion base, cut in v-shape, was fitted in the slit.
2. *Wedge micrografting on the stump*: The rootstock was decapitated to remove all leaves. A wedge was cut in the stump, and the scion base cut in a v shape, was gently fit into the wedge.
3. *Wedge micrografting in the leaf axil*: The rootstock was decapitated leaving a single leaf. A wedge was cut off in the leaf axil after removing the leaf blade and the scion base gently sloped to fit into the wedge.

In all three methods, the micrografted seedlings were maintained in the medium similar to the seed germination medium. In the subsequent experiments, slit micrografting was elected for grafting. In this method, the rootstock was decapitated to remove all leaves and a vertical slit was made on the stump, and the scion base, cut in v-shape, was inserted in the slit (Figure 1C).

Different scion sizes were tested: <0.5, 0.5–1, 2–4, 4–6 and >10 mm long. There were differences in the frequencies of successful micrografts between the treatments applied (Onay et al., 2004b). Success increased with the size of the scions up to 6 mm but then decreased with size. All shoot tips <0.5 mm became necrotic and nearly a third of the scions measuring 0.5–1 mm survived.

The effects of culture medium on micrograft development were also tested in three different media: (1) BCM containing 30 g l^{-1} sucrose, 0.5 mg l^{-1} BA and 7.0 g l^{-1} (Micrograft Development Medium; MDM) agar (2) BCM supplemented with 30 g l^{-1} sucrose, 0.5 mg l^{-1} IBA and 7.0 g l^{-1} agar; and (3) BCM containing 30 g l^{-1} sucrose and 5.7 g l^{-1} agar. Differences in successful micrografts and shoot development were observed between the treatments tested.

Steps of the slit micrografting:

1. Decapitate the developed 14-day-old seedlings to remove all leaves.
2. Make a vertical slit on the stump (3–5mm).
3. Use 4–6 mm sterile shoot tips as scions.
4. Cut in v-shape in the scion base.
5. Insert the v-shape scion in the vertical slit.
6. Culture carefully the micrografts on micrograft development medium (MDM). Shoots with a pair of leaves were observed 6 weeks after micrografting (Figure 1D).

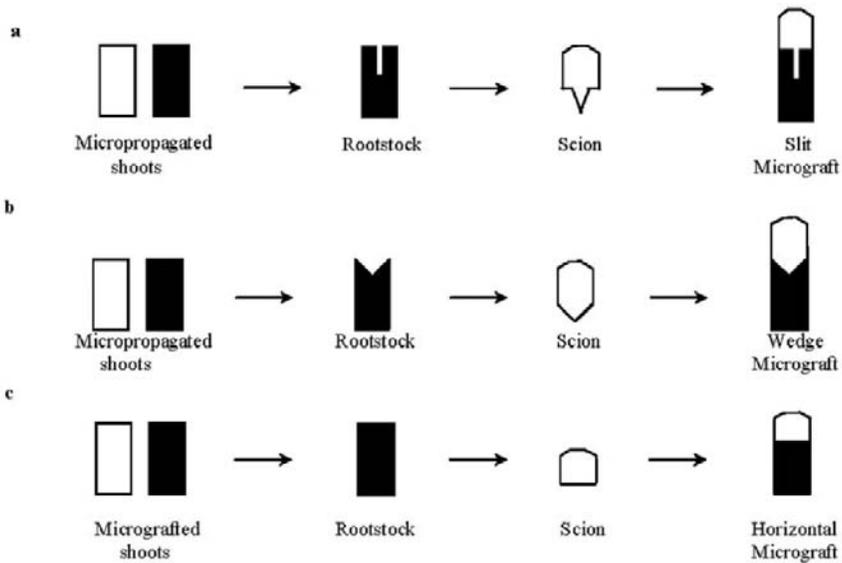


Figure 2. *In vitro* micrografting of pistachio plantlets with slit a), wedge b) and horizontal micrografts c).

2.2.5. Acclimatization and Field Testing

1. Remove plantlets from culture vessels and submerge them in a beaker containing enough water for overnight.
2. Gently rinse the plantlets under a slow stream of tap water to remove residual agar medium from the root region.
3. Transfer the morphologically normal plantlets with both shoots and roots that developed from micrografts to plastic pots containing autoclaved peat: perlite (1:1 v/v) or peat and soil (2:1 v/v) mixture located in the growth room.
4. Water the transplants with N-P-K 20-20-20 water soluble fertilizer and subsequently as needed.
5. Keep potted plantlets (Figure 3A) in clear plastic enclosures or glass beakers under culture room conditions a photoperiod of 16 h at $25 \pm 2^\circ\text{C}$.
6. Transfer plantlets to ambient conditions by gradually uncovering the plastic enclosures or the beakers over a period of 8 weeks. Eight weeks after planting the survival rate of regenerated plants can be determined.
7. Then, the acclimatized plantlets can be transferred to the growth room or to the greenhouse conditions after transplanting to soil.
8. After 2–4 months, transfer the plantlets to bigger pots and maintain in the greenhouse. Vigorous shoots with three or four buds developed from the scion 12 weeks after micrografting (Figure 3B).

9. The regenerated plantlets can be transferred to the shadehouse and nurtured with N-P-K fertilizer for several months. Then, the plants can be planted in the field.
10. The survival rate of micrografts is dependent upon the acclimatization time. The highest survival rate of regenerated plantlets was obtained when minimum acclimatization time was 6 weeks.

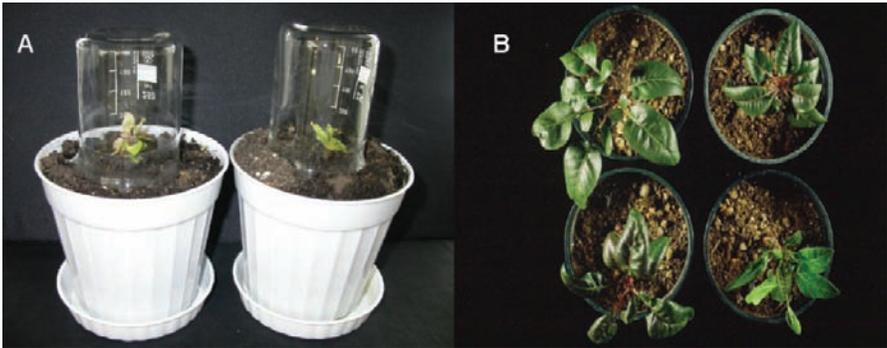


Figure 3. A) Plantlets undergoing acclimatization in a mixture of peat and soil (2:1). B) Micrografted plantlets successfully established in soil after 12 weeks from potting.

3. CONCLUSION

This regeneration protocol via *in vitro* micrografting is an excellent system for mass-scale production of plants since the genetic constitution can be maintained and no somaclonal variation among *in vitro* grown plants is observed. *In vitro* shoot tip micrografting technique can be considered as an alternative to organogenesis and somatic embryogenesis for *P. vera* L., especially for plant material collected from the selected mature trees. Thus, the micrografting technique detailed in this protocol for clonally propagating true-to-type mature *P. vera* genotypes has a potential for restoring proliferation and rooting competence in mature scions.

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CHAPTER 28

PROTOCOL FOR MICROPROPAGATION OF *CASTANEA SATIVA*

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1. INTRODUCTION

Since the 1970s, natural resources the world over have been progressively re-evaluated in accordance with the principles of sustainable agriculture. The European chestnut, *Castanea sativa* Mill., a hardwood species belonging to the family Fagaceae, has not been unaffected by this process. It is an important resource in many parts of the world because of its economic and environmental role in many agroforestry systems, and in Europe it has been gaining in value as a source of timber and nut production and due to the contribution of chestnut groves to the landscape (Bounous, 2005).

Like the American chestnut *C. dentata*, *C. sativa* has been plagued for more than a century by ink disease and chestnut blight, caused by the fungi *Phytophthora cinnamomi* and *Cryphonectria parasitica*, respectively. A great deal of research on chestnut focuses on the development of vegetative propagation systems capable of satisfying the demand for elite genotypes that provide both high-quality timber and/or nuts and resistance to these diseases. Since chestnut is a difficult-to-root species, grafting is the most frequent conventional propagation technique, although methods for layering and cutting have recently been improved and are widely used in nurseries to propagate ink-disease-resistant Euro-Japanese hybrids. However, as an alternative to conventional vegetative propagation methods, efforts are being made to establish reliable *in vitro* regeneration systems that allow clonal propagation. The two major systems are based on embryogenesis or on micropropagation of axillary shoots.

Several studies have shown the potential of somatic embryogenesis of chestnut, not only for clonal propagation but also for genetic engineering programmes

(Corredoira et al., 2006). However, although somatic embryogenesis is theoretically more efficient for clonal mass propagation than propagation via axillary shoots, several difficulties need to be overcome in order to make it commercially viable, particularly when cultures originate from adult tissues. By contrast, chestnut can currently be micropropagated from both juvenile and mature material using the axillary shoot development method. In the last years, efforts have been concentrated on the regeneration systems allowing clonal propagation of mature chestnut trees. Large-scale propagation is still in many cases challenging, because it is common for the protocol to require optimization for a specific cultivar; but there are nevertheless several European companies that now produce thousands of plants a year. In this chapter we describe the various steps of the typical protocol for *C. sativa*, with occasional reference to other chestnut species.

2. EXPERIMENTAL PROTOCOL

The micropropagation of chestnut via axillary shoots involves four stages: 1) initiation (*in vitro* shoot growth on primary explants); 2) shoot proliferation; 3) shoot rooting; and 4) plantlet acclimatization (hardening). In the interests of efficiency, the rooting and acclimatization stages are combined whenever possible. Before examining each stage in detail, we present the culture media that have been found to be most appropriate for each of the *in vitro* stages.

2.1. Culture Media

Of the various culture media that have been assayed for micropropagation of European chestnut, most studies have adopted Gresshoff and Doy mineral medium (GD; 1972) for material of both juvenile and mature origin. Apical necrosis and chlorosis are best avoided, and general appearance improved, with either GD or Murashige and Skoog medium (MS; 1962) with half strength nitrates (Vieitez et al., 1986; Sánchez et al., 1997a; Gonçalves et al., 1998; Ballester et al., 2001). However, in protocols developed for *C. dentata*, Woody Plant Medium (Lloyd & McCown, 1981) has been used both for culture initiation (supplemented with 1 mg/l 6-benzyladenine, BA) and for shoot proliferation (supplemented with 0.2 mg/l BA) (Xing et al., 1997). The protocols described here for the various stages of micropropagation use GD, modified and/or supplemented as detailed in Table 1.

2.2. Explant Preparation and Culture Initiation

The primary explants from which chestnut shoot cultures are initiated are generally shoot tips and nodes bearing 1 or 2 axillary buds. After excision from the source plant (juvenile or mature tree), they must be sterilized and established *in vitro* on the initiation medium.

Table 1. Culture media used in the initiation, shoot multiplication and rooting stages of the micropropagation of European chestnut via axillary shoots.¹

Culture medium	Initiation	Multiplication	Rooting
Macronutrients	GD	GD	1/3 GD
Micronutrients ²	Modified GD	Modified GD	Modified GD
Fe-EDTA	MS	MS	MS
m-Inositol (mg/l)	10	10	10
Thiamine HCl (mg/l)	1	1	1
Nicotinic acid (mg/l)	0.1	0.1	0.1
Pyridoxine-HCl (mg/l)	0.1	0.1	0.1
Glycine (mg/l)	0.4	0.4	0.4
BA (mg/l)	0.5	0.1–0.2	–
IBA (mg/l)	–	–	3 (5-7 days) 25 (24 h) 1000 (1–2 min)
Sucrose (g/l)	30	30	30
Bacto Difco agar (g/l)	7	7	7 ³
pH	5.6–5.7	5.6–5.7	5.6–5.7

¹ Culture conditions: All cultures are kept in a growth chamber with a 16 h photoperiod (50–60 $\mu\text{mol m}^{-2} \text{s}^{-1}$, cool-white fluorescent lamps) and 25°C day, 20°C night temperatures.

² Modified GD (mg/l): 3 BO_3H_3 ; 10 $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 3 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.25 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.25 $\text{MnO}_4 \cdot \text{Na}_2 \cdot 2\text{H}_2\text{O}$; 0.25 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; 0.75 KI.

³ Agar used in the root induction medium (5–7 days or 24 h) before transfer to *ex vitro* substrate.

BA, 6-benzyladenine; GD, Gresshoff and Doy; IBA, indole-3-butyric acid; MS, Murashige and Skoog.

2.2.1. Juvenile Plant Material

When the initial explants are obtained from juvenile plants, the micropropagation of chestnut is fairly unproblematic. Seedlings obtained conventionally should be grown in the greenhouse or, preferably, in a climate chamber so as to reduce explant contamination rates (Vieitez et al., 1986). Active growing shoots are collected from plants between a few weeks and a few months old and are treated as described below in Section 2.2.2 (steps 5–10). Alternatively, initial explants may be taken from seedlings obtained by *in vitro* germination of zygotic embryonic axes (Vieitez & Vieitez, 1980). In either case, the frequencies of responsive explants, shoot multiplication rates and rooting rates are generally high.

2.2.2. Juvenile Parts of Mature Trees

The micropropagation of chestnut from tissues taken from adult trees appears usually to be feasible when these tissues retain physiologically juvenile characteristics, as is the case of basal shoots and stump sprouts (Vieitez et al., 1986; Sánchez & Vieitez, 1991; Sánchez et al., 1997a). In general, cuttings 15–20 cm long are taken in winter from shoots emerging from the base of the trunk, and are stored at 4°C

until forced to flush in a climate chamber, primary explants then being taken from the flushed shoots. The use of a climate chamber is both logistically advantageous, allowing primary explants to be obtained throughout the year, and also helps keep contamination rates low (5–15%).

Steps

1. Basal shoots or stump sprouts are harvested from selected trees growing in the field in late autumn or winter, and cuttings 15–20 cm long are taken.
2. The cuttings are immersed for 1 h in a 3.4 g/l solution of Cupravit (50% copper oxychloride), left to dry for 24 h, packed tightly in plastic bags, and stored at 4°C for 2–6 months, until use.
3. The cuttings are taken out of cold storage, arranged upright in water or in moistened perlite in trays, and forced to flush (Figure 1A) in a growth cabinet at 24°C and 90% relative humidity (RH) under a 16 h photoperiod (95–100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool-white fluorescent lamps).
4. After 15–20 days of flushing, shoots 1–4 cm long are collected as the source of explants (Figure 1B).
5. The collected shoots are stripped of leaves and surface-sterilized by successive immersion for 30 s in 70% ethanol and for 8–10 min in sodium hypochlorite solution (Millipore Chlorine Tablets, 0.6% active chlorine) containing 2 or 3 drops of Tween 80.
6. The shoots are rinsed three times with sterile distilled water.
7. The disinfected shoots are sectioned at 5 mm long shoot tips and nodes (primary explants).
8. The explants are placed upright in 20 × 150 mm culture tubes containing 15 ml of initiation medium (Table 1).
9. After 24 h, the primary explants are moved to a different site within the same tube to reduce the deleterious effects of exudation and blackening of the medium. Thereafter, the explants are transferred to fresh medium every 2 weeks until 6 weeks after the initiation of culture.
10. Shoots produced on primary explants (Figure 1C) are excised and subcultured to achieve clonal shoot multiplication cultures.

Clones differ in their response to establishment *in vitro*. Although this may be attributed largely to their genetic differences, differences in age among the source trees may also be responsible.

2.2.3. Crown Material of Mature Trees

When the material of origin has been taken from the crown of mature chestnut trees, micropropagation has achieved very limited success, shoot multiplication and rooting rates having been poor (Sánchez & Vieitez, 1991). However, the reactivity of mature material can be considerably improved by reinvigorating it before primary explants are excised, either by pre-harvest etiolation (Ballester et al., 1989) or by grafting it onto seedling rootstocks, with or without repeated subsequent spraying with cytokinins (Sánchez et al., 1997b).

Localized etiolation of branches can greatly facilitate the establishment of mature chestnut material *in vitro*, allowing successful explant response rates of up to 79% as against 27% for unetiolated material (Figure 1D). Partial etiolation (blanching) is applied in late May, when 10–15 cm segments of the new year's growth on lower crown branches are stripped of their leaves and wrapped in aluminium foil. In late September, cuttings are taken from the etiolated segments and stored at 4°C until use. These cuttings are forced to flush in a growth cabinet, and the new shoots are used as the source of primary explants as described in steps 5–10 of Section 2.2.2.

Successful establishment *in vitro* can also be achieved if 3–4 cm long scions taken directly from the crown are grafted onto 2-week-old seedlings and kept in a growth cabinet or in the greenhouse for 5 weeks, where for the last 2 weeks they may be sprayed three times a week with 50 mg/l BA solution (Sánchez et al., 1997b). The lateral shoots that emerge during this time are used as the source of primary explants (Figure 1E) as described in steps 5–10 of Section 2.2.2. However, serial grafting may be necessary: Giovanelli and Giannini (2000) reported successful establishment *in vitro* after having successively grafted shoots four times onto 10-month-old seedling rootstocks over a 4-year period.

It should be noted that even when mature material is reinvigorated by one of the above techniques, the cultures established generally have lower shoot proliferation and rooting rates than those of shoot cultures derived from juvenile parts of the tree (Figure 1F). Material that retains juvenile characteristics should therefore be used whenever possible (see Section 2.2.2).

2.3. Shoot Proliferation and Maintenance

New shoots obtained *in vitro* by 6–8 weeks of culture of primary explants are excised, divided into 0.8–1.0 cm segments (including shoot tips), and subcultured into 500 ml glass jars (9 explants/jar) containing 70 ml of shoot multiplication medium (Table 1). The indicated carbon source of the multiplication medium, 3% sucrose, has been chosen on the basis of comparison of the results obtained with sucrose (Figure 2A), glucose and fructose at concentrations of 1–4%: although 3–4% fructose or glucose afford the greatest number of shoots, these shoots exhibit high hyperhydricity rates and poor growth. A feasible alternative to 3% sucrose is 2% glucose.

Clonal shoot multiplication is effected by subculturing at intervals of 4–5 weeks. *In vitro* stabilization is achieved within 4–8 subculture cycles, and cultures can be maintained for years if healthy, vigorous shoots are always used for subculture. When shoots have not elongated satisfactorily after 3–4 weeks of culture, their length and vigour can be promoted by spending 2–3 weeks more on fresh multiplication medium with a lower BA concentration (0.05 mg/l), giving a 6–7 week multiplication cycle.

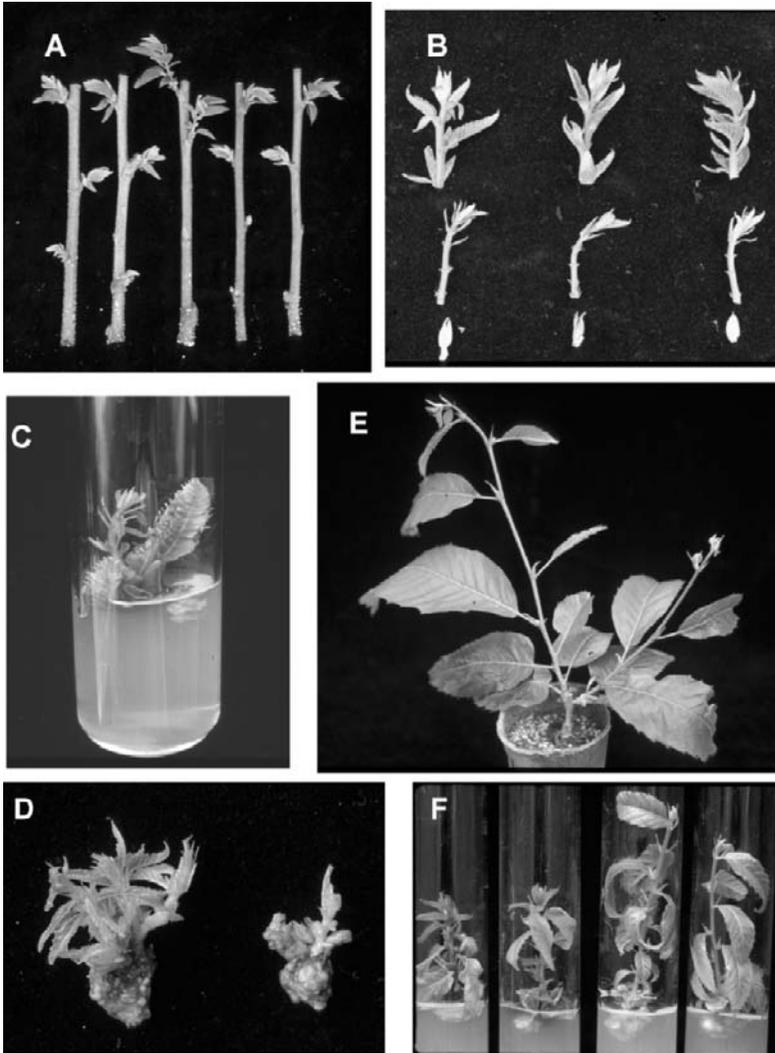


Figure 1. Shoot culture initiation from buds of mature chestnut trees. A) Forced flushing of cuttings taken from basal sprouts of an adult tree. B) Shoots excised from flushed cuttings (upper), sterilized shoots (middle) and sectioned shoot tip explants to be established in vitro (lower). C) Shoot development from a primary explant of basal sprout origin 1 month after culture initiation. D) Initial cultures of etiolated (left) and unetiolated (right) crown-derived material. E) Plant grafted onto seedling rootstock, exhibiting new shoots to be used as source of crown-derived explants. F) Proliferating shoot cultures originated from crown branches (left) and basal sprouts (right), after 4 weeks in multiplication medium.

Shoot proliferation is influenced by both genotype and the type of explant. Genotype is one of the most important determinants of all relevant parameters (number of shoots, shoot length and overall multiplication rate). Multiplication rates are also influenced by explant type, basal nodal explants (which bear 2–3 axillary shoots) affording higher multiplication rates than shoot tip explants (Sánchez et al., 1997a). Shoot growth on basal segments is more vigorous if their basal callus is retained. Therefore, shoots taken for rooting should have their basal 1 cm removed and this tissue, with its callus, can be recycled to the proliferation stage.

2.4. Rooting

Chestnut is a difficult-to-root species, and the rooting and acclimatization of shoots obtained *in vitro* are crucial for mass production of viable plants by micropropagation. As indicated in Table 1, there are three main options for inducing roots on micropropagated chestnut shoots (Sánchez et al., 1997a; Gonçalves et al., 1998): 1) culture for 5–7 days in rooting medium containing 3 mg/l indole-3-butyric acid (IBA); 2) dipping the basal end of the shoots for 1–2 min in 1 g/l IBA solution; and 3) culture for 24 h in rooting medium containing 25 mg/l IBA. All three can afford rooting rates of 80–95%, and although the 24 h treatment generally affords better shoot quality than the quick dip, which option is best will depend on genotype. After one of the three procedures has been carried out, the auxin-treated shoots are transferred either to a substrate mixture (Figure 2B), or to auxin-free root expression medium consisting of basal rooting medium containing 1% activated charcoal (Figure 2C). When possible, the former procedure is clearly the more cost-effective: whereas *in vitro* rooting is labour-intensive, rooting in a substrate in the greenhouse allows rooting and acclimatization to be achieved in one step instead of two.

One of the main threats to the survival of plantlets during acclimatization is the occurrence of shoot tip necrosis during rooting (Vieitez et al., 1989; Piagnani et al., 1996), and it is chiefly to reduce necrosis and to promote shoot growth that charcoal is included in the root expression medium (Sánchez et al., 1997a). When shoot tip necrosis does occur, the role of apical dominance is generally taken over by one of the axillary buds, usually the one nearest the apex, and the plantlet survives. The rapid development of an axillary bud can be promoted by the removal of the shoot tip before rooting (Vieitez et al., 1989; Gonçalves et al., 1998).

2.5. Hardening

The adaptation of micropropagated chestnut plants to *ex vitro* conditions is generally long and difficult. Survival rates are higher if microcutting rooting has been carried out directly in substrate mixture rather than *in vitro* in root expression medium. Survival depends heavily on genotype, with values ranging from 50% to 90%, and the best period for stimulating rapid stem elongation is February–August. For *C. sativa* × *C. crenata* plantlets, survival rates higher than 80% have been obtained under a range of irradiance and CO₂ availability conditions. However, under high

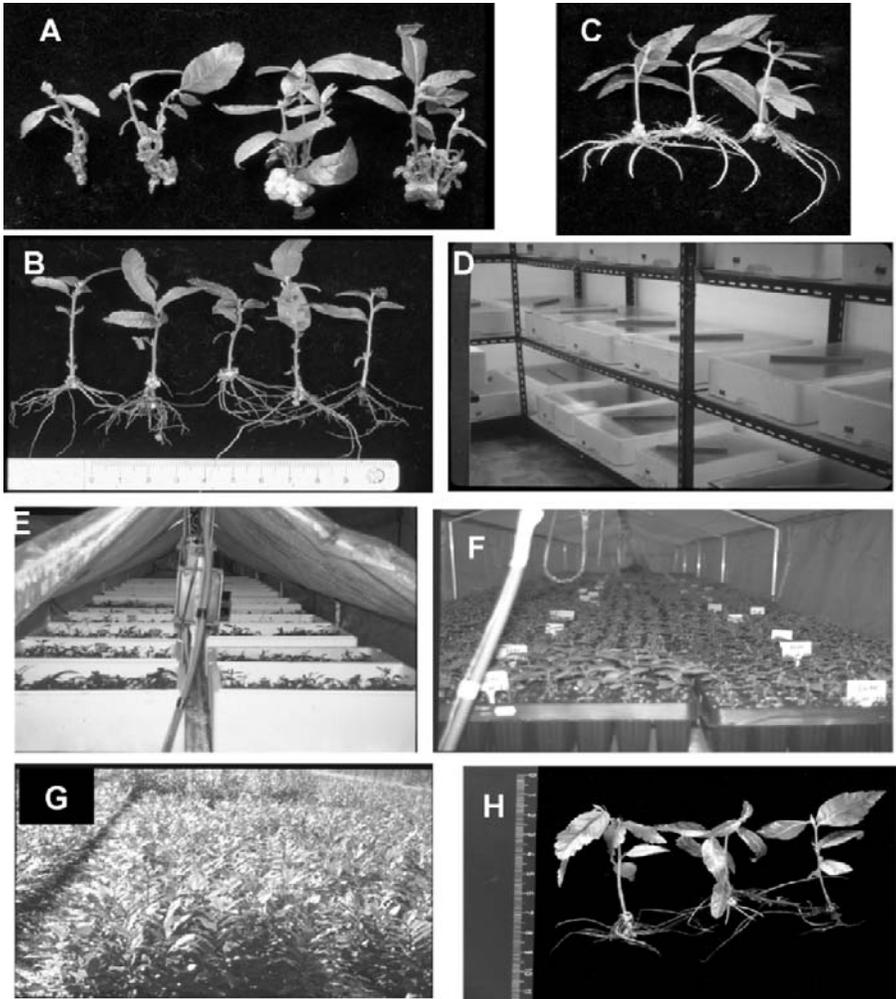


Figure 2. Rooting, hardening and cryopreservation of chestnut. A) Morphology of shoots after 4 weeks of culture in sucrose-supplied multiplication media (from left to right, 1, 2, 3 and 4%). B) Rooting of auxin-treated shoots following transfer to a mixture substrate. C) Root development in shoots treated with 25 mg/l IBA for 24 h and subsequent culture for 1 month in activated charcoal medium. D) Polystyrene boxes filled with substrate and covered with plastic lids, where IBA-treated microcuttings are transferred for rooting. E) Plantlets in open polystyrene boxes placed in the acclimatization tunnel. F) Rooted plants transferred to trays and maintained in the acclimatization tunnel. G) Micropropagated chestnut plants growing in the outdoor nursery. H) Rooted plantlets derived from cryopreserved shoot apices.

irradiance ($300 \mu\text{mol m}^{-2} \text{s}^{-1}$) combined with high CO_2 concentration ($700 \mu\text{l/l}$) growth yields increased consistently as well as the efficiency in inducing an autotrophic behaviour (Carvalho & Amâncio, 2002; Carvalho et al., 2005). These authors suggested that these conditions are worth to be tested as an acclimatization protocol for recalcitrant genotypes.

Steps for Direct Rooting and Hardening

1. Shoots 2.5–5.0 cm long are excised from shoot proliferation cultures.
2. The basal end (1 cm) of the shoots are dipped for 1–2 min in 1 g/l IBA solution in a glass beaker (50 shoots per vessel). Alternatively, the shoots can be cultured for 24 h in rooting medium containing 25 mg/l IBA.
3. The IBA-treated microcuttings are transferred to polystyrene boxes (100 microcuttings/box) filled with a moistened 1:2 (v:v) mixture of pine bark and perlite that has previously been treated with antifungal solution (Figure 2D). The boxes are each covered with a transparent polyethylene lid and are kept in the growth chamber for 2 months under standard conditions (Table 1), lids with ventilation holes being used during the second month so as to decrease the RH inside the boxes. During this time the microcuttings are watered twice a week, once with antifungal solution and once with water.
4. After 2 months the boxes are placed without lids in plastic acclimatization tunnels in the greenhouse, where they stay for 1 month (Figure 2E). The tunnels are provided with mist and fog systems to maintain 90% RH, and during autumn and winter artificial lighting ensures a 16 h photoperiod.
5. The rooted plantlets are transferred to multi-cell trays filled with a 1:2:1 (v:v:v) mixture of pine bark, perlite and peat supplemented with Osmocote[®], a slow-release fertilizer. The trays are kept in the tunnels for 1 month (Figure 2E), during which time RH is gradually lowered to normal greenhouse conditions.
6. By the end of the month, average plant height is 25 cm. The plants are removed from the tunnels, transplanted to pots or to trays with larger cells, and kept in the greenhouse for a further 1–2 months.
7. Still in their pots, the plants are placed outdoors under shade. When the plants have reached a height of 40–50 cm they can be planted out in the field (Figure 2G).

2.6. Storage of In Vitro Cultures

Tissue culture techniques not only offer an opportunity for rapid propagation, they also constitute an approach to genotype conservation. However, the maintenance of large collections in conventional *in vitro* culture systems, which involve subculture at regular intervals, exposes the cultures to increasing risks of contamination and somaclonal variation. In the case of medium-term storage of chestnut cultures, these risks can be reduced by conservation under slow growth conditions (cold storage), which moreover reduces maintenance costs by prolonging subculture periods. For long-term conservation of micropropagated chestnut germplasm, cryopreservation has been used.

2.6.1. Cold Storage of In Vitro Shoot Cultures

Janeiro et al. (1995) reported the possibility of keeping chestnut cultures at 2–4°C for up to 1 year without subculture. Explant necrosis is common following cold storage, but even apparently necrotic explants should spend one subculture cycle in the growth chamber under standard conditions, as some brown cultures can proliferate, especially from lateral buds that remained immersed into the agar throughout cold storage. Also, both the survival rate and the post-storage proliferation capacity of the explants are significantly better if transfer to the cold is due to 10 days after the last subculture rather than immediately after it.

Steps for Cold Storage of in Vitro Shoot Cultures

1. Nodal segments 10–12 mm long bearing 2–3 axillary buds are excised from shoot multiplication cultures.
2. These explants are placed 6 explants to a jar in 300 ml glass jars containing 50 ml of multiplication medium and are maintained in the growth chamber for 10 days under standard growth conditions (Table 1). Most of the length of the explant should be immersed in the medium in order for it to protect lateral buds.
3. After 10 days of subculture, the jars are stored in the dark at 3–4°C in Sanyo Medicool Cabinets.
4. The cultures can be kept in the cold for up to 12 months. When removed from the cabinets they are immediately transferred to fresh multiplication medium and kept in a growth chamber under standard conditions (Table 1).
5. After 1 month of culture, new green shoots will have developed from axillary buds, and shoot multiplication can be carried out as described in Section 2.3.

2.6.2. Cryopreservation of Shoot Tips

A vitrification procedure involving successive treatments with Loading Solution (LS; Matsumoto et al., 1994) and modified Plant Vitrification Solution (PVS2; Sakai et al., 1990) allows micropropagated chestnut shoot apices to be cryopreserved fairly successfully for subsequent plant regeneration (Vidal et al., 2005). Factors influencing success include the source of the shoot tips (terminal buds are better than axillary buds), the duration of exposure to PVS2 (optimum 120 min), the size of the shoot tip (0.5–1 mm shoot apices isolated from terminal buds), and the composition of the post-cryostorage recovery medium (should include 0.5 mg/l indole-3-acetic acid, IAA, in addition to cytokinin). Depending on genotype, shoot recovery rates of 33–54% have been obtained (recovery being defined as greening, leaf expansion and shoot production), and shoots derived from the cryopreserved material show no significant difference in rooting ability (Figure 2H) from shoots of the same clones that have not been subjected to cryopreservation.

Steps for Cryopreservation of Shoot Tips

1. Terminal shoot buds 1 cm long are excised from shoot cultures 4–5 weeks old that have been maintained in multiplication medium (Table 1).

2. Cold hardening. The terminal buds are transferred to GD medium containing 0.05 mg/l BA dispensed in 9 cm diameter Petri dishes, and are cold-hardened for 2 weeks at 3–4°C under dim light.
3. Shoot apices 0.5–1.0 mm long are dissected from terminal buds.
4. Preculture. The shoot apices are precultured in Petri dishes for 48 h at 3–4°C on GD medium containing 0.2 M sucrose but no BA.
5. Loading. Following preculture, the shoot apices are immersed for 20 min at room temperature in cryoprotectant mixture (LS: 2M glycerol + 0.4 M sucrose) in 2 ml cryotubes.
6. Dehydration. The LS solution is replaced by modified PVS2 (30% glycerol, 15% ethyleneglycol, 15% dimethylsulfoxide, in GD medium containing 0.4 M sucrose). The shoot apices are treated with this mixture for 120 min at 0°C.
7. Cryopreservation. The apices are finally suspended in 0.6 ml of PVS2 in cryotubes, and are plunged into liquid nitrogen (LN).
8. Thawing. For recovery of the cryostored shoot tips, the cryotubes are rapidly warmed for 2 min in a water bath at 40°C.
9. Unloading. The PVS2 solution is drained off and replaced with liquid GD medium containing 1.2 M sucrose. The shoot apices are left in this medium for 10 min, and then for another 10 min in a fresh dose of the same medium.
10. Regrowth. The apices are transferred to sterilized filter paper discs placed in Petri dishes on a recovery medium consisting of GD medium supplemented with 0.5 mg/l BA, 0.5 mg/l IAA and 0.2 mg/l zeatin.
11. Subsequently (24 h, 2 weeks and 4 weeks later), the apices are transferred to fresh recovery medium without paper discs. Standard subculture is carried out 8 weeks after thawing.

Cryopreserved chestnut shoot tips turn blackish-brown within 1 day after thawing, but culture on recovery medium induces surviving tips to resume growth within 2–3 weeks, leaf development becoming evident by the fourth week. Eight weeks after thawing, shoots are an average 2.6–5.0 mm long, depending on genotype. After two 1-month periods on multiplication medium (Table 1), these shoots have achieved 2–3 cm and are thus suitable for shoot multiplication or rooting as described in Sections 2.3 and 2.4.

2.7. Molecular Marker Analysis

The genetic stability of *in vitro* regenerated plants is an essential requisite to maintain clonal identity. Although shoot tips are in principle genetically stable, micropropagation protocols should include confirmation that true-to-type plants are being produced. Methods for early detection of genetic variation include morphological observations, cytological methods and DNA analysis (Rani & Raina, 2003). In the case of chestnut, screening for DNA polymorphism by RAPD analysis has provided evidence supporting the genetic stability of *Castanea sativa* × *C. crenata* hybrids propagated *in vitro*; after *in vitro* multiplication of axillary shoots for more than 4 years, comparison of RAPD patterns of *in vitro* cultures with those

of the plants of origin showed no polymorphism between the former and the latter (Carvalho et al., 2003).

Assessment of genetic stability is also required following cryopreservation (Harding, 2004). For chestnut, the analysis of genetic integrity of cryopreserved shoots has been performed by RAPD analysis (San-José et al., 2005): in experiments on three genotypes using DNA obtained from fresh young leaves, RAPD profiles generated with 40 10-mer primers showed no differences between non-cryopreserved cultures and cultures that had been recovered following cryopreservation.

Steps for Molecular Marker Analysis

1. Young leaves (100 mg) are harvested from individual shoots, and are immediately frozen in LN and stored at -70°C until used for DNA isolation.
2. The frozen leaves are ground to a fine powder in LN in a precooled mortar, and genomic DNA is extracted using a DNeasy Plant Minikit (Quiagen GmbH). The DNA is quantified by spectrophotometry, and its quality is assessed by electrophoresis in agarose gels (1.2% in $0.5\times$ TBE).
3. PCR reactions are carried out in a Biometra Thermocycler using 25 μl of a reaction mixture composed of Tris-HCl (67 mmol/l, pH 8.8), $(\text{NH}_4)_2\text{SO}_4$ (16 mmol/l), MgCl_2 (6 mmol/l), dNTPs (200 $\mu\text{mol/l}$ each), bovine serum albumin (0.4 g/l), decanucleotide primer (0.4 $\mu\text{mol/l}$, from kits A and S of Operon Technologies Inc.), 20 ng of genomic DNA, and 0.6 U of BioTaq DNA polymerase (from Bioline).
4. Amplification programme: 1 min at 94°C , followed by 45 cycles of 10 s at 94°C (denaturation), 15 s at 36°C (annealing) and 90 s at 72°C (elongation), and finally 7 min at 72°C . At least two independent PCR runs are performed per sample and selected primer.
5. The amplification products are resolved in agarose gels (1.4% in $0.5\times$ TBE buffer containing 0.15 $\mu\text{g/l}$ ethidium bromide) alongside a 100 bp ladder for size estimation. The bands are visualized under UV light for photography and capture of digitalized images.
6. For each primer, RAPD bands are scored as present or absent by visual inspection of gel photographs. The molecular weight of each fragment can be estimated from digitalized gel images using Bio-Rad Quantity One one-dimensional analysis software.

3. CONCLUSION

Micropropagation has been adopted for commercial production of chestnut cultivars and hybrids selected for desirable qualities such as resistance to ink disease. One of the major problems preventing wider use of micropropagation technology has been the difficulty in using explant source material from mature trees. Whenever possible, the material used should be taken from stump sprouts or epicormic shoots, which retain juvenile characteristics. When this is not possible, non-juvenile material can be reinvigorated by techniques involving pruning, grafting or etiolation, although the results are poorer than with juvenile material, especially as regards rooting and

plantlet acclimatization. It must be stressed that results in any case depend heavily on genotype, which not only influences shoot proliferation and rooting rates, but also the vigour and acclimatization of regenerated plantlets.

Advances in breeding and genetic engineering programmes create a need for means of preserving valuable new genotypes. Storing chestnut shoot cultures at 3–4°C reduces the risk of contamination and genetic variation by lengthening subculture cycles to up to 1 year, constitutes a convenient method for medium-term storage of germplasm, and facilitates its safe distribution. For long-term storage, cryopreservation of chestnut shoot apices is made feasible by the vitrification procedure.

For confident use of micropropagated chestnut plants, they must be known to be genetically identical to their trees of origin. As yet, relatively little research has been done on verification of genetic stability, but RAPD analyses have found no evidence of genetic variation in chestnut cultures propagated *in vitro* by multiplication of axillary shoots. At present, however, technologies based on molecular markers need to be supported by cytological methods and by morphological observations at field testing level.

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CHAPTER 29

MICROPROPAGATION OF CASHEW (*ANACARDIUM OCCIDENTALE* L.)

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1. INTRODUCTION

Cashew (*Anacardium occidentale* L.) is an important nut crop belonging to the family Anacardiaceae and is extensively grown in tropical regions of South America (Brazil), Africa and India. The edible portion (kernel) is quite nutritious and even the apple (false fruit) has many uses. In India it is economically important crop as it earns sizable foreign exchange and provides employment to more than 10 lakh population directly or indirectly. As cashew is a woody perennial, efforts made for improving its productivity has met with limited success. For achieving the desired progress and to make rapid strides in improvement of this crop, application of biotechnological tools are needed. Establishment of an efficient, fast and reproducible plant regeneration protocol is a prerequisite in this direction. Cashew can be propagated both by seeds and vegetative means but the seedling progenies have disadvantage of variable performance due to heterozygosity of this crop. Hence, vegetative propagation by softwood grafting has proved useful for large-scale multiplication of cashew (Swamy & Nayak, 2003). Micropropagation is useful for multiplication of breeders stock or hybrids and can augment the supply of bud-wood which is required in large quantity for grafting and thus supplementing the propagation needs of nursery industry. Besides this it has applications in *in vitro* screening against biotic and abiotic factors and regeneration of transgenic plants.

Micropropagation work in cashew has been initiated first by Philip (1984) and reported direct plantlet regeneration from mature cotyledonary segments. Shoot bud proliferation on double phase medium (Lievens et al., 1989) and shoot multiplication in cotyledonary nodal segments of cashew seedlings have also been reported (D'Silva & D'Souza, 1992). Although shoot multiplication was possible but rooting was

limited and hence *in vitro* rooting using *Agrobacterium rhizogenes* was reported by Das et al. (1996). Boggetti et al. (1999) and Mneney and Mantell (2002) have also attempted micropropagation in cashew. Thimmappaiah and Shirly (1999) have been able to regenerate cashew completely using explants from juvenile source and the protocol standardized in our laboratory is described in this chapter. Our success with regeneration from explants of mature tree source has been limited owing to poor growth, elongation and rooting of shoots (Thimmappaiah et al., 2002a). Micrografting as a technique to rejuvenate cashew and overcome rooting has also been described (Thimmappaiah et al., 2002b). Earlier attempts on micrografting in cashew were reported by Mantell et al. (1997) and Ramanayake and Kovoov (1999).

Regeneration of cashew through embryogenesis from cotyledon (Hegde et al., 1994; Jha, 1988; Sy et al., 1991), immature embryos (Cardoza & D'Souza, 2000; Gogate & Nadgauda, 2003) and nucellus (Ananthakrishnan et al., 1999; Gogte & Nadgauda, 2000; Cardoza & D'Souza, 2002; Shirly & Thimmappaiah, 2005) have been reported but complete regeneration and establishment of plantlets are yet to be achieved.

2. EXPERIMENTAL PROTOCOL

2.1. Materials

1. Mature cashew seeds, *in vitro* raised seedlings, defoliated terminal shoots.
2. Sodium hypochlorite (NaOCl) (4% available chlorine), mercuric chloride, concentrated hydrochloric acid, rectified spirit, sterile distilled water, Tween-20.
3. Laminar flow hood, stainless steel sterile surgical blades, scalpel handle, spirit lamp, sterile Petri plates, forceps, tissue paper, rimless test tubes (25 × 150 mm), screw cap bottles (200 ml capacity), baby food jars (Sigma).
4. Tissue culture chamber, portable autoclave, microwave oven, environmental shaker.
5. Thidiazuron (TDZ), naphthalene acetic acid (NAA), N⁶-benzyl adenine (BA), indole-3-butyric acid (IBA), polyvinylpyrrolidone (PVP-360), L-glutamine, casein hydrolysate, activated charcoal (AC).
6. Media: Murashige & Skoog (1962) (MS) and Raj Bhansali (1990) (RBM) basal media (Table 1) and their formulations (Table 2).

2.2. Methods

The technique of regeneration involves 1) raising of *in vitro* seedlings or seedlings in greenhouse as a source of explants, 2) explant preparation and sterilization, 3) initiation of shoot cultures on medium, 4) axillary shoot bud proliferation on multiplication medium, 5) shoot bud elongation phase on hormone free medium, 6) root induction phase and 7) hardening and potting stages.

2.2.1. Explant

Shoot explants like nodal cuttings (1–2 nodes), shoot-tips and coteledonary nodes with and without cotyledons excised either from 1 month old *in vitro*-raised seedlings or greenhouse seedlings are used. *In vitro*-raised seedlings are obtained by germinating mature seeds *in vitro* after sterilizing the seeds. Similarly to raise seedlings in greenhouse the seeds are sterilized and germinated in aluminum trays containing sterile sand or pot mixture.

Sterilization and In Vitro Germination of Seeds

1. Soften fresh mature cashew seeds in concentrated hydrochloric acid for 10 min in a fume hood.
2. Rinse seeds several times in sterile distilled water (SDW).
3. Surface sterilize the seeds in the flow hood first by immersing in 70% ethanol (1 min).
4. Rinse in SDW (1–2 times).
5. Sterilize seeds second time in 50% sodium hypochlorite solution (4% available chlorine) with 1 to 2 drops of Tween-20 for 20 min by constant stirring (in a shaker).
6. Rinse at least thrice in SDW.
7. Inoculate the disinfected seeds into sterile screw cap bottles (200 ml capacity) (5.5 × 10.5 cm OD × H) laid with moist (in SDW) absorbent cotton (surgical grade) at bottom.
8. Germinate the seeds at $25 \pm 2^\circ\text{C}$ in a growth chamber in dark.
9. The seedlings emerged 25–30 days after inoculation are used as a source of explants (Figure 1A).

Explant Sterilization

Shoot explants collected from 3 to 4-week-old greenhouse raised seedlings are sterilized following the procedure described below.

1. Collect actively growing terminal shoots (5–8 cm long) in a clean beaker.
2. Shoots are defoliated in laboratory and segmented into one or two cuttings. Petioles are removed close to the axils without damaging the dormant buds in the axils.
3. Dip shoot segments in 70% ethanol for 20–30 seconds to do a preliminary disinfection.
4. Wash the shoot segments several times in SDW after adding and rinsing in 0.01% detergent and pinch of carbendazim (50% WP).
5. Surface sterilization of explants is carried out in a sterile container in 0.1% mercuric chloride containing a drop of Tween-20 (wetting agent) by constant agitation for 5–7 min. Explants of cashew from nursery or greenhouse source are prone to high contamination. Mild sterilants were found not effective.
6. Rinse in SDW minimum of three to five times.
7. Segment the explants to size (1.0–1.5 cm) in SDW and inoculate into tubes.

Explant Preparation and Culture Initiation

To prepare explants from *in vitro* seedlings (3–4 weeks), the seedlings are carefully removed from bottles into a big sterile Petri plate (14.5 cm) and detopped to collect the terminal shoot and cotyledonary node with root. Cotyledonary nodal explant (2.5–3.0 cm long) is prepared with and without expanded cotyledons discarding the root portion and retaining about 0.5–1.0 cm of hypocotyl and epicotyl portion on either side of cotyledonary node junction. Single or double nodal cuttings and shoot tip (1–2 cm length) explants are prepared by segmenting the terminal shoot after defoliation. The explants thus prepared are used directly for culturing without sterilization. From the sterilized shoot segments (greenhouse raised seedlings) final explants (1.0–1.5 cm long) are prepared by cutting in SDW as single node cuttings and shoot-tips and are cultured in tubes (25 × 250 mm) for up to 3–4 weeks and then in baby food jars.

2.3. Culture Media

The explants are initiated into cultures on hormone free MS medium which was modified with three-fourth strength major nutrients, full supplements of minor salts, vitamins, organics, 3% sucrose, 0.2% activated charcoal (AC) and solidified with 2.25 g/l phytigel (Sigma). The pH of the medium was adjusted to 5.8 ± 0.1 prior to autoclaving at 1.1 kg cm^{-2} at 121°C for 20 min. Fifteen ml and 35–40 ml of the medium was dispensed into 25 × 100 mm test tubes and baby food jars respectively.

2.4. Culture Incubation

Cultures are maintained at $25 \pm 2^\circ\text{C}$ following 16 h light duration. Initially for 2–3 weeks, low intensity of light ($16 \mu\text{mol m}^{-2} \text{s}^{-1}$) is provided. Subsequently, the light intensity is increased ($45\text{--}55 \mu\text{mol m}^{-2} \text{s}^{-1}$) and provided from cool daylight fluorescent lamps (Philips, 40 W). Subculturing is done at weekly intervals until the 3rd week and then at an interval of 3 weeks. Shoot-tip cultures turn dark and necrotic if they are not frequently cultured.

2.5. Axillary Shoot Bud Proliferation

Shoot cultures on hormone free medium start growing by 3 weeks with axillary bud swelling and turning green with sprouting as well. At this stage, the cultures are transferred to a multiplication medium consisting of MS major salts (three-fourth strength), full strength minor nutrients, vitamins, organics, 3% sucrose and supplemented with 1 g/l PVP-360 instead of AC, 0.1 mg/l thidiazuron (TDZ) (Sigma) and 0.1 mg/l of NAA or IBA and solidified with phytigel (2.25 g/l). After 3 to 4 weeks the cultures are transferred from multiplication medium to hormone-free half-strength MS medium (Shoot expression medium). Several axillary buds start appearing and proliferating around the axils of nodes (Figure 1B). The number of

axillary buds formed may vary from 1 to 13 with an average of 4–5 buds/culture in majority (80–100%) of the cultures. Usually 1 or 2 subcultures on expression medium are required for emergence of axillary buds. Prolonged culture on TDZ medium beyond 3–4 weeks turns shoots dark and necrotic.

Among all the cytokinins tried TDZ was more potent for shoot bud proliferation in cashew. However, cotyledonary nodal explants from *in vitro* source also showed multiple shoot induction on full-strength MS medium containing 2.2 mg/l BAP, 0.2 mg/l IBA and 1 g/l PVP 360. Initial number of the induced axillary buds varied between 4 and 8 per culture (Figure 1C) and this increased to many fold (40–60 buds/explant) when the multiple shoot buds were divided and subcultured (3–4 times) on the same medium at 3 week intervals. The elongation of 2 to 3 shoots was also observed. The long shoots observed in the clumps are harvested and used for rooting.

2.6. Shoot Bud Elongation

Axillary buds induced on the multiplication medium containing TDZ are to be cultured on either half-strength semi-solid MS hormone-free medium supplemented with 0.2% AC and 400 mg/l l-glutamine or on hormone free Raj Bhansali (1990) semi-solid medium supplemented with 0.2% AC, 500 mg/l glutamine and 500 mg/l casein hydrolysate for elongation. Multiple shoots with proliferating buds are divided in 2 to 3 separate clusters and cultured at an interval of 2 to 3 weeks. At each stage long shoots are harvested and rest of buds were put back on same medium for elongation. By the end of 8 to 10 weeks the shoot elongation is observed to a frequency of 70–80% (Figure 1D). The shoot buds in cotyledonary nodes elongated faster than those ones of other explants.

2.7. Root Induction

Long microshoots (>2 cm) obtained are rooted following *in vitro* method of culturing on half-strength semi-solid MS medium supplemented with 1 g/l PVP 360, 3% sucrose along with either 5 mg/l of NAA or 2.5 mg/l each of NAA and IBA in combination. The cultures are incubated in light and with the appearance of root initials; they are transferred to hormone free liquid half-MS medium containing 1 g/l AC on a filter paper bridge. Here initiation of rooting is seen as early as 10 days and up to 40 days. Although the number of roots formed vary (1–6/culture) the majority has one to two prominent roots growing up to a length of 12–15 cm. The percentage of rooting varies from 50–80%. Reduction of salt concentration, sucrose and use of liquid medium are found better for rooting. Shoots harvested from cotyledonary nodes showed higher percentage of rooting.

Microshoots are also rooted (60%) by pulsing shoots for few seconds either in 10 mM NAA or 10 mM IBA and culturing on hormone free semi-solid half-MS medium containing 0.2% AC. *Ex vitro* rooting (12–15%) is also observed when

microshoots are given a slow dip in 250 ppm of either NAA or IAA for 48 h in dark and potting the treated shoots in mixture of soil rite and sand (2:1 v/v). The duration of rooting is longer (3–4 months) in the latter method. Spontaneous rooting up to 30% is also observed on shoots cultured on medium for elongation.



Figure 1. Micropropagation of cashew. A) *In vitro* germination of seedling. B) Axillary shoot bud proliferation in nodal explant. C) Axillary shoot bud proliferation in cotyledonary node. D) Shoot bud elongation. E) *In vitro* rooted shoots being hardened. F) Potted micropropagated plants in the greenhouse. G) Micropropagated plant in the field. H) Micropropagated plant in bearing. I) Fruits harvested from micropropagated plants.

Table 1. Media used for cashew micropropagation.

Media	MS	RBM
Constituents	mg/l	mg/l
NH ₄ NO ₃	1237.5	278.38
KNO ₃	1425.0	2337
MgSO ₄ ·7H ₂ O	277.5	185
KH ₂ PO ₄	127.5	85
CaCl ₂ ·2H ₂ O	440.0	220
MnSO ₄ ·4H ₂ O	22.3	8.4
ZnSO ₄ ·4H ₂ O	8.6	4.3
H ₃ BO ₃	6.2	3.1
KI	0.83	0.415
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.125
CuSO ₄ ·5H ₂ O	0.025	0.0125
CoCl ₂ ·6H ₂ O	0.025	0.0125
Na ₂ EDTA·2H ₂ O	37.3	37.3
FeSO ₄ ·7H ₂ O	27.8	27.8
Glycine	2.0	2.0
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	0.1	1.0
Myo-inositol	100	500
Sucrose	30000	30000
Phytigel	2250	2250
Activated charcoal	2000	2000
L-Glutamine	–	500
Casein hydrolysate	–	500
pH	5.8	5.8

Table 2. Media formulations used at different stages of micropropagation.

Media additives	Culture initiation	Culture multiplication	Shoot bud elongation	Rooting	Hardening
Basal medium	¾ MS	¾ MS	½ MS	½ MS	½ MS
TDZ	—	0.1mg/l	—	—	—
NAA	—	0.1 mg/l	—	2.5 mg/l	—
IBA	—	—	—	2.5 mg/l	—
Sucrose	30 g	30 g	30 g	30 g	30/15/0 g
AC	2 g	—	2 g	—	1 g
PVP-360	—	1.0 g/l	—	—	—

2.8. Hardening, Acclimatization and Potting

1. In the first stage of hardening the *in vitro* rooted shoots are cultured on liquid half-strength MS medium containing 1 g/l AC and a reduced percentage of sucrose (1.5%) for 2 to 3 weeks (Figure 1E).
2. Transfer the shoots into the same medium without sucrose for another 2–3 weeks by siphoning out the old medium. This facilitated shoot elongation and secondary root formation. One of the roots becomes prominent at this stage and assumes the role of tap root when planted in field.
3. Prior to potting the plantlets are taken out of the container carefully and rinsed gently in SDW to remove traces of media and then immersed for 20 min in 0.2% bavistin (carbendazim 50% WP) solution for preventing contamination of plantlets in pots.
4. In the second stage of hardening, the *in vitro* hardened plantlets are transferred to 4" plastic pots containing sterile pot mixture of sand and soil rite (2:1 v/v) (autoclaved for 2 h at 121°C) (Figure 1F) and plantlets are covered with 200 gauge transparent polythene bags for maintaining humidity.
5. The potted plantlets are irrigated initially for 10 days with only SDW and then with 1/10 MS macrosalt solution (pH 6.8–7.0).
6. The humidity is gradually reduced by punching holes and increasing the number of holes in polythene bags and finally by fourth or fifth week the bags are completely removed.
7. Over-irrigation needs to be avoided and sometimes drenching of pots with 0.2% bavistin is required to prevent any residual contamination if any in plantlets. The survival of plantlets during this stage varies from 80 to 100%.
8. After hardening in pots for 4–6 weeks in laboratory, the plantlets are carefully transferred to 20 cm earthen pots containing mixture of red earth, sand and compost or FYM (1:1:1 v/v) and kept in greenhouse under partial

shade and provided with drip irrigation. The survival at this stage was 100% without any mortality.

9. Under the greenhouse conditions, the tissue culture derived plants are maintained for 4 to 6 months until they are planted in the field in rainy season. The approximate time required from explanting to planting varied from 40 to 50 weeks.

2.9. Field Planting and Evaluation of Micropropagated Plants

Micropropagated plants (40 to 50 weeks old) maintained in greenhouse are finally planted in pits (75 cm³) filled with soil and compost and rock phosphate added to root zone (100 g/pit). If planted during rainy season additional irrigation is not required; otherwise, hose or pot watering is required for initial establishment. The establishment was found to be 100% in the micropropagated plants planted in field (laterite soil) (Figure 1G).

In the first year of planting of micropropagated plants, 33% of the plants flowered and in the second year 60% flowered and bearing of fruits was observed in some tissue cultured plants of VRI-1 variety (Figures 1H, I). Flowering and fruiting behavior was normal in micropropagated plants. Root-biomass of micropropagated plants and grafted plants was studied by sectorial method of destructive sampling of one-year-old trees and it was observed that root biomass of micropropagated plants was four times that of grafted plants. The tissue culture plants had predominantly lateral root system while the grafted plant had a strong tap root and poor lateral root system.

3. CONCLUSION

Although micropropagation technique is available in cashew, it needs to be scaled up before taking it up on a commercial scale besides working the production cost. Micropropagation may be useful for producing clonal root stock and to multiply the breeders stock at a faster rate. The technique needs to be perfected in shoot explants of mature tree origin in order to have a wider application.

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CHAPTER 30

IN VITRO MUTAGENESIS AND MUTANT MULTIPLICATION

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1. INTRODUCTION

Induced mutations technique is a valuable tool not yet fully exploited in fruit breeding. Tissue culture makes it more efficient by allowing the handling of large populations and by increasing mutation induction efficiency, possibility of mutant recovery and speediness of cloning selected variants. Some vegetatively-propagated species are recalcitrant to plant regeneration, which can be a limit for the application of gene transfer biotechnology, but not for mutation induction breeding. Mutagenesis offers the possibility of altering only one or a few characters of an already first-rate cultivar, while preserving the overall characteristics. Traits induced by mutagenesis include plant size, blooming time and fruit ripening, fruit color, self-compatibility, self-thinning, and resistance to pathogens (Predieri, 2001). The combination of *in vitro* culture and mutagenesis is relatively inexpensive, simple and efficient (Ahloowalia, 1998). The availability of suitable selection methods could improve its effectiveness and potential applications. The molecular marker technology available today already provides tools to assist in mutation induction protocols by investigating both genetic variation within populations and early detection of mutants with desired traits. However, cost still represents a major limitation to their application.

Among the techniques and sources of genetic variation available for tissue culture mutation induction, physical mutagens have already shown potential for application in fruit breeding. The types of radiation suitable for mutagenesis are ultraviolet radiation (UV) and ionizing radiation (X-rays, gamma-rays, alpha and beta particles, protons, and neutrons). X-rays and gamma-rays are the most convenient and easiest types of radiation to use with regards to application methods and handling (Sanada & Amano, 1998), and have been both the most widely used ionizing radiation types and the most effective for fruit breeding purposes. Furthermore, physical mutagens

have some technical advantages over chemical mutagens. With regards to safety and environmental issues there is no need for manipulation of hazardous substances and production of toxic residues. Physical mutagen post-treatment manipulation is simpler and allows for a more precise determination of exposure time.

This manuscript describes methodologies for *in vitro* mutation induction using physical mutagens, and in particular γ -ray technology, on fruit tissue culture.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

Explants should preferably derive from certified virus-free mother plants. This initial choice strengthens the complete protocol, including useful mutant identification and requirements for its release as a cultivar. Cultures should be free of latent microbial contaminants, since radiation may stimulate microorganism proliferation while weakening the plants. Mutation induction should be performed on well-established *in vitro* cultures that show providing a consistent proliferation rate through subcultures. Generally, after explant establishment, 5–6 subcultures are necessary to have cultures growing at a consistent rate on the proliferation medium. Proliferation media suitable for apple, pear, and plum are presented in Table 1. Cultures are incubated at $23 \pm 2^\circ\text{C}$ with a 16-h photoperiod and subcultured every 3–4 weeks.

Table 1. Culture media composition.

<i>Medium composition</i>	<i>Apple</i>	<i>Pear</i>	<i>Plum</i>
Mineral Salts	MS*	MS	MS
Thiamine-HCl (μM)	1.2	1.2	1.2
Myoinositol (mM)	0.55	0.55	0.55
BAP benzyladenine (μM)	4.4	6.6	3.3
IBA indole-3-butyric acid (μM)	0.5	0.5	0.5
Sucrose (w/v)	2	2	2
Agar (w/v)	0.65	0.65	0.65
pH	5.7	5.7	5.7

*MS: (Murashige & Skoog, 1962)

When an efficient regeneration protocol from plant tissue is available, treatment can be performed on tissue before inducing regeneration. However, if a species or a cultivar is recalcitrant to regeneration, treatment can be performed directly on prolixerating shoots. Actually, when the aim of the breeding program is to maintain all the traits of a cultivar and improve only one or a few specific traits, the irradiation and propagation of *in vitro* axillary shoots may be the most adequate and easiest method. Efficient micropropagation protocols are available for nearly all species of horticultural importance. Furthermore, without the passage through undifferentiated growth, the undesired influence of somaclonal variation could be avoided (van Harten, 1998). On the other hand, when mutagens are used on undifferentiated tissues and organs, without preformed axillary buds, either prior to regeneration, or in different

stages of adventitious meristem differentiation, somaclonal variation is added to the mutagen effects. This system has the advantage that when regeneration is achieved from single cells the risk of obtaining chimaeras is reduced.

2.2. Radiosensitivity Assessment

The first step of a breeding program with physical mutagens is the assessment of treatment dose. Before executing the actual mutagenic treatment is advisable to perform a preliminary study on the specific sensitivity of the material to be used. In fact, the difference in radiosensitivity can be explained not only by inherent genetic differences between cultivars but also by physiological differences. Literature is used to provide generic information for setting an experiment for assessing specific radiosensitivity. The choice of the most suitable dose is commonly based on growth reduction as a result of the treatment. The aim of the preliminary investigation is to assess the dose that results in a 50% reduction of growth (LD50 = lethal dose 50%). Growth is determined, depending on the material tested, on the basis of the number of regenerated shoots or proliferation achieved in the first subculture after treatment, indicating apical and axillary meristems survival rate. For most micropropagated fruit trees a dose of 60 Gy is expected to be higher than LD50. Thus four doses of 0, 20, 40, or 60 Gy can provide the basis for calculating LD50 of the specific material to submit to treatment.

2.2.1. Radiosensitivity Assessment on Adventitious Buds from Leaf Tissues

To provide reliable data for LD50 calculation, thirty leaves per treatment must be subjected to irradiation and four doses tested, including the unirradiated control, on which performance 50% growth reduction is calculated. Following the regeneration protocol set up by Predieri and Fasolo (1989) for apple (*Malus pumila* L.), leaves are taken from 30-day-old cultures, and only the first three apical unfurled leaves are used. Three transverse cuts are made to the midrib and the petioles are removed. Each leaf blade is then placed with the adaxial face touching the medium (Figure 1A).

The regeneration medium, suitable for a number of apple (Fasolo & Predieri, 1990) and pear (Predieri et al., 1989) cultivars, contains MS salts (Murashige & Skoog, 1962), LS vitamins (Linsmaier & Skoog, 1965), 22.2 μM benzyladenine (BA), 1.1 μM α -naphthaleneacetic acid (NAA), 2% (w/v) sucrose, agar concentration ranges from 0.65 to 0.75% (w/v) depending on brand, pH 5.7. Leaves are placed six per Petri dish and submitted to treatment (Figure 1B).

The use of acute irradiation allows rapid treatment of the plant material. Predieri and Gatti (2003) tested total doses of 0, 10, 20, 30, or 40 Gy. Acute irradiation (42.7 Gy/min) with gamma rays from a cobalt (Co^{60}) source was provided by "Gammacell 220" (Atomic Energy Canada Limited, Ottawa, Ontario, Canada) at the Institute of Photochemistry and High Energy Radiations (ISOF—CNR, Bologna) (Figure 1C).



Figure 1. A) Leaves are taken from 30-day-old pear cultures. After three transverse cuts are made to the midrib, leaf blades are placed with the adaxial face touching the regeneration medium. B) Petri dishes containing six leaves each are placed inside a gamma cell for treatment. C) “Gammacell 220” (Atomic Energy Canada Limited, Ottawa, Ontario, Canada) with Petri dishes inside, is closed for having plant material subjected to mutagenic treatment. D) Shoots taken from 30-day-old pear cultures are placed in a Petri dish for treatment. E) After mutagenic treatment shoots are transferred to a fresh proliferation medium. F) Proliferation rate is determined by counting the number of shoots developed from control (left) and from irradiated shoots (right: 60 Gy).

The number of adventitious shoots regenerated was recorded after culture in the dark on regeneration medium for 45 days. LD50 was calculated as the dose of γ -radiation that reduces the number of shoots regenerated per irradiated leaf to 50% of

unirradiated control leaves, based on linear regression (Wu et al., 1978). Supposing to have four treatments of 0, 10, 20, and 30 Gy, yielding average regenerated shoots per leaf of respectively 8.8, 6.8, 3.4, 0.2, the linear regression equation results $y = -0.2911x + 9.174$ with an $R^2 = 0.9889$ (Figure 2). The Gy dose inducing DL 50 (x) is calculated with the equation, by substituting 'y' with the value of 50% of control (0Gy) regeneration: 4.4. Calculated DL50 will be: $x = (4.4 - 9.174)/-0.2911 = 16.40$.

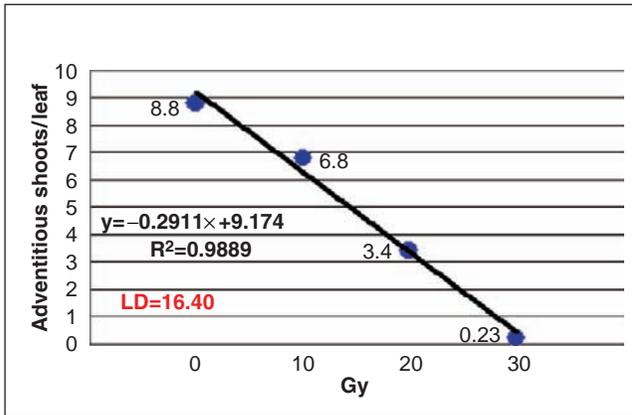


Figure 2. Example of LD50 calculation on the regeneration response of leaves subjected to different doses of gamma ray.

2.2.2. Radiosensitivity Assessment on Axillary Shoots from Microcuttings

When shoots are used, the protocol is aimed to support their growth after treatment and to induce the maximum proliferation of axillary buds. Microcuttings 1.5–2 cm long are cut from 30-day-old proliferating cultures and placed horizontally in plastic Petri dishes (10 cm diameter) with a few drops of sterile water added to protect shoots from dehydration during treatment (Figure 1D). After irradiation, shoots are transferred to jars containing proliferation medium (Figure 1E). Proliferation rate is recorded after 30 days of culture (Figure 1F). LD50 is calculated as the dose of γ -radiation that reduces the proliferation rate of irradiated shoots to 50% of unirradiated control shoots, based on linear regression (Wu et al., 1978), as described for regeneration in 2.2.1.

2.3. Treatment

Cultures are prepared for treatment following the protocol described for radiosensitivity determination. After the estimation of the dose inducing LD50, the most convenient dose for treatment can be chosen. The actual dose to be applied in a particular breeding project is chosen based on the breeder's experience with the specific plant material, its genetics, and its physiology, with the aim of having the highest probability of useful mutant rescue. Heinze and Schmidt (1995) suggested as

a starting point for the experimental protocol doses giving $LD50 \pm 10\%$). Doses lower than $LD50$ favour plant recovery after treatment, while the use of higher doses increases the probability to induce mutations (either positive or negative).

Once treatment dose is chosen, more information is needed to decide how many shoots or leaves to submit to treatment. The number of plants (P) to be obtained is calculated on the basis of the expected frequency of induction of the desired trait. Expected mutations frequencies for single trait can be expected to appear with a frequency of 0.1–1.0%. Predieri and Zimmerman (2001) report for a number of variation in fruit traits in different cultivars of pear, frequencies ranging from 0.14 to 1.93%.

To stay on the safe side, we set an expected frequency of 0.5%, out of 1000 plants 5 individuals would be carrying the desired trait. The breeder's experience and a thumb rule that must also take in account economic costs should be applied. However, plan to work with less than 500 plants limits the possibility of successful selection. Eight hundred or better thousand plants (P) appears to be the minimum to provide reasonable opportunities for selection.

Some basic information is also needed: a) expected regeneration/proliferation rate of the irradiated material; b) number of subcultures after treatment before rooting microcuttings; c) expected percent shoot rooting; d) expected percent plant survival. Expected proliferation rate should be calculated with the same regression equation used for calculating $LD50$. It presumably will increase in the subcultures following the first, but it is advisable to stay on the safe side, and plan to produce more plants to face unexpected contamination, rooting or survival problems.

The number of subcultures needed after treatment vary from a minimum of 3 to a maximum of 5 depending of the care exercised on avoiding chimaeras. Rooting percentage can be calculated to be about 80% of the regular frequency obtained for the material or more directly obtained by observing cultures treated for LD determination. Plant survival must be calculated on the basis of experience with the specific plant material.

To calculate how many shoots (X) to submit to treatment use the following formula:

$$X = P / ((a * b) * c) * d.$$

e.g. Number of plants planned for field selection: 1000. a) expected proliferation rate 3.3; b) number of subcultures 4; c) expected rooting percent 0.85; d) expected plant survival 0.90. $X = 1000 / ((3.3 * 4) * 0.85) * 0.90 = 99$.

2.4. Post-treatment Handling

2.4.1. Post-treatment Care

The use of acute irradiation allows rapid treatment of the plant material, less than 1 min for reaching the $LD50$ in the cases described in Predieri and Gatti (2003). The irradiation of a high number of meristems is easy to manipulate and transport vessels, such as Petri dishes, allows to save space and to use even small irradiation

facilities (e.g. gamma cells), thus facilitating also the execution of the required post-irradiation handling. Undesired negative primary effects of radiations, causing tissue browning, necrosis or chlorosis, are unavoidable. However, appropriate handling of plant material can provide the desirable limitation of physiological effects that favors mutant survival and the emergence of phenotypic variation determined by genetic changes. Cultures irradiated either during proliferation or regeneration should be transferred rapidly to a fresh medium, to avoid the formation of toxic compounds (Ahloowalia, 1998). The basal part of irradiated shoots (1 mm) should also be removed for the same reason. Avoiding to exposure of the culture to high light intensity in the first hours after treatment may also help in limiting negative physiological effects induced by irradiation.

2.4.2. Irradiated Shoot Proliferation

In vitro techniques allow for the rapid execution of propagation cycles of subculture aimed to separate mutated from non-mutated sectors (Ahloowalia, 1998). After transfer to fresh regeneration medium, irradiated tissues can be subjected to standard regeneration procedures, since their aim is already to maximize shoot differentiation and growth (Figure 3A).

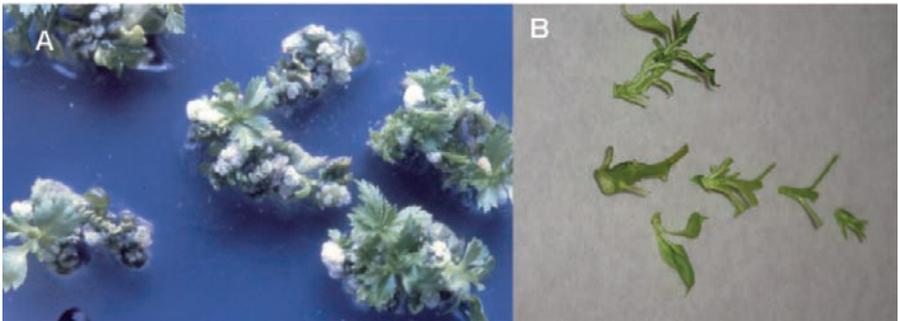


Figure 3. A) Shoots are grown on the regeneration medium until developed enough (2–3 mm) to be transferred to proliferation medium. B) At each subculture propagules should be separated in 1–2 nodes cuttings to stimulate axillary bud development.

However, when microcuttings already provided of meristems are irradiated, after treatment culture should be aimed to help all treated meristems to develop shoots. This is done through repeated cuttings to avoid apical dominance effects (Figure 3B), coupled with a convenient choice of growth regulators to support shoot growth. Cytokinins can be reduced to one half of the standard concentration for the first subculture after treatment, and increased to the full concentration for the subsequent subcultures.

Following mutagen treatments, cultures are chimaeras, composed of non-mutated cells and cells carrying different mutations. To avoid chimaeras is important to guarantee that all the treated shoots undergo at least three subcultures. The protocol

proposed by Predieri (2001) (Figure 4) can be adopted and adapted to the requirements of the different species of interest.

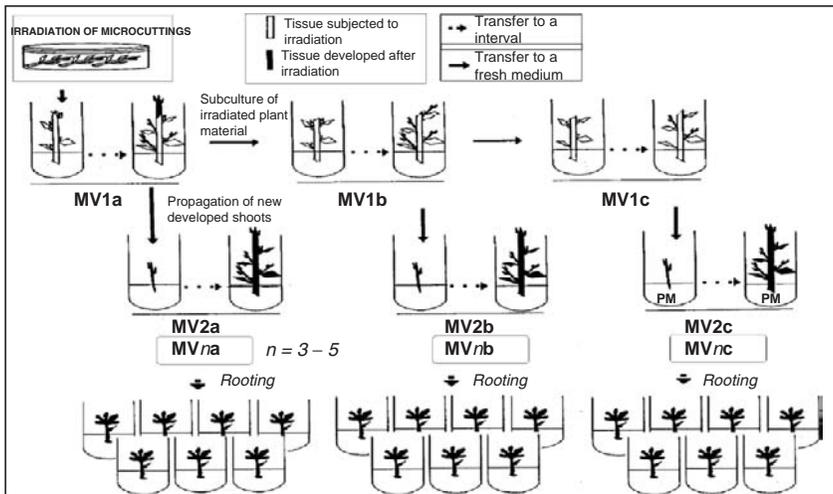


Figure 4. Protocol for post-irradiation culture multiplication (modified from Predieri, 2001).

2.4.3. Rooting

Undesired physiological effects can be observed even at the rooting stage, where they negatively affect the microcutting's rooting capacity (Jain, 1997; Predieri & Gatti, 2000). Particular care should be exercised at this stage and during the following acclimation to prevent the loss of useful mutants due to impaired rooting ability. For a number of species a short, acute, auxin treatment (2–8 days) followed by transfer to auxin-free medium can help in increasing rooting as compared to performances achieved by maintaining microcuttings for all the rooting period (4–5 weeks) on the same rooting medium.

2.5. Selection

2.5.1. *In Vitro* Selection

The identification and selection of desired mutants should be performed on non-chimaeric plants. As compared to methodologies involving treatment of *in vivo* buds, *in vitro* culture provides a wider choice of plant material composed of a few or even just one cell for mutagenic treatment (Maluszynsky et al., 1995). This results in a lesser risk of obtaining chimaeric plants and an higher probability of obtaining homohistonts having mutated cells expressing the mutation in the phenotype. When effective selection methods are available, they can be used directly *in vitro* on the population obtained from propagation performed after mutagenic treatment. Several screening methods have been developed with regards to resistance to pathogens,

tolerance to high pH or elevated metal concentration, and selection of reduced vigor individuals (Predieri, 2001). Screening performed *in vitro* allows for handling of large populations, avoiding the problem of working with a low number of individuals. Different selection pressures can be applied, e.g. to reduce population to 10%. *In vitro* selected variants should always undergo specific *ex-vitro* testing to confirm the existence of improved selected traits and to exclude the possible emergence of other undesired traits.

2.5.2. *Ex Vitro Selection*

When *in vitro* selection methods are not available, mutant identification must be postponed to field observation. Predieri et al. (1997) set up a protocol for the selection of compact pears in a population subjected to mutagenic treatment. Given the impossible burden of measuring thousands of plants, three groups were defined representing about 1/10 of the whole population: 1) control, which consisted of non-irradiated, micropropagated plants; 2) sample, which consisted of every tenth plant in the row; 3) selected, which consisted of trees chosen because they exhibited variation in vegetative growth characteristics. On these plants phenotypic data were collected on shoot length and diameter and number of nodes, spurs and lateral shoots, plant height and trunk diameter. Cluster analysis was performed on data to select trees carrying interesting traits. As related to pomological traits, data were collected for selecting: a) early bearing small trees; b) high productivity combined with a high production efficiency; c) consistent production and high fruit weight.

2.6. *Advances in Mutant Identification*

The range of methods available for the identification of mutations is widening (Ahloowalia & Maluszynski, 2001). However, none of the techniques can guarantee the identification of mutants carrying a single random mutation in the genome. Two methods showing potential application have been discussed by Karp (2000). These methods are based on the detection of changes known to be induced at high frequency in tissue culture: (1) AFLPs with methylation-sensitive enzymes and (2) detection transposon insertional polymorphisms. When markers linked to traits of interest are available, a marker-assisted selection can be performed. For this purpose, highly saturated linkage maps can provide a choice of markers closely linked to a specific trait. An increasing number of markers linked to agronomically important traits are now being identified, providing even more opportunities for marker-assisted selection. Recently developed technologies such as TILLING (Targeting Induced Local Lesions IN Genomes) (Slade & Knauf, 2005) appear to provide new opportunities for early mutant identification. Another promising technique is S-SAP (sequence-specific amplified polymorphism), which has been applied to study genetic bases of bud mutations in apple (Venturi et al., 2006).

3. CONCLUDING REMARKS

Mutation induction treatments performed on *in vitro* shoots or on plant tissues prior to regeneration have a potential for contributing to fruit breeding, allowing for single-trait changes in the improvement of a cultivar, while retaining yield parameters. Induced mutagenesis with the use of physical mutagens appears to be a ready-to-use technique, since a large number of fruits would benefit from the improvement of single specific key traits. The interest in results of mutations breeding is demonstrated the impact of mutation derived varieties (Ahloowalia et al., 2004) and by the continuing interest in “clones” of fruit cultivars (Sturm et al., 2003; Venturi et al., 2006), often simply selected by chance in the field, which have generated new patented varieties. The first steps of such a protocol are easy and relatively cheap. *In vitro* mutation induction results could be increased by the use of effective early screening methods for biotic agents and abiotic factors. However, only the planning of extensive field trials could allow a real evaluation of selected mutants' potential.

The molecular approach offers tools for increased understanding of specific DNA changes caused by mutagenic treatments. This can be of great help in reducing the unpredictability of the results of breeding protocols based on nuclear technology. Jain (2005) recommends the development of a molecular database as an instrument for predicting expected mutations. Mutation-assisted breeding programs can become a reliable method for tropical and subtropical fruit crop improvement in countries where enhanced food production and sustainability are of great importance. However, often tissue culture can be applied only if it is cost-effective. The research of low-cost procedures for tissue-cultured plant production is crucial for the development of new opportunities of exploitation. Research efforts should be focused also into the development of new regeneration and selection methods, e.g. those based on cell suspension culture (Ahloowalia & Maluszynski, 2001). Tissue culture, supported by expertise on genetics, plant pathology, and molecular biology can effectively use induced mutation procedures as a tool in plant improvement. Every advance in the understanding of *in vitro* plant physiology and the improvement of tissue culture efficiency can open new opportunities for the development of successful mutation breeding programs.

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CHAPTER 31

IN VITRO PROPAGATION OF NUTMEG, *MYRISTICA FRAGRANS* HOUTT.

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1. INTRODUCTION

Nutmeg (*Myristica fragrans* Houtt.) is a tropical evergreen dioecious tree which is a native of Molucca islands of Indonesia and is also now cultivated in Grenada. It yields the spices, nutmeg (endosperm) and mace (the reddish aril), highly coveted for their aromatic, culinary and medicinal properties. Nutmeg has a rich diversity of phytochemicals (Mallavarapu & Ramesh, 1998). The essential oil is an active ingredient of several pharmaceutical products and cosmetics. The medicinal value of nutmeg in the treatment of several ailments ranging from nervous to digestive disorders (Van Gils & Cox, 1994) has been recognized worldwide since ancient times and it was regarded as a cure for plague. Recent studies relating to its anti-bacterial (Rani & Khullar, 2004), anti-viral (Goncalves et al., 2005), anti-cancer (Mahady et al., 2005), anti-proliferative (Lee et al., 2005), anti-oxidant (Singh et al., 2005), hepatoprotective (Morita et al., 2003) and neuroprotective (Ban et al., 2004) effects reveal a wide scope for its application in the health sector.

Myristica fragrans is of very limited geographical distribution owing to its characteristic ecological requirements. The factors that restrict its propagation are recalcitrant seeds, a long juvenile phase and scarcity of propagules (Nair & Ravindran, 1988). Though the development of *in vitro* technology for the propagation of nutmeg is essential in view of its commercial importance and its immense potential as a source for new leads for drugs there are very few reports available. Induction of somatic embryogenesis in nutmeg has been achieved (Iyer et al., 2000). Metabolite profiling of the embryogenic cultures revealed the presence of the phytochemicals α & β -pinene, sabinene, myristicin, safrole, amphetamine derivatives and other

compounds (Iyer et al., 2004, 2005a,b) of potent clinical value, some of which had not been reported earlier *in vivo*. Elicitation of the production of anthraquinones *in vitro* by chitosan was obtained (Iyer et al., 2005b). There was strong anti-bacterial activity against the human pathogens *Salmonella typhi* and *Staphylococcus aureus* (Iyer et al., 2005a) in the spent medium of the embryogenic cultures and nucleic acid base analogues could be detected in it. This reveals the potential of the embryogenic cultures for continuous production of valuable bioactive phytochemicals. The development of *in vitro* propagation techniques is done with the culture of different explants including zygotic embryos, leaf segments, nodal explants, and stem segments.

2. EXPERIMENTAL PROTOCOL

2.1. *In vitro* Propagation from Zygotic Embryos

Zygotic embryos have been used as explants in many woody plants since these are relatively contaminant-free being enclosed in the fruit and seed tissues. Zygotic embryos of nutmeg are surrounded by the massive endosperm. Somatic embryogenesis was obtained from zygotic embryos in media with activated charcoal (Iyer et al., 1994, 2000). The extent of somatic embryogenesis was dependent on the size of the zygotic embryos.

2.1.1. Explant Preparation

The zygotic embryos from ripe, freshly-harvested nutmeg fruits were used as explants.

Growing conditions of mother plants. The fruits were obtained from State Horticultural Farm, Kallar (11.18°N, 76.5°E), Coimbatore, India with rainfall of 150–200 cm well distributed throughout the year. The soil is well-drained, deep, loamy and rich in organic matter.

Explant excision and sterilization

1. Fully ripe split fruits (about 5 cm diameter) were harvested from the trees.
2. Husk of the fruit was removed.
3. Seed was then freed from the surrounding aril. All further operations were carried out in a laminar flow cabinet.
4. Seeds were swabbed with ethanol.
5. They were surface-sterilized by immersing in 0.1% HgCl₂ for 3 min, followed by several rinses with sterile distilled water.
6. Shell was removed to facilitate embryo removal.
7. Embryos were excised with a sharp scalpel and a pair of forceps.
8. The intact zygotic embryos and the pieces of zygotic embryos fragmented during excision were used as explants.

Table 1. Concentration (mg/L) of inorganic salts in the basal media.

Constituents	Medium A	Media B ₁ – B ₇
Ammonium nitrate	400	1650.0
Boric acid	6.2	6.2
Calcium chloride, anhydrous	332.2	332.2
Cobalt chloride, 6 H ₂ O	0.025	0.025
Cupric sulphate, 5 H ₂ O	0.025	0.025
Disodium EDTA	74.5	37.26
Ferrous sulphate, 7 H ₂ O	55.7	27.8
Magnesium sulphate	180.7	—
Magnesium sulphate, 7 H ₂ O	—	370.0
Manganese sulphate, H ₂ O	16.9	16.9
Potassium iodide	0.3	0.83
Potassium nitrate	480.0	1900.0
Potassium phosphate, monobasic	—	170.0
Sodium molybdate, 2 H ₂ O	0.25	0.25
Sodium phosphate, monobasic, anhydrous	330.6	—
Zinc sulphate, 7 H ₂ O	8.6	8.6

Table 2. Organics, growth regulators (mg/L) and other supplements added to the different inductive media.

Components (mg/L)	A*	B ₁	B ₂	B ₃	B ₄	B ₅	B ₆	B ₇
Myo-inositol	1000	100	100	100	100	100	100	100
Sucrose	20000	30000	30000	30000	30000	30000	30000	30000
Thiamine	8.5	10	0.5	0.5	0.5	0.5	0.5	0.5
HCl								
Nicotinic acid	5	1	0.4	0.4	0.4	0.4	0.4	0.4
Pyridoxine	5	1	0.5	0.5	0.5	0.5	0.5	0.5
HCl								
2,4-D	10	1	4	—	—	—	—	—
IAA	10	1	2	—	—	—	—	—
NAA	—	—	—	—	5	5	5	5
GA ₃	—	—	—	5	—	—	1	—
BAP	—	4	—	—	1	1	—	1
Kinetin	10	—	—	—	—	—	—	—
TDZ	—	—	—	—	—	0.1	—	0.05
Activated charcoal	250	250	250	250	250	250	250	250
PEG 4000	—	—	—	—	—	—	50,00	—
							0	

* To medium A, 10% (w/v) potato extract was added. Potato extract was prepared from 100 g potato boiled in water, mashed, cooled and filtered.

Abbreviations. 2,4-D: 2,4 dichlorophenoxyacetic acid; IAA: indole-3-acetic acid; NAA: naphthaleneacetic acid; BAP: benzylaminopurine; GA₃: gibberellic acid; TDZ: thidiazuron; PEG: polyethylene glycol.

2.2. Culture Medium

There was severe browning and necrosis, even in darkness, owing to the heavy leaching of phenolics in the culture medium when zygotic embryos were used as explants. The problem was overcome by addition of activated charcoal to the medium which absorbs all the phenolic substances resulting in survival, growth and massive proliferation of the cultured tissues. Somatic embryos from intact and fragmented pieces of zygotic embryos could be obtained (Iyer et al., 2000) regardless the use of the basal media and plant growth regulators after addition of activated charcoal in the medium. Table 1 describes the mineral salt composition of the basal media A (Anderson, 1978) and media B₁– B₇ (Murashige & Skoog, 1962) used for various steps of plant regeneration.

2.2.1. Medium Preparation

Medium preparation involved the following steps:

1. Stock solutions with the mineral salts and vitamins were mixed in the appropriate proportion.
2. Various growth regulators were added singly or in combinations at different levels to the media together with sucrose and other growth supplements (Table 2).
3. The pH of the nutrient media was adjusted to 5.7. After addition of activated charcoal (0.25%), the media were solidified with agar or gelrite and sterilized in an autoclave for 15 min at $121 \pm 2^\circ\text{C}$.
4. Cultures were incubated in a temperature-controlled room at $25 \pm 2^\circ\text{C}$ in continuous light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) from cool white fluorescent lamps.

2.2.2. Regeneration and Maintenance

Direct formation of green somatic embryos (12–14 per explant) without any intervening callus stage (Figure 1A) was obtained from intact medium-sized (9–10 mm long) zygotic embryos in the culture media supplemented with activated charcoal (Iyer et al., 2000) in 3–5 weeks. Direct somatic embryogenesis was also obtained from zygotic embryos of large (13–14 mm long) size (Figure 1B) as well as from broken zygotic embryos. There was no response from embryos of very small size (3–4 mm long). The embryogenic competence could be maintained by repeated subculture in the same or different media for about 10 months and establishment of long term embryogenic cultures that could serve as stable sources of the various secondary metabolites of nutmeg could be achieved. High frequency somatic embryogenesis (Figure 1C) could be obtained from fragmented zygotic embryos. Light was absolutely essential for induction of somatic embryogenesis.

2.2.3. Establishment and Maintenance of Embryogenic Cultures

1. The intact and fragmented zygotic embryos are placed on media with activated charcoal (Table 2).
2. The cultures are maintained in continuous light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) from cool white fluorescent lamps at $25 \pm 2^\circ\text{C}$.
3. Formation of green somatic embryos takes place after 3–5 weeks.

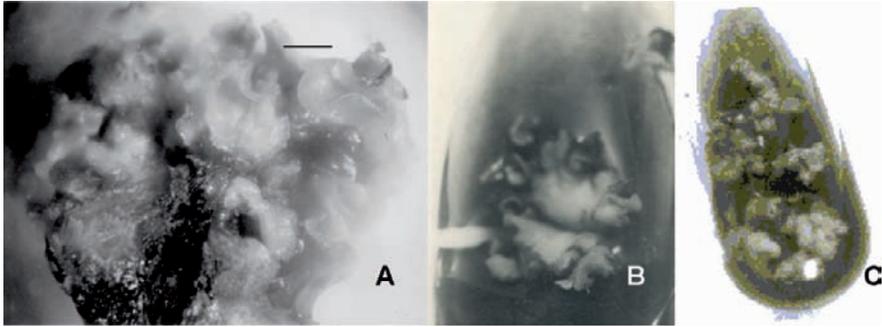


Figure 1. Somatic embryogenesis of nutmeg (*Myristica fragrans* Houtt.). A) Proliferation of somatic embryos over the surface of intact zygotic embryos of medium size in medium A (bar = 1 mm). B) Somatic embryos arising from the margins of intact zygotic embryos of large size in medium B₁. C) High frequency somatic embryogenesis from fragmented zygotic embryos in medium B₂.

4. The embryogenic mass is subcultured by transfer to fresh media of the same or different composition at intervals of 4–5 weeks depending on the growth rate. Massive proliferation of somatic embryos could be elicited on subculture in medium B₃.
5. A high proportion of abnormal forms are observed when fragmented zygotic embryos are used as explants.
6. The potential for continuous production of somatic embryos could be maintained for more than 10 months involving eight to nine subcultures.

2.2.4. Maturation of Somatic Embryos, Rooting and Regeneration of Plantlets

Somatic embryos germinated by production of roots after two or three sub-cultures. In some cases initially there was shoot emergence (Figure 2A).

1. The embryogenic cultures are transferred to B₅ media with TDZ regularly at 4 week intervals for 3 months at increased light intensity ($37 \mu\text{mol m}^{-2} \text{s}^{-1}$).
2. *In vitro* rooting (Figure 2B) is obtained after about 3 months in medium B₅. Germination was also observed in media B₆ with PEG 4000.

The number of somatic embryos showing root formation was higher than those showing partial germination by shoot formation. Axillary shoot proliferation and multiple shoot formation (Figure 2C) were observed in media B₇ and B₅ with TDZ.

2.2.5. Storage of *In Vitro* Cultures

1. The cultures are maintained in continuous light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) from cool white fluorescent lamps at $25 \pm 2^\circ\text{C}$.
2. Subcultures are carried out at intervals of 4–5 weeks by transfer to fresh media of the same or different composition.

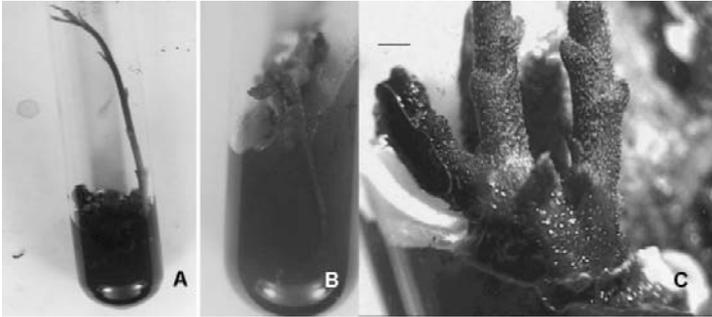


Figure 2. Formation of somatic embryo plants of *Myristica fragrans*. A) Plantlet formation by shoot emergence. B) *In vitro* rooting in medium B₅. C) Formation of multiple shoots in medium B₅ (bar = 1mm).

2.3. Organogenesis and Somatic Embryogenesis from Other Explants

In vitro propagation from other explants has been attempted using nodal explants, stem segments, laminar pieces and petioles from 4-year-old juvenile plants. Somatic embryogenesis from leaves was achieved (Iyer et al., 1995, 2002). Regeneration of single axillary shoots from nodal explants and formation of adventitious buds from stem segments and petioles were some of the other responses obtained (Iyer et al., 1995, 2002). Though, there are reports of attempts of *in vitro* propagation using nodal explants (Mallika et al., 1997; Rao et al., 1997) no shoot elongation and plantlet formation has been obtained. A protocol for regeneration of single axillary shoots from nodal explants and induction of somatic embryogenesis from immature leaves is being described.

2.3.1. Explant Preparation

Growing conditions of mother plants. Leafy shoots obtained from 4-year-old juvenile plants growing in Lalbagh Botanical Garden, Bangalore, India (12.58°N, 77.38°E), with predominantly lateritic soil were used as the sources of nodal explants, stem segments and leaf pieces.

Explant excision and sterilization. The leafy shoots were washed in running tap water for half an hour. All further operations were carried out in a laminar flow cabinet.

Nodal explants

1. Shoot was trimmed and cut transversely into 3 or 4-node segments .
2. Segments were then sterilized with 0.1% HgCl₂ for 5 min followed by several rinses with sterile distilled water.

3. They were then cut into several single nodes about 1 cm in length or 2-node explants.
4. Nodal explants were cultured upright with the lower end inserted into the culture medium.

Stem segments

1. Shoot was cut into segments.
2. Stem segments were then sterilized with 0.1% HgCl₂ for 5 min followed by several rinses with sterile distilled water and cultured.

Leaves

1. Tender, immature leaves were cut transversely after discarding 2–3 mm of the margin, into pieces of about 1.5 cm width with midrib.
2. Segments were then sterilized with 0.1% HgCl₂ for 3 min followed by several rinses with sterile distilled water.
3. Segments were placed mostly with the adaxial surface in contact with the culture medium.
4. Petioles was cultured with the lower end inserted into the culture medium. In some cases the petiole was placed horizontally on the surface of the medium.

2.3.2. Culture Medium and Growth Conditions

The media used were based on those devised by Murashige and Skoog (1962). Various growth regulators were added singly or in combination at different levels to the media together with sucrose (Table 3). After adjustment of the pH to 5.7, the media were solidified with agar or gelrite and sterilized in an autoclave for 15 min at $121 \pm 2^\circ\text{C}$. The cultures were incubated in a temperature-controlled room at $25 \pm 2^\circ\text{C}$ in continuous light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) from cool white fluorescent lamps.

Table 3. Several concentrations of plant growth regulators were tested in the culture media used for culturing explants from juvenile leafy shoots.

<i>Sl. No.</i>	<i>Plant growth regulator concentration (mg/L)</i>	<i>C₁</i>	<i>C₂</i>	<i>C₃</i>	<i>C₄</i>
1	BAP	5.0	—	—	—
2	Kinetin	—	1.0	3.5	—
3	2,4 - D	—	—	5.0	2.0
4	NAA	—	5.0	—	—

2.3.3. Regeneration

Formation of axillary shoot from nodal explants. Axillary bud break was obtained after 3 weeks in medium C₁. Single axillary shoots were formed in nodal explants (Figure 3A) in medium C₁.

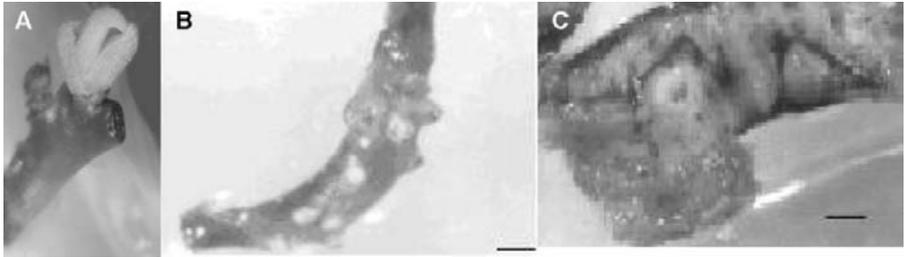


Figure 3. Formation of axillary A) and adventitious B) shoots as well as somatic embryogenesis in leaves C) of nutmeg. Bars 1 mm.

Formation of adventitious buds in stem explants. Formation of adventitious buds in stem explants (8 per explant) taken from the apical region was obtained with a frequency of 20% in medium C₂.

Formation of adventitious buds in leaf petioles. Formation of adventitious buds (10 per explant) in leaf petioles (Figure 3B) cultured horizontally was obtained in medium C₃.

Somatic embryogenesis from immature leaf explants. In median segments from tender leaves formation of globular and elongated somatic embryoids at the margin on the adaxial surface was obtained after 11 days with a frequency of 90% (Figure 3C) in medium C₄.

3. CONCLUSION

A protocol for direct, repetitive somatic embryogenesis and establishment of long-term embryogenic cultures from zygotic embryos of nutmeg has been developed. In view of the difficulties of multiplication of this plant by conventional methods the developed *in vitro* techniques constitute an important progress, which has a great potential to generate biomass source for valuable nutmeg compounds with pharmaceutical values. The extent of the embryogenic response was influenced by the developmental stage of the zygotic embryos. Activated charcoal and light were identified as the critical factors for the survival of explants and induction of somatic embryogenesis. The protocol still needs refinement for the transfer of plantlets to soil. A protocol for regeneration of single axillary shoots from nodal explants and somatic embryogenesis from immature leaves has also been described.

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CHAPTER 32

MICROPROPAGATION OF ELITE NEEM TREE (*AZADIRACHTA INDICA* A. JUSS.)

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1. INTRODUCTION

Neem (*Azadirachta indica* A. Juss.) is one of the most beneficial species for the mankind. The importance of neem, as a source of medicines and biopesticides, was known for centuries in India. Recently, the U.S. National Academy of Sciences has recognized its value (Anonymous, 1992).

Neem seeds, seed kernels, twigs, stem, root and bark possess insect antifeedant, insecticidal, insect growth inhibitor, nematocidal, fungicidal (Schmutterer et al., 1981; Schmutterer & Ascher, 1984, 1987; Jacobson, 1989; Randhawa & Parmar, 1993), bactericidal (Ara et al., 1989), anti-inflammatory (Fujiwara et al., 1982, 1984; Dhawan & Patnaik, 1993), anti-tumor (Fujiwara et al., 1982, 1984), immunostimulating (Van der Nat et al., 1987, 1991) properties. Many virtues of this tree are attributed to different chemicals it synthesizes in its root to tree-top. Since the early investigations by Qudrat-i-Khuda et al. (1940), more than 100 compounds have been isolated from various parts viz. neem seeds, seed kernels, twigs, leaves and bark (Warthen, 1979; Taylor, 1987; Siddiqui et al., 1988; Jones et al., 1989; Koul et al., 1990; Lee et al., 1991; Champagne et al., 1992; Devakumar & Sukh Dev, 1993). Most of the active principles belong to the group of tetranortriterpenoids. Among them, 'azadirachtin' (C₃₅H₄₄O₁₆) is a well-known compound, which was first isolated by Butterworth and Morgan (1968) from neem seeds. This has been found to be the most active compound in neem and is responsible for its medicinal and biopesticidal roles.

Among 2,400 plants known to yield insecticidal and insect repellent constituents, neem is the only taxon, which holds out a promise of providing highly effective, non-toxic and eco-friendly means of controlling or eliminating insect pests (Govindachari et al., 1992). About 413 species of insects, 44 of nematodes, almost equal number of fungi, a few bacteria and viruses are susceptible to neem (Singh, 1997). Though the

margosa tree is regarded as a major source of biopesticides and medicines, its other properties are equally significant.

Neem can be grown in several types of soil. It flourishes in dry areas, in waste lands, as shade tree near roadside and as avenue trees in dry and moderately dry climates, surroundings of villages and towns, as windbreaks and shade trees, near banks of rivers for checking soil erosions, etc. Its foliage is used as fodder and wood for fuel. With specific gravity of 0.6, and being termite proof, its wood is also used for making furniture.

It is estimated that we need nearly 200 million neem trees to fulfill the increasing world demand while we have only about 20 million (Rawat, 1994; Singh, 1997). Therefore, a huge quantity of planting material is required. To meet the large demand, especially when neem seeds are known to lose viability within a few days (Ezumah, 1986; Maithani et al., 1989), clonal propagation seems to be the only alternative. Micropropagation protocols reported earlier on neem tree have mostly dealt with juvenile tissues like seedlings, seeds and cotyledons but very few on mature tree-derived tissues (Rangaswamy & Promila, 1972; Narayan & Jaiswal, 1985; Muralidharan & Mascarenhas, 1989; Ramesh & Padhya, 1990; Joarder et al., 1993a, 1993b; Gautam et al., 1993; Su et al., 1997; Murthy & Saxena, 1998; Tomar & Rathore, 1998). Quite a few micropropagation reports have appeared as abstracts in proceedings of seminars, symposia and conferences (Drew, 1993; Joshi & Thengane, 1993; Nirmala et al., 1993; Wewetzer & Schulz, 1994; Biswas & Gupta, 1995; David et al., 1998; Gill & Gosal, 1998; Sinha & Roy, 1998; Syamal & Singh, 1988).

2. EXPERIMENTAL PROTOCOL

The protocol described here for micropropagation is based on an extensive study carried out on neem trees during 1992–2000 at the University of Delhi, India. One hundred and eight profusely fruit bearing trees were selected after visual screening of several hundred trees growing in North Delhi area (approximately 14 sq. kms).

In these trees, azadirachtin content varied from 0.220 to 0.746% of dry weight. The tree producing the highest percentage of azadirachtin was named 'elite' tree. It is nearly 10 meters in height and 35 years old. It is growing as an avenue tree on Probyn Road, University of Delhi. The yearly temperatures of Delhi (Elev. 218m, Lat. 28N, Lon. 77E) being maximum: 20–40°C and minimum: 8–28°C. Humidity varies from 28 to 72% and yearly rainfall is 72 mm. The only neem species growing in Delhi is *Azadirachta indica*.

2.1. Selection

The elite tree was micropropagated since its seed kernels contain the highest azadirachtin content ever known in the world. Thus, the value-added offsprings have been developed. The azadirachtin levels were estimated through TLC and HPLC.

2.1.1. Plant Material

Select profusely fruit bearing neem trees by visual observation, serially number them, collect the yellowish green fruits and wrap them in aluminium foil. Store in liquid nitrogen at -96°C until the azadirachtin is extracted.

Sample Preparation. Shift the fruits from liquid nitrogen to room temperature and after some time separate the seed kernels manually from the testa and pericarp. Dry the seed kernels at 35°C for 18 h. When they turn light brown in colour. Crush 3.3 g (equivalent to 1 mg of active ingredients contained in kernels according to the Bureau of Indian Standards) of each neem sample and dissolve it in 5 ml methanol. Centrifuge at 15,000 rpm at 4°C for 30 min. Collect the supernatant into a fresh tube and again centrifuge at 20,000 rpm at 4°C for 30 min to remove all debris. Collect the supernatant and filter it using 0.2 μm Millipore filter. Use this as a sample solution.

2.1.2. Standard Solution

Precisely weigh 1 mg pure azadirachtin 'A' powder (with 99% purity) and dissolve it in 5 ml methanol. Use it as the reference solution during TLC and HPLC analyses. Test the presence of azadirachtin in sample solutions by TLC with standard azadirachtin solution, prior to HPLC estimations.

2.1.3. Thin Layer Chromatography

Confirm the presence of azadirachtin in methanol extracted sample solutions using TLC plates (5×10 cm aluminium sheets coated with silica gel 60F₂₅₄, E. Merck, Darmstadt, Germany). Load standard azadirachtin solution 'A' as well as sample solutions at one end (1 cm above the lower end) of TLC plate using micropipettes (Dhummond, U.S.A.). Air-dry the plate and place it in a beaker (500 ml) having sufficient amount (50–60 ml) of ethyl acetate (100%) so that the loaded spots on the TLC plates are not dipped in the solvent but are in its close vicinity. Cover the beaker with aluminium foil to avoid evaporation of solvent and keep it undisturbed. The solvent will move upwards and let it run until the solvent front reach almost the upper end of the TLC plate. Remove the plate from the beaker and keep it in an iodine jar for 5–10 min to visualize the spots. They can then be compared with the azadirachtin's control spot. Trace the impression on a butter paper for calculating the R_f (Resolution factor) values of different spots.

2.1.4. High Performance Liquid Chromatography

Apparatus. The HPLC unit of SHIMADZU-4A type, equipped with ultraviolet (UV) detector and printer-plotter-cum-integrator can be operated under the following parameters:

- | | | | |
|-------|----------------|---|--|
| (i) | Column | — | C ¹⁸⁻¹⁵ cm \times 4.6 mm ss |
| (ii) | Column packing | — | Zorbax ODS |
| (iii) | Solvent | — | Methanol:Water (65:35, v/v) |

(iv)	Injection volume	—	5 μ l
(v)	Flow rate	—	0.5 ml min ⁻¹
(vi)	Detector	—	SPD – 2AS
(vii)	Detection at	—	UV – 215 nm

Reagents. Use the routine reagents for estimation of azadirachtin, such as (i) HPLC grade methanol, (ii) HPLC grade water, and (iii) azadirachtin of at least 99% purity.

Azadirachtin Estimation. Inject 5 μ l of standard azadirachtin solution as well as sample solutions separately into HPLC unit to get area reproducibility for two consecutive injections. The percentage of azadirachtin in samples can be calculated, on the basis of HPL chromatograms by employing the following formula:

Azadirachtin content (% by mass) = $A_1/A_2 \times m_2/m_1 \times P$. Where, A_1 = peak area of azadirachtin in sample solution injected, A_2 = peak area of azadirachtin in standard injected, m_1 = mass in gm, of the sample taken for the test, m_2 = mass in g, of the standard azadirachtin, P = purity of reference azadirachtin. Prepare HPL chromatograms for all samples and select the tree having highest azadirachtin content in its seed kernels.

2.2. Micropropagation

2.2.1. Explant Collection

Though micropropagation has been presently accomplished using nodes, rachis, leaf, apical meristem and cotyledons but protocols only for nodes and apical meristem culture are described below. Collect young shoots from the selected tree, growing orthotropically either on the main tree trunk or on the primary and secondary branches spreading horizontally, as well as those arising on coppiced branches.

2.2.2. Surface Sterilization

Remove the leaves from the twigs and thoroughly wash under running tap water for 45–60 min. Cut the twigs into 10–15 cm long pieces and treat with Polysan detergent for 30–45 min with continuous shaking on a shaker at 110 rpm. Wash again in running tap water for 10 min and treat with freshly prepared chlorine water (30–45 min) or 0.1–0.2% HgCl₂ (15–30 min). The duration and method of treatment will depend on the type of twigs, i.e. if very hard and old, then treat for a longer duration (chlorine water for 45 min and HgCl₂ for 30 min). Give a dip in 70% ethanol for 1–3 min. Finally, wash them 3 or 4 times with sterilized distilled water.

2.2.3. Node

Subsequently, cut the twigs into 1–1.5 cm long pieces each comprising only one node and inoculate two explants in each culture tube. To minimize cross contamination, in some cases, it is advised to culture only one explant per test tube (Figure 1a). Some nodal explants may still develop pink bacterial infection on the medium even

after 1–2 months of culture but that does not harm the shoot regeneration (Figure 1b). Such shoots can be excised carefully, dipped in 70% ethanol for 30 sec and used for subsequent experiments.

2.2.4 Apical Meristem

Excise apical meristem from shoot apices of horizontally growing branches, as they have well-developed shoot tips which are easy to dissect than those developing on orthotropically growing shoots. Dissect the shoot apices under stereoscopic binocular microscope with the help of very sharp pointed pieces of razor blades under a laminar flow cabinet. Remove all leaf primordia from the shoot apices one by one to expose light greenish white meristematic tissue. Cut 0.5 mm long meristem tissue and inoculate one in culture tube.

2.3. Culture Medium

Selection of an optimum culture medium is necessary for healthy growth as nutrient requirement of a plant species or even organ depends on its physiology. For neem micropropagation, MS (Murashige & Skoog, 1962), B5 (Gamborg et al., 1968), and N (Nitsch, 1969) media have been used frequently. Nitsch's basal nutrient proved better in the present experiments for shoot regeneration. For root induction, in micropropagated shoots, half strength of Nitsch's (rooting medium 1) or Knop's medium (Knop, 1865) with activated charcoal (rooting medium 2) proved better, ofcourse, without any auxin. Other media for neem shoot regeneration, multiple shoot induction and shoot elongation are mentioned in Table 1.

Table 1. Culture media used for neem micropropagation based on Nitsch's and Knop's basal media augmented with culture stage-specific plant growth hormones and activated charcoal.

Culture stage	Culture stage-specific media					
	Basal Salts	Final concentrations (mg/l)				
		BA	Kn	GA ₃	Ad.S.	Activated charcoal (%)
Shoot regeneration (NB1)	NB	0.1	0.1	—	—	—
Multiple shoot induction (NB2)	NB	0.1	0.1	—	1.0	—
Shoot elongation (NB3)	NB	1.0	.0	1.0	—	—
Meristem culture medium (NB4)	NB	0.5	—	—	—	—
Rooting medium 1	½ strength Nitsch	—	—	—	—	—
Rooting medium 2	½ strength Knop	—	—	—	—	0.02

2.3.1. Medium Preparation

Prepare medium with Nitsch's basal salts, add 3% sucrose and 0.8% agar (Qualigens, India). Adjust the pH of the medium to 5.8 by means of 0.1 N NaOH or HCl whichever necessary. Pour 15 ml medium into each culture tube (2.5 × 15 cm) and autoclave at 1.02 kg cm⁻² pressure at 121°C for 16 min. to ensure sterilization. Use 0.2 µm Millipore filters for sterilizing the thermolabile solutions and add them to the sterilized medium just before pouring into pre-sterilized test tubes.

2.4. Incubation of Cultures and Maintenance

Maintain cultures in the culture room at $25 \pm 2^\circ\text{C}$ with $55 \pm 5\%$ relative humidity. Programme for 16 h photoperiod, emitted by 40 W Philips tubes. Transfer explants to a freshly prepared (same) culture medium after every 8 weeks. In some cases, frequent subculturing may be helpful. Allow explants to grow for a longer duration, even up to 16–18 weeks, without subculturing, if the medium does not dry up.

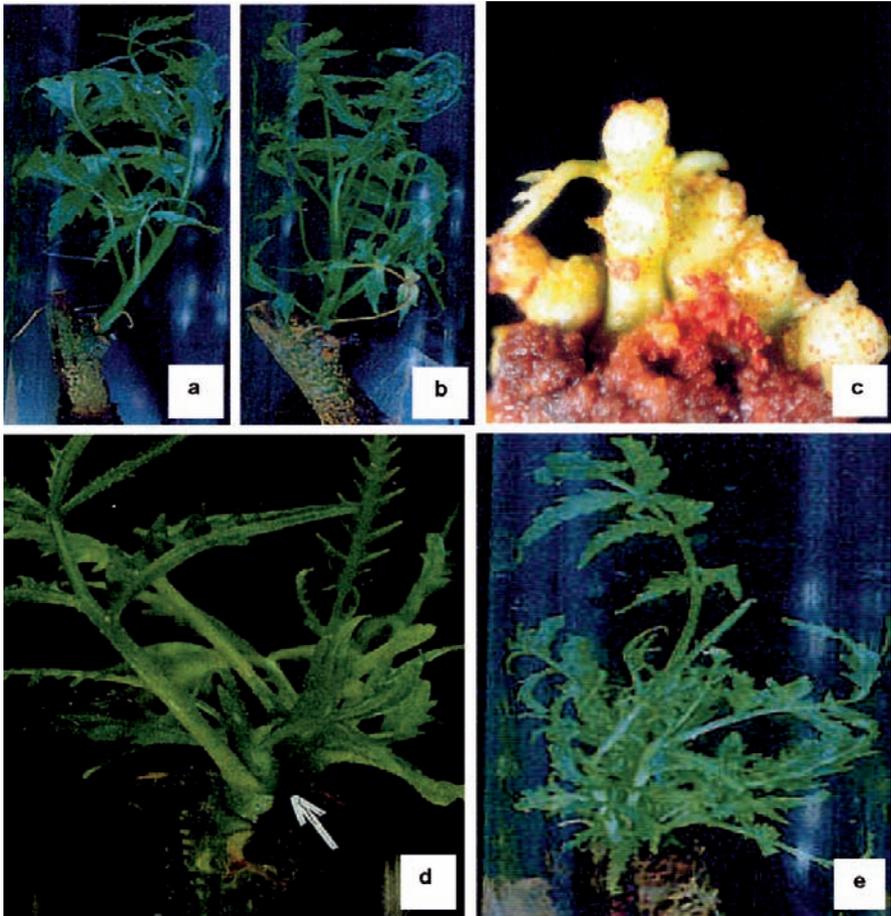


Figure 1. Shoot regeneration in *Azadirachta indica* through node culture. a) Single shoot developed on a node in leaf axil as a result of bud break. b) Bacterial infection appeared on medium sometimes even after 1–2 months of culture which is not much harmful for shoot growth. c) Multiple shoot induction in nodal region of neem on multiple shoot induction medium. d) Pseudo-multiple shoots on shoot regeneration medium as a result of secondary branching on main stem (arrow). e) Multiple shoot development on a node on multiple shoot induction medium.

2.5. Regeneration

2.5.1. Node

Culture nodal explants either on Nitsch's medium for bud break (Figure 1a) or on shoot regeneration medium (NB1) for 6 weeks where multiple (Figure 1c) or pseudo-multiple shoots (Figure 1d) will regenerate. Transfer these explants to multiple shoot induction medium (NB2) for another 6–8 weeks. The nodal explants will develop numerous accessory shoots on this medium (Figure 1e).

Multiple shoots will differentiate in nodal regions in two ways, i.e. (i) several shoots will be induced in the nodal region in a cluster (Figure 2a) where it will be difficult to count the exact number of shoots, and (ii) in some explants, a shoot will emerge through bud break, later other shoot buds will develop near its base (Figure 2b). The percentage of explants forming multiple shoots and the number of shoots per explant, will be higher on medium containing adenine sulphate (Ad.S., NB2 medium).

2.5.2. Microcuttings

Cut the axenically raised shoots into 1 - 1.5 cm long pieces (Figure 2c) and use them as microcuttings. Inoculate them on multiple shoot induction medium where they multiply profusely (Figures 2d,e). Culture the *in vitro* raised smaller shoots on shoot elongation medium (NB3) before inducing roots (Figures 2h,i).

2.5.3. Apical Meristem

Excise the apical meristem (Figure 2f), specially if the nodal explants are infested with inborn bacterial infection which is a very common problem in tree tissue cultures, and rear them on meristem culture medium (NB4). Within 10–15 days, shoot buds will be visible (Figure 2f) which will grow into healthy shoots (Figure 2g).

2.5.4. Elongation of Shoots

Subculture *in vitro* raised small shoots on gibberellic acid supplemented medium (NB3) for elongation (Figures 2h,i). Within 20–25 days of culture, explants will start elongating and after 8 weeks explants will have several healthy elongated shoots.

2.6. Rhizogenesis

Excise 2–3 cm long *in vitro* raised or elongated shoots (on elongation medium) and culture on rooting medium.

2.6.1. Rooting of Excised Shoots

Culture *in vitro* raised shoots on either (i) ½ strength Nitsch's (Figure 3a) or (ii) ½ strength Knop's medium supplemented with 0.02 mg l⁻¹ activated charcoal (Figure 3b). Roots will be induced at the base of shoots, within 15–20 days of inoculation.

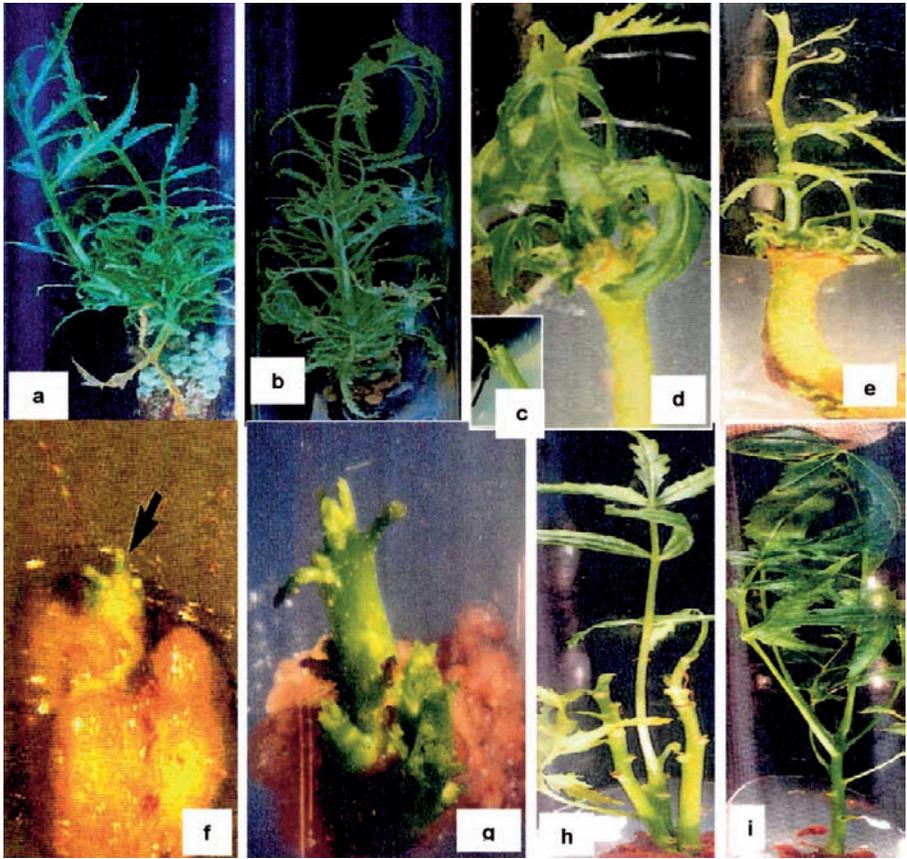


Figure 2. Induction, multiplication and elongation of shoots in *Azadirachta indica*. a) A bunch of shoots growing in the nodal region. b) Multiple shoots developed at the base of the main shoot on multiple shoot induction medium. c) Microcutting. d,e) Multiple shoots developed in microcuttings on multiple shoot induction medium. f) Apical meristem (arrow). g) Multiple shoot induction in apical meristem culture. h,i) Elongation of shoots on shoot elongation medium.

2.6.2. Transfer of Plantlets to Soil

Wash the *in vitro* raised plantlets carefully after 30–45 days of root induction to remove the adhering agar and transfer to small pots containing (2:1) sand:neopeat (Sterling, Cochin, India) or vermiculite. During the first week of acclimatization maintain high humidity (almost saturated). Transfer these plants to soil in a medium-size earthen pot (Figure 3c). Regular irrigation is necessary during this period. These potted plantlets will have to be gradually transferred to field conditions. Initially, they will have to be maintained in a glasshouse (Figure 3d).

2.7. Genomic Fidelity of Offsprings

Genetic fidelity of tissue culture-raised plants has been tested to ascertain if they remain true to type (Biswas, 2000; Biswas et al., 2007). Somaclonal variations are known to develop variations in micropropagated plants. It is but natural to ascertain if they retain the capacity of synthesizing azadirachtin as that of the parent



Figure 3. Rooting and organization of 'elite' neem plantlets. a) Root initiation on rooting medium 1 along with some callus growth at the base of the shoot. b) Root initiation on rooting medium 2 without any callus growth. c) Soil transfer of plantlet. d) Plantlets in a glasshouse later used as source of leaves for DNA extraction.

tree. Unfortunately, one will have to wait for a few years to check it because the *in vitro* raised plants should first reach physiological maturity so that they can flower and set seeds, since seeds possess the highest azadirachtin content in a plant. Therefore, the only viable alternative is to assess genetic fidelity of the offsprings raised from the elite neem tree through tissue culture. Though this will not guarantee the same azadirachtin levels in the seeds produced by the offsprings but will certainly detect if any change takes in the genomic DNA.

2.7.1. Total DNA Extraction

Extract DNA from the leaves of selected elite tree and its *in vitro* raised offsprings. Extract total genomic DNA following Cetyl Trimethyl Ammonium Bromide (CTAB) method of Murray and Thompson (1980).

Procedure

1. Grind 5 g leaves to fine powder in liquid nitrogen using a mortar and pestle.
2. Transfer the powdered tissue into a 50 ml sterile screw capped centrifuge tube.
3. Add 20 ml CTAB extraction buffer to the tube, mix thoroughly and then incubate the homogenate at 60°C for 1 h.
4. Add equal volume of chloroform:isoamyl alcohol (24:1) to the homogenate and mix gently by inverting it for 10 min.
5. Centrifuge at 15,000 rpm for 10 min at 25°C.
6. Then transfer aqueous phase to 50 ml sterile glass tube.
7. Add 0.7 volume chilled isopropanol to the aqueous phase, cover with parafilm and mix gently for about 2 min.
8. Observe the precipitation of DNA into the tube and then transfer the DNA to 1.5 ml Eppendorf tube.
9. Add 0.75 ml 70% ethanol and keep at room temperature for 1 day.
10. Dry DNA either in vacuum drier for 30–45 min or keep the tube open for several hours on the working bench.
11. Dissolve DNA in 1.5 ml TE buffer and store at –20°C.

2.7.2. RAPD Analysis

RAPD Analysis of Micropropagated Offsprings. To test the genomic fidelity of micropropagated neem plants through random amplified polymorphic DNA (RAPD), take the genomic DNA of mother plant (Figure 4a). and of micropropagated plants (Figure 4b). Presently, 13 micropropagated plants along with their parent tree have been analysed. Isolate the DNA as described earlier. Take a set of random decamer primers (viz. OPA, OPC and OPK, from Operon Technologies, Alameda, California, USA) to generate comparative RAPD profiles in AP-PCR mode.

Prepare the master mix as shown in Table 2 and perform the following thermocycler program for the amplification.

Table 2. Reaction mixture and thermocycler parameters used to amplify genomic DNA in AP-PCR mode of elite neem tree and its in vitro-raised offsprings.

Reaction components	Concentration	
Amplification buffer, 10×	1×	
MgCl ₂	2.0 mM	
Genomic DNA template	60 ng	
dNTP's mix	200 μM	
Primers each	5 pM	
Taq DNA polymerase	1 U	
Final volume with water	25 μl	

Thermocycler program	Temp.	Time
Initial cycle		
Denaturation	94°C	1 min
Annealing	36°C	30 sec
Extension	72°C	1 min
Number of cycle	1	
Amplification cycle		
Denaturation	94°C	5 sec
Annealing	36°C	15 sec
Extension	72°C	1 min
Number of cycles	35	
Final extension	72°C	7 min

2.7.3. Electrophoresis

Analyze the amplification products by electrophoresis in 1.8% agarose gel stained with 0.5 μg ml⁻¹ ethidium bromide.

Agarose Gel Preparation. Prepare agarose gel by melting 4.5 g agarose powder in 250 ml 1× TAE buffer, cool it to 60°C and before casting add 0.5 μg ml⁻¹ ethidium bromide to the gel. Load the gel with PCR products and use 10 kb DNA ladder as molecular standard. Run the electrophoresis, visualize the bands under a gel documentation system and take the photograph of the DNA bands. As expected, a uniform pattern of monomorphic bands is seen with primer OPA-13 (Figure 4c) and primer OPC-14 (Figure 4d) indicating no variation among the mother plant and 13 micropropagated offsprings.

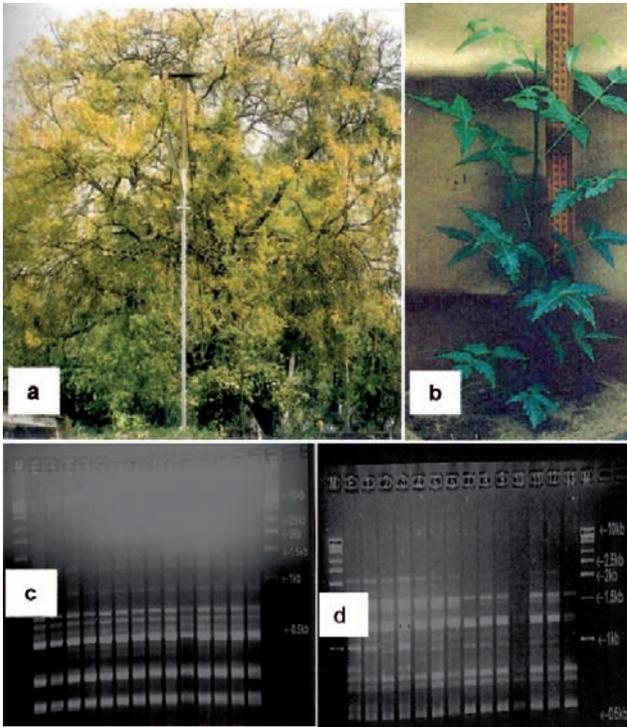


Figure 4. DNA finger printing of elite mother tree and in vitro raised plants. a) Mother plant growing as an avenue tree on Probyn Road, Delhi. b) In vitro raised plants after 1 month of transfer to soil in greenhouse. c,d) Evaluation of genetic fidelity of micropropagated neem plants through RAPD analysis. RAPD profiles generated using primers OPA-13 (c) and OPC-14 (d).

3. CONCLUSIONS

This chapter describes biochemical analysis of azadirachtin to select a 35-year-old elite tree of *Azadirachta indica* A. Juss., its clonal propagation protocol and assessment of genetic fidelity of the regenerants by RAPD analysis. The neem tree, of Indian origin, is highly valued worldwide as a source of medicine and a potent biopesticide, whose active principle is azadirachtin – a triterpenoid. The selection of the elite neem tree, based on azadirachtin content determination in its seed kernels using TLC and HPLC, has increased the significance of this study.

The highest azadirachtin content (0.4%) has been reported from Myanmar while presently a neem tree with 0.746% in its seed kernels has been identified which is the highest ever known record in the world.

Most of the published protocols on neem involve the juvenile tissues while the success with physiologically mature explants has been limited. However, it is essential

that any reliable micropropagation protocol should use mature tissues to minimize the chances of variations. It is not certain if the seedling-derived juvenile tissues will also possess the same genetic make up as that of the mother plant. The protocol described here involves the nodal explants and apical meristems of an old value-added neem tree where callus is not formed during caulogenesis.

Surface sterilization is a major challenge in tree tissue culture, particularly if the donor is the solitary elite tree and that too shows high bacterial infection. A serious problem faced presently in establishing aseptic cultures of elite neem tree was the inborn bacterial infection. This has been resolved by culturing the apical meristems.

Earlier, on a single cytokinin (BA, Kn, 2iP or zeatin) containing medium multiple shoots have been regenerated which is, of course, species specific. A combination of two cytokinins (BA+Kn, Kn+2iP, Kn+zeatin and BA+zeatin) has yielded appreciable caulogenesis. Addition of lower concentrations of adjuvants with a combination of two cytokinins has supported best morphogenesis presently. Adenine sulphate has enhanced the number of shoot buds if used in combination with cytokinins, BA and Kn.

In vitro raised neem shoots have been presently rooted best on half-strength Nitsch's or Knop's basal salts, which are cost effective, compared to the earlier reports using auxins. A significant point in favor of this protocol is that the tissue culture raised offsprings have maintained genetic fidelity with the mother elite *A. indica* tree as demonstrated by RAPD analysis. Thus, the clonal propagation protocol of the value-added neem tree has a great potential for commercial production, specially when scientists are keen to produce biodegradable pesticides in large quantities.

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CHAPTER 33

MICROPROPAGATION PROTOCOL FOR MICROSPORE EMBRYOGENESIS IN *OLEA EUROPAEA* L.

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1. INTRODUCTION

Olive tree (*Olea europaea* L.) has a high economic value. The Mediterranean region produces about 99% of the olives in the world and almost 2000 cultivars have been identified and gathered in germplasm collections in various Mediterranean countries. Spain is the leading olive growing country, with 25% of the world area of olive groves, which provides for 39 and 35% of the total olive oil and table olive production, respectively.

The Olive (*Olea europaea* L.) belongs to the family Oleaceae. The leaves are opposite. The flowers are borne in racemes which emerge from the axils of the leaves, and produce large quantities of pollen. The fruit is a drupe. Olive trees grow very slowly and rarely reach more than 15 m in height, but they may have a lifespan of hundreds of years. To date, modern molecular technologies in plant breeding have not been applied extensively in olive, but using biotechnology may provide profitable results. As has been demonstrated in other crops, biotechnological methods can improve the efficiency and increase the speed of breeding. Microspore embryogenesis through *in vitro* culture is a widely-used method to generate genetic variability by obtaining microspore-derived embryos and double-haploid plants, with many applications for plant breeding (Chupeau et al., 1998). Gametic and haploid regenerants are also very important in breeding because the single set of chromosomes allows the isolation of mutants and the production of homozygous doubled-haploids, through chromosome doubling.

Efficient protocols for microspore embryogenesis induction in culture have been developed in recent years for many herbaceous crops (reviewed in Maluszynski et al., 2003), and in a lesser extent for some woody plants (Bueno et al., 1997, 2005; Germana & Chiancone, 2003; Höfer, 2004). In trees, with a long reproductive cycle, high levels of heterozygosity and sometimes self-incompatibility, methods for obtaining homozygous plants are of strong interest, as their production through conventional methods requires several generations which is difficult to realize in woody plants. In contrast, gametic embryogenesis by isolated microspore culture allows the single step development of complete homozygous lines from heterozygous parents.

With the aim of developing a gametic embryogenesis system for olive, a protocol for efficient *in vitro* microspore culture and proembryo production has been developed (Bueno et al., 2004, 2005). Different pollen developmental stages and stress conditions (heat or cold shock) were evaluated. The correlation of inflorescence and anther morphology and the suitable stage of microspore development were analysed. The morphology of responsive buds was identified, which corresponded with microspores from the late uninucleate to early binucleate pollen stages. Symmetrical divisions of microspores as well as resulting multinucleate structures and pro-embryos were observed. Microspore reprogramming, the induction of embryogenic divisions and the formation of multicellular proembryos have been achieved as the basis for developing gametic doubled-haploid plant production in olive.

2. EXPERIMENTAL PROTOCOL

2.1. Induction of Gametic Embryogenesis

The induction of gametic embryogenesis in *Olea europaea* L. was obtained from microspore culture. During the process, several factors which may affect pollen embryogenesis *in vitro* were considered.

2.1.1. Physiological Condition of the Donor Plants

Although the physiological status of donor plants may influence dramatically the androgenic process, this parameter has not been investigated in depth in woody plants. In herbaceous crops, growth conditions (temperature, photoperiod, light irradiance, soil traits and culture techniques) have an influence on the endogenous levels of plant growth regulators and on the nutritional status of anther tissues. However, these factors are difficult to control in woody plants in the field.

2.1.2. Plant Genotype

The genotype of the donor plant affects the frequency of pollen plant induction and therefore it is important to analyse the response of various genotypes to achieve a high frequency in haploid plant production. The genotype determines the number of embryogenic pollen grains, playing a decisive role on pollen embryogenesis. This genetic ability controls the development of multicellular structures with symmetric divisions in the microspores. (Barro & Martin, 1999). In this work, floral buds were taken from trees of the cultivar 'Arbequina' (from the Olive World Germplasm Bank in Córdoba, Spain).

2.1.3. Correlation of Floral and Male Gametophyte Development

In most species, embryogenic anthers contain either late uninucleate microspores or early binucleated pollen grains. In some tree species, such as olive tree and cork oak, the transition from the microspore to the pollen grain is greatly asynchronous. Floral buds similar in morphologic traits such as colour or size may contain microspores at very different stages, e.g. tetrads, uninucleate microspores or even mature pollen.

The appropriate microspore developmental stage is crucial in pollen embryogenesis. In our study in olive, developmental stages from tetrad to mature pollen are presented with the corresponding anther, bud and inflorescence (Figure 1). A weak relationship between bud size and microspore stage was observed. Inflorescences were selected at different phenological stages. Buds and anthers were dissected and the respective microspores were stained with 4'-6-diamidino-2-phenylindole (DAPI) for the determination of the developmental stage (Figure 1A–F). The anthers were placed on a glass slide in a few drops of 1mg/l DAPI in PBS plus 1% Triton X-100, and tapped softly through the coverglass. Microspores were examined under a Nikon fluorescence microscope and photographed under ultraviolet light ($\lambda = 360$ nm) with a digital Coolpix 4500 Nikon camera. Open floral buds with yellow-greenish anthers that began to emerge from the flower were identified as those with higher proportion of microspores from the late uninucleate to the binucleate pollen stage. About 100 microspores for each development stages and a total of 50 floral buds were tested.

2.1.4. Bud Selection in Olive

Bud and anther length are similar in olive. It appeared that for pollen embryogenesis, the optimal microspore developmental stage is quite short-lived and microspores at an inappropriate developmental stage will neither divide nor produce embryos. We found that the selection of bud morphology was more consistent than bud (or anther) size. This parameter is probably maintained with other cultivars since flower size is influenced by the number of flowers in each peduncle.

The best stage for the induction of gametic embryogenesis in cultivar Arbequina is when the buds are approximately 2 mm long. This size corresponds to half-opened flowers with yellowish anthers containing microspores at late uninucleate – early binucleate pollen stage (Figure 1D,E). These microspores are at the optimal stage for the induction of divisions which lead to proembryo formation

2.1.5. Pretreatment of Olive Buds

Generally cold and heat pretreatments are applied to flower buds in order to achieve high frequencies of gametic embryogenesis (Bueno & Manzanera, 2003). Therefore, the effect of cold and heat pretreatments on pollen embryogenesis were also evaluated in olive. Stress pretreatments may be applied either *in vivo* and/or *in vitro*, to floral buds, isolated anthers or even to isolated microspores. Stress can be applied in different ways, physical, physiological or chemical. Typically, heat or cold shocks, starvation, sugar or nitrogen depletion etc., yield good results (Zheng, 2003). In the present work, a pretreatment was applied *in vivo* to trigger microspore embryogenesis in olive. Small branches of olive bearing flowers were harvested. The harvested olive branches (50 cm long) were wrapped in aluminium foil, with damp cotton wool at the base of the stem and maintained in darkness at 4°C for 9 days.

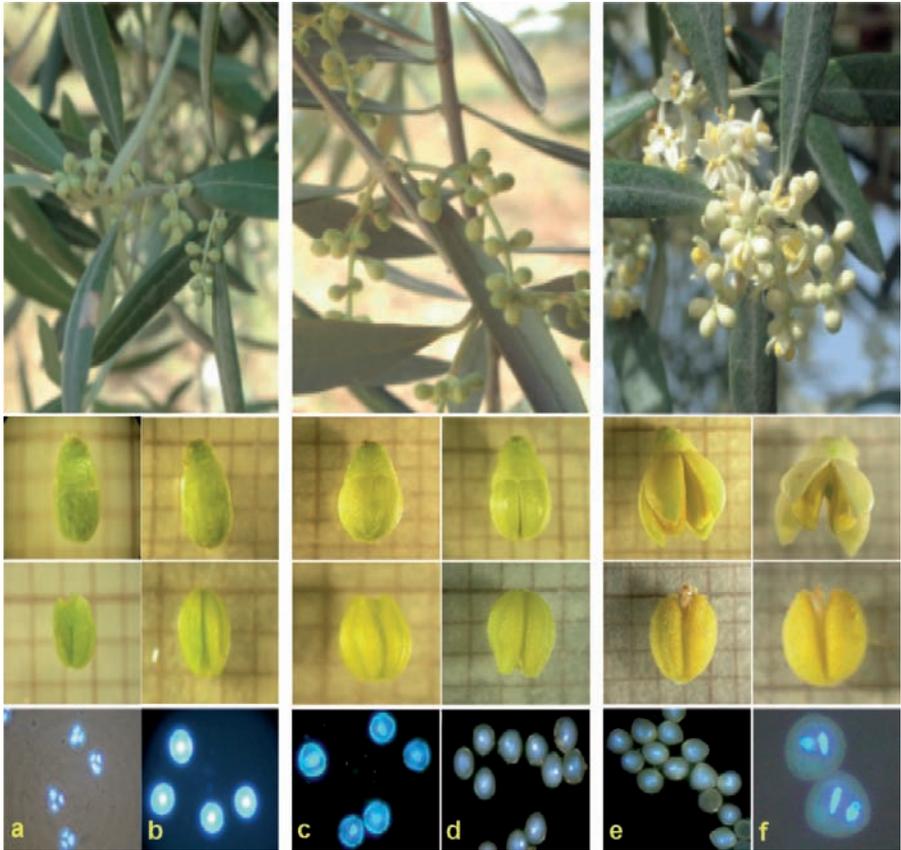


Figure 1. Inflorescence, bud, anther and microspore development stages in olive (cv. Arbequina). a) Tetrads. b) & c) Early uninucleate microspores. d) Late uninucleate microspores. e) Early binucleate pollen grains. f) Mature pollen grains.

2.1.6. Sterilization Procedure

Before the anthers were isolated, flower buds are submitted to vacuum using a HydroTech™ Vacuum Pump for 1 h, washed in fungicide Benomilo-50 (Aragro) solution ($200 \text{ mg}\cdot\text{l}^{-1}$) for 1 min, in 70% Ethanol for 1 min, and then surface-sterilized in sodium hypochlorite solution (2% active chlorine), with a few drops of Tween-20 for 30 min. Finally flower buds were rinsed several times in sterile distilled water. Anthers were dissected from flower buds under aseptic conditions in a horizontal laminar air flow.

2.1.7. Microspore Viability

Microspore viability was determined by FDA staining ($0.05 \mu\text{g}\cdot\mu\text{l}^{-1}$) both immediately after the isolation of microspores and after cold and heat-shock treatment. Three

hundred microspores with a minimum of twenty replicates for each treatment were tested. The viability of microspores determined immediately after isolation was 50–77%. Microspore viability after cold-shock was 30–68%, while heat-shock treatments reduced viability to 6–43% (Figure 2).

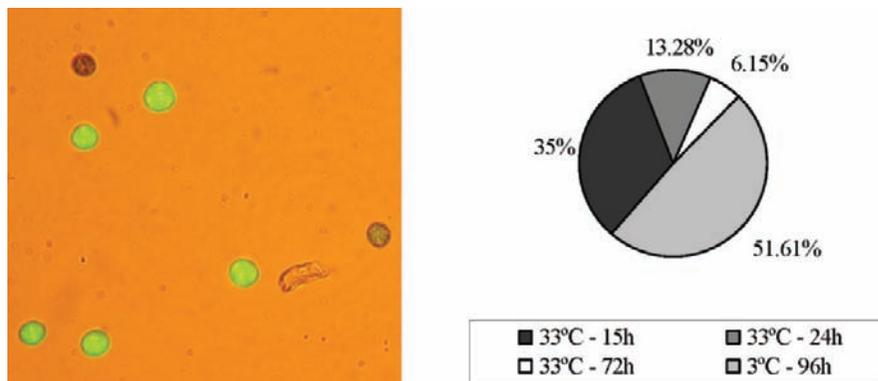


Figure 2. A) Microspore viability was determined by FDA staining. B) Survival of *in vitro* olive microspore subjected to different shock treatments: cold (3°C) for 96 h or heat (33°C) at different duration (15, 24 and 72 h) for the induction of pollen embryogenesis.

2.1.8. Isolation of Microspores

The composition of culture medium is a very important factor for haploid plant induction. Generally, the basal medium consists of salts, vitamins, sugars and a gelling agent. Many species require plant growth regulators such as auxins, cytokinins and ABA in the culture medium for the production of haploid plantlets. In some species, haploid induction frequency was enhanced with the addition of polyamines, amino acids and silver nitrate in the induction medium. Different sugars like sucrose, maltose, glucose and fructose also enhance the frequency of microspore embryogenesis in some species (Bueno et al., 2000). Furthermore, in cultures of isolated microspores, cell density may be critical. Best results have generally been obtained between 5×10^3 and 2×10^4 microspores per ml (Zheng et al., 2002).

Olive microspores were isolated from anthers using the following protocol (Bueno et al., 2005). A total of 200 anthers were placed in 2 ml medium B adjusted at pH 7.0 (Table 1). Anthers were agitated on a magnetic stirrer at 250 rpm for 1 min. The released microspore population was filtered through a 30 μ m sieve which retained the anthers. Next, a further 2 ml of medium B was added to the remaining anthers. This extraction process was repeated a total of four times. The pooled filtrate containing microspores was centrifuged at room temperature at 1200 rpm for 5 min. The microspore pellet obtained was re-suspended in 5 ml medium B. This process was also repeated for four times.

Culture density was adjusted to $1-5 \times 10^5$ microspores/ml by adding medium B to the pellet obtained after the final centrifugation step. A volume of 1.5 ml was dispensed into each 35×10 mm sterile Petri dishes, leaving about 500 μ l to determine microspores viability and concentration. Seven Petri dishes were used for each stress treatment.

Table 1. Formulation of culture medium used for the isolation of microspores from anthers of olive. The culture medium is based on B medium (Kyo & Harada, 1986).

Components	Chemical formula	Stock (g/L)	Medium (mg/L)
<i>Macro nutrients, 10 \times stock, use 100 ml per L medium</i>			
Potassium chloride	KCl	1.49	149
Calcium chloride-2H ₂ O	CaCl ₂ ·2H ₂ O	0.146	14.6
Magnesium sulfate-7H ₂ O	MgSO ₄ ·7H ₂ O	0.25	25
Potassium bi-phosphate	KH ₂ PO ₄	0.14	140
<i>Other additives</i>			
Mannitol			55000

2.1.9. Heat Shock Stress Treatments of the Microspores

Chilling has been used in microspore culture of citrics, apple and cork oak. Low temperature may trigger the stimulus that changes microspore development from the gametophytic to the sporophytic pathway, although this mechanism is not well understood.

High temperatures (32°C or higher) in combination with sucrose starvation increase the production of embryos. Some species require a combination of heat, chilling, sucrose starvation and even chemicals to increase the efficacy of embryo production (Touraev et al., 1997). In this work with olive, seven Petri dishes for each stress treatment, were placed in heat or cold stress conditions: at 33°C in the dark for 15 h, at 33°C in the dark for 24 h, at 33°C in the dark for 72 h or at 3°C in the dark for 96 h. Each treatment was repeated three times. The optimal treatment for microspore embryogenesis induction was 3°C for 96 h.

The cold shock at 3°C in darkness for 96 h caused "swelling" of about 16% of the microspores after 1 day of the culture. Microspore diameter increased by 100–150% over the original size. "Swollen" microspores continued embryogenic development (Figure 3 A,B). Heat shock treatments produced "swollen" microspores but at lower frequency (0.3%). Some reports suggest that low temperatures slow microspore metabolism and arrest cell cycle in preparation for the resumption of mitosis once microspore are cultured at normal temperatures (25–28°C) (Zheng, 2003). In the present study, both low and high temperature pre-treatments induced a switch from the normal development pathway of microspores to pollen embryogenesis. The first manifestation of this change is swelling of microspores and cytoplasm structural reorganization, which were more evident after cold treatment.

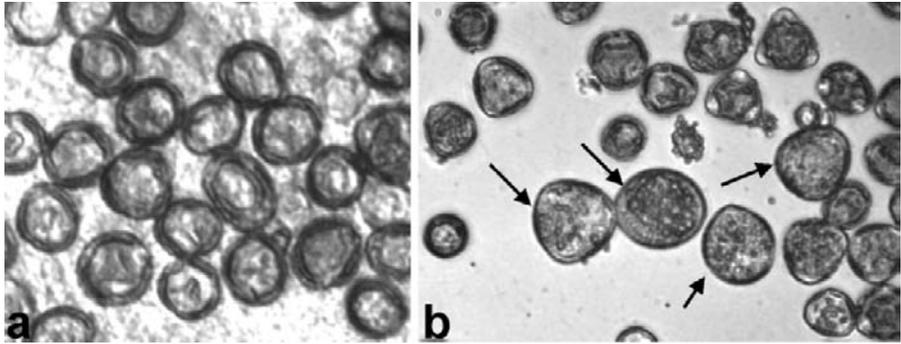


Figure 3. Normal microspores a) and “swollen” microspores b) after cold treatment in the dark at 3°C for 96 h.

2.1.10. Induction of Microspore Embryogenesis

After stress treatment, the microspore population in medium B was centrifuged for 5 min at 1200 rpm at room temperature. The microspore pellet was resuspended in induction medium AT3 I (Höfer et al., 1999) (Table 2) in 4-well plates with the final density of the microspores being determined using a haemocytometer (Neubauer chamber) and adjusted to $4-8 \times 10^4$ or $1-2 \times 10^5$ microspores/ml. The microspores were incubated in a culture chamber at $25 \pm 1^\circ\text{C}$ in the dark.

The frequencies of microspore division and multicellular structure formation were scored by DAPI staining from the first week to the fourth week of culture using an inverted microscope. About 1200 microspores were counted for each treatment. The first sporophytic binucleate microspores (Figure 4) were observed after 1 week of culture. Multinucleate structures are produced following the interruption of gametophytic pollen grain development and at the beginning of embryogenetic pathway. These microspores continued to divide and the number of nuclei per microspore increased to three, four, six or more fold during the next weeks. After the 5 weeks in culture, further development of these proembryos was limited. In *Lupinus* spp. the development of multicellular structures in isolated microspore cultures appears to be limited by the rigid outer exine layer (Bayliss et al., 2004) which could also be the case in olive.

The induction of microspore embryogenesis is usually achieved by a stress treatment, and it is suggested to be associated with a symmetric microspore division producing two equal nuclei as opposed to the differentiated generative and vegetative nuclei resulting from asymmetric division in the normal gametophyte pathway (Zaki & Dickinson, 1991). The influence of heat and cold shock treatments on the initiation of microspore division (induction of embryogenesis) and the development of multicellular structures are illustrated on Table 3. The sporophytic development pathway induced by cold treatment of isolated olive microspores was also seen in cultures subjected to heat shock although with much lower efficiency. The optimal treatment was 3°C for 96 h (32.4% multicellular structures per dividing microspore) while the best heat treatment was at 33°C for 24 h (15.2% multicellular structures per dividing microspore).

Table 2. Formulation of culture medium used for the induction of microspore embryogenesis in olive. The culture medium is based on AT3 I medium (Höfer et al., 1999).

Components	Chemical formula	Stock (g/L)	Medium (mg/L)
<i>Macronutrients, 100 × stock, use 10 ml per L medium</i>			
Potassium nitrate	KNO ₃	195	1950
Calcium chloride-2H ₂ O	CaCl ₂ ·2H ₂ O	16.6	166
Magnesium sulfate-7H ₂ O	MgSO ₄ ·7H ₂ O	18.5	185
Ammonium sulfate	(NH ₄) ₂ ·SO ₄	27.7	277
Potassium bi-phosphate	KH ₂ PO ₄	40	400
<i>Micronutrients, 100 × stock, use 10 ml per L medium</i>			
Potassium iodide	KI	0.083	0.83
Boric acid	H ₃ BO ₃	0.62	6.2
Manganese sulfate-H ₂ O	MnSO ₄ ·4H ₂ O	2.23	22.3
Zinc sulfate-7H ₂ O	ZnSO ₄ ·7H ₂ O	0.86	8.6
Sodium molybdate-2H ₂ O	Na ₂ MoO ₄ ·2H ₂ O	0.025	0.25
Cupric sulfate-5H ₂ O	CuSO ₄ ·5H ₂ O	0.0025	0.025
Cobalt chloride-6H ₂ O	CoCl ₂ ·6H ₂ O	0.0025	0.025
<i>Iron-EDTA, 100 × stock, use 10 ml per L medium</i>			
Iron sulfate-7H ₂ O	FeSO ₄ ·7H ₂ O	2.78	27.8
Ethylenediamine tetraacetic acid disodium	Na ₂ EDTA	3.72	37.2
<i>Vitamins, 100 × stock, use 10 ml per L medium</i>			
Myo-Inositol		10	100
Nicotinic acid		0.1	1
Pyridoxine hydrochloride		0.1	1
Thiamine hydrochloride		0.1	10
<i>Other additives</i>			
Maltose			90000
MES			1950
Glutamine			1256

Table 3. The effect of temperature and duration of treatment on olive microspore division (%) and development of multicellular structures per divided microspores (%) measured after 3 weeks of culture.

Treatments	Microspore division (%)	Multicellular structures/divided microspores (%)
33°C 15 h	0.09 ± 0.00	0.09 ± 0.00
33°C 24 h	8.23 ± 1.24	15.23 ± 1.67
33°C 72 h	3.16 ± 2.00	2.54 ± 1.59
3°C 96 h	12.6 ± 1.15	32.44 ± 4.20

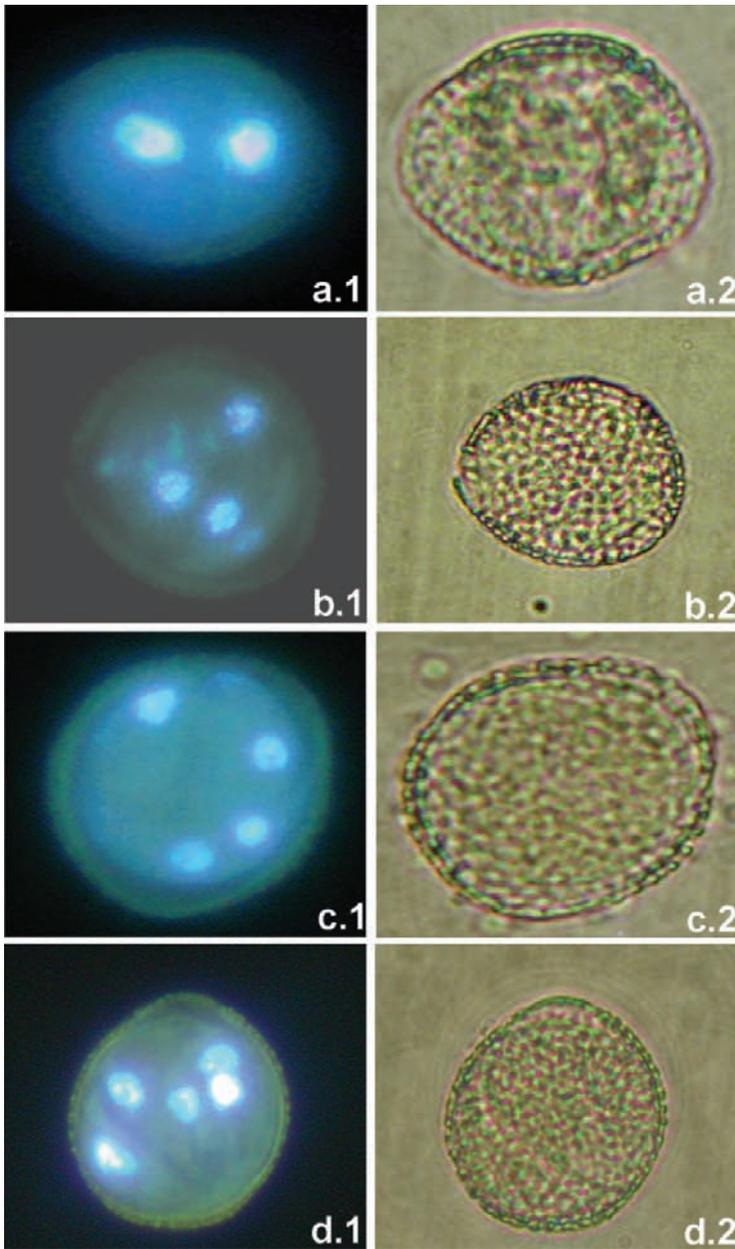


Figure 4. Sporophytic divisions of olive microspores in culture. Multinucleate microspores with 2(A), 3(B), 4(C), 5(D) nuclei after 15 days of culture. a1–d1: Microspores observed under fluorescence microscope. Nuclei were stained DAPI. a2–d2: The same microspores under phase contrast.

3. CONCLUSION

We present here a new protocol to produce an isolated microspore culture that leads to cell divisions and proembryo formation in *Olea europaea* L., cv. Arbequina. The morphology of floral buds containing responsive anthers and microspores (open buds with yellow-greenish anthers) from trees grown in the field has also been described. Moreover, also the optimum microspore stage for the induction of pollen division has been determined (from late uninucleate microspore to binucleate pollen stage) as well as the culture conditions for the production of proembryoids from isolated olive microspores have been defined (33°C for 24 h or 3°C during 96 h). In this work, the obtained multicellular olive microspores (proembryos) represent the first step towards haploid olive embryo production.

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CHAPTER 34

MICROPROPAGATION OF *PRUNUS DOMESTICA* AND *PRUNUS SALICINA* USING MATURE SEEDS

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1. INTRODUCTION

European plum (*Prunus domestica* L.) and Japanese plum (*Prunus salicina* L.) are two major fruit species among *Prunus* plants. These two species are widely grown across the world and are important economic plants (Okie & Ramming, 1999; Bellini et al., 2002; Kaufmane et al., 2002; Szabo & Nyeki, 2002; Capote et al., 2006).

European plum and Japanese plum are related but they are different in several aspects: The fruits of these two plum plants mature at different times during the season depending on varieties, have different appearances and colours, have different textures, and share different markets. The genetic composition of these two species is also different. *P. domestica* is a hexaploid plant; however, *P. salicina* (and many other plum species), has a diploid genetic background (Scorza et al., 1995; Okie & Ramming, 1999).

In this chapter, plant micropropagation of both European plum and Japanese plum using epicotyls and hypocotyls of mature seeds is described. The protocol has been used for propagation of various varieties of both species.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Sampling from Mother Plants

Harvest fruit from mature European and Japanese plum trees as early as three weeks before full maturity of the fruit. Shoot induction efficiency may decrease when

younger or over-mature fruit is used. Store fruit at 4°C, and process (see below) as soon as possible after collection, preferably within 2 months. Longer storage will result in an overgrowth of microorganism contamination.

Remove the flesh (mesocarp) from the endocarp (stone) with a paring knife. Wash the endocarp containing the seed with 0.05% sodium hypochlorite (1% commercial bleach) for approximately 10 minutes with stirring, and rinse several times with water until no further flesh is removed and the water remains clear. Allow the endocarps to air dry on absorbent paper on the lab bench for 2 days before storing the endocarps in plastic mesh bags at 4°C. The endocarps containing the seeds can be stored under this condition and be used for micropropagation as needed over the year.

2.1.2. Surface Disinfection

Crack stored endocarps with a hammer to release seeds. Care must be taken to avoid damaging the seed. Surface-disinfect seeds in a flask containing a solution of 0.5% sodium hypochlorite with 0.005% Tween 20 for 15 minutes while stirring. Under aseptic conditions, remove the bleach solution and wash the seeds three times with sterile deionized water to remove any remaining bleach. Soak seeds overnight in sterile water at room temperature to soften the tissue for easier dissection.

2.1.3. Explant Dissection

The following day in a laminar flow hood, cut seeds in half crosswise in a Petri dish, remove the seed coat (Figure 1A) and split open the cotyledons to reveal the embryonic axis (Figure 1B). Remove the embryonic axis using a scalpel (#11 blade preferred) and dissect into three segments: epicotyl, hypocotyl and radicle. Discard the radicle since it generally fails to produce shoots. Cut the hypocotyl into three slices as shown in Figure 1C. Epicotyls are used for propagation via axillary bud development and hypocotyls are used for propagation via adventitious shoot induction (Scorza et al., 1995; Tian et al., 2004; 2005; Sibbald et al., 2006).

2.2. Culture Medium

Three different types of media can be used for plum micropropagation, including MS medium (Murashige & Skoog, 1962), B5 medium (Gamborg et al., 1968) and woody plant medium (WPM) (Lloyd & McCown, 1981). The current protocol is based on the use of Murashige & Skoog's medium.

Shoot induction medium (Table 1) contains MS salts, 100 mg/L myo-inositol, 0.4 mg/L thiamine HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.5 mg/L indolebutyric acid (IBA), 25 g/L sucrose, 7 g/L Bactoagar (Difco) (Mante et al., 1991). Adjust pH to 5.9 with the addition of HCl (or KOH if the pH is too low). Place the medium in 1L glass bottles and autoclave at 121°C for 30 minutes. After cooling to approximately 45°C, add 1.66 mg/L filter-sterilized thidiazuron (TDZ). Pour approximately 35 ml of the medium into 25 × 100 mm petri plates and allow to solidify.

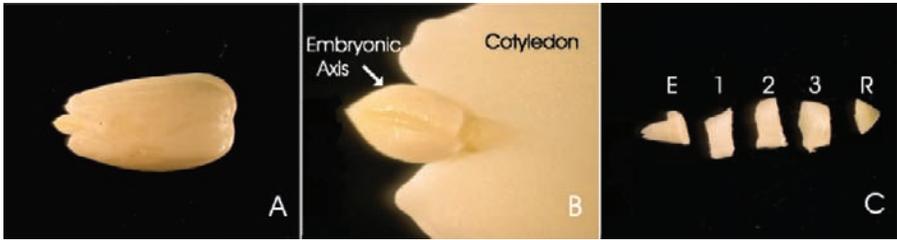


Figure 1. Explant excision. A) Seed without seed coat. B) Cotyledon with embryonic axis attached. C) Embryonic axis dissected into segments (the epicotyl, the three slices of hypocotyl and the radicle) as indicated.

Table 1. Composition of media used for plum micropropagation.

Component	Final concentration (mg/l)	
	Induction medium	Rooting medium
MgSO ₄ ·7H ₂ O	370.0	185.0
MnSO ₄ ·H ₂ O	16.9	8.45
CaCl ₂ ·2H ₂ O	444.0	222.0
NH ₄ NO ₃	1650.0	825.0
KNO ₃	1900.0	950.0
KH ₂ PO ₄	170.0	85.0
H ₃ BO ₃	6.2	3.1
ZnSO ₄ ·7H ₂ O	8.5	4.25
CuSO ₄ ·5H ₂ O	0.025	0.0125
KI	0.88	0.44
CoCl ₂ ·6H ₂ O	0.025	0.0125
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.125
Fe(III)EDTA	0.0425	0.0213
Myo-Inositol	100	100.0
Thiamine-HCl	0.4	0.4
Nicotinic Acid	0.5	0.5
Pyridoxine-HCl	0.5	0.5
Indole-3-butyric Acid (IBA)	0.5	—
Naphthaleneacetic Acid (NAA)	—	1.0
Kinetin	—	0.002
Sucrose	25.0	10.0
Agar	7.0	7.0
Thidiazuron (TDZ)	1.66	—

Rooting medium (Table 1) consists of ½ strength MS salts (Murashige & Skoog, 1962), vitamins as above, 1.0 mg/L naphthaleneacetic acid (NAA), 0.002 mg/L kinetin, 10 g/L sucrose, 7 g/L Bactoagar (Difco) with the pH adjusted to 5.9 (Gonzalez Padilla et al., 2003). After autoclaving, the medium may be poured into 25 × 100 mm Petri plates (35 ml) or into 10 cm Magenta containers (60 ml).

Murashige & Skoog basal salt mixture may be purchased from Phytotechnology Laboratories (Shawnee Mission, KS, Catalogue # M524). Vitamins and hormones are prepared as stock solutions (Table 2) to accurately add small quantities to the final media.

Table 2. Stock vitamin and hormone solutions.

Component	Solvent	Stock concentration (mg/ml)
Thiamine-HCl	Water	10
Nicotinic Acid	Water	10
Pyridoxine-HCl	Water	10
Indole-3-butyric Acid (IBA)	1 M Sodium Hydroxide	1
Naphthaleneacetic Acid (NAA)	1 M Sodium Hydroxide	1
Kinetin	1 M Sodium Hydroxide	1
Thidiazuron (TDZ)	Dimethyl Sulfoxide	1

2.3. Shoot Regeneration and Maintenance

Place approximately 20 epicotyl or hypocotyl segments on the surface of the induction medium in a 100 × 25 mm petri dish and maintain the culture at 25° ± 2°C under a 16 hour photoperiod of fluorescent Sylvania “Cool White” light with a Photosynthetic Photon Flux of about 50 μmol m⁻² s⁻¹ (Figure 2A).

On induction medium, the primary shoot of the epicotyl segment elongates and turns green after 1 week in culture. Axillary shoots initiate around the lower area of the primary shoot. The shoots develop and become visible after 2–3 weeks. The primary shoot is usually longer compared to axillary shoots and distinguishable. The majority of axillary shoots develop and elongate between 4 to 8 weeks (Figure 2B).

Hypocotyl slices enlarge after 1 week in culture on induction medium. Shoot initials are generally visible after 3 weeks. Most shoots develop around 4 weeks after the explants are introduced into the culture (Figure 2C). Some new shoots may continue to form up to week 16. Transfer explants to fresh medium every 4 weeks for shoot elongation and for new shoot formation, if it is desired.

The percentage of explants which respond for shoot development is dependent on the variety of plum. No variety of European plum nor Japanese plum showed 100% response. The average number of shoots induced on each responding explant ranges from 2 to 9. The shoot induction rate using hypocotyls is usually higher for European plum compared to Japanese plum. In addition, it appears that European plum varieties are generally more responsive to regeneration (Tian et al., 2007a) and Japanese plum regeneration is more genotype dependent (Tian et al., 2007b). Screening and identifying responding varieties may be needed for micropropagation of varieties of a particular plum germplasm using the hypocotyl approach.

When shoots reach approximately 4 mm in length, cut them from the explant and transfer either to rooting medium for rooting and plant recovery, or to fresh induction medium for new shoot initiation. Trim all callus tissue from the base of the shoot and divide any multiple shoots to single shoots before transferring for rooting (Figure 2D).

Secondary shoot development can be induced by transferring single shoots developed from epicotyls or from hypocotyls to fresh induction medium. Again, all callus tissue must be removed from the base of the shoot since the callus tissue becomes toxic to the development of the shoots with time. Over the period of a month, this single shoot can generate up to 12 more new shoots which can then be divided again (Figure 2E). Almost all primary shoots will produce new multiple shoots in this manner.

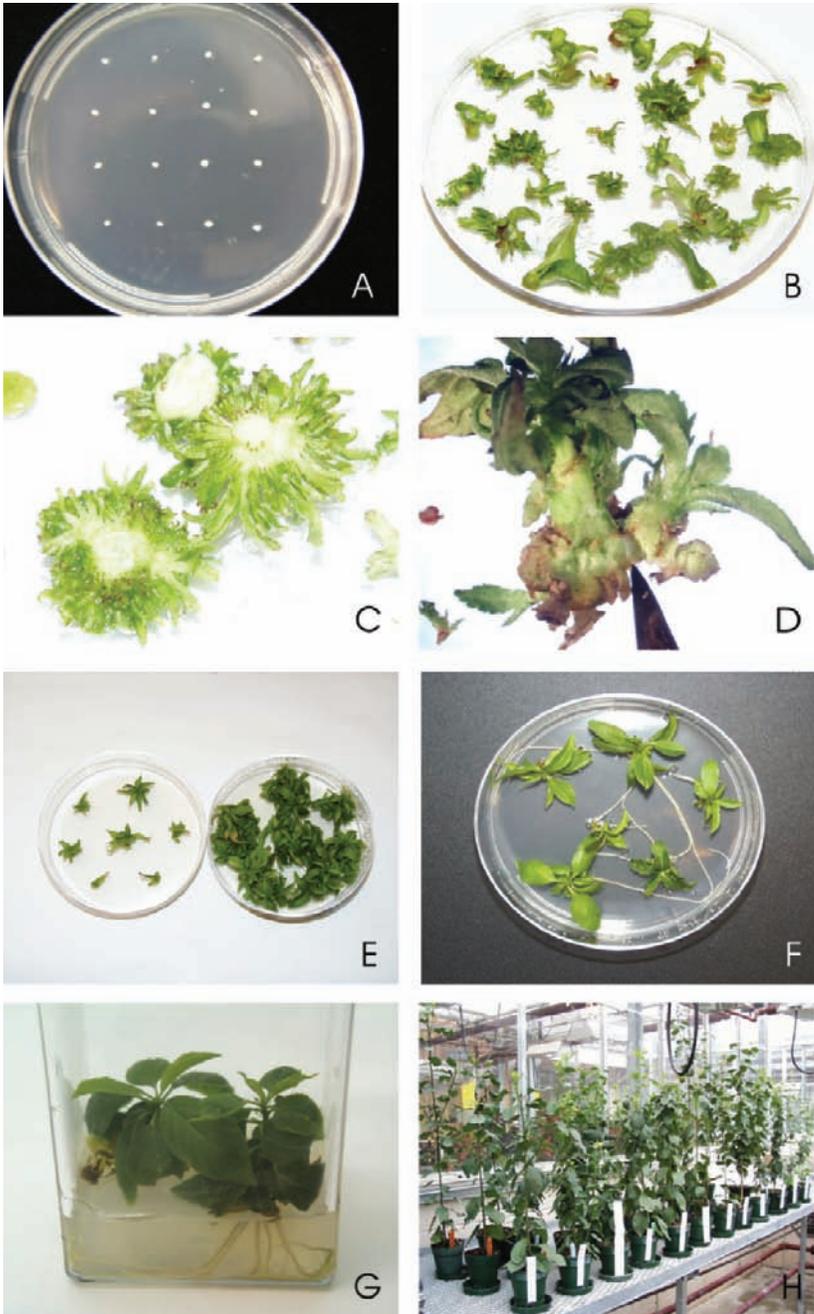


Figure 2. Shoot induction and plant recovery of plum. A) hypocotyls after excision. B) Shoot formation from a epicotyl segment at 25 days. C) Shoot formation from a hypocotyl slice at 25 days. D) Division of clump of multiple shoots. E) Secondary shoot production at day 0 and day 28. F) Root development from induced shoots. G) Plantlet development in magenta box. H) Plum recovery in the greenhouse.

2.4. Rooting

Transfer individual shoots to rooting medium (Table 1) in a 100 × 25 mm petri dish for rooting. Rooting efficiency varies with each plum variety. Use of NAA can increase the rooting efficiency, especially for the varieties with poor rooting efficiency (Tian et al., 2007a). Roots generally appear after 4 to 8 weeks (Figure 2F). Each shoot produces 1 to 2 main roots with a few small rootlets.

Carefully move rooted plantlets to 10 cm Magenta containers (Magenta Corp., Chicago) containing the same rooting media for plant development (Figure 2G). Roots are prone to breaking off at this point if care is not taken to first loosen the media around them.

2.5. Hardening

When plantlets have reached 3–5 cm in height, gently remove them from the Magenta containers and remove any agar from the roots. Transfer the plantlets to 12-cm pots containing moistened commercially available potting mix. Keep soil moist and the plantlets covered with empty Magenta containers or transparent plastic bags during the first week. After this period of acclimation, remove the Magenta containers and expose the plantlets to standard greenhouse conditions. Plants usually establish in the greenhouse in about 2 weeks. Fertilize weekly with N-P-K fertilizer (20-20-20).

2.6. Field Testing

Plants that are well established in the greenhouse (Figure 2H) and with a size of about 1 meter can be transferred to the field.

2.7. Storage of *In Vitro* Cultures

In vitro cultures can be maintained for months or years as shoot cultures. Each month, divide multiple shoots into single shoots and transfer to fresh induction medium (Figure 2D) in order to keep the cultures in a healthy condition. Over the month, these shoots will produce multiple shoots and the process can be repeated. In this fashion, cultures have been maintained for several years, with up to 98% of the shoots continuing to multiply after one year.

3. CONCLUSION

Although different in taxonomy and other aspects, Japanese plum and European plum can be regenerated via epicotyls and/or hypocotyls using a similar approach. Mature seeds are used as the source of explants and thus the research materials can be supplied year around. The plant recovery time is relatively quick. It usually takes about 4 to 5 months from the introduction of the explant into culture to plant establishment in the greenhouse. Plants can be regenerated from seeds of different plum varieties. New shoot development from induced primary shoots is a useful way for maintaining the culture in the laboratory. A large number of plants can be produced via culture of epicotyls or hypocotyls, or via secondary shoot formation from the developed shoots.

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CHAPTER 35

MICROPROPAGATION OF *JUGLANS REGIA* L.

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1. INTRODUCTION

Micropropagation of *Juglans regia* and hybrids may either be initiated from zygotic embryos, shoots, branches or adult plant scions (Leslie & McGranahan, 1992). Like several other woody species, walnut tree explants obtained from embryonic tissues are easily established *in vitro* and multiplied by direct organogenesis; however, this often happens with little early care. Explants obtained during the fruit production phase can also be multiplied this way (Leslie & McGranahan, 1992). The experience with *Juglans regia* cv. Chandler shows that individuals reproduced *in vitro* by culture of nodal segments have earlier fruit production as compared with those obtained by traditional grafting techniques, besides presenting a stronger rooting system and avoiding incompatibility between rootstock and scions (Hasey et al., 2001; López, 2001).

The major problem associated with *in vitro* introduction of walnut mature material is the endogenous bacterial contamination and phenolic compound exudation. However, the use of renewed material from consecutive pruning or macrografting allows us to develop shoot tips with high growth rates (Claudot et al., 1992; Leslie & McGranahan, 1992), a practice that could reduce such problem in later phases of the culture. The use of annual growth shoot tips (Leslie & McGranahan, 1992), meristems (Meynier, 1984) as well as tips obtained from epicormic sprouts appear to be the best alternatives for introduction of explants from field to *in vitro* conditions (Heile-Sudholt et al., 1986).

McGranahan & Leslie (1988) introduced nodal segments from Sunland, Vina and Chandler cultivars and concluded that their commercial propagation is possible. On the other hand, Navatel and Bourrain (2001) detected differences between genotypes with

respect to multiplication rates and elongation of internodal zones. Regarding the culture media used, and did not detect any quantitative difference in the multiplication rates, and concluded that larger diameter and more homogeneous microshoots were obtained in the DKW media (Driver & Kuniyuki, 1984). On the other hand, Marques and Dias (2001) reported that sucrose is the most appropriate carbohydrate source for *in vitro* walnut culture. Furthermore, time of cultivation does not affect the multiplication rate of mature material, but has an effect on the incidence of apical necrosis after the third subculture period.

Regarding gelling agents, Saadat and Hennerty (2002) obtained higher shoot proliferation both in MS media plus phytigel and DKW media plus phytigel (2.7 new shoot per explant). Best proliferation results were obtained with 1 mgL^{-1} of benzylaminopurine (BAP) plus 0.01 mgL^{-1} of indole-3-butyric acid (IBA).

With respect to the rooting phase, Saadat and Hennerty (2001) obtained higher percentage of rooted microshoots with IBA as compared to naphthalene acetic acid (NAA). They also recommended carrying out the root induction under dark conditions (9 days) in presence of auxins in the culture media, which permit to obtain up to 83% rooted microshoots. On the other hand, Sánchez-Olate (1996) indicates that the best rhizogenic response comes from apical shoot segments 2-cm long, showing a close relationship between auxin concentration and exposure time. Rooting rates of 85% are achieved inducing microshoots in MS media with major nutrients diluted to 25% and in complete darkness. The time of induction will depend on the auxin concentration applied. For a concentration of 3 mgL^{-1} the induction phase should last 7 days while for a concentration of 5 mgL^{-1} the induction phase should be 3 days. However, there are differences in rooting rates depending on the cultivar used (Vahdati et al., 2004).

This work presents the method for micropropagation of mature tissues of *Juglans regia* L. growing in field conditions, and describes the establishment, multiplication, rooting and microplant acclimatization phases.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Growing Conditions of Mother Plants

Plants growing in the field. Material showing glabrous shoot tips with 2–4 week growth, 0.5–0.8 cm diameter, 2–5 cm internodes and presence of vegetative leaf buds is collected from selected trees growing during the spring season (Figure 1A). The material must be transferred to the lab using an antioxidant solution (cysteine and ascorbic acid at concentrations of 20 and 5 mgL^{-1} , respectively) and a fungicide solution (Captan and Benomil at concentration of 1 gL^{-1} each).

Greenhouse plants. *Juglans regia* plants grafted over *Juglans nigra* are maintained in the greenhouse conditions with biweekly applications of a fungicide mixture of Benlate (Dupont®), Kasumin (Hokko Chemical®, Tokyo) and Sanagráfica (Agrocros S.A., Spain), in order to reduce superficial contamination levels. Scion comes from adult trees (25 years), while the rootstock is a 7-year-old plant (Figure 1B).



Figure 1. A) Walnut mother trees where initial material for *in vitro* vegetative multiplication is obtained are growing in the field. B) Grafted walnut plant growing in the greenhouse conditions.

2.1.2. Explant Excision and Sterilization

Excision. Plants growing in the field: Late winter (August) and early spring (September), semi-woody branches 2–4 years old, 20 cm long and having active vegetative leaf buds from mature walnut tree are collected (Figure 2A). They are submitted to a superficial disinfection process with a mixture of fungicides (Captan and Benomil, 1 gL^{-1} each) in a laminar flow chamber and continuous stirring for 20 min. Branches are then washed three times with sterile distilled water and placed on filter paper in the laminar flux chamber until dry. Subsequently, they are left in 200 cc bottles under sterile conditions, with 50 ml water and sealed with translucent plastic bags (Figure 2A). The system is left in cultivation chamber during a 16-hour photoperiod ($40\text{--}45 \mu\text{Em}^{-2}\text{s}^{-1}$) at $25 \pm 1^\circ\text{C}$ until vegetative leaf bud shooting.

Greenhouse plants. Nodal segments containing 2 or 3 vegetative leaf buds are extracted from grafted plants maintained in greenhouse. They are transferred to the lab in an antioxidant aqueous solution as explained previously in section 1.1. Sprouts are cut in laminar flux chamber keeping one vegetative leaf bud per knot (Figure 2B) and making superficial washing in solution of Kasumin[®] (0.5 g L^{-1}) and Captan[®] (3 g L^{-1}).

2.1.3. Sterilization

Plants growing in the field. Vegetative leaf buds approximately 3 cm long are cut from branches under laminar flux chamber conditions. Superficial asepsis is carried out in hypochlorite solution (1.5 gL^{-1} active chlorine) in continuous stirring for 10 min. They are then washed three times in distilled water and put into an antioxidant



Figure 2. Type of plant material to be introduced in vitro. A) Branches collected from adult tree growing in field and induced to vegetative leaf bud development. B) Nodal segments obtained from grafted plants growing in greenhouse conditions.

aqueous solution containing 100 mgL^{-1} polyvinylpyrrolidone (PVP) until transferred to culture media.

Greenhouse plants. The shoot tips excised from the donor plant are dried at temperature of 50°C for 1 hour in a double boiler. Afterwards, they are submerged for 30 min into a mixture of fungicides (Captan and Benomil, 1 gL^{-1} each) in a laminar flux chamber. By this time, the shoot tips are washed in sterile distilled water and transferred into stirred sodium hypochlorite solution (2.5 gL^{-1} of active chlorine) for a 10 min period. Finally, they are washed three times with sterile distilled water. The nodal segments, approximately 3 cm long, and vegetative leaf buds are placed in a Petri dish containing an aqueous solution with 2 gL^{-1} of DIECA (diethyl carbamate acid, tri-hydrated sodium salts, Aldrich®).

2. 2. Culture Medium

2.2.1. Media Composition

Explants must be dried first on sterile filter paper before they can be cultivated in test tubes containing 10 ml DKW (Driver & Kuniyuki, 1984) (Table 1), diluted macro-nutrients at 25% concentration, without hormones and solidified with a 2.5 g L^{-1} gelrite (Phytigel, Sigma®). Prior sterilization, pH must be adjusted to 5.8.

2.2.2. Media Preparation

In order to prepare the DKW-based media variants, powdered media supplied by DUCHEFA Biochemie B.V. were used (Table 1). Stock solutions of major and minor nutrients, vitamins, Na and Fe EDTA, ten times their concentration for easy culture media shall be prepared. The high amount of auxin (1 mg ml^{-1} IBA) was dissolved separately with a few ml of under natured ethanol (70%) before it was

added to the total quantity. After adjusting the pH, the media were heated until all compounds were completely dissolved. After adjusting pH, phytigel was added and the heated media until all compounds were completely dissolved. While stirring carefully, the media was distributed in cultivation bottles. Twenty five ml media were filled into 100 ml culture vessels and 15 ml media were filled into 150 mm long and 25 mm diameter test tubes. After closing with aluminum foil, the media were autoclaved at 121°C, at a pressure of 105 KPa for 20 min.

2.3. *In Vitro* Shoot Multiplication

Stretched leaf buds are cultivated in test tubes with 10 ml DKW medium containing BAP (1 mgL^{-1}), IBA (0.01 mgL^{-1}), 100 mgL^{-1} PVP, 50 mg L^{-1} streptomycin and solidified with phytigel (2.5 gL^{-1}). Tubes are kept in a growth chamber for a 16-hour photoperiod ($40\text{--}45 \text{ }\mu\text{Em}^{-2}\text{s}^{-1}$) at $25 \pm 1^\circ\text{C}$. In the establishment phase, 50 mgL^{-1} of streptomycin are added to avoid endogen bacteria to arise. The establishment of proliferation phase is carried out by selecting explants with the best proliferation and lowest contamination rate (Figure 3A). Sub-cultivation of basal and apical tips and entire shoots in fresh culture media occurs every 30 days in order to increase the production of microshoots (Figure 3B) followed by the rooting phase. *Juglans regia* does not present spontaneous rooting but induced one instead, so it is considered to be a recalcitrant species.



Figure 3. *In vitro* introduction of adult walnut plant material on DKW culture medium. A) Nodal segment with vegetative leaf bud activation after 15 days in cultivation. B) Production of multiple shoots after 30 days of culture.

Table 1. Formulation of culture medium used for walnut micropropagation based on modified DKW salt augmented with culture stage-specific plant growth regulators.

Stock	Component	Chemical formula	Stock concentration (g L ⁻¹)	Medium concentration (mg L ⁻¹)
<i>Major nutrients, 10 × stock, use 100 ml per L medium</i>				
A	Ammonium nitrate	NH ₄ NO ₃	14,16	1416,0
	Calcium chloride- 2H ₂ O	CaCl ₂ × 2H ₂ O	1,47	147,0
	Calcium nitrate- 3H ₂ O	Ca(NO ₃) × 3H ₂ O	18,11	1811,0
B	Potassium orthophosphate	KH ₂ PO ₄	2,58	258,0
C	Potassium sulfate	K ₂ SO ₄	15,6	1560,0
D	Magnesium sulfate- 7H ₂ O	MgSO ₄ × 7H ₂ O	7,4	740,0
<i>Minor nutrients, 500 × stock, use 2 ml per L medium</i>				
E	Boric Acid	H ₃ BO ₃	6,2	12,4
	Cupric sulfate- 5H ₂ O	CuSO ₄ × 5H ₂ O	0,025	0,05
	Manganese sulfate-H ₂ O	MnSO ₄ × H ₂ O	16,9	33,8
	Sodium molybdate-2H ₂ O	Na ₂ MoO ₄ × 2H ₂ O	0,25	0,5
	Zinc sulfate-7H ₂ O	ZnSO ₄ × 7H ₂ O	10,6	21,2
	Potassium iodide	KI	0,83	1,66
	Cobalt chloride- 6H ₂ O	CoCl ₂ × 6H ₂ O	0,025	0,05
<i>Amino acids 100 × stock, use 10 ml per L medium</i>				
F ₁	Glycine		0,1	1,0
	Cysteine		0,1	1,0
<i>Vitamins 100 × stock, use 10 ml per L medium</i>				
F ₂	Nicotinic acid		0,1	1,0
	Tiamine HCl		0,1	1,0
	Pyridoxine		0,1	1,0
	Calcium Pantenonate		0,1	1,0
	Biotin		0,001	0,00001
<i>Myo-Inositol, 500 × stock, use 2 ml per L medium</i>				
G	Myo-Inositol		50	100,0
<i>Iron 100 × stock, use 10 ml per L medium</i>				
H	Ethylenediamine tetraacetic acid disodium and iron	Na and Fe EDTA	3,67	36,7

<i>Other additives</i>	<i>Medium Concentration (g L⁻¹)</i>	
sucrose	30	
Polyvinylpyrrolidone	1	
Phytigel	2,5	
<i>Plant growth regulators add according to culture stage</i>		
	<i>BAP(mg L⁻¹)</i>	<i>IBA (mg L⁻¹)</i>
Establishment	—	—
<i>In vitro</i> shoot multiplication	1	0,01
Rooting	—	4

2.4. *In Vitro* Rooting

Upon clone establishment, rooting essays are conducted by adding 4 mgL⁻¹ IBA to the MS medium (Murashige & Skoog, 1962) during a 3-day darkness induction period, followed by a 27-day manifestation period under a 16-hour photoperiod in DKW medium, where macronutrients are diluted at 25%, mixed with vermiculite:perlite (220/250 v/v) and solidified with phytigel (Sigma[®]).

Microshoot type and size used are essential to get optimum rhizogenic response. Microshoots more than 2.5 cm long with apical leaf bud have higher rooting rates and the new roots formed are more fibrous (Figure 4).

The rhizogenic capacity of walnut depends on the endogenous content of auxin and the exogenous concentration to be used (Feito et al., 1997; Ríos et al., 1997, 2002, 2005) and is always preceded by basal callus formation. However, their minimum

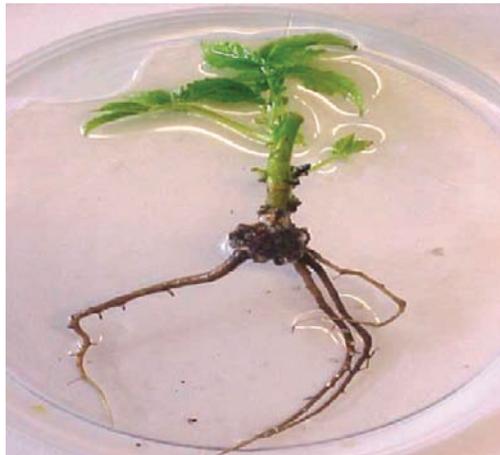


Figure 4. Induced rooting system in *Juglans regia* microshoots, after 30 days of culture in a mixture of perlite/vermiculite substrate.

formation favors the expression of root primordia, giving place to emergency of a significantly larger number of roots, which assures higher survival rate during the acclimatizing and hardening phases of the new microplants (Sánchez-Olate et al., 1997, 2005).

2.5. Microplant Acclimatization

Upon completion of the rooting manifestation phase, the microplants are extracted from the nutritive medium and washed in running water in order to eliminate all the substrate adhered to their root system. Subsequently, they are planted in polystyrene expanded trays with 84 cavities of 100 cc of sterilized substrate, which consists of a mixture of perlite:vermiculite (1:1, v/v), and maintained under greenhouse conditions (Figure 5). During the first culture week, the plants are irrigated by nebulization using a fogger, at a frequency of 15-min and 10-sec duration, ensuring that humidity is kept close to 100%. Upon observation of apical leaf bud growth, irrigation frequency must diminish (e.g. every 30–45 min for 10 sec, according to the greenhouse conditions) and ventilation must increase. Finally, during the full growth phase, irrigation events become more distanced and the nursery adaptation phase begins gradually.



Figure 5. Micropropagated plantlets of *Juglans regia*, 6 weeks after transfer to soil. A) Acclimatized plants. B) Induced root system view after 30 days in greenhouse acclimatization.

After hardening, the micropropagated plants are transplanted to the nursery if they show sufficient level of lignification. Either growing in containers or rows in the ground, the plants must be kept in nursery for later use in establishment of clonal orchards.

3. CONCLUSIONS

The main problem of propagation of *in vitro* mature material of walnut is the endogenous contamination of initial explants, where the key is the use of antibiotics in the means of cultivation. Tissue oxidation during the superficial asepsis of explants induces a low production of microshoots when obtaining microplants. Difficulties related to material induction are, at a great extent, solved by selecting shoots and nodal segments with semi-woody tissue, which makes them resistant to disinfection products. Although the rate of proliferation of material in the first subcultures is initially low, it will increase over the maintenance period and during the transfer to fresh cultures every 30 days. Finally, successful rooting rate can be reached using both *in* and *ex vitro* treatments, beginning the acclimatization period of microplants under greenhouse conditions (relative humidity of 100% and temperature in the range from 18 to 20°C).

The future appears promising for walnut biotechnology, particularly in commercial production. Although the sale price of *in vitro* walnut is high and has not met yet the consumer expectations, it may be reduced by research in automation, bioreactor, and synthetic seed technologies.

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CHAPTER 36

TISSUE CULTURE PROPAGATION OF MONGOLIAN CHERRY (*PRUNUS FRUTICOSA* L.) AND NANKING CHERRY (*PRUNUS TOMENTOSA* L.)

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1. INTRODUCTION

Mongolian cherry (*Prunus fruticosa* L.) and Nanking cherry (*P. tomentosa* L.), the fruit-bearing shrubs, are well adapted to severe winter conditions of Canadian Prairie Provinces. Although native to China, both species are well known in North America and are transcontinental in distribution (Knowles, 1975). Beside their ornamental value, there are selections that have relatively large, good quality fruits suitable for processing. Both *P. fruticosa* and *P. tomentosa* are not self-fruitful, two or more genotypes are needed for cross-pollination and good fruit set. Fruits in both species are cherry-like, round, fleshy and red in color (Figure 1). There are additional two phenotypes of Nanking cherry that bear pinkish-white and very dark-red, almost black fruit. The fruit growers in Western Canada are currently considering these two species as new fruit crops that could be commercially grown in the severe climate of the prairies. This new commercial interest in Mongolian and Nanking cherry cultivation, based solely on the selection of superior clones from wild shrubs, indicates possibilities in the genetic improvement and utilization of these species. To make large scale cultivation and an effective breeding viable, an efficient multiplication method of the superior clones will have to be established. Micropropagation has become a reliable and often routine method for a mass production of newly developed plant cultivars.

Micropropagation has been successfully used for production of other fruit-bearing, native prairie shrubs such as *Amelanchier alnifolia* Nutt., the Saskatoon berry (Pruski et al., 1990, 1991), *Prunus pensylvanica* L., the pincherry (Pruski et al., 2000), and *P. virginiana*, the chokecherry (Pruski et al., 2000; Zhang et al., 2000). Recently, successful micropropagation methods for *P. fruticosa* L. and *P. tomentosa*

L. have also been reported (Ping et al., 2001; Pruski et al., 2005). Tissue culture protocols have been developed for propagation of several other species of *Prunus* including *P. cerasus* L. (Boxus & Quoirin, 1977; Borkowska, 1985), *P. tenella* (Alderson et al., 1987), *P. insititia* L. (Loreti et al., 1988), *P. avium* L. Hammatt & Grant, 1996), *P. armeniaca* (Murai & Harada, 1997), 'Hally Jolivette' cherry (Lineberger, 1983), several cherry rootstocks, including *P. cerasifera* × *P. munsoniana* (Dalzotto & Docampo, 1997), *P. persica* × *P. amygdalus* peach rootstock (Marino & Ventura, 1997) and *Prunus* spp. (rootstocks: MrS 2/5, Ferdor Julior and San Julia Hybrid N-01) (Radice et al., 1999). There is very limited information on work on tissue culture propagation of Mongolian cherry and Nanking cherry. Most of the work on these two cherry species has been done on micropropagation, and there is no information available on embryo, fruit, anther, or callus culture. Although tissue culture propagation has been used for a number of cherry species for over 20 years, the summary below is the first compilation of the experimental protocols for micropropagation of *P. fruticosa* and *P. tomentosa*.

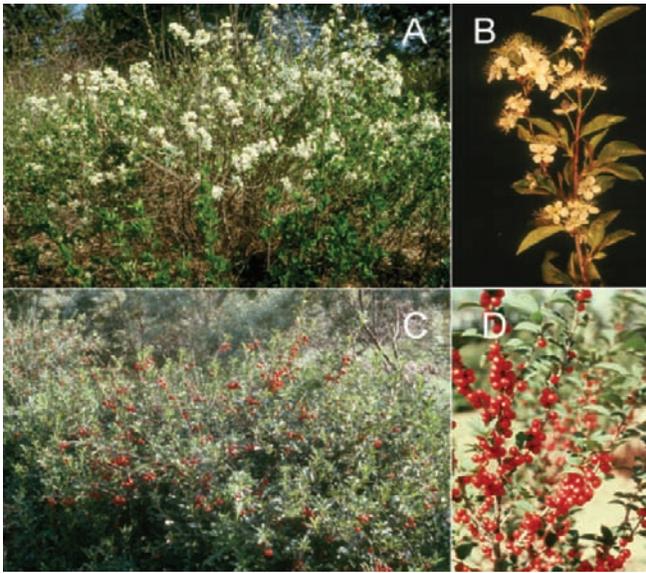


Figure 1. A) Mongolian cherry shrub in bloom, B) A branch of Nanking cherry with flowers, C) Fruit of Mongolian cherry, D) Fruit of Nanking cherry.

2. EXPERIMENTAL PROTOCOL

2.1. Materials

1. Sodium hypochlorite (NaOCl) in a form of diluted commercial bleach, Javex 5% chlorine, Tween-20 (surfactant), sterile distilled water.

2. Flow hood, orbital shaker, sterile Petri dishes, Erlenmeyer flasks, glass test tubes (15 × 150 mm) with plastic caps, Magenta GA7 vessels, pipettes, forceps, scalpels, stainless steel surgical blades.
3. Fully controlled environment tissue culture growth-room.
4. Culture media (MSMO – see Table 1), Bacto agar.
5. Plant Growth Regulators (PGRs): 6-benzylamino purine, Thiadizuron (TDZ), indole-3-butyric acid (IBA), indole-acetic acid (IAA), naphthalene acetic acid (NAA), gibberellic acid (GA₃).
6. Terminal dormant buds, shoot tips, regenerated apical or axillary shoots.

2.2. Methods

The protocol would require: 1) the establishment of *in vitro* cultures from either dormant or active buds, or shoot tips; 2) multiplication of shoots, 3) rooting either *in vitro* or *ex vitro*, 4) acclimatization and 5) transplanting to the field.

2.2.1. Explant Preparation

To obtain true-to-type propagated plants via tissue culture methods, care must be taken in both the choice of explant and the method of multiplication. Most often shoot tips and meristems, as well as dormant (winter) or active (spring) terminal buds are the explants of choice due to their genetic stability. Explant size is not that important for micropropagation purposes as for obtaining disease-free plants. A general approach is: the smaller the explant, the less the possibility of bacterial and fungal contamination. In our laboratory, cultures of *P. tomentosa* (a black fruit clone) were established from terminal buds and dissected up to 3 mm by removing the outer scales (Figure 2A) showed no sign of contamination after over 1 year of continued culture, while cultures obtained from stem pieces with lateral buds developed bacterial contamination after several months in culture. Once established, explants of both Mongolian and Nanking cherries form small rosettes and these are later moved to the multiplication stage.

2.2.2. Growing Conditions of Mother Plants

Explants of Mongolian cherry and Nanking cherry used in our work were taken from 10-year-old plants grown in the shelterbelt row at Crop Diversification Centre North, Edmonton, Alberta, Canada (53° N latitude, 113° W longitude). The plants were derived from rooted cuttings taken from F1 seedlings selected for fruit size and flavor. Terminal (dormant) buds were dissected from current year growth in December. December collection was chosen to avoid winterkill conditions that occur on the Canadian prairies between January and March due to frequent freezing and thawing periods (Pruski et al., 2000). In subsequent experiments, active young buds collected in early May and actively growing young shoot tips collected in early June were also used as explant sources with various success (Pruski, unpublished). Actively growing stem tips of *P. fruticosus* field grown trees (Liaoning, China) were also successfully used as explants by Ping et al. (2001).

2.2.3. Explant Excision and Sterilization

1. Collect the dormant buds (winter) or active buds (early spring) or actively growing shoot tips, 1–2 cm long (spring to early summer) by cutting off short twigs and take the twigs to the laboratory.
2. Remove buds with sharp scalpel, place them in a small paper bag in a small container (i.e. a baby food jar) and place the container under the stream of tap water for one hour; if shoot tips are used, remove outer leaves and proceed as with to bud explants.
3. Peel off outer scales from the buds. If shoot tips are used, proceed to step 4.
4. Surface-sterilize by agitation (orbital shaker) in 0.5% sodium hypochlorite (10 X diluted commercial bleach, Javex) containing 0.1% Tween-20 (surfactant) for 10 min.
5. Rinse the explants three times in sterile distilled water.
6. Trimmed to approximately 3 mm length.
7. Place the explants on 10–12 ml medium in culture tubes (25 × 150 mm) and close with plastic caps.
8. Incubate cultures for 4 weeks in a growth-room under conditions described in the next section (2.1.2).

2.2.4. Culture Medium, Its Preparation and Culture Conditions

The basic culture medium contained MS salts and vitamins (Murashige & Skoog, 1962) (MSMO medium – Murashige and Skoog Minimal Organic) supplemented with sucrose at 30 g l⁻¹ is used for growing cultures of Mongolian and Nanking cherry. The components are listed in Table 1. Vitamins and organic compounds other than plant growth regulators (PGR) are generally not changed in the different stages and are as in Table 1. Different auxins and cytokinins at various concentrations are used depending on the stage of micropropagation. One cytokinin, 6-benzylaminopurine (BA) is used in establishment of cultures, generally at 4.4–8.8 µM, together with an auxin, indole-3-butyric acid (IBA), or naphthalene acetic acid (NAA), at 0.5 µM. In multiplication stage the concentration of BA in culture media is generally higher, 8–20 µM. Also another cytokinin, thidiazuron (TDZ) can be used at 0.5–1.0 µM concentration. At the rooting stage, IBA alone (at 5–10 µM) or in combination with NAA (at 2.7 µM) is used and cytokinin is omitted. Prior to rooting stage, proliferating cultures of both *P. fruticosa* and *P. tomentosa* are placed on PGR-free media for the elongation and growth of both shoots and possible root initials.

Culture conditions follow a common pattern for most of the woody plant species. Culture media are adjusted to pH 5.5–5.7 prior to autoclaving, and are solidified with agar at 0.6–0.7%. Various cultures containers are used to grow the cultures. For initiation of cultures, test tubes are used, while for multiplication the Magenta G7 containers (50 ml of medium) or baby food jars (30 ml of medium) with Magenta caps are the most common. Cultures are incubated usually for 4 weeks in environmental chambers at 24/22°C day/night temperature, 16-h photoperiod at 150 µEm⁻²s⁻¹ mixed fluorescent and incandescent illumination. Subcultures of plant material to fresh medium are made every 3–4 weeks. If kept too long without transfer, for example over 6 weeks, senescence process starts and the cultures go to dormancy after 7–8 weeks.

Table 1. Murashige and Skoog Minimal Organic (MSMO) medium (Murashige and Skoog, 1962).

	<i>Compound</i>	<i>Amount [mg l⁻¹]</i>
<i>MS Macro Nutrients</i>	NH ₄ NO ₃	1650
	KNO ₃	1900
	CaCl ₂ · 2H ₂ O	440
	MgSO ₄ · 7H ₂ O	370
	KH ₂ PO ₄	170
<i>MS Micro Nutrients</i>	H ₃ BO ₃	6.2
	MnSO ₄ · H ₂ O	16.9
	ZnSO ₄ · 7H ₂ O	8.6
	KI	0.8
	Na ₂ MoO ₄ · 2H ₂ O	0.25
	CuSO ₄ · 5H ₂ O	0.025
	CoCl ₂ · 6H ₂ O	0.025
<i>Iron Sources</i>	FeSO ₄ · 7H ₂ O	27.8
	Na ₂ EDTA	37.3
<i>Vitamins</i>	Thiamine HCl	0.4
	Pyrodixin HCL	0.5
	Nicotinic acid	0.5
<i>Organic constituents</i>	Inositol	100
<i>Sucrose</i>		Varies
<i>Agar</i>		Varies

2.2.5. Shoot Regeneration and Maintenance

As in other woody plant species, genetically uniform plants of Mongolian and Nanking cherry are obtained from cultures established from shoot tips, buds or meristems. Other organs and callus would provide a risk of inducing variability (Druart & Gruselle, 1986). Thus, suitable PGR combinations and concentrations have to be used to differentiate new axillary buds (rosettes) and to develop into shoots without callus formation. In our work, MSMO media were supplemented with combinations of various PGRs for initiation and establishment of cultures. The optimal combinations for culture establishment and performance have been determined following a number of experiments and are presented in Table 2. The combination of IBA and/or NAA with 8.9 µM BA is the best for initiation of both Mongolian and Nanking cherry cultures. The number of rosettes produced on media with 8.9 µM BA is significantly higher than on media with 4.4 µM BA.

Initially, one explant (dormant bud) was placed on medium to establish the culture. Following 4 weeks in culture, approx. 90% explants survived. Most of them produced a single rosette and a few produced two rosettes (out of 30 explants). For Mongolian cherry media supplemented with lower concentration of BA (4.4 µM)

can also be used (Table 2). Prediction of the number of rosettes per explants is an important element in multiplication scheduling for both Mongolian and Nanking cherry cultures.

Table 2. Effects of growth regulators on culture initiation and performance (rosette production) and % survival of Nanking cherry and Mongolian cherry (Pruski et al., 2005).

Treatment	Number of rosettes per established culture			
	Mongolian cherry		Nanking cherry	
NAA 0.5 μ M + BA 4.4 μ M	1.00	(39 ¹ , 90% ²)	0.97	(28, 90%)
NAA 0.5 μ M + BA 8.9 μ M	1.40	(45, 93%)	1.20	(37, 96%)
IBA 0.5 μ M + BA 4.4 μ M	1.30	(30, 96%)	0.93	(27, 80%)
IBA 0.5 μ M + BA 8.9 μ M	1.50	(42, 100%)	1.23	(36, 86%)

The results are given as the mean number of rosettes per explant. In brackets are the total number of produced rosettes and the percentage survival; n = 30 per each treatment and species.

¹ Total number of rosettes produced out of 30 explants.

² Survival percentage of cultures.

In general, the Nanking cherry cultures are more difficult to establish than the Mongolian cherry. The percentage survival varied from 80% to 96% while the Mongolian cherry was from 90% to 100% being the highest on media recommended above. Rosettes produced on initiation medium (Figure 2B) are placed on solid media in Magenta GA7 vessels for multiplication. In our experiments, shoot proliferation was conducted on MSMO medium with various concentrations of BA and TDZ. Following a number of repetitions we were able to determine optimal concentrations of BA for multiplication of both Mongolian and Nanking cherries. For each species, we calculated the value of BA concentration that maximizes the number of good quality shoots produced by proliferating cultures. Proliferating cultures and their rosettes are shown in Figure 2C. Summary of the regression analysis for shoot proliferation responses as affected by BA concentration is presented in Figure 3.

Controls (zero BA) usually produce ca. 1.5–2 shoots, approx. 25 mm long per rosette in both species. With increasing BA concentration in the medium, more but shorter shoots are produced. Above BA concentration of 15.00 μ M number of shoots per culture in both species starts to decrease. With increasing BA concentration in the medium length of shoots decreases. Hyperhydricity starts to show on media with high concentrations of BA where cultures become dark green, having short and fragile shoots with poorly developed leaves. In related species, shoot deformations of cultures grown on media with a high content of BA (22.5 μ M and above) are known and were reported by Linebeger (1983) for *Prunus* \times Hally Jolivette, Pruski et al. (1990) for Saskatoon berry (*Amelanchier alnifolia* Nutt.), and Pruski et al. (2000) for chokecherry (*P. virginiana* L.) and pincherry (*P. pennsylvanica* L.). For both Mongolian and Nanking cherries, BA concentrations 8–15 μ M are optimal for

shoot proliferation. Approximately 3–5 good quality, 16–20 mm long shoots, with well developed leaves (15–25 mm), are produced after 4 weeks in culture (Figure 3). The 8–15 μM level of BA in the medium is generally considered high, although it has been reported to be optimal for a number of related species including *Prunus* \times Hally Jolivette (Lineberger, 1983), *Prunus cerasifera* \times *Prunus munsoniana* rootstocks (Dalzotto & Docampo, 1997) and *P. virginiana* (Pruski et al., 2000). Thiadizuron (TDZ)



Figure 2. A) and B) Initiation of Mongolian cherry cultures, C) Proliferating cultures of Nanking cherry, D) In vitro rooted culture of Nanking cherry (black fruit selection, BNC).

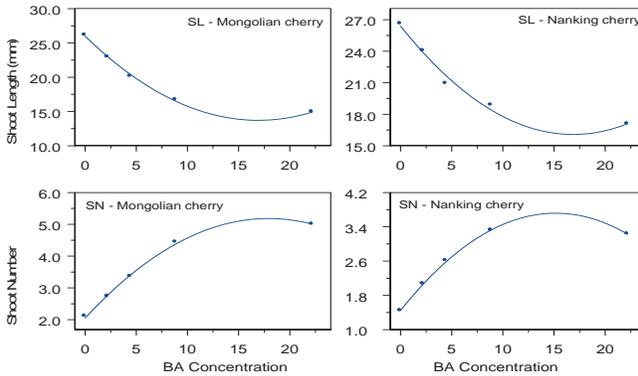


Figure 3. BA concentration effect on shoot number and shoot length in Mongolian Cherry and Nanking Cherry. Actual values (solid circles) and the fitted (solid lines) (Pruski et al., 2005).

can also be used successfully for shoot proliferation of Mongolian and Nanking cherry cultures, however at a very low level. As indicated in Table 3, compared to controls (no TDZ), the number of shoots is almost doubled on media containing 0.45 μM TDZ in both species after 4 weeks in culture. Higher TDZ concentrations induce much more shoots, but their length decreases drastically. Besides these short shoots, a mass of stunted dark green dwarf shoots are produced by cultures. Such responses to TDZ have been reported by a number of researchers in a variety of woody plant species (Bates et al., 1992; Sarwar and Skirvin, 1997; Thengane et al., 2001). Based on the observations from our studies, a TDZ concentration of 0.5 μM is optimal for production of good quality Mongolian and Nanking cherry shoots. TDZ has been reported to be a potent growth regulator of *in vitro* plant morphogenesis (Mante et al., 1989; Bates et al., 1992; Murthy et al., 1998).

Procedure for multiplication of shoots:

1. Transfer the established rosette cultures from initiation media in test tubes to proliferation media in Magenta GA7 vessels: MSMO supplemented with 18 μM of BA for Mongolian cherry and with 15 μM of BA for Nanking cherry, or with TDZ at 0.5 μM for both species.
2. Place five rosettes per vessel.
3. Incubate cultures for 4 weeks in a growth-room under conditions described in section 2.2.
4. Repeat the procedure until the desired number of shoots/rosettes for rooting is achieved.
5. Place the proliferating cultures on hormone-free media 4 weeks prior to the rooting stage.

Table 3. TDZ concentration effect on shoot number and shoot length in Mongolian Cherry and Nanking Cherry (Pruski et al., 2005).

Species	Treatment TDZ (μM)	Average number of shoots	Average length of shoots (mm)
Mongolian cherry	0.00	2.1	26.1
	0.45	4.2	20.6
	0.90	4.5	15.4
	1.80	5.0	13.7
Nanking cherry	0.00	1.4	26.6
	0.45	2.1	23.5
	0.90	3.2	18.4
	1.80	4.5	15.4

2.2.6. Rooting

Root differentiation is stimulated by auxins that usually are applied to the base of the shoots. Root induction may take place in different ways, the most often IBA is included in the rooting medium and the cytokinin is omitted. Also, when rooting *in vitro*, the concentration of salts in rooting media is reduced significantly, often by 50%. Figure 2D presents *in vitro* rooted plantlet of black fruiting Nanking cherry.

Similar to other fruit-bearing woody species (Maene & Debergh, 1983; Pruski et al., 1991; Harada & Murai, 1996; Pruski et al., 2000), *in vitro* produced shoot rosettes of both Mongolian and Nanking cherry produce roots readily *ex vitro* when they are treated with auxins. On average, shoots of Mongolian cherry are somewhat easier to root (average 73% rooting) than shoots of Nanking cherry (63%). Treatment with auxin(s) definitely enhances rooting percentage compared to untreated controls. Dai et al. (2001) reported effectiveness of IBA application for rooting of Mongolian cherry. The best rooting has been observed when IBA/NAA combination is used (Table 4). In our experiments, rooting was performed in two ways, *in vitro* and *ex vitro*. The rooting *ex vitro* method was superior to the *in vitro* system. The peat moss/perlite (1:1 v/v) mixture was packed in Hillson-type plastic rootainers with 32 cells per tray and one *in vitro* derived shoot was placed in each cavity. Trays were kept on the bottom heated (25°C) greenhouse bench equipped with an intermittent misting system. The auxin treatments were introduced as daily watering for the first week of the rooting period. A commercial rooting powder, Rootone F containing 0.057% IBA/0.067% NAA mixture, was applied as a basal dip on microcuttings prior to planting.

Rootone F proved to be almost equally effective as IBA/NAA combination. Mongolian and Nanking cherry plantlets, rooted on the greenhouse bench under mist,

can tolerate transplant stress better than the plantlets rooted *in vitro* that lack well developed root hairs. Poor adventitious root formation is a major obstacle in micropropagation systems (de Klerk, 2002). The conditions during *in vitro* rooting treatment(s) may have a profound effect on performance of plantlets after transfer *ex vitro*. Particularly, accumulation of ethylene during *in vitro* rooting can have a devastating effect (de Klerk, 2002). This problem is avoided when rooting is performed *ex vitro*. Auxin concentrations optimal for root initiation during rooting *in vitro* can be inhibitory for root elongation (Maene & Debergh, 1983). Rooting *ex vitro* under intermittent mist creates favorable leaching conditions, where the initial concentration of auxin is high and decreases with each application of mist.

Table 4. Effects of the auxin treatment (Control, IBA, IBA + NAA and Rootone F) on percentage of rooted plantlets.

Treatment	% Rooting	
	Mongolian cherry	Nanking cherry
Control	58.5	59.1
IBA	63.4	64.0
IBA + NAA	79.0	72.3
Rootone F	73.6	67.8

IBA concentration 9.8 μM , NAA concentration 2.7 μM .

Procedure for Rooting of Shoots:

1. Following the elongation stage on hormone-free media (4 weeks), separate the rosettes to individual shoots.
2. If rooting *in vitro*: transfer these shoots to the rooting media, $\frac{1}{2}$ MSMO supplemented with 20 g l^{-1} sucrose and 2.4–4.8 μM IBA and incubate in the growth room for 4–5 weeks under conditions described above.
3. If rooting *ex vitro*: transfer the individual shoots into rootainers trays filled with moist soilless mix and place them on the bottom heated (25°C) greenhouse bench under intermittent mist.
4. For the first week, water the trays with IBA/NAA solution at 9.8/2.7 μM .
5. If using the rooting powder: dip the bottom end of microcuttings in powder and place them individually into the trays' cavities; place the trays the bottom heated (25°C) greenhouse bench under intermittent.
6. Keep the plantlets in the mist bench for 3–4 weeks gradually reducing frequency of misting with time; start watering the plants (as needed) with a water soluble fertilizer NPK 20-20-20 at 1 g l^{-1} towards the end of the second week in the misting bench; misting is usually not needed by the end of the third week.

2.2.7. Dormancy Factor and Hardening

Poor winter survival of rooted cuttings is a common problem when shoot extension growth ceases following rooting. Harris (1976) observed that softwood cuttings of a Saskatoon berry (*Amelanchier alnifolia* Nutt.) behave in this fashion. He also noted (Harris, 1980) that *in vitro* – rooted Saskatoon plantlets cease growth and loose their leaves right after transplantation to the greenhouse bench. Furthermore, we observed that sometimes plantlets in culture tend to loose leaves and stop growth even during the rooting period before transferring them to the greenhouse (Pruski et al., 1990, 1991). We have made similar observations with the Mongolian and Nanking cherries, although the dormancy symptoms were not that severe as with Saskatoon berry cultures.

Rooting in non-sterile conditions helps significantly in solving the dormancy problem since the *ex vitro* rooted plantlets do not require acclimatization to greenhouse conditions. However, some of the newly *ex vitro* rooted Mongolian and Nanking cherry plantlets can still show dormancy symptoms. Changes in levels of plant growth regulators are correlated with the onset of bud dormancy. Investigation of endogenous levels of PGRs in a number of plants showed a decrease in growth promoters, gibberellins and cytokinins, and an increase of the growth inhibitor, the abscisic acid (ABA) during the development of dormancy. With the breaking of dormancy, the activity of growth promoters increases. Experiments with exogenously applied PGRs have shown that dormancy in many species can be overcome by the use of both cytokinins and gibberellins (Forshey, 1982; Pruski et al., 1990, 2000). With Mongolian and Nanking cherry, a single foliar application (spray) of gibberellic acid (GA₃) at 200 ppm was beneficial in breaking dormancy of newly *ex vitro* rooted (6 weeks-old) plantlets (Pruski unpublished). The effect is noticeable within 48 h after application; terminal buds in newly rooted plantlets that were showing dormancy symptoms start to open. The rooted plants can be grown in root trainers until field transplant or can be transplanted to larger pots for the container production. The peat moss/perlite (1:1 v/v) mixture or a commercial Promix is usually used for growing the transplants.

2.2.8. Field Testing

The newly rooted plants were grown for 10–12 weeks under standard greenhouse conditions 24/20°C day/night temperature. This growing period seems to be sufficient, since size of plants after 10 weeks is adequate (15–25 cm) and such plants could be even be directly transplanted to the field.

There are many reports that a short acclimatization period of 5–10 days under shade, prior to field planting greatly improves transplant survival in the field (Harris, 1980; Struve & Lineberger, 1985; Pruski et al., 1990; Murai & Harada, 1997). Our data showed that the 10-day outdoor acclimatization period in the lath-house with 50% shade, enhanced field-transplant survival of Mongolian cherry transplants from 89.5% to 98% (data for Nanking cherry unavailable) (Table 5).

Table 5. The effect of outdoor acclimatization on % survival of field transplants of *Prunus fruticosa* (Pruski unpublished).

Acclimatization Period (days)	% Survival 3 month. after planting	% Winter survival
0	89.5	100
5	95.3	100
10	98.0	100

2.3. Molecular Marker Analysis

The search for biochemical and molecular markers for the genus *Prunus* was initiated relatively recently. Since morphological markers are time consuming and prone to equivocal interpretations the work on development of DNA markers (RFLPs, RAPDs) for fruit trees has been encouraged (Eldridge et al., 1992).

Initially, isoenzymes markers in *Prunus* crops like peach, almond and cherry were developed (Monet & Gribault, 1991; Arús et al., 1994; Boskovic & Tobutt, 1994). In addition to the isozyme markers RAPD, RFLP and AFLP (Arús et al., 1994) which are used to clearly distinguish the different stone fruit cultivars, additional markers, such as SSRs have recently been developed for peach (Cipriani et al., 1999). Although both the Mongolian cherry and the Nanking cherry belong to a genus group of *Prunus cerasus*, there is no reported work on development of DNA markers for these two species, most likely due to their lesser importance as fruit crops.

2.4. Cytology

2.4.1. Taxonomy

Both *Prunus fruticosa* and *P. tomentosa* belong to the subfamily *Prunoide*, and the family *Rosaceae*. The seven subgenera in *Prunus* are determined basically by how the leaves are rolled up in the bud, whether the flowers are organized in cymes or in racemes and by morphological characteristics of the generative organs (for example the color and size of flowers, fruit, stone and seed traits) (Strasburger et al., 1991). Both Mongolian and Nanking cherry belong to subgenus *Cerasus* and are included in the list below:

- AMYGDALUS (almonds): *P. amygdalus*, *P. bucharica*, *P. kuramica*, *P. nana*, *P. orientalis*, *P. webbii*
- PERSICA (peaches): *P. davidiana*, *P. ferganensis*, *P. kansuensis*, *P. mira*, *P. persica*
- ARMENIACA (apricots): *P. armeniaca*, *P. brigantiaca*, *P. mandshurica*, *P. mume*, *P. sibirica*
- PRUNUS (plums and prunes): *P. cerasifera*, *P. divaricata*, *P. domestica*, *P. insititia*, *P. italica*, *P. spinosa*, *P. syriaca*, *P. salicina*, *P. simony*,

- P. ussuriensis*, *P. Americana*, *P. angustifolia*, *P. hortulana*, *P. maritime*,
P. mexicana, *P. munsoniana*, *P. nigra*, *P. rivularis*, *P. subcordata*
 – CERASUS (sweet and sour cherries): *P. avium*, *P. cerasus*, ***P. fruticosa***,
P. japonica, *P. maackii*, *P. mahaleb*, *P. pseudocerasus*, *P. pumila*, *P. serrulata*,
P. tomentosa
 – PADUS (bird cherries): *P. padus*, *P. serotina*
 – LAUROCERASUS (bay-cherries).

2.4.2. Number of Chromosomes

In *Prunus*, the basic number in vegetative cells is eight chromosomes (Crane & Lawrence, 1952; Iezzoni et al., 1991). Polyploidy, due to interspecific hybridization, took place during the phylogeny of the genus and is responsible for self-sterility and intersterility. Polyploidy is important since plants with this character are often very vigorous and may possess a natural resistance to frost, diseases or insects. Among cherries, the sweet (*P. avium*) and sour (*P. cerasus*), flowering ornamental cherries, and the other that are used as rootstocks for cherries are important. The earliest description of so called 'keration' (the name of the fruit, *Ceratonia silqua* or carobtree) comes from Theophrastus about 300 B.C. *Prunus fruticosa*, the Mongolian cherry (also called the ground cherry), is considered the most probable parent of both *P. avium* and *P. cerasus* (Fogle, 1975).

The chromosome number of *P. fruticosa* is $2n = 32$ (tetraploid) (Izzoni et al., 1991). On the other hand, *Prunus tomentosa* is a diploid with the chromosome number of $2n = 16$ (Darlington & Janaki, 1945). For comparison, both Mongolian and Nanking cherry are included in Table 6 which illustrates the chromosome numbers for species classified in subgenus *Cerasus*.

Table 6. The number of chromosomes and degrees of ploidy for genus *Cerasus*.

Species	Chromosome number	Reference
<i>Prunus avium</i>	$2n=16$ (diploid), 24, 32	Darlington & Janaki, 1945 Bennett & Leitch, 1995
<i>P. besseyi</i>	$2n=16$ (diploid)	
<i>P. cerasus</i>	$2n=32$ (tetraploid)	Missouri Bot. Garden, 1985; Bennett & Leitch, 1995
<i>P. fruticosa</i>	$2n=32$ (tetraploid)	
<i>P. mahaleb</i>	$2n=16$ (diploid)	Darlington & Janaki, 1945
<i>P. pumila</i>	$2n=16$ (diploid)	Darlington & Janaki, 1945
<i>P. serrulata</i>	$2n=16$ (diploid), 24	Darlington & Janaki, 1945
<i>P. subhirtella</i>	$2n=16$ (diploid)	Darlington & Janaki, 1945 Bennett & Leitch, 1995;
<i>P. tomentosa</i>	$2n=16$ (diploid)	Darlington & Janaki, 1945

2.5. Storage of *in Vitro* Cultures

To stay competitive and adjust propagation scheduling to the market demands, the tissue culture laboratories store large number of stock cultures for various periods of time. Low-temperature storage of the *in vitro* stock material, commonly used in conservation of plant germplasm (Bessembinder et al., 1993; Withers & Engelmann, 1998), is being recommended. This method, if properly adjusted to specific genotypes, can substantially reduce labor and media costs (Westcott, 1981a,b; Withers & Engelmann, 1998). Temperature, light illumination (photosynthetic photon flux density or PPF), and medium composition need to be adjusted (Heines et al., 1992; Kubota & Kozai, 1993, 1994, 1995; Hansen & Kristiansen, 1997). Most of the commercial micro-propagation laboratories in Canada usually store their stock cultures for up to 3 months, either in refrigerators or small coolers with low light, at 4°C +/-1. Our studies on *in vitro* storage of Saskatoon berry and chokecherry cultures (Pruski et al., 2000) also included cultures of Mongolian and Nanking cherry (Pruski unpublished). Sucrose in media, light and the length of the storage period have had an impact on capacity to re-grow of the cultures, relative growth rate (RGR) as well as on quality of shoots produced by these cultures following the storage period. *P. virginiana* and *A. alnifolia* (Saskatoon) cultures stored very well without sucrose; although chokecherry benefited from sucrose in the storage medium when the stock cultures were kept at the low temperature for over 12 weeks. Similar observations were made with Mongolian and Nanking cherry cultures (Figure 4), even when stored for over

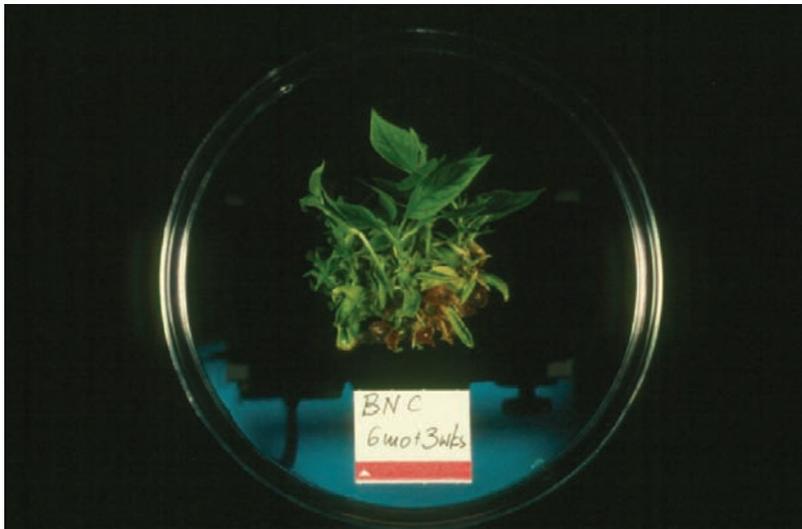


Figure 4. A culture of Black Nanking Cherry (BNC) stored for 6 months and re-grew for 3 weeks on proliferation media (Pruski unpublished).

6 months. In general, low light during storage improves quality of cultures. The chokecherry and Saskatoon cultures grew during storage, and the longer the stock plants were stored, the more vigorous cultures they re-grew. This indicates that a prolonged exposure to the low temperature reduces dormancy (Pruski et al., 2000). Cultures of Mongolian and Nanking cherry followed the pattern showing a strong capacity to re-grow after 6 months of storage. It took only 3 weeks to fully re-grow black Nanking cherry cultures (Figure 4).

3. CONCLUSION

Tissue culture techniques have been applied successfully to various types of cherries with different aims: breeding, propagation, and obtaining virus/disease-free plants. Although the Mongolian (*P. fruticosa* L.) and the Nanking (*P. tomentosa* L.) cherries are of lesser importance among the cherry crops, their adaptation to severe winter conditions of Canadian Prairie Provinces has made them important for fruit growers in these regions. Selections of both species became of economical importance to small fruit producers on the prairies increasing a variety of fruit crops that can commercially be grown in cold regions of the country. The demand for their fruit is growing steadily, so is the demand for true-to-type plant material. The tissue culture propagation offers a possibility to produce the plant material in a relatively short time and in required numbers. Also, the *in vitro* methods could be useful in new breeding programmes. Improvement programmes will likely focus on high productivity and fruit quality, resistance to diseases, and dwarf rootstocks. This micropropagation protocol is a powerful tool for a mass production of Mongolian and Nanking cherry plants that are coming from selection programmes aimed at fruit quality. It offers a possibility for a mass production of true-to-type plants that maintain fruit quality.

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CHAPTER 37

MICROPROPAGATION OF FIG TREE (*FICUS CARICA* L.)

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1. INTRODUCTION

Fig tree plantations cover 426.244 hectare area worldwide with an overall yield of 1.070.676 million tones. The largest fig producing countries are Turkey, Egypt, Iran, Greece and Algeria (FAOSTAT, 2006). The planting of the species occurs predominantly with vegetatively propagated plants, especially by the rooting of cuttings. This fact contributes significantly to the dissemination of pathogens which affect the yield potential of the crop. Through micropropagation of fig tree it is possible to obtain pathogen free plantlets and this is one of the basic requirements for a successful commercial orchard. Furthermore, the technique has the advantage of large-scale production, providing plantlets whenever needed. Aside the advantages of micropropagation, *in vitro* culture has also supported the research on transgenic plants obtained by genetic transformation.

2. EXPERIMENTAL PROTOCOL

2.1. Conditions of the Mother Plant

The mother plants must be in good phytosanitary and nutritional conditions in order to facilitate the decontamination of the explants during isolation. Spraying systemic agents solutions such as benomyl (Benlate) or Terramycin (oxytetracycline) with streptomycin sulfate (Agrimycin) is effective, when compared with non-systemic fungicides containing different active principles. These applications must follow a rigorous chronogram and the explants must be collected preferably between 24 and 48 h after the last spraying.

2.2. Establishment and Sterilization of Explants

The use of new shoots has been effective in establishing *in vitro* fig plants. Pruning is therefore recommended to induce sprouting or shoot collection after the dormancy period. The maintenance of the mother plants in the greenhouse allows the control and manipulation of the photoperiod, light intensity and temperature which stimulates new shoots, independent of the season of the year. In the greenhouse, the treatments with fungicides, bactericides and insecticides are convenient and helpful. The pruned branches should be covered with plastic bags and the newly developed branches are immediately recovered.

The material collected must be kept in the running tap water for some hours to wash away the dust particles, other contaminants, and remove phenolic substances that cause oxidation of the explants.

Several substances with germicidal action can be used for explant sterilization, such as ethanol and chlorine-based compounds, e.g. sodium (0.5–2%) and calcium hypochlorite. A spreader-sticker surfactant, e.g. Tween 20 (1 to 2 drops/100 ml), is normally used in order to increase the tissue penetration capability of the chlorine-based solutions and to improve the contact of the latter with the tissues. Ethanol 70% and 80% is generally used for a few seconds, since at higher concentrations it is less effective and can dehydrate the tissues quickly. Apart from the germicidal action, ethanol is also a surfactant and when applied in the beginning of the sterilization procedure, it may facilitate the action of the other compounds. After sterilization the explants are washed three to five times with distilled or deionized autoclaved water and cultured in the culture medium.

2.3. Culture Medium

The most adequate and effective culture medium for *in vitro* cultivation of fig tree depends on the explant type, cultivar and cultivation stage: establishment, multiplication and rooting. For *in vitro* cultivation of fig the MS (Murashige & Skoog, 1962) and the WPM (Woody Plant Medium) media are being used.

The Murashige & Skoog (MS) culture medium is widely used for plant tissue culture. Its high salt concentrations are responsible for significant gains in tissue and cell growth. The WPM is the second most used medium for *in vitro* cultivation (Lloyd & McCown, 1981). It was developed for culturing shoots of woody plants and has found widespread use in the propagation of bushes and trees.

Several culture media were tested for the micropropagation of 'Roxo de Valinhos' fig cultivar by Brum (2001), including MS, Knudson, WPM, and White and B5 media. Each medium was tested with four different sucrose concentrations (0, 15, 30 and 45 g L⁻¹). The results indicated a high number of shoots and excellent growth of roots and aerial parts for the WPM medium supplemented with 20 g sucrose. It is thus highly recommended for micropropagation of fig tree.

2.4. Culture Establishment by Shoot Tips

For the production of virus-free fig plants, the collection of shoot tips in different seasons of the year does not affect the viability and proliferation of sprouts. However, the addition of activated charcoal in the culture medium improved shoot development (Demiralay et al., 1998).

The age of the mother plant does not affect the establishment of shoot tip culture either. The taken of shoot tips from 10-year-old trees and its culture in MS medium supplemented with 1 mg L^{-1} 6-Benzylaminopurine (BA) and 1 mg L^{-1} 1-naphtalene acetic acid (NAA) was recommended by Gunver et al. (1998).

The following protocol has been efficient for 'Roxo de Valinhos':

1. Select the healthy mother plant (s) with young shoots (Figure 1A).
2. Collect young shoots and remove the infected leaves (Figure 1B).
3. Wash shoots under running tap water for 3 h, and then immerse them in 40% commercial sodium hypochlorite solution (0.2% NaOCl) containing 2 drops of Tween-20 as a surfactant for 20 min.

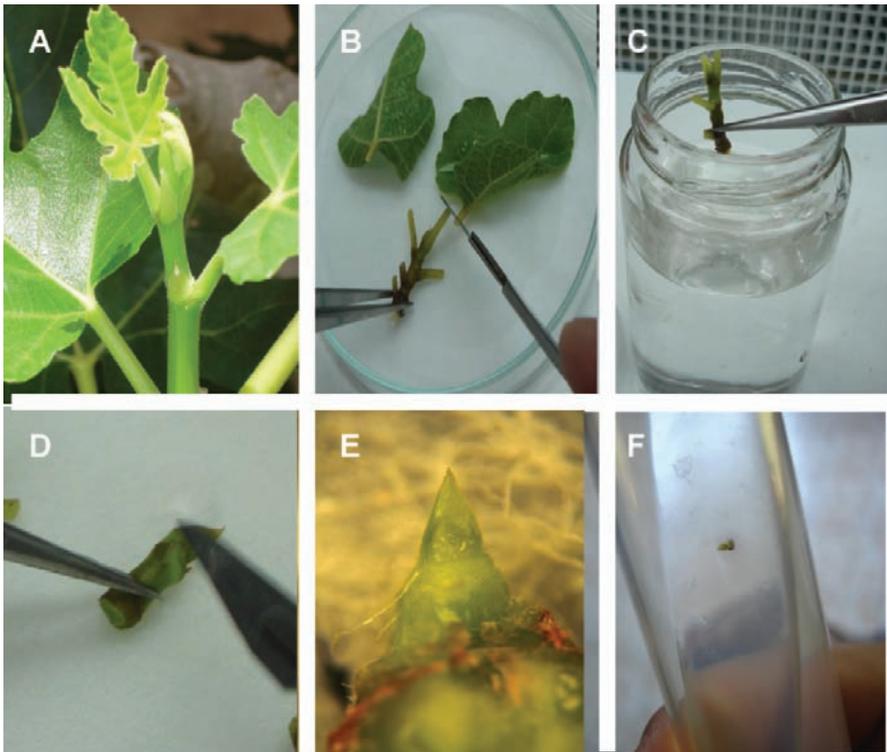


Figure 1. Some steps for shoot tip isolation from a fig tree. A) Young shoots of the mother tree, B) Sample preparation in the flow chamber C) Washing of the explant in the flow chamber, D) Excision of the leaf primordia, E) The shoot apex region amplified 70 times, F) Shoot tips in culture medium.

4. Rewash the shoots in autoclaved distilled water under laminar flow chamber in order to remove the excessive NaOCl (Figure 1C).
5. Remove the leaf primordial under a stereomicroscope to expose the shoot apex (Figure 1D).
6. Inoculate shoot explants as fast as possible in MS medium containing 0.39 mg L^{-1} BA. (Figure 1E).
7. Grow shoots at a temperature of $25 \pm 1^\circ\text{C}$, under a 16-h photoperiod with $35 \mu\text{mol m}^{-2} \text{ s}^{-1}$ of irradiance.
8. Transfer shoot tips to a fresh culture medium every time oxidation occurs until it is not found anymore.

2.5. Culture Establishment by Apical Buds

The *in vitro* culture establishment from apical buds is similar to that of shoot tips. However, for the selection of mother tree and shoots as well as for the explant preparation careful attention is required.

Oxidation (i.e. phenol leakage) in apical buds is less usual than in shoot tips and the frequent transfers of explants to the fresh culture medium at 2–3 day interval, for 5–7 weeks can minimize this fact. Add 0.39 mg L^{-1} BA to the MS culture medium, when there is no more sign of oxidation. The stages of apical bud development are presented in Figure 2. However, for different cultivars the concentrations of supplemented growth regulators and other ingredients may differ as presented in Table 1.

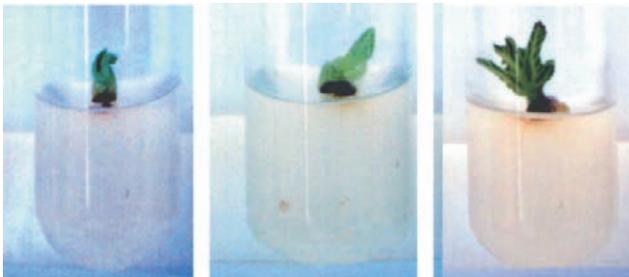


Figure 2. Development of apical buds of fig tree Roxo de Valinhos when cultivating on MS medium.

2.6. Culture Establishment by Leaf Segments

Leaf fragments of fig tree (*Ficus carica* L. ‘Masui Dauphine’) obtained from *in vitro* shoot cultures were cultured on MS medium supplemented with different combinations of 2, 4-D, TDZ, and 0.5 mM phloroglucinol. The addition of 2, 4-D induced root formation and the presence of phloroglucinol increased root formation significantly. When the combination of 2, 4-D and TDZ was added to MS medium

containing phloroglucinol, the explants produced adventitious buds on the edges. The addition of phloroglucinol was also effective in inducing adventitious bud formation. Regenerated plantlets were successfully established in soil after a short period of acclimatization. This is the first protocol of organogenesis and plant regeneration from vegetative organs of *Ficus carica* L. (Yakushiji et al., 2003).

Table 1. Plant growth regulators and other substances added to a MS medium on the *in vitro* establishment of different fig cultivars.

Cultivars	Growth regulator	Other compounds	Observations
Roxo de Valinhos ¹	0.39 mg L ⁻¹ BA		Apical buds transferred to a new medium weekly
Bursa	1 mg L ⁻¹ BA		Cultures in darkness
Siyahi ²	1 mg L ⁻¹ NAA		
Bursay	0.2 mg L ⁻¹ GA ₃		
Siyahi e Alkuden ³	0.5 mg L ⁻¹ BA		
Roxo de Valinhos ⁴	1 mg L ⁻¹ BA 3 mg L ⁻¹ GA ₃ 1 mg L ⁻¹ NAA	10 mg L ⁻¹ thiamine 2 mg L ⁻¹ nicotinic acid 12 mg L ⁻¹ pyridoxine 100 mg L ⁻¹ Myo-inositol 80 mg L ⁻¹ cysteine 3 g L ⁻¹ activated charcoal	Cultures in darkness

¹Anjos Sobrinho (1998); ² Gunver et al. (1998); ³ Demiralay et al. (1998); ⁴ Barbosa et al. (1992).

2.7. Oxidation

For almost all approaches of *in vitro* fig plant micropropagation the darkening of culture medium is very common and it is caused by the oxidation of phenolic compounds. It can be reduced by the application of some prevention practices, as listed below. It is worth highlighting that oxidation depends on the species and that no method is effective for all species. When the problem is more severe, a combination of treatments is recommended.

Precautions before establishment

1. Removal of phenolic compounds – The removal or dispersion of these substances as they are formed is an effective control method. The substances released by damaged cells must be removed by washing. The explants must be washed or left to stand in sterilized water after isolation and before culturing. Careful rinsing after sterilization is necessary to remove the

chemical substances used for decontamination. The traces of these chemicals can lead to the synthesis of phenolic substances. The rinsing of the sterilized explants also removes any accumulating oxidise phenols.

Precautions during the establishment

1. Frequent explant transfer – when the explants do not show any sign of growth after 3 to 4 weeks and there are signs of oxidation, the chances of their survival can be increased by frequent transfer of explants to a fresh culture medium. Similarly, frequent subculture of explants is required when the culture medium around the explant becomes discoloured or darkened. Darkening is most visible in solid medium and it is concentrated on the edges of the explant. The interval of transference must be adjusted according to the severity of the problem. Generally, subculture of explants is necessary every 1 to 7 days.
2. Removal of darkened tissue – in each subculture the darkened tissue must be removed.
3. Addition of activated charcoal to the medium – the addition of activated charcoal hinders the accumulation of phenolic inhibitors. However it can also adsorb growth regulators and other components of the medium and be toxic to some tissues. Activated charcoal is normally added to the culture medium at concentrations varying from 0.2% to 3%, although it can promote or inhibit growth *in vitro*, depending on the species and tissue.

2.8. Shoot Multiplication

For 'Roxo de Valinhos' fig cultivar, WPM culture medium supplemented with 0.5 mg L⁻¹ BA or 0.5 mg L⁻¹ kinetin is efficient for shoot multiplication. Long shoots of *Ficus carica* 'Kalamon' were obtained after 8 weeks on the medium containing 0.5 mg L⁻¹ BA (Pontikis & Melas, 1986). For the *in vitro* proliferation of cultivars 'Brown Turkey' and 'Smyrna' and their subsequent use in genetic transformation, Yancheva et al. (2005) used MS medium supplemented with 0.25 mg L⁻¹ BA, 0.05 mg L⁻¹ IBA and 0.05 mg L⁻¹ GA₃. The shoots were placed horizontally in shoot proliferation medium. The cultures were maintained in the culture room, illuminated by white fluorescent light (32 μM m⁻¹ s⁻¹) for 16-h photoperiod at 25°C for 4 to 5 weeks before removing the explant leaves. The highest shoot regeneration rate achieved was on the basic MS medium amended with 1 mg L⁻¹ thiamine-HCl, 4% sucrose, 2.0 mg L⁻¹ thidiazuron (TDZ), and 2 mg L⁻¹ IBA. The regeneration depended directly on the explant orientation (Table 2).

2.9. Rooting

For root induction and growth of 'Roxo de Valinhos' fig cultivar, the addition of auxins to the culture medium is not necessary (Brum, 2001; Fráguas, 2004). However, the rooting of 'Sarilop' cultivar clones is successful on MS medium amended with 1.2 and 2.5 μM IBA or NAA (Hepaksoy & Aksoy, 2006) (Table 3).

Table 2. Plant growth regulator concentrations in MS medium used for shoot multiplication of different fig cultivars.

Cultivar	Growth regulator	Reference
Berbera	2.2 μM BA	Nobre et al. (1998)
Lampa	1 mg L^{-1} IBA	Hepaksoy & Aksoy (2006)
Sarilop	1 mg L^{-1} GA3 5 mg L^{-1} BA	

Table 3. Growth regulators and other substances added to a MS medium on rooting of different fig cultivars.

Cultivar	Growth regulators	Other substances	Reference
Berbera	2.5 μM IBA		Nobre et al. (1998)
Lampa			
Sarilop	1.2 and 2.5 μM IBA or NAA	100 g L^{-1} thiamine 2 mg L^{-1} nicotinic acid 12 mg L^{-1} pyridoxine 100 mg L^{-1} myo-inositol 3 g L^{-1} activated charcoal	Hepaksoy and Aksoy (2006)

2.10. Acclimatization

Substantial amounts of *in vitro*-micropropagated plants do not survive direct transfer to the greenhouse or field conditions. The greenhouse and field have substantially lower relative humidity, high light intensity, and aseptic conditions that are stressful to the micropropagated plants compared to *in vitro* conditions. Most species grown *in vitro*, including the fig tree, require an acclimatization process before transferring them to the greenhouse as well as to the field. This practice ensures the high survival rate and vigorous growth of *in vitro* plantlets when transferred to soil. Researchers are successful in improving the survival index of *in vitro* fig plantlets during acclimatization. A 90–95% survival rate was obtained for fig plantlets according to the following steps.

1. Cultivation for 30 days in WPM medium without growth regulators.
2. Opening of flasks 3 days prior to transplanting to the greenhouse.
3. Increased light intensity ($40 \mu\text{M m}^{-2} \text{s}^{-1}$) in the growth chamber.
4. Use of commercial substrate Plantmax[®] from Eucatex, São Paulo – Brazil.

Plantlets of the ‘Berbera’ and ‘Lampa’ fig cultivars, acclimatized for 3 months in the greenhouse by Nobre et al. (1998), produced fruit 2 years after transfer to the field. Plantlets of ‘Roxo de Valinhos’, ‘Radotta’, ‘Uruguai’ and ‘Celeste’ cultivars produced fruit 4 years after initiation of *in vitro* cultures Amaral (2002).

3. CONCLUSION

This chapter covers *in vitro* propagation protocol for cv ‘Roxo de Valinhos’ from shoot tips and apical buds. Since several cultivars are cultivated worldwide a short review of some research results on micropropagation stages of some other fig varieties was presented. Even though tissue culture has been successful in fig micropropagation for many cultivars, more research is still needed in order to optimise the protocol and also to support others biotechnologies researches.

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CHAPTER 38

HIGH FREQUENCY SHOOT FORMATION OF YELLOW PASSION FRUIT (*PASSIFLORA EDULIS* F. *FLAVICARPA*) VIA THIN CELL LAYER (TCL) TECHNOLOGY

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1. INTRODUCTION

The genus *Passiflora* belongs to Passifloraceae family and includes the passion fruit, is the largest and the most widespread genus of tropical flora. About 400 species of this genus are grouped into 21 subgenera (Cronquist, 1981). More than 350 species have been found in tropical regions and rain forests of South America (Killip, 1938) and 60 of them are edible species. Passion fruit is an important fruit crop in many tropical and subtropical countries due to its edible fruits, ornamental use and medicinal properties. Some species (*P. edulis*, *P. quadrangularis*, *P. ligularis*) are chiefly cultivated for the production of fruit juice. *P. icarnata* is reputed for its sedative properties and several other species are known for their ethnobotanical uses. *P. amethystina* Mikan, found in the rain forest of the Brazilian south-eastern coast (Fouqué, 1972), is a wild passion fruit species. It has purple blue flowers which are very aromatic. The genus also contains some species of ornamental use and medicinal properties as sedatives, antispasmodics and antibacterials (Coppens et al., 1997). *P. mollissima* Bailey, for example, is resistant to the bacterium *Xanthomonas campestris* f. *passiflorae* and *P. maliformis* L. is resistant to *X. passiflorae*. Furthermore, *P. giberti* has been used as a rootstock for *P. edulis* which is susceptible to a fungal disease caused by *Fusarium oxysporum* f. *passiflorae* (Oliveira, 1987). *P. cincinnata* Mast was found from east of Brazil to Paraguay and Argentina (Fouqué, 1972), and it is tolerant to *Xanthomonas campestris* f. *passiflorae*, a limiting disease in passion fruit orchards (Yamashiro, 1991). Due to

its economical importance, propagation of passion fruits in general and yellow passion fruit in particular is of interest of many researchers and breeders.

Passion fruit is a perennial plant that can be propagated by seeds, cuttings, air-layering or grafting. Most commercial passion fruit producers worldwide use seedlings to establish plantations, because they do not spread the woodiness virus (Nakasone and Paull, 1998). However, seed propagation results in undesirable variability, inadequate and seasonal supply (Isutsa, 2004). Propagation using cuttings and grafting is occasionally practiced, but these methods risk spreading the woodiness virus (Nakasone and Paull, 1998). Although this species is resistant to *Fusarium* wilt (Gardner, 1989) and nematodes (Drew, 1997), other fungi, viruses and bacteria, are responsible for significant losses in passion fruit production. Therefore, a reliable method for *in vitro* passion fruit propagation would have considerable benefits. *In vitro* regeneration of passion fruit has been obtained from axillary shoots or internodal segments (Robles, 1978, 1979), shoot apices or nodal segments cultivation (Kantharajah & Dodd, 1990; Drew, 1991; Faria & Segura, 1997a; Biasi et al., 2000; Monteiro et al., 2000; Reis et al., 2003) or from adventitious buds developed from leaf discs (Dornelas & Vieira, 1994; Appezzato-da-Glória et al., 1999), hypocotyls, leaves and cotyledons (Dornelas & Vieira, 1994; Faria and Segura, 1997b), leaf discs (Monteiro et al., 2000; Othola, 2000; Becerra et al., 2004; Trevisan & Mendes, 2005), mesophyll and cotyledon-derived protoplasts (Dornelas & Vieira, 1993; d'Utra Vaz et al., 1993; Otoni et al., 1995), and internodal segments (Biasi et al., 2000). Several studies indicated that various juvenile tissues from *Passiflora* spp. can be used as explants to obtain adventitious shoots (Kantharajah & Dodd, 1990; Dornelas & Vieira, 1994; Kawata et al., 1995). Moreover, the regeneration after protoplast fusion and micropropagation (Kawata et al., 1995) has also been described. A mature endosperm culture was reported for *P. foetida* (Mohamed et al., 1996). Embryo and endosperm cultured from seeds of several *Passiflora* species mainly collected in the wild has also been attempted (Guzzo et al., 2004). Optimized protocols have been established mainly by using different combinations of plant growth regulators (Dornelas & Vieira, 1994; Kawata et al., 1995; Faria & Segura, 1997b), and different salt solutions (Faria & Segura, 1997b; Monteiro et al., 2000). Though many reports on *in vitro* culture of passion fruit have been published, there are no reports on the use of TCL technology to micropropagate this species. Therefore, in this chapter, a novel protocol on shoot formation of *P. edulis* f. *flavicarpa* via TCL technology is presented.

2. THIN CELL LAYER TECHNOLOGY IN MICROPROPAGATION OF *P. EDULIS* F. *FLAVICARPA*

Schematic representation of the protocol for micropropagation of yellow passion fruit is described in Figure 1.

2.1. Preparation of *tTCL* for Shoot Regeneration

Nodal segments of 3-year-old plant grown in the greenhouse, Dalat Institute of Biology are used as primary explants. After removal of leaves and axillary buds, the

stems are washed and shaken in running tap water for 1 h, and disinfected in a solution of commercial bleach (0.7%) and two drops of Tween 20® (0.1%) for 20 min, followed by rinsing three times (2 min each) in sterile distilled water. The plant materials are then treated with 70% (v/v) ethanol for 15 s in the laminar flow cabinet, and rinsed three times again in sterile distilled water (2 min each). Immerse the stems in 0.1% (w/v) mercuric chloride solution and shake for 5 min, then rinsing three times in sterile distilled water to wash out the sterilizing agent.

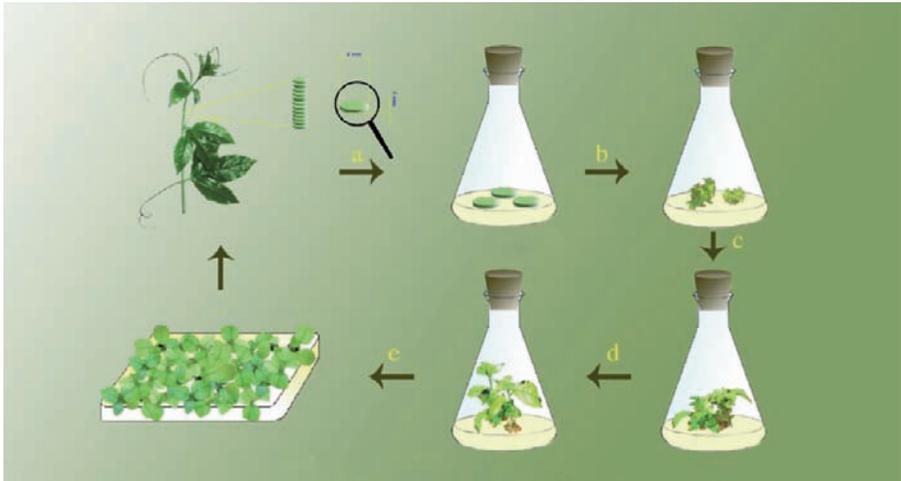


Figure 1. Schematic representation of the protocol for micropropagation of yellow passion fruit. a) preparation of tTCLs from nodal segments of 3-year-old plant grown in the greenhouse; b) culture of tTCLs on regeneration medium; c) shoot elongation; d) rooting; e) acclimatization.

The stems are then sectioned transversally into 1 mm thick explants, called tTCL (transverse thin cell layer) explants, and cultured in 100 ml glass vessels containing 20 ml shoot regeneration medium (Table 1). The cultures are placed in a growth chamber, $45 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity for 10 h per day, $25 \pm 2^\circ\text{C}$, and 75–80% relative humidity.

2.2. Culture Media

All of the culture media for shoot regeneration, shoot elongation, and root formation containing 6-benzyladenine (BA), kinetin (Kn), indole-3-acetic acid (IAA), gibberellin (GA_3), α -naphthaleneacetic acid (NAA) singly or in combination, agar, and sucrose are described in Tables 1, 2 and 3.

Table 1. Shoot regeneration media.*

Plant growth regulators	Concentrations (mg l ⁻¹)				
	0	0.5	1.0	1.5	2.0
BA	B ₁	B ₂	B ₃	B ₄	B ₅
Kn	K ₁	K ₂	K ₃	K ₄	K ₅
GA ₃	G ₁	G ₂	G ₃	G ₄	G ₅

*All of the medium used were based on MS medium (Murashige & Skoog, 1962).

2.3. Shoot Regeneration from tTCL Explants

After 4 weeks of culture, the regeneration of *P. edulis* f. *flavicarpa* shoots was observed on the media supplemented with BA. Figure 2 shows the effects of different BA concentrations on shoot and callus formation of tTCL explants.

For shoot regeneration, 1.0 mg l⁻¹ BA is optimal from tTCL explants. At this concentration, 100% explants regenerated shoots. However, at higher concentration 2.0 mg l⁻¹ of BA the percentage of newly formed shoots decreased. Organogenesis of tTCL explants was shown in Figure 3.

2.4. Shoot Elongation

Vigorous shoots regenerated from tTCL segments were transferred into shoot elongation medium (Table 2). Cultures were maintained in the same condition as that of regeneration stage for more than 8 weeks. The results of shoot elongation with the affect of BA and GA₃ are shown in Table 4.

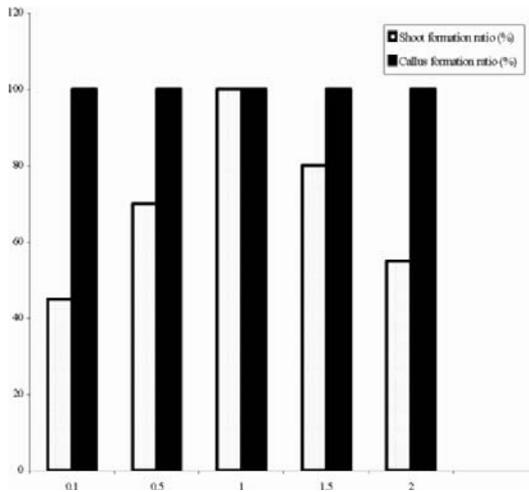


Figure 2. Effects of different BA concentrations on shoot and callus formation rate of explants.

Table 2. Shoot elongation media.

Treatments	Medium	BA (mg l ⁻¹)	GA ₃ (mg l ⁻¹)
EM ₁	1/2MS	5	3
EM ₂	1/2MS	5	4
EM ₃	1/2MS	5	5
EM ₄	1/2MS	5	6

Table 3. Rooting media.

Treatment	Medium	NAA (mg l ⁻¹)	IAA (mg l ⁻¹)	Sucrose (g l ⁻¹)
MR ₁	MS	0	0	30
MR ₂	MS	0.5	0	30
MR ₃	MS	1.0	0	30
MR ₄	MS	2.0	0	30
MR ₅	½MS	0	0	30
MR ₆	½MS	1.0	0	30
MR ₇	MS	0	1.0	20

The shoot elongation was best observed on the medium containing 5 mg l⁻¹ BA and 6 mg l⁻¹ GA₃ (EM₄). Higher concentration of GA₃ in the culture medium resulted in longer shoots (Table 4). Shoots elongated rapidly and grew 3–4 cm long after 8 weeks of culture (Figure 4C,D). The elongation of explants cultured on EM1 and EM2 media was only observed after 3 months of culture on ½ MS medium containing 3 mg l⁻¹ GA₃ and 5 mg l⁻¹ BA.

Table 4. Effects of BA and GA₃ on shoot elongation.

Treatments	Average of shoot length (cm)
EM ₁	2.0
EM ₂	0.6
EM ₃	4.1
EM ₄	4.7

2.5. Root Formation

Shoots, 3–4 cm long were placed on the rooting media (Table 3) for 6 weeks to induce roots. The results indicated that all hormone-free media (MR1 and MR5) could not induce rooting (Figure 5A, E). Various rooting responses of tested shoots were recorded in both full strength MS and half-strength MS medium. All explants were rooted in MS medium containing 1.0 mg l⁻¹ NAA (MR3) (Figure 5C), reduced of macro-nutrients in this medium results in lower root formation rate. In MR2, MR4, and MR6 media, all explants exhibited light yellowish green callus with roots (Figure 5B,D,F). No shoot formation was observed on MR4 and MR6 media. Shoots exhibited vigorous roots without callus formation phase when cultured on MS medium contained 1.0 mg l⁻¹ IAA (Figure 5G).

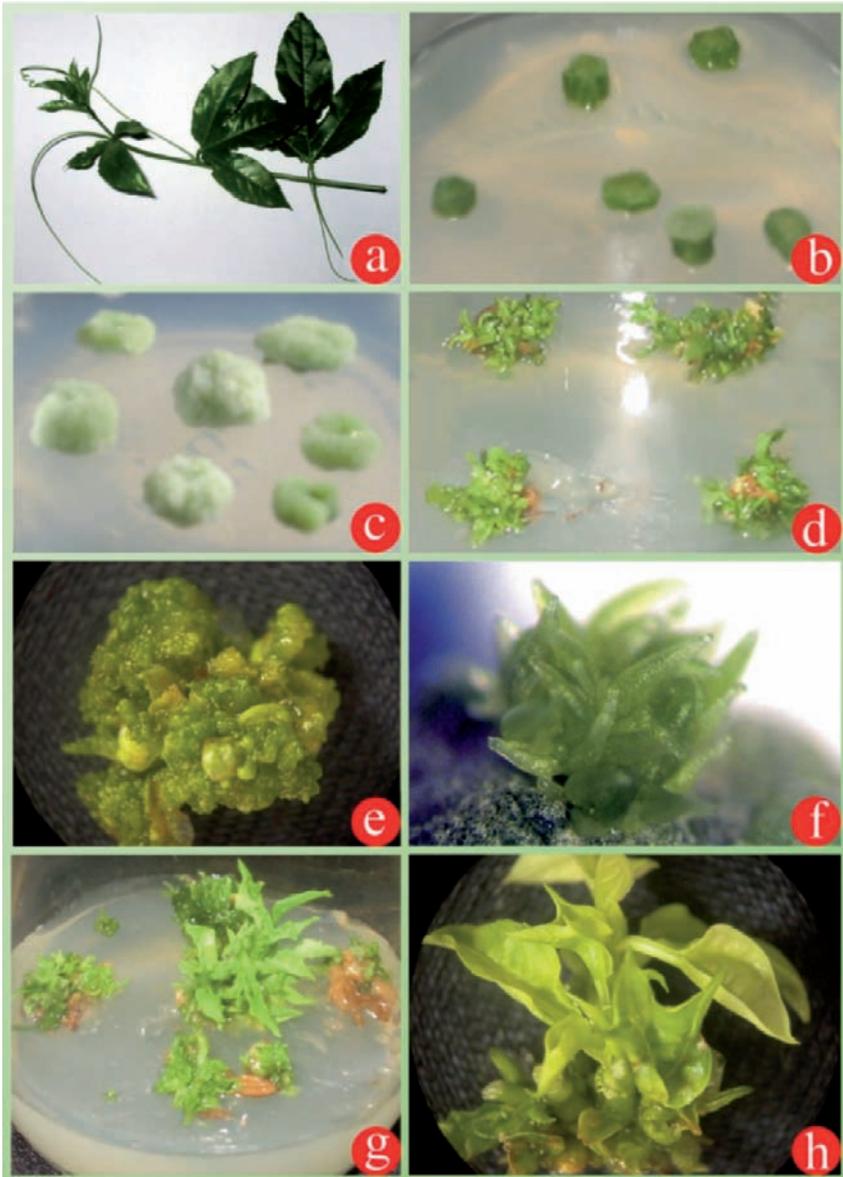


Figure 3. Organogenesis of *tTCL* explants. a) initial explant; b) *tTCLs* explants; c) calogenesis; d), e), f) caulogenesis; g), h) shoot development after 2 months.

2.6. Ex Vitro Acclimatization

Plantlets (3.5–4.0 cm tall) with healthy roots were removed from culture flasks. They were washed gently with running tap water to remove agar sticking to the roots and transplanted into plastic pots filled with soil and sand (ratio of 1:1, v/v) (Figure 5H). For the first 2 weeks, plantlets were kept under natural light, 75% relative humidity, and irrigated with tap water three times a day, and kept acclimatizing in the same condition but irrigated once a day for another 4 weeks before transferring into the field.

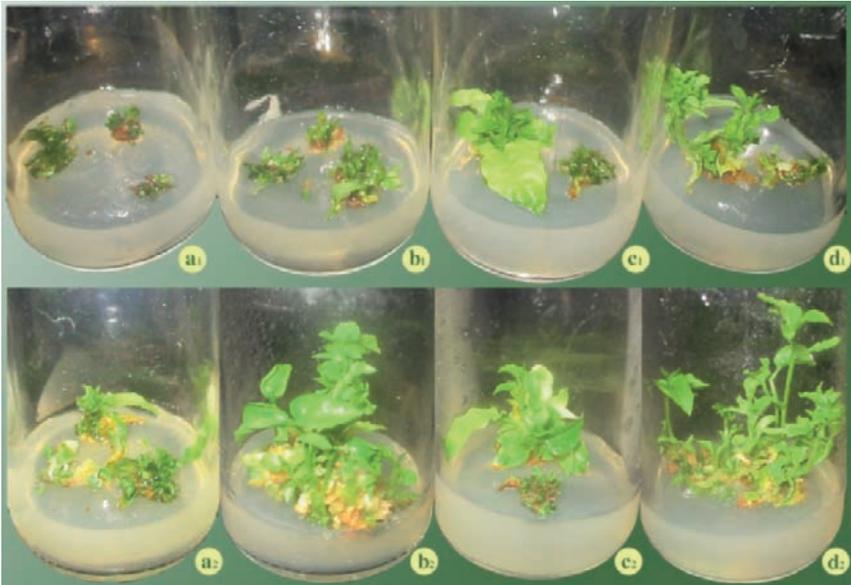


Figure 4. Effects of BA and GA₃ on shoot elongation of yellow passion fruit after 2 and 3 months. a₁, a₂) $\frac{1}{2}$ MS + 5 mg l⁻¹ BA + 4 mg l⁻¹ GA₃; b₁, b₂) $\frac{1}{2}$ MS + 5 mg l⁻¹ BA + 5 mg l⁻¹ GA₃; c₁, c₂) $\frac{1}{2}$ MS + 5 mg l⁻¹ BA + 3 mg l⁻¹ GA₃; d₁, d₂) $\frac{1}{2}$ MS + 5 mg l⁻¹ BA + 4 mg l⁻¹ GA₃.

3. CONCLUSION

In this protocol, tTCL is efficient in enhancing shoot regeneration rate and number of shoots per explant of yellow passion fruit. The optimal PGR for shoot formation from tTCL explants was 1.0 mg l⁻¹ BA. Shoot elongation should be carried out on the culture medium containing 5 mg l⁻¹ BA, and 6 mg l⁻¹ GA₃. NAA and IAA, each added at the concentration of 1 mg l⁻¹ in the MS medium were highly suitable for rooting. The callus formation from shoot could be induced by culturing shoots in the culture media containing high concentration of NAA.

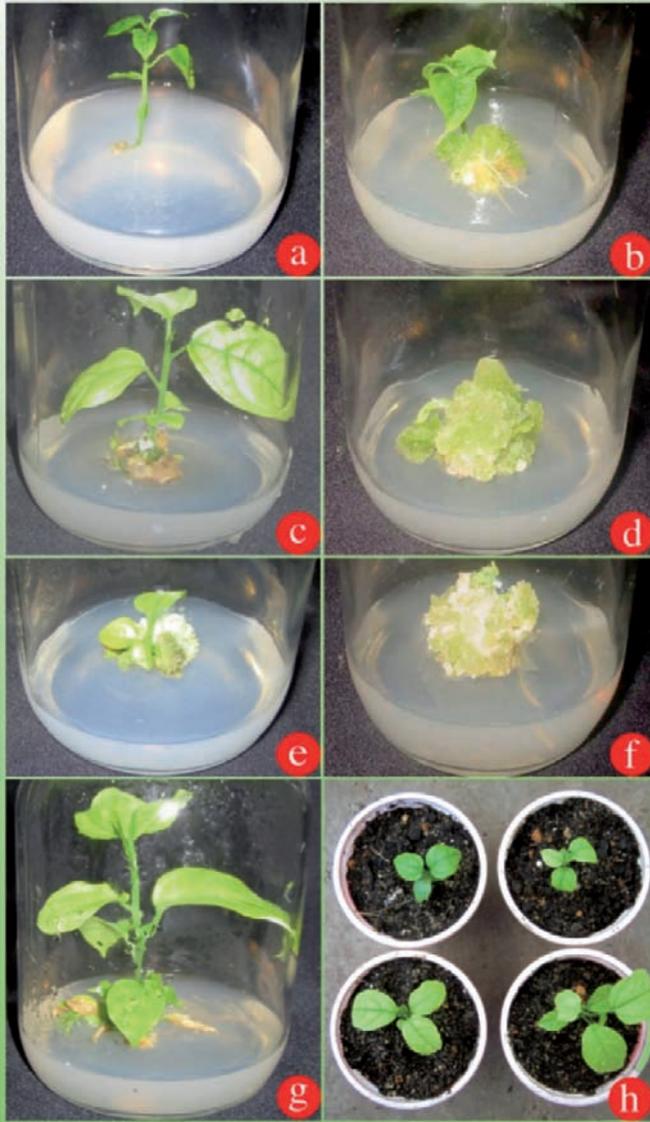


Figure 5. Effects of various media on rhizogenesis. a) MS + 30 g l⁻¹ sucrose; b) MS + 0.5 mg l⁻¹ NAA + 30 g l⁻¹ sucrose; c) MS + 1.0 mg l⁻¹ NAA + 30 g l⁻¹ sucrose; d) MS + 2.0 mg l⁻¹ NAA + 30 g l⁻¹ sucrose; e) ½MS; f) ½MS + 1.0 mg l⁻¹ NAA + 30 g l⁻¹ sucrose; g) MS + 1.0 mg l⁻¹ IAA + 20 g l⁻¹ sucrose; h) rooted plantlets in the greenhouse.

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CHAPTER 39

MICROPROPAGATION OF CALABASH TREE *CRESCENTIA CUJETE* L.

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1. INTRODUCTION

The Calabash tree, *Crescentia cujete* L., belongs to the Bignoniaceae, a large and primarily tropical family with few genera in temperate regions (Elias & France, 1978). As shown in Figure 1, it is a small tree up to 10 m high, with spreading branches and a broad head. It has a rough bark, simple leaves and cauliflorous flowers that appear directly from nodes on the trunk and branches. The flowers are 1–3 cm long with yellowish corolla, which have red or purple veins. It produces large green spherical or elliptical fruits up to 13 to 20 cm in diameter with small flat seeds embedded in the pulp. The plant is pollinated by bats (Bestmann et al., 1997).

This evergreen tree is used throughout the world as a source of medicines. It is used as an aperient, diuretic and febrifuge, to clean wounds, and for the treatment of headaches (Binutu, 1997; Romero, 2001). Extracts of the plant have been found to be active against Gram positive bacteria, but not notably active against Gram negative bacteria and have the neutralizing ability against the haemorrhagic effect of *Bothrops atrox* venom (Otero et al., 2000). In Vietnam, dried fruit of the Calabash tree are sold by the name 'Dao Tien' for use as a laxative and an expectorant (Kaneko et al., 1998). Medicinally active constituents from *C. cujete* include: furanonaphthoquinones (Heltzel et al., 1993), iridoids, iridoid glucosides (Kaneko et al., 1997), n-alkyl glycosides and p-hydroxybenzoyloxy glucose (Agarwal & Popli, 1992; Kaneko et al., 1998).

C. cujete L. is commonly grown throughout Costa Rica. However, the Calabash tree is not native to North America and is grown only in a relatively small number of nurseries in the United States and Canada. *C. cujete* is cultivated in Costa Rica in yards and fences. The shell of *Crescentia* fruit are used as containers, cups and spoons. The cups, referred to as “jícara”, were specially used for drinking chocolate. The indigenous people also used the fruits to make artwork. The name “jícara” arose from the Nahuatl language “xicalli”, a word used by the Native Americans to refer to cups for drinking chocolate (León & Poveda, 2000).

Mass propagation of elite trees for commercial use as well as for the extraction of chemically consistent medicinal preparations requires that the plants be grown in an environment with consistent light, nutrition and temperature as well as in isolation from abiotic and biotic contaminants (Murch et al., 2001; Saxena, 2001). This chapter provides details of various steps involved in the micropropagation of *C. cujete* including:

1. establishment of *in vitro* seedling cultures of *C. cujete*,
2. development of protocols for proliferation and maintenance of axenic cultures in perpetuity,
3. protocols for *de novo* regeneration, and
4. determination of the optimal configuration of bioreactors for large-scale production.

2. PROPAGATION PROTOCOLS

2.1. Plant Materials

Mature fruits of *C. cujete* can be used in micropropagation experiments. Plant material employed in our experiments was collected from mature trees (Figure 1) in March 2001 from Mercedes Sur, Puriscal, in the Pacifico Central Conservation Area, Costa Rica.

2.2. Explant Preparation from Donor Trees for *in vitro* Cultures

1. Collect mature fruits from donor trees.
2. Place the fruit explants in a 1 L beaker and wash thoroughly with running tap water.
3. Clean the fruits with a rigorous washing them using water and soap followed by surface flaming using 90% alcohol.
4. Sterilize seeds from opened fruits in a 3% sodium hypochlorite solution for about 15 min with occasional swirling of the seeds to ensure uniform exposure to the sterilant.
5. Remove the unhealthy seeds with a pair of forceps.
6. Wash the seeds 4–5 times with sterile distilled water.



Figure 1. *Crescentia cujete* L. mature tree.

2.3. Tissue Culture Media

C. cujete is propagated on media containing MS (Murashige & Skoog, 1962) salts, B5 (Gamborg et al., 1968) vitamins and various growth regulators (Table 1). Media are prepared by dissolving all chemical components in a flask containing distilled water placed on a stirring/hot plate and the pH of the solution adjusted to 5.7. For solidification, 2.2 g l^{-1} of gelling agent (Gellan gum, Sigma Chemical Co., St. Louis, MO) is added to the medium and dissolved by heating the medium on the stirring/hot plate. The medium is dispensed into tissue culture vessels using a dispenser and then autoclaved at 121°C for 20 min (1.19 kg cm^{-2}).

2.4. In vitro Seedling Cultures from Seeds

1. Place the surface sterilized seeds in Petri dishes (90 mm) containing 25 ml MS medium devoid of growth regulators.
2. If bacterial or fungal contamination is observed, sterilize the seeds from contaminated cultures again by dipping the intact or germinated seeds in 70% alcohol for 30 s, then immerse in 10% bleach for 3–5 min, and rinse 5 times with sterilized distilled water.
3. Culture the re-sterilized seeds individually in Petri-dishes each containing 25 ml of MSO medium (Table 1) and examine the cultures regularly to ensure that they are free of contamination.
4. Incubate the cultures in a culture growth room at $25^\circ\text{C} \pm 2^\circ\text{C}$ with a 16 h photoperiod ($25\text{--}40 \mu\text{mol m}^{-2} \text{ s}^{-1}$) provided by cool white fluorescent lamps (Figure 2A).
5. Seeds germinate after 2 weeks of culture on MS medium, and are transferred to fresh medium every month.

Table 1. Composition of the culture media used for *C. cujete* L. micropropagation based on MS salts, B5 vitamins augmented with culture stage-specific plant growth regulators.

Components	MSO ^a	CS ^b	SCR ^c	LCR ^d
<i>Major elements (mg l⁻¹)</i>				
NH ₄ NO ₃	1,650	1,650	1,650	1,650
KNO ₃	1,900	1,900	1,900	1,900
CaCl ₂ × 2H ₂ O	440	440	440	440
MgSO ₄ × 7H ₂ O	370	370	370	370
KH ₂ PO ₄	170	170	170	170
<i>Minor elements (mg l⁻¹)</i>				
H ₃ BO ₃	6.2	6.2	6.2	6.2
MnSO ₄ × 4H ₂ O	22.3	22.3	22.3	22.3
ZnSO ₄ × 4H ₂ O	8.6	8.6	8.6	8.6
KI	0.83	0.83	0.83	0.83
Na ₂ MoO ₄ × 2H ₂ O	0.25	0.25	0.25	0.25
CoCl ₂ × 6H ₂ O	0.025	0.025	0.025	0.025
CuSO ₄ × 5H ₂ O	0.025	0.025	0.025	0.025
<i>Iron source (mg l⁻¹)</i>				
FeSO ₄ × 7H ₂ O	27.8	27.8	27.8	27.8
Na ₂ EDTA	37.3	37.3	37.3	37.3
<i>Organic constituents (mg l⁻¹)</i>				
Sucrose	30,000	30,000	30,000	30,000
Glycine	2	2	2	2
Myo-inositol	100	100	100	100
Nicotinic acid	0.5	1	1	1
Pyridoxine-HCl	0.5	1	1	1
Thiamine-HCl	0.1	10	10	10
<i>Growth regulators (μmol l⁻¹)</i>				
TDZ		5		
Kinetin		2.5	1	1
<i>Solidifying agent (mg l⁻¹)</i>				
Gellan gum	2,200	2,200	2,200	

Note: ^aMSO – medium used for seed germination and seedling culture; ^bCS – shooting medium; ^cLCR – liquid rooting medium; ^dSCR – solid rooting medium.

2.5. Initiation of Aseptic Stock Cultures

1. Individual seedlings are used to establish axenic cultures.
2. Seedlings are maintained on a medium containing MS salts, B5 vitamins, 3% sucrose, and 1.0 μM kinetin (Figure 2A).
3. Once aseptic cultures are established, *de novo* regeneration is induced on petiole explants from the axenic cultures.
4. Transfer petiole sections to *C. kujete* shooting (CS) medium containing 10 μM thidiazuron (TDZ) in combination with 2.5 μM 2, 4-dichlorophenoxyacetic acid (Figure 3), and subculture every 3 weeks.
5. Grow cultures for about 6 weeks in a culture growth room adjusted to 25°C \pm 2°C with a 16 h photoperiod (25-40 $\mu\text{mol m}^{-2} \text{s}^{-1}$) provided by cool white fluorescent lamps (Figure 2B–D).

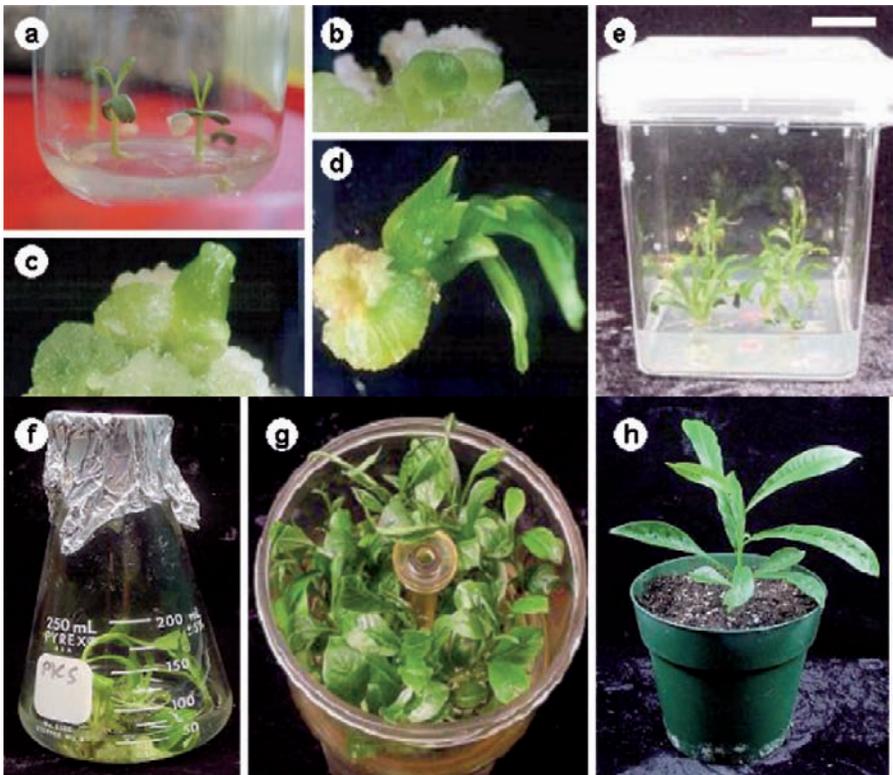


Figure 2. Micropropagation of *Crescentia kujete* L. a) *In vitro* germinated seedlings (bar = 5 cm). b) Callusing of the petiole explants after 28 days (bar = 5 mm). c) *De novo* shoot organogenesis on petiole explants on a medium supplemented with 5 μM TDZ (bar = 5 mm). d) Protrusion of shoot and root primordial (bar = 5 mm). e) Plantlets grown on solid MS medium with 1.0 μM kinetin (Bar = 3 cm). f) Plantlets grown in liquid MS medium with 1.0 μM kinetin (bar = 3 cm). g) Plantlets grown in temporary immersion system with liquid MS medium with 1.0 μM kinetin (bar = 3cm). h) Mature plant grown in the greenhouse 1 month after transplanting (bar = 5 cm).

6. Excise the shoots developing from the petiole cultures with sterile scalpels and place them in Magenta boxes each containing 50 ml CS culture medium. The shoots should be pushed 0.5–1.0 cm deep into the medium with shoot tip well above the surface of the medium.
7. Maintain stock cultures by subculturing shoots to fresh medium every 3 weeks. Up to four shoots can be cultured per Magenta box.

2.6. Large-scale Propagation

Mass production of rooted *C. cujete* plants can be achieved by placing differentiated nodes from aseptic stock cultures on a solid medium, in liquid medium or in a bioreactor. The solid, liquid, and bioreactor culture systems can also be utilized in combination to maximize the plantlet proliferation and growth rates.

2.6.1. Solid Culture System for Plant Production

1. Excise 10 ± 2 mm long plant nodes from the aseptic stocks. Leave one intact leaf at the top of the node and remove all other leaves of the node.
2. Place up to four plant nodes in Magenta boxes containing 50 ml of solid *C. cujete* rooting (SCR) medium supplemented with 1.0 μM kinetin. Ensure that the nodes are in contact with the culture medium by pushing the bottom portions (0.5–1.0 cm) of the explants gently into the medium. Incubate the cultures under the same growth conditions as the stock cultures.
3. Examine the cultures for rooting response after 2 weeks. Normally, the roots differentiate from the nodal section in contact with the medium within 2 weeks of culture.
4. Whole plantlets with well established root systems develop after 5 weeks of culture (Figure 2E). Representative samples of plantlets are rinsed, blotted dry, and growth parameters including weight, shoot height, leaf number, and rooting frequency are measured (Figure 4). Transfer single plantlets to Magenta boxes each containing 50 ml SCR culture medium for future culture.

2.6.2. Flask-liquid Culture System for Plant Production

1. Prepare 10 ± 2 mm long plant nodes from the aseptic stocks. Leave one intact leaf at the top of the node removing the other leaves of the node.
2. Place up to four nodes in flasks containing 50 ml of liquid *C. cujete* rooting (LCR) medium with 1.0 μM kinetin. Ensure that the node explants are not entirely submerged in the liquid culture medium.
3. Place the cultures on a shaker at a speed of 80 rpm and incubate under the same conditions as the stock cultures.
4. Examine the cultures for rooting response after 2 weeks. Normally, the roots differentiate from the nodal section in contact with the medium within 2 weeks of culture.

5. After 5 weeks of culture, shoots with well developed roots (Figure 2F) are transferred to Magenta boxes each containing 50 ml SCR culture medium for future use.
6. Select uniform plantlets, rinsed, blot dry, and analyze for weight, shoot height, leaf number, and rooting frequency (Figure 4).

2.6.3. Bioreactor Culture System for Plant Production

1. Select 10 ± 2 mm long nodes with one single intact leaf.
2. Put 16 nodes into each RITA temporary immersion system (RITA, Cirad Biotrop, France) with 200 ml liquid *C. cujete* rooting (LCR) medium with $1.0 \mu\text{M}$ kinetin.
3. Circulate the medium to immerse the plants completely by positive pressure from an air compressor with a 3 min cycle at 3 h interval between cycles.
4. Growth conditions in the culture room are set to provide a 16 h photoperiod with an intensity of $25\text{--}40 \mu\text{mol m}^{-2} \text{s}^{-1}$ at a temperature of 25°C .
5. After 5 weeks of culture, all explants develop into clumps of shoots with well developed roots (Figure 2G). Collect data for the weight, shoot height, leaf number, and rooting frequency (Figure 4).
6. Separate the rooted shoots from clumps and transfer them to Magenta boxes each containing 50 ml SCR culture medium for future culture.

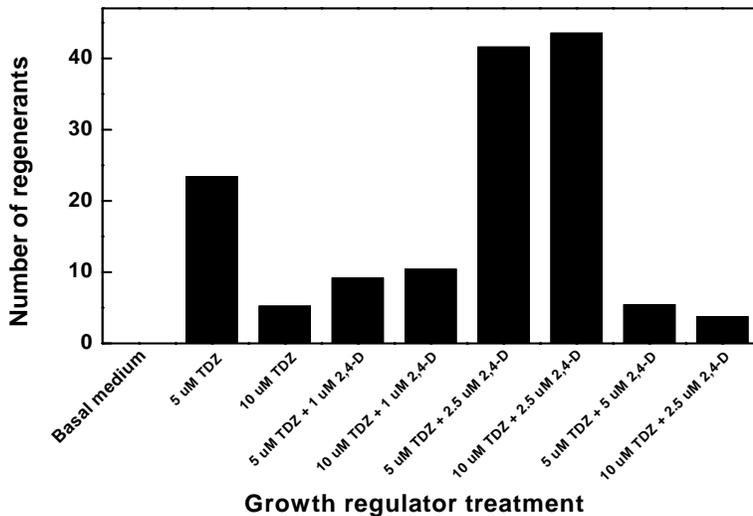


Figure 3. Effect of plant growth regulators on regenerant formation in petiole cultures of *Crescentia cujete* L. after 42 days.

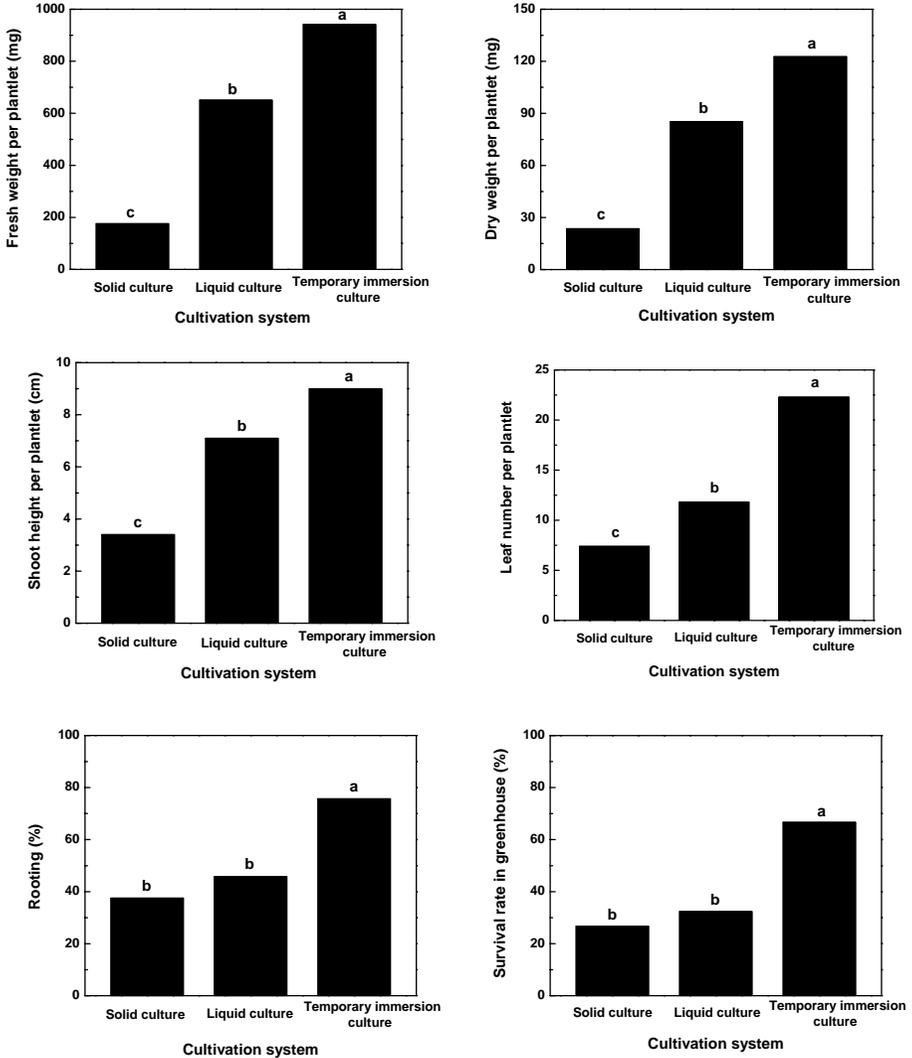


Figure 4. Effect of various culture systems (Magenta box, liquid-flask and temporary immersion bioreactor) on growth and development of *Crescentia cujete* plantlets.

2.7. Transfer of Plantlets to the Greenhouse

1. Plantlets with well formed roots obtained from solid, flask-liquid, and bio-reactor culture systems are suitable for transplanting. *In vitro*-rooted plants are acclimatized before transfer to the greenhouse as described in the next step.
2. When the plantlets reach a height of 5 to 8 cm, gently remove the plantlets from culture vessels and wash them with tap water to eliminate nutrient media

- and then transplant into 4-inch plastic pots containing a soil-less mix (Promix BX, Premier Horticulture Ltd, Quebec, Canada).
3. After potting, water the plants with an N-P-K (20-20-20) fertilizer solution (Plant Products Co. Ltd, Brampton, On, Canada) weekly.
 4. Place the pots in a growth chamber set for 16 h light at 26°C, 8 h dark at 24°C, and 95% relative humidity. Reduce the relative humidity by 5% every week for 5 weeks and then keep it constant at 70%.
 5. At the end of acclimatization period, transfer the plants to 1-gallon pots and place the potted plants in the greenhouse. Most of the transplants survive and grow to the size of juvenile trees, which can be planted outdoors, 1 month after transplanting (Figure 2H).

3. CONCLUSION

C. cujete L. is a widely distributed medicinal tree commonly known as the Calabash Tree. The current study was undertaken to establish protocols for *in vitro* maintenance and multiplication of axenic cultures of *C. cujete* and to develop a large-scale production system for biochemical characterization and phytomedicine production. The results of this study provide an optimized system for mass multiplication, and production of sterile, consistent plant material of *C. cujete*.

The culture system significantly influenced the micropropagation of *C. cujete* shoot cultures. Differences were observed between solid and liquid culture as well as between the two liquid culture systems, the flask and temporary immersion bioreactors. Liquid culture proved to be more effective in promoting shoot growth than solid medium and no hyperhydricity of *in vitro* grown plantlets was observed with liquid culture. Plantlets obtained from the temporary immersion liquid culture were longer and of better quality than those obtained from both solid medium culture and flask-liquid culture. Temporary immersion culture systems for micropropagation have been shown to be beneficial for many plant species, and offer significant advantages including: 1) efficient supply of liquid nutrition and oxygen transfer, 2) less shear and hydrodynamic forces, 3) minimal hyperhydricity of regenerants in the liquid medium, 4) improvement of plant quality and survival during acclimation, and 5) automation at a relatively low cost (Etienne & Berthouly, 2002). The results with the temporary immersion bioreactor culture of *C. cujete* shoots provide further evidence that enhanced atmospheric gas exchange and reduced stress and hydrodynamic forces can improve shoot proliferation and quality in a scaled-up micropropagation culture system with the liquid medium.

The principle prerequisite for the development of high-quality phytopharmaceutical products is a consistent source of high-quality plant material. The development of a regeneration protocol and scaled-up micropropagation system for *C. cujete* represents a significant advancement for phytopharmaceutical production of this medicinal species. The bioreactor multiplication can provide mass quantities of sterile, consistent, standardized and optimized plant material which can be utilized for the biochemical characterization of medicinally active constituents. Finally, the *de novo* regeneration protocol may be useful for genetic improvement using *Agrobacterium* and other gene transfer methods.

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CHAPTER 40

MICROPROPAGATION OF PAPAYA (*CARICA PAPAYA* L.)

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1. INTRODUCTION

Papaya (*Carica papaya* L) belongs to family Caricaceae which consists of several genera including *Carica*, *Cylicomorpha*, *Horovizia*, *Jacartia*, *Jarilla* and *Vasconcella* (Van Droogenbroeck et al., 2003). Major papaya producing countries are Brazil, Nigeria, Congo, Indonesia, Malaysia and India. The papaya has become an important fruit having great nutritive and commercial value. It is a fair source of iron and calcium, a good source of vitamins A, B and has high vitamin C (Sampson, 1986). The latex of the papaya plant has commercial use, as it is the source of two proteolytic enzymes, papain and chymopapain.

Vegetative propagation of papaya through conventional methods has not been successful. To overcome this limitation, papaya can be propagated through *in vitro* propagation. Papaya Ring Spot and Papaya Leaf Curl are two major viral diseases that threaten papaya cultivation worldwide. An efficient *in vitro* regeneration protocol is therefore imperative for papaya improvement (virus resistance) through recombinant DNA technology.

Micropropagation of papaya began three decade ago. Papaya is a polygamous fruit crop and both dioceous as well as gynodioceous varieties of papaya are being cultivated. Cloning of female papaya plants through *in vitro* shoot bud culture is an ideal approach. *In vitro* regeneration with shoot tip, excised from mature papaya plant, has been attempted (Ali & Hogan, 1976; Litz & Conover, 1978; Rajeevan & Pandey, 1986; Drew, 1988, 1992). However, most of the protocols are genotype dependent and could not be reproduced. Callus culture has also been reported in papaya (Arora & Singh, 1978; Litz et al., 1983). Papaya is recalcitrant to tissue culture. Slow rate of proliferation, poor establishment of axenic cultures and high mortality of plants during acclimatization are some of the main problems of papaya

micropropagation. Most of the reports on papaya micropropagation using mature explants are therefore conflicting. Somatic embryogenesis system using hypocotyl (Fitch, 1993) or immature zygotic embryos (Fitch & Manshardt, 1990) has been successful *in vitro* regeneration.

The micropropagation protocol described herein is based on direct somatic embryogenesis using immature zygotic embryo for somatic embryo induction and subsequent plant regeneration.

2. EXPERIMENTAL PROTOCOL

2.1. Sterilization and Explant Preparation

Select 90- to 120-day-old immature green papaya fruit from the mid position of fruit bunch. Wash it in the running tap water. Surface sterilize the fruit with 1.05% sodium hypo chloride (NaOCl) solution containing 1 drop of Tween 20 for 1 h and wash with autoclaved distilled water five times. Bisect the fruit under aseptic condition and then scoop out white, plump immature seeds. With forceps and scalpel remove the testa, cut it from one side and press the immature seed from mid portion to take out immature zygotic embryos (Figure 1).



Figure 1. Immature zygotic embryos of papaya. Left a group of embryos, right a single embryo.

2.2. Culture Medium

2.2.1. Culture Medium Preparation

Either commercially available pre-packaged MS (Murashige and Skoog, 1962) salt formulation or in-house prepared stock solutions can be used for culture medium preparation. Table 1 lists the ingredients of MS medium and specifies other inorganic and organic additives. Appropriate amount of stock solution of salts and plant growth regulators should be transferred to a one-litre flask and made up volume to one litre (Table 1). The medium is solidified with 8 gm/l agar. Prepare half strength MS basal medium containing all additives except plant growth regulators, which are added according to culture stage as shown in Table 1. Adjust pH of the medium to 5.7 with 1M NaOH and HCL and dispense in culture vessels and autoclave them for 20 min at 121°C at 20 kg cm⁻² pressure. Autoclavable polycarbonate or glass transparent bottles are suitable for papaya tissue culture work. Inoculate tissue after 48 h in the medium to ensure contamination free conditions.

Table 1. Formulation of culture medium (pH 5.7) used for papaya micropropagation based on MS salt augmented with different plant growth regulators at different stages.

Components	Chemical formula	Stock (g/l)		
<i>Macro nutrients, 10 × stock, use 100 ml/l medium</i>				
Ammonium nitrate	NH ₄ NO ₃	16.5		
Potassium nitrate	KNO ₃	19.0		
Calcium chloride-2H ₂ O	CaCl ₂ ·2H ₂ O	4.4		
Magnesium sulfate-7H ₂ O	MgSO ₄ ·7H ₂ O	3.7		
Potassium orthophosphate	KH ₂ PO ₄	1.7		
<i>Micro nutrients, 100 × stock, use 10 ml/l medium</i>				
Potassium iodide	KI	0.083		
Boric acid	H ₃ BO ₃	0.62		
Manganese sulphate	MnSO ₄ ·4H ₂ O	2.23		
Zinc sulphate	ZnSO ₄ ·7H ₂ O	0.86		
Sodium molybdate	Na ₂ MoO ₄ ·2H ₂ O	0.025		
Cobaltous chloride	CoCl ₂ ·6H ₂ O	0.0025		
<i>Iron –EDTA, 100 × stock, use 10 ml/l medium</i>				
Ferrous sulphate	FeSO ₄ ·7H ₂ O	2.78		
Ethylenediamine tetraacetic acid disodium	Na ₂ EDTA·2H ₂ O	3.36		
<i>Vitamins, 100 × stock, use 10 ml/l medium</i>				
Myo-Inositol		10.0		
Nicotinic acid		0.05		
Pyridoxine hydrochloride		0.05		
Thiamine hydrochloride		0.01		
Glycine		0.2		
<i>Other additives</i>				
Glutamine		0.4		
Sucrose		60		
Agar		8.0		
<i>Plant growth regulators and activated charcoal, add according to culture stage</i>				
	Final concentrations (mg/l)			
Culture stage	2,4-D	BAP	NAA	IBA
Embryogenic callus	10			
Embryogenesis	10			
Regeneration		0.2	0.1	
Rooting				1.0

2.3. Somatic Embryogenesis from Immature Zygotic Embryo

2.3.1. Somatic Embryo Induction and Multiplication

Inoculate excised immature zygotic embryos on Petri dishes containing ½ strength MS medium supplemented with 6% sucrose, 400 mg/l glutamine, MS vitamins, 10 mg/l 2, 4-D and 0.8% Difco bacto-agar having 5.7 pH (induction medium) (Table 1). In

each plate inoculate 10 zygotic embryos. Incubate cultures at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in the dark for 4 weeks. Globular embryos appeared after 4 weeks of inoculation, which will form bipolar-shape. Subculture somatic embryos on this medium (after 4 weeks), this will result in proliferation of embryos (Figure 2 a, b, c).

2.3.2. Somatic Embryo Maturation and Germination

After 4 weeks, several creamy white to light yellow thread like embryos will appear. Transfer the clumps of somatic embryos on half strength MS medium containing 3% sucrose and 0.8% Difco bacto-agar, 400 mg/l glutamine, MS vitamins, 0.2 mg/l BAP and 0.1 mg/l NAA (Regeneration medium). (Table 1) Incubate the cultures under 16 h light ($25 \mu\text{mol m}^{-2}\text{s}^{-1}$) at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Allow somatic embryos to germinate. If callus formation starts at the base of the shoots, than transfer the cultures on MS medium devoid of any plant growth regulators. This will check the callus growth at the base of shoot.

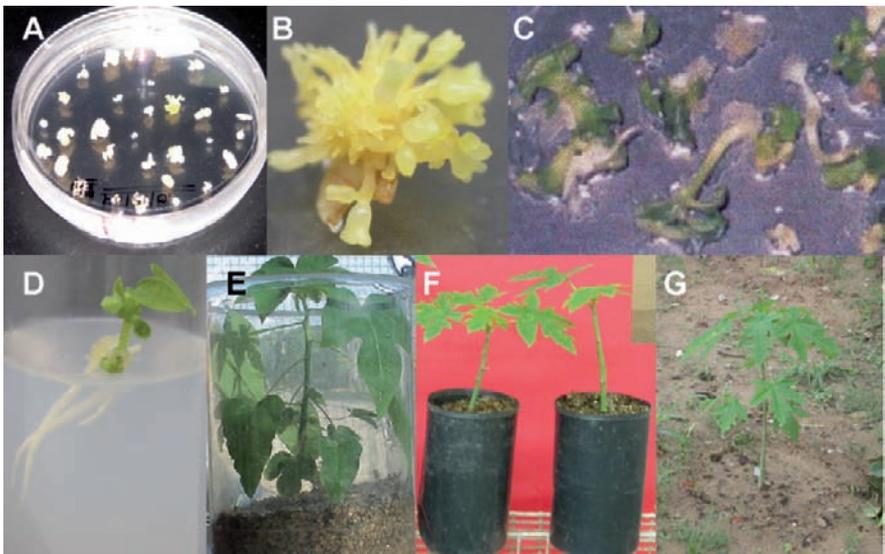


Figure 2. Somatic embryogenesis in papaya. A) Immature zygotic embryo, B) Somatic embryo, C) Germination of embryos, D) Rooting of plantlets, E) Acclimatization of plantlets, F) Acclimatized plants in poly bags, G) Establishment of plants in field.

2.3.3. Rooting and Acclimatization

Transfer regenerated plantlets (5 cm) in transparent culture bottles containing autoclaved vermiculite supplemented with $\frac{1}{4}$ MS liquid medium, and incubate them under light ($25 \mu\text{mol m}^{-2}\text{s}^{-1}$) illumination) at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2–3 weeks. Sometimes good rooting is a problem. In such case, add 1 mg/l IBA solution in vermiculite for better root growth. Gradually loosen the cap of bottle as soon as plant starts touching the cap of bottle. Transfer the plantlets into a poly house provided with 50% shade

net and misting. Once plant develops 4–6 fully opened leaves, transfer them into poly bags filled with Soil + Sand + Farm Yard Manure (1:1:1) (Figure 2).

2.3.4. Somatic Embryogenesis from Hypocotyls

Select 10-day-old *in vitro* grown seedling. Cut the hypocotyl sections and inoculate them on half strength MS (Induction) medium. Incubate the cultures in the darkness for 4 weeks at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for callus induction and subsequently somatic embryo formation. For embryo proliferation, germination, rooting and acclimatization follow the above mentioned protocol (2.3.1 to 2.3.3).

3. CONCLUSION

Papaya is an important cash fruit crop owing to its fast maturation rate and continuous fruit production in subtropical and tropical climates. There are reports on micropropagation of papaya from mature field grown trees. However, the technique could not be reproduced for mass cloning of papaya at commercial scale. Latex oozing, in borne contamination, and poor *in vitro* rhizogenesis are some of the bottlenecks in micropropagation of papaya.

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Section C

CHAPTER 41

PROTOCOL FOR MICROPROPAGATION OF SELECTED *VACCINIUM* spp.

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1. INTRODUCTION

The highbush blueberry (*Vaccinium corymbosum* L.) belongs to the genus *Vaccinium* L. (*Ericaceae*) and is widely cultivated in various parts of the world. Most of the high bush blueberry cultivars (*V. corymbosum*) grow in the midwestern, eastern and central United States and are also cultivated in Europe, Australia and New Zealand. They are commercially and biologically important as small fruits for their high content of vitamins, bioactive organic substances with antibacterial and anticancer effects, anthocyanin pigments, and also as an excellent source of antioxidants. There is an increase in both interest and efforts to establish the production plantations of some of the cultivars of this species, suitable for cultivation as an alternative fruit crops, as medicinal plants and landscape ornamental ground cover plants. Large-scale production of high quality plants is needed for commercial plantation establishment. Traditional vegetative propagation of high bush blueberry by cuttings has not been successful due to poor rooting ability, considerable demand for large amount of mother plants, their limited seasonal growth and relatively high price. Generative reproduction does not produce homogeneous progeny.

In *Vaccinium* species, *in vitro* techniques are quite effective for rapid mass production of high quality planting material for large-scale cultivation, germplasm improvement, gene conservation and research purposes (Jaakola et al., 2001; Cao et al., 2003; Gajdošová et al., 2006). Micropropagation via direct shoot regeneration

from meristems is well suited to obtain genetically homogeneous planting material, identical with mother genotype. *In vitro* plant regeneration via adventitious organogenesis is an effective system for the production of plant material, as well as for genetic engineering (Song & Sing, 2004). For the confirmation of clonal fidelity of vegetatively propagated plant material, cytological, biochemical and molecular analyses are efficient and rapid when compared with the traditional methods based on morphological and physiological assays.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Growing Conditions of Mother Plants

The mature mother plants of *Vaccinium corymbosum* L. were cultivated in sandy-clay soil on the plantation of Research Station Krivá na Orave, Slovak Republic. Since 1994, several cultivars of this species were tested for their suitability to grow under local climatic conditions. The Research Station is located at 700 m above sea level, with an average annual temperature 6°C and annual precipitation 800–900 mm, the highest during June and July. Each plant was planted into acid peat-made bed, with the volume of 10 l peat per plant, and pH 3.8.

2.1.2. Explant Excision and Sterilisation

As an initial plant material stem cuttings with dormant buds were collected from the selected cultivars of mature mother plants during the month of February and beginning of March. Cuttings with apical and axillary buds were washed under running water for 1 h followed by 70% ethanol treatment for 2 min and 0.1% mercuric chloride with 3 drops of Tween for 6 min, and finally washed with sterile distilled water for 3×15 min.

2.2. Culture Medium and Conditions of Cultivation

For primary explant cultivation Anderson's Rhododendron medium-AN (Anderson, 1980) was prepared from packaged powder macro and micro mineral salt mixture including vitamins supplied by Duchefa, The Netherlands (Table 1). For 1 liter medium preparation, 2 g powder was completely dissolved in double distilled water. Heat stable supplements, such as 30 g sucrose, 8 g Phyto agar, desired plant hormones, etc. were added and medium raised to the final volume by adding double distilled water. Medium pH was adjusted with HCl and KOH and heated until the solution was clear. Medium was autoclaved at $1 \text{ kg}^{-1} \text{ cm}^{-2}$ (15 psi), 121°C, for 20 min. Heat labile constituent zeatin was filter sterilized and added to the medium after autoclaving. All used plant growth regulators and Phyto agar were supplied by Duchefa, The Netherlands. Cultures were maintained in the growth chamber at $23 \pm 2^\circ\text{C}$ under 16/8 light and dark photoperiod and light intensity $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$ provided by white fluorescent lamps.

Table 1. Anderson's *Rhododendron* medium composition.

AN medium (Anderson, 1980)	Shoot regeneration from dormant apical and axillary buds	Adventitious shoot induction from leaf explants	Microshoot proliferation	<i>In vitro</i> rooting of microshoots
Macroelements	mg l ⁻¹	mg l ⁻¹	mg l ⁻¹	mg l ⁻¹
CaCl ₂	332.02	332.02	332.02	332.02
KNO ₃	480.00	480.00	480.00	480.00
MgSO ₄	180.54	180.54	180.54	180.54
NaH ₂ PO ₄	330.60	330.60	330.60	330.60
NH ₄ NO ₃	400.00	400.00	400.00	400.00
Microelements				
CoCl ₂ .6H ₂ O	0.025	0.025	0.025	0.025
CuSO ₄ .5H ₂ O	0.025	0.025	0.025	0.025
FeNaEDTA	73.40	73.40	73.40	73.40
H ₃ BO ₃	6.20	6.20	6.20	6.20
KJ	0.30	0.30	0.30	0.30
MnSO ₄ .H ₂ O	16.90	16.90	16.90	16.90
Na ₂ MoO ₄ .2H ₂ O	0.25	0.25	0.25	0.25
ZnSO ₄ .7H ₂ O	8.60	8.60	8.60	8.60
Vitamins				
Adenine hemisulphate	80.00	80.00	80.00	80.00
Myo-Inositol	100.00	100.00	100.00	100.00
Thiamine-HCl	0.40	0.40	0.40	0.40
Plant growth regulators and other components				
Zeatin	2.00 mg l ⁻¹	0.50 mg l ⁻¹	0.50 mg l ⁻¹	–
IBA	–	–	–	0.80 mg l ⁻¹
charcoal	–	–	–	0.80 g l ⁻¹
Sucrose	30.00 g l ⁻¹	30.00 g l ⁻¹	30.00 g l ⁻¹	30.00 g l ⁻¹
Phyto agar	8.00 g l ⁻¹	8.00 g l ⁻¹	8.00 g l ⁻¹	8.00 g l ⁻¹
pH	4.5–5.0	4.5–5.0	4.5–5.0	4.5–5.0

2.3. Shoot Regeneration and Maintenance

2.3.1. Shoot Regeneration from Dormant Apical and Axillary Buds in *Vaccinium corymbosum*

Elaboration of efficient shoot regeneration by axillary branching in selected cultivars was aimed for needs of practice. For direct shoot regeneration dormant apical and axillary buds were used, from which the upper scales were removed after sterilisation. AN shoot regeneration medium supplemented with different cytokinin concentrations was used for the cultivation. Influence of cytokinin zeatin on shoot

regeneration ability was evaluated after 5 weeks. Data evaluation was performed using Statgraphic analysis of variance and multiple range analysis. The optimal composition of shoot regeneration medium and zeatin concentration for the majority of tested cultivars is presented in Table 1.

The results showed that shoot regeneration ability is highly depending on cultivar and cytokinin type and concentration. Zeatin at concentration 2 mg l^{-1} was the most favourable for shoot regeneration in *V. corymbosum* cultivars (Figure 1A). On an average 10.16–15.23 numbers of shoots were regenerated on the medium supplemented with 2 mg l^{-1} zeatin with the highest number of shoots in cv. ‘Brigitta’ (Ostrolucká et al., 2002).



Figure 1. Micropropagation of *V. corymbosum*, cv. ‘Berkeley’ A) Shoot regeneration from dormant apical and axillary buds on AN medium with zeatin 2 mg l^{-1} . B) Adventitious shoot regeneration on leaf explants after 4 months of cultivation on medium with 0.5 mg l^{-1} zeatin. C) Shoot proliferation on medium with 0.5 mg l^{-1} zeatin.

2.3.2. Shoot Regeneration via Adventitious Organogenesis in *Vaccinium corymbosum* L.

Adventitious organogenesis is very efficient to scale up clonal production of selected genotypes of several *Vaccinium* species (Gajdošová et al., 2006). *Vaccinium corymbosum* L. – cv. ‘Berkeley’ was tested for adventitious shoot induction. As a primary explants leaves of *in vitro* plants with cut margins, were placed horizontally with adaxial surface on the culture medium. AN medium of the same composition supplemented with 0.5 mg l^{-1} zeatin (Table 1) was used for adventitious bud induction from leaf tissue. After 5 weeks of cultivation explants were transferred on AN medium of the same composition for shoot regeneration and multiplication. Percentages of explants regenerating shoots and number of regenerated shoots per explant after 3 subcultures, with subculture interval 5 weeks, were recorded.

Multiple adventitious shoots were formed on leaf explants cultivated on AN medium with 0.5 mg l^{-1} zeatin, produced via direct regeneration. Regeneration ability of the cultivar ‘Berkeley’ was high (18.0 shoots/explant) what is in correlation with regeneration ability achieved via culture of apical and axillary buds. After 3 subcultures on the same medium high number (237.14) of vigorous shoots with good elongation growth was obtained per explant in cv. ‘Berkeley’ (Figure 1B).

2.3.3 Influence of Medium pH on Micropropagation of *Vaccinium* spp.

It is known that by changing pH levels nutrient availability and plant metabolism are affected. *Vaccinium* spp. production is limited to soils with naturally low pH or soils treated with acidifying amendments (Finn et al., 1991). For understanding of pH tolerance, *in vitro* screening with modification of pH values in AN medium with 0.5 mg l⁻¹ zeatin within a tested pH range 3.0–6.0 was performed in selected cultivars of *V. corymbosum* during shoot formation from apical and axillary buds (Ostrolucká et al., 2004).

With increasing pH values of the medium in the range 3.0–5.0, also the number of shoots per explant increased in the range 2.33–7.34 in ‘Duke’ and 4.02–11.03 in ‘Brigitta’ as a mean number of shoots after 3rd subculture. At pH 5.5 considerable decreasing in the shoot number was observed. Statistically significant differences in the shoot proliferation were found among all tested pH variants with optimum medium pH 5.0.

2.3.4 Shoot Proliferation

For long-term proliferation of *in vitro* regenerated axillary or adventitious shoots AN medium supplemented with 0.5 mg l⁻¹ zeatin was successfully used. On this medium formation of vigorous multiple shoot cultures was observed with different intensity of shoot proliferation in tested cultivars. Increasing of shoot proliferation intensity can be achieved by segmentation of regenerated, elongated microshoots on one-node segments and their further cultivation on medium with 0.5 mg l⁻¹ zeatin (Figure 1C).

2.4. Rooting and Hardening

Satisfactory rooting of isolated microshoots is achieved by following steps:

1. *ex vitro* rooting by short (2–3 min) dipping of 15–20 mm long microshoots into 0.8 mg l⁻¹ IBA solution followed by direct planting in containers 50 mm in diameter filled with peat substrate
2. *in vitro* rooting on AN medium supplemented with 0.8 mg l⁻¹ IBA and 0.8 g l⁻¹ activated charcoal.

With both approaches the percentage of rooting reached 80–90–95% in dependence on cultivar. The rooted plantlets transplanted into peat substrate were subsequently acclimatized. Hardening of plantlets was performed under greenhouse conditions without any special light treatment, with regular irrigation, in containers at the beginning (2 weeks) covered by lid, later opened. Plantlets in length 100–150 mm (after 2 months) were replanted into bigger containers (120 mm in diameter) and transferred to Research Station Krivá, where they were placed under open-air conditions. Transfer of regenerants from *in vitro* to *ex vitro* conditions and their acclimatization was successful, as almost 80–90% of transferred plants survived (Figure 2A,B).

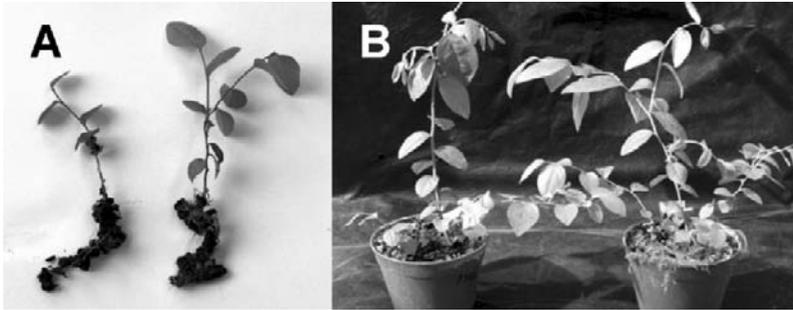


Figure 2. Rooting and hardening of micropropagated *V. corymbosum*, cv. 'Berkeley' plants A) Rooting of microshoots in peat substrate. B) Hardening of plants in containers under open-air conditions.

2.5. Field Testing

Transferred plants in length 100–150 mm were kept in containers (120 mm in diameter) placed under open-air conditions for 2 years (Figure 3A). Two years-old plants were planted into acid peat-made bed, with the volume of 10 l peat per plant, and pH 3.8 in experimental orchard of Research Station Krivá (Figure 3B). *In vitro* plants were from the beginning characterized by more synchronous growth. No morphological variations and anomalies were observed among them. In general, during the first 2 years after transfer to soil, *in vitro*-derived plants formed more shoots and had a bushier growth habit with a higher number of flower buds. The beginning of breeding was slightly delayed for *in vitro*-derived plants. This is an advantage for commercial growers, because during the first 2 years after planting fruit setting is not desirable. On the contrary, the vegetative growth should be supported in order to strengthen full fertility during following years. Later no obvious morphological differences were visible between *in vitro* and by cuttings propagated plants. Regarding fertility and berry quality the plants are also equivalent.

2.6. Molecular Marker Analysis

Selected *in vitro* obtained clones of *V. corymbosum* cultivars, after several months of cultivation, were used for the detection of undesirable genetic variation by Inter-Simple Sequence Repeats (ISSR) markers.

In total nine *in vitro*-derived clones of *V. corymbosum* and one clone of *V. vitis-idaea* L., grown on AN medium supplemented with 0.5 mg l⁻¹ zeatin (Table 2), were tested by ISSR markers (Inter-Simple Sequence Repeats).

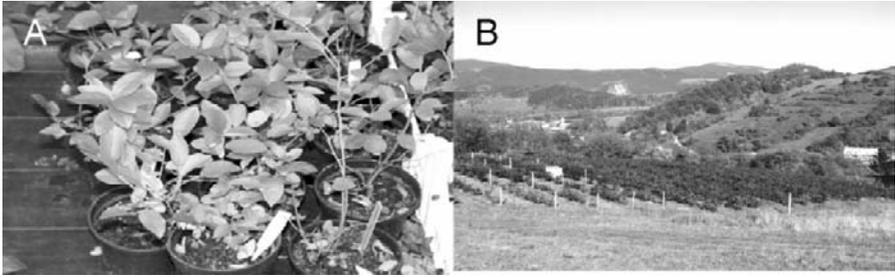


Figure 3. A) *Vaccinium corymbosum* *in vitro*-derived plants kept in containers under open-air conditions. B) *Vaccinium corymbosum* plants growing in experimental orchard in Research Station, Krivá.

Table 2. Characterization of samples.

Genus	Sample number	Cultivar	Origin of <i>in vitro</i> clone
<i>Vaccinium corymbosum</i> L.	1	Berkeley	Meristem
	2	Berkeley	Leaf (L-1)
	3	Berkeley	Leaf (L-2)
	4	Berkeley	Leaf (L-3)
	5	Bluecrop	Meristem
	6	Bluecrop	Leaf (L-1)
	7	Bluecrop	Leaf (L-4)
	8	Blueray	Meristem
<i>V. vitis-idaea</i> L.	9	Darrow	Meristem
	10	Linnea	Meristem

2.6.1. DNA Isolation

A total genomic DNA was isolated by Qiagen kit (Qiagen™) from leaves of *in vitro* plants derived by meristem cultivation and/or adventitious organogenesis from leaf tissue. Approximately 100 mg of leaf tissue was grounded in liquid nitrogen by pestle in mortar. Tissue powder was transferred into pre-chilled test tube (1.5 ml) and the next steps were performed according to the instructions in manufacturer's manual.

2.6.2. DNA Quantification

Human Genomic DNA (Promega®) with concentration $245 \text{ ng} \times \mu\text{l}^{-1}$ was used as a control DNA (sample of known concentration). Several control DNA dilutions of 500, 250, 125, 62.5 and $31.5 \text{ ng} \mu\text{l}^{-1}$ were prepared. $1 \mu\text{l}$ of tested DNA samples and dilutions of control DNA were loaded on 1% (w/v) agarose (3:1, Amresco™) gel (80 mA, 120 V). Consequently, isolated DNA quantity was determined by visual comparison to control DNA samples.

2.6.3. PCR-ISSR

For ISSR technique 3'OH anchored primers [(CA)₆GT, (GAG)₃GC, (GT)₆CC, (GA)₉C] and 5'OH anchored primer GT(CA)₄ were used. Amplification reactions were performed in 25 µl of reaction volume containing 20 mmol × dm⁻³ Tris-HCl (pH 8.0), 50 mmol × dm⁻³ KCl, 3 mmol × dm⁻³ MgCl₂, 0.1 mmol × dm⁻³ dNTPs, 0.4 µmol × dm⁻³ primer, 1U *Taq* polymerase and 30 ng of DNA. All reactions were heated to 94°C for 2 min, followed by 45 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. Polymerisation was followed by final extension cycle of 72°C for 10 min.

2.6.4. Electrophoresis and PCR Products Evaluation

The amplification PCR-ISSR products were separated by electrophoresis on 2% (w/v) agarose gel (120 V, 80 mA). Electrophoregrams were evaluated by KODAK EDAS 190 1D software (Figure 4) where the size (in bp) of individual DNA bands was recorded. DNA bands were retrieved based on their size and these information was transferred into Microsoft Excel Application where the size was replaced by binary 1 (for present band on specific position) or 0 (for absent band). Consequently, Nei and Li (1979) similarity index (SI_{NL}) based on ISSR profiles was calculated in the MS Excel according to the $SI_{NL} = 2 \times \text{sum of common bands of lane A and B} / (\text{sum of the bands in the lane A} + \text{sum of the bands in the lane B})$.

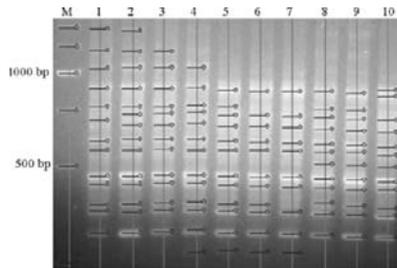


Figure 4. ISSR profile using primer (GT)₆CC evaluated by KODAK EDAS 190 1D software. M-250 bp ladder, 1–4 Berkeley, 5–7 Bluecrop, 8 Bluegray, 9 Darrow, 10 Linnea.

Distance index (DI_{NL}) values were calculated according to the $DI_{NL} = 1 - SI_{NL}$. Dendrograms were constructed using UPGMA (Unweighted Pair-Group Method Using Arithmetic Averages) analysis for the objective position of ISSR in the statistical program SYNTAX. Dissimilarity of every single cluster is showed by the Euclidian Distance Averages of Clusters (EDAC).

The group of tested genotypes was divided into two main clusters (Figure 5). First group consists of 'Bluecrop' and 'Berkeley' genotypes with an average GI_{NL} 0.308. Second group consist of three individual genotypes, two of species *V. corymbosum* ('Bluegray' and 'Darrow') and one of species *V. vitis-idaea* ('Linnea') with an average GI_{NL} 0.552. The polymorphism of analyzed samples varied from 96% to 99% depending on the used primer. Unique bands appeared in very low

number (1–3) in all ten genotypes. The number of synthesized DNA fragments was in average 8 per genotype while the size of synthesized fragments reached 250–1550 bp. A high DNA polymorphism level and low distance index values determined by DNA microsatellite polymorphism have pointed out a stable *in vitro* cultures of *Vaccinium* spp., however independently from the plant origin. Two primers out of five have clustered as the closest genotypes number 8 and 9 ('Blueray' and 'Darrow') originated from meristematic tissue. Three out of five primers have clustered genotypes 3 and 4 ('Berkeley') and two have clustered genotypes 6 and 7 ('Bluecrop'). In both cases a leaf tissue was the primary explant. It seems that a primary explant origin might play a role in stability of *in vitro* culture.

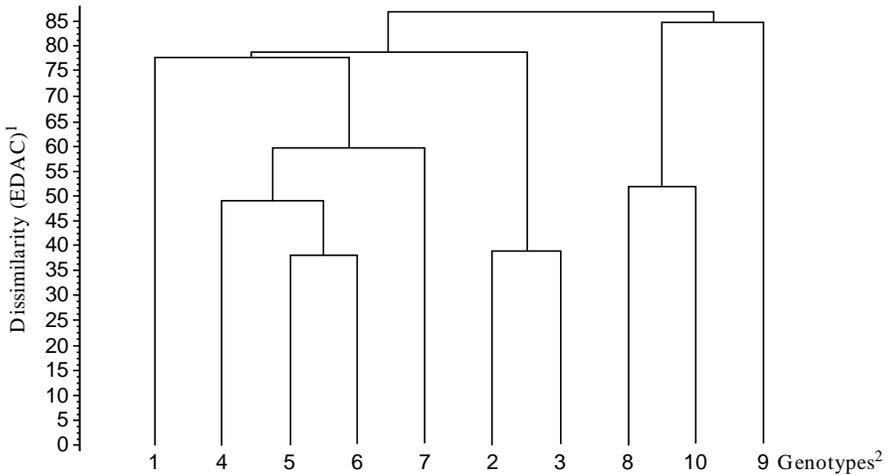


Figure 5. Dendrogram of *Vaccinium* genotypes analyzed by ISSR using five primers. ⁽¹⁾EDAC – Euclidian Distance Averages of Clusters, ⁽²⁾Genotypes, 1–4 Berkeley, 5–7 'Bluecrop', 8 'Blueray', 9 'Darrow', 10 'Linnea'.

2.7. Flow Cytometry Analysis

Flow cytometry analysis was performed with the aim to determine undesirable variations in genome size induced during *in vitro* regeneration process. Three clones of *V. corymbosum* cv. Berkeley derived by adventitious regeneration and one meristem-derived clone were analysed. Flow cytometry analyses were performed on Ploidy analyzer PA II, with mercury arc lamp using UV excitation. The samples were prepared from young leaves of *in vitro* plants of *Vaccinium corymbosum* L. by two step procedure using reagent kit Partec CyStain UV precise P (Partec GmbH, Münster, Germany) containing Extraction Buffer and Staining Buffer with DAPI:

1. Approximately 20 mg of young leaf tissue were chopped with a sharp razor blade in a plastic petri dish containing 400 μ l Extraction Buffer for 30–60 s. After incubation in Extraction Buffer for 1–2 min at the room temperature the sample was filtered through a 50 μ m filter.
2. Staining solution 1.6 ml was added to the test tube and sample was incubated for 30–60 s. Samples were analysed in flow cytometer in the blue fluorescence channel.

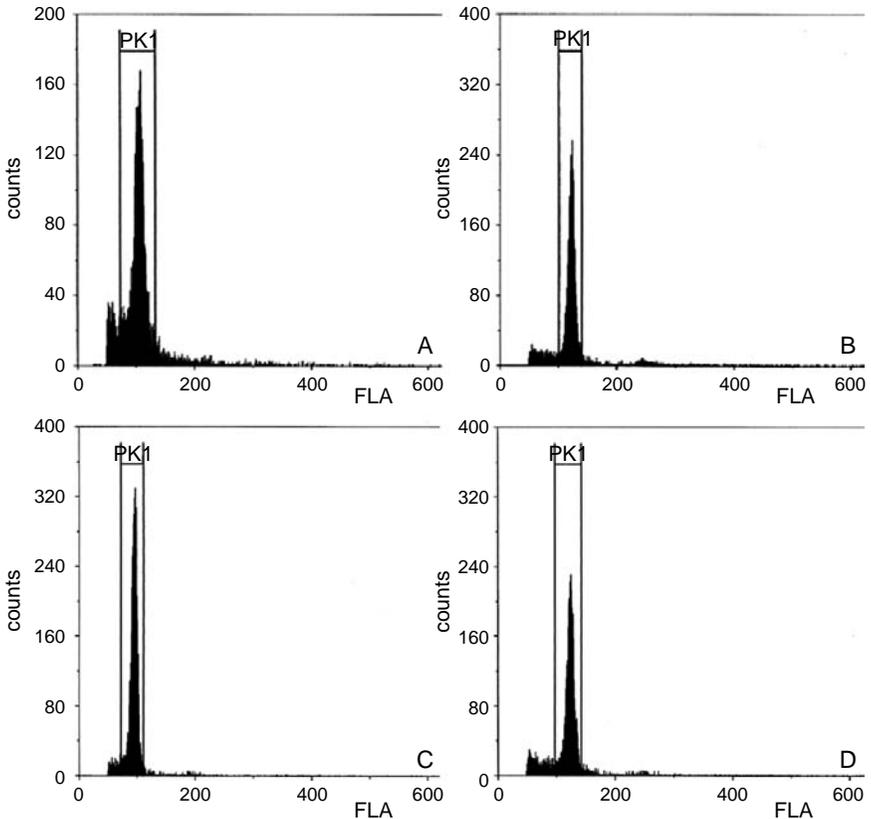


Figure 6. Flow cytometry histograms of blueberry cv. 'Berkeley' (A – meristem-derived clone, B–D – adventitious regeneration-derived clones).

The histograms obtained after nuclear DNA analysis contained a single dominant peak corresponding to nuclei in G phase of the cell cycle with 2C DNA content. Comparison of DNA histograms of tested *in vitro*-derived clones (Figure 6A–D)

originated from meristems and/or adventitious buds shown that tested clones are characterized by significant genetic stability, as flow cytometry analyses detected any changes in ploidy level among tested clones (Gajdošová et al., 2006).

According to flow cytometry analyses, as well as a low DNA polymorphism level and distance index values determined by DNA microsatellite polymorphism it can be stated that the presented regeneration protocol is suitable for production of true to type plants.

3. CONCLUSION

In conclusion we can state, that an efficient cloning protocol was developed for *Vaccinium corymbosum* L. cultivars, which enables large-scale propagation of high quality, true to type plants for need of practice. Our results, as well as results of other authors, prevailingly confirmed that *in vitro* regeneration ability is highly genotype depending, therefore small protocol modifications may be necessary in different cultivars.

Acknowledgement. The work was supported by Slovak Grant Agency VEGA, project no. 2/5128/25, COST 863 Action and APVT-20-026002.

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CHAPTER 42

PROTOCOL FOR MICROPROPAGATION OF *VACCINIUM VITIS-IDAEA* L.

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1. INTRODUCTION

Berry fruit species are in many countries considered as important, biologically and economically valuable species of small fruits. They acquired the great importance in human nutrition because of their high nutritional value and high antioxidant content important for human health. Berry fruits have the broad use in food and pharmaceutical industry. In lingonberry (*Vaccinium vitis-idaea* L.) the original forms are considerably reduced on their natural localities caused by intensive and harsh collection. The production of berry should be therefore replaced by growing of new productive cultivars. Lingonberries are already cultivated in many northern latitude countries in Europe and in the USA, as alternative and non-conventional fruit crops, and are also grown as landscape ornamental ground cover plants. The trends are to use them more intensively for growing in mountain and sub-mountain areas, which are in the complex climate – soil suitable for their cultivation. Large-scale production of high quality planting material of new productive cultivars is necessary for establishment of production plantations. Traditional vegetative propagation of *Vaccinium* species by cuttings has not been successful due to poor rooting ability, considerable demand for large amount of mother plants, their limited seasonal growth and relatively high price.

The problems which are connected with traditional propagation methods, e.g. rapid spread of diseases, low propagation coefficient, can be eliminated by using *in vitro* techniques. In fruit species these techniques assume high practical importance because they enable not only rapid and effective plant propagation, but also elimination of pathogens and this way production of healthy planting material.

Disadvantages of *in vitro* propagation in *Vaccinium vitis-idaea* L. is higher cost of *in vitro* plants in comparison with classically propagated plants, mainly in cultivars with natural rich shooting ('Koralle', 'Linnea'). However, this technique could be effectively used for a production of cultivars characteristic by weak shooting ('Ida', 'Sanna') (Gustavsson & Trajkovski, 1999 Ondrušková et al., 2006).

The present paper is focused on description of efficient *in vitro* regeneration and propagation protocol for *Vaccinium vitis-idaea* L.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Growing Conditions of Mother Plants

The mature mother plants of *Vaccinium vitis-idaea* L. were cultivated in sandy-clay soil on the plantation of Research Station Krivá na Orave, Slovak Republic. Since 1994, several cultivars of this species were tested for their suitability to grow under local climatic conditions. The Research Station is located at 700 m above sea level, with an average annual temperature 6°C and annual precipitation 800–900 mm, the highest during June and July. Each plant was planted into acid peat-made bed, with the volume of 10 l peat per plant, and pH 3.8.

2.1.2. Explant Excision and Sterilisation

As an initial plant material stem cuttings with dormant buds were collected from the selected cultivars of mature mother plants during the month of February and beginning of March. Cuttings with apical and axillary buds were washed under running water for 1 h followed by 70% ethanol treatment for 2 min and 0.1% mercuric chloride with 3 drops of Tween for 6 min, and finally washed with sterile distilled water for 3×15 min.

2.2. Culture Medium and Conditions of Cultivation

For primary explant cultivation Anderson's Rhododendron medium – AN (Anderson, 1980) was prepared (Table 1) from packaged powder macro and micro mineral salt mixture including vitamins (Duchefa, The Netherlands). For 1 liter medium preparation, 2 g powder was completely dissolved in double distilled water. Heat stable supplements, such as 30 g sucrose, 8 g Phyto agar, desired plant hormones, etc. were added and medium raised to the final volume by adding double distilled water. Medium pH was adjusted with HCl and KOH and heated until the solution was clear. Medium was autoclaved at $1 \text{ kg}^{-1} \text{ cm}^{-2}$ (15 psi), 121°C, for 20 min. Heat labile constituent zeatin was filter sterilized and added to the medium after autoclaving. All used plant growth regulators and Phyto agar were supplied by Duchefa, The Netherlands. Cultures were maintained in the growth chamber at $23 \pm 2^\circ\text{C}$ under 16-h photoperiod with light intensity $50 \mu\text{mol m}^{-2}\text{s}^{-1}$ provided by white fluorescent lamps.

Table 1. Composition of Anderson's (AN) *Rhododendron* medium.

AN medium (Anderson, 1980)	Shoot regeneration from dormant apical and axillary buds	Adventitious shoot induction on leaf explants through callus phase	Adventitious shoot regeneration and Microshoot proliferation	<i>In vitro</i> rooting of microshoots
Macroelements	mg l⁻¹	mg l⁻¹	mg l⁻¹	mg l⁻¹
CaCl ₂	332.02	332.02	332.02	332.02
KNO ₃	480.00	480.00	480.00	480.00
MgSO ₄	180.54	180.54	180.54	180.54
NaH ₂ PO ₄	330.60	330.60	330.60	330.60
NH ₄ NO ₃	400.00	400.00	400.00	400.00
Microelements				
CoCl ₂ ·6H ₂ O	0.025	0.025	0.025	0.025
CuSO ₄ ·5H ₂ O	0.025	0.025	0.025	0.025
FeNaEDTA	73.40	73.40	73.40	73.40
H ₃ BO ₃	6.20	6.20	6.20	6.20
KJ	0.30	0.30	0.30	0.30
MnSO ₄ ·H ₂ O	16.90	16.90	16.90	16.90
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	0.25	0.25
ZnSO ₄ ·7H ₂ O	8.60	8.60	8.60	8.60
Vitamins				
Adenine hemisulphate	80.00	80.00	80.00	80.00
Myo-Inositol	100.00	100.00	100.00	100.00
Thiamine-HCl	0.40	0.40	0.40	0.40
Plant growth regulators and other components				
Zeatin	0.75 mg l ⁻¹	2.19 mg l ⁻¹	0.5 mg l ⁻¹	–
IBA	–	–	–	0.80 mg l ⁻¹
charcoal	–	–	–	0.80 g l ⁻¹
Sucrose	30.00 g l ⁻¹	30.00 g l ⁻¹	30.00 g l ⁻¹	30.00 g l ⁻¹
Phyto agar	8.00 g l ⁻¹	8.00 g l ⁻¹	8.00 g l ⁻¹	8.00 g l ⁻¹
pH	4.5–5.0	4.5–5.0	4.5–5.0	4.5–5.0

2.3. Shoot Regeneration and Maintenance

2.3.1. Shoot Regeneration from Dormant Apical and Axillary Buds in *Vaccinium vitis-idaea* L.

Elaboration of efficient shoot regeneration by axillary branching in *Vaccinium vitis-idaea* L. – cv. 'Red Pearl', was aimed for needs of practice. For direct shoot regeneration dormant apical and axillary buds were used, from which the upper scales were removed after sterilisation. AN shoot regeneration medium (Table 1) supplemented with different cytokinin concentrations was used for the cultivation.

Influence of cytokinin zeatin on shoot regeneration ability was evaluated after 5 weeks. Data evaluation was performed using Statgraphic analysis of variance and multiple range analysis. The optimal composition of shoot regeneration medium and zeatin concentration for the tested cultivar is presented in Table 1.

Positive influence of zeatin on multiple shoot induction was confirmed in *V. vitis-idaea*. From tested zeatin concentrations the most effective was 0.75 mg l^{-1} zeatin (Figure 1A). On an average 6.8 numbers of shoots in cultivar 'Red Pearl' were regenerated on the medium supplemented with 0.75 mg l^{-1} zeatin (Ostrolucká et al., 2002).

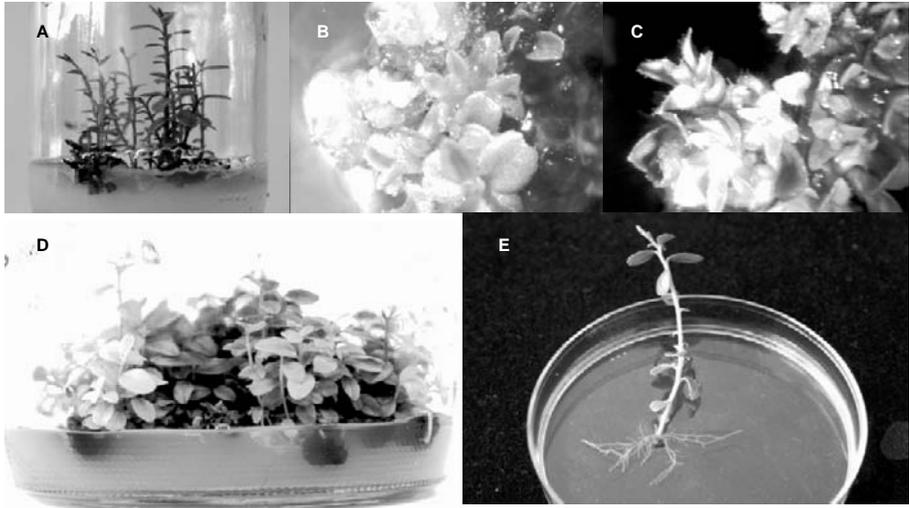


Figure 1. *In vitro* propagation of *V. vitis-idaea*. A) Shoot regeneration from dormant apical and axillary buds on AN medium with 0.75 mg l^{-1} zeatin. B) Adventitious shoot regeneration on leaf-derived callus after 8 weeks of cultivation on AN medium with 0.5 mg l^{-1} zeatin. C) Adventitious shoot regeneration on AN medium with 0.5 mg l^{-1} zeatin after 12 weeks of cultivation. D) Intensive shoot proliferation on AN medium with 0.5 mg l^{-1} zeatin. E) *In vitro* rooting of *V. vitis-idaea*, cv. 'Red Pearl' microshoot.

2.3.2. Shoot Regeneration via Adventitious Organogenesis in *Vaccinium vitis-idaea* L.

Adventitious organogenesis is very efficient to scale up clonal production of selected genotypes of several *Vaccinium* species (Gajdošová et al., 2006). *Vaccinium vitis-idaea* L. cv. 'Red Pearl' was tested for adventitious shoot induction. As a primary explants leaves of *in vitro* plants with cut margins, were placed horizontally with adaxial surface on the culture medium. AN medium supplemented with 2.19 mg l^{-1} zeatin was used for adventitious bud induction from leaf tissue (Table 1). After 5 weeks of cultivation explants were transferred on AN medium containing 0.5 mg l^{-1} zeatin for shoot regeneration and multiplication. Percentages of explants regenerating shoots and number of regenerated shoots per explant after 3 subcultures, with subculture interval 5 weeks, were recorded. Shoot regeneration via callus phase was

recorded in *V. vitis-idaea*. On media supplemented with 2.19 mg l^{-1} zeatin callus formation started on leaf explants of cultivar 'Red Pearl'. After transfer of callus on AN medium with 0.5 mg l^{-1} zeatin (Table 1) adventitious buds began to appear on the callus surfaces. After 3 subcultures the number of adventitious shoots regenerated from the leaf-originated callus was considerable high and reached number 122.46 shoots per explant (Figure 1B,C).

2.3.3. Influence of Medium pH on Micropropagation of *Vaccinium vitis-idaea* L.

It is known that by changing pH values nutrient availability and plant metabolism are affected. *Vaccinium* spp. production is limited to soils with naturally low pH or soils treated with acidifying amendments (Finn et al., 1991). For understanding of pH tolerance *in vitro* screening with modification of pH values in AN medium with 0.5 mg l^{-1} zeatin within a tested pH range 4.0–6.0 was performed in selected cultivars of *V. vitis-idaea* cvs. 'Red Pearl' and 'Koralle' during shoot formation from apical and axillary buds (Ostrolucká et al., 2004).

In cv. 'Koralle' with increasing pH values in the range 4.0–5.5 the increasing tendency in shoot proliferation intensity was observed during 1–3 subcultures, with the highest shoot number at the third subculture on the medium with pH 5.5 (9.63 shoots/explant). At higher medium pH 6.0, the considerable decreasing in shoot number was noted (4.63 shoots/explant).

The higher tolerance to medium pH exhibits cv. 'Red Pearl', where the highest value in shoot number was noted at pH 4 (7.83 shoots/explant). In the pH range 4.5–5.5 the number of formed shoots was similar (4.73–5.10), while at medium pH 6 the number of shoots declined to 3.13. We can state that cv. 'Red Pearl' is much more tolerant to the substrate pH, what is favourable for the growing in the less demanding natural conditions. In general, the medium pH value in the range 4–5.5 may be recommended for micropropagation of lingonberry.

2.3.4. Shoot Proliferation

For long-term proliferation of *in vitro* regenerated axillary or adventitious shoots AN medium supplemented with 0.5 mg l^{-1} zeatin was successfully used. On this medium formation of vigorous multiple shoot cultures was observed with different intensity of shoot proliferation in tested cultivars. Increasing of shoot proliferation intensity can be achieved by segmentation of regenerated, elongated microshoots on one-node segments and their further cultivation on medium with 0.5 mg l^{-1} zeatin (Figure 1D).

2.4. Rooting and Hardening

Satisfactory rooting of isolated microshoots, alike in *Vaccinium corymbosum*, is possible to achieve by two approaches:

1. *ex vitro* rooting – by short (2–3 min) dipping of 15–20 mm long microshoots into 0.8 mg l^{-1} IBA solution followed by direct planting in containers 50 mm in diameter filled with peat substrate;

2. *in vitro* rooting on AN medium supplemented with 0.8 mg l^{-1} IBA and 0.8 g l^{-1} activated charcoal.

With both approaches the percentage of rooting reached 80–90–95% in dependence on cultivar. The rooted plantlets transplanted into peat substrate were subsequently acclimatized. Hardening of plantlets was performed under greenhouse conditions without any special light treatment, with regular irrigation, in containers at the beginning (2 weeks) covered by lid, later opened. Plantlets in length 100–150 mm (after 2 months) were replanted into bigger containers (120 mm in diameter) and transferred to Research Station Krivá, where they were placed under open-air conditions. Transfer of regenerants from *in vitro* to *ex vitro* conditions and their acclimatization was successful, as almost 80–90% of transferred plants survived (Figure 1E, 2A,B).

2.5. Field Testing

Transferred plants in length 100–150 mm were kept in containers (120 mm in diameter) placed under open-air conditions for 2 years. Two years-old plants were planted into acid peat-made bed, with the volume of 10 l peat per plant, and pH 3.8 in experimental orchard of Research Station Krivá. *In vitro* plants were from the beginning characterized by more synchronous growth. No morphological variations or anomalies were observed among them. In general, during the first 2 years after transfer to soil, *in vitro*-derived plants formed more shoots and had a bushier growth habit with a higher number of flower buds. The beginning of breeding was slightly delayed for *in vitro*-derived plants. This is an advantage for commercial growers, because during the first 2 years after planting fruit setting is not desirable. On the contrary, the vegetative growth should be supported in order to strengthen full fertility during following years. Later no obvious morphological differences were visible between *in vitro* and by cuttings propagated plants. Regarding fertility and berry quality the plants are also equivalent.



Figure 2. Hardening and acclimatization of *in vitro*-derived *V. vitis-idaea* plants in containers under A) greenhouse conditions and B) under open-air conditions.

2.6. Flow Cytometry Analysis

Flow cytometry analysis was performed to determine potential undesirable variations in genome size induced during *in vitro* regeneration process. Three clones of *V. vitis-idaea* cv. 'Red Pearl' derived by adventitious regeneration and one meristem-derived clone were analysed. Flow cytometry analyses were performed on Ploidy analyzer PA II, with mercury arc lamp using UV excitation. The samples were prepared from young leaves of *in vitro* plants of *Vaccinium vitis-idaea* L. by two step procedure using reagent kit Partec CyStain UV precise P (Partec GmbH, Münster, Germany) containing Extraction Buffer and Staining Buffer with DAPI:

- Approximately 20 mg of young leaf tissue were chopped with a sharp razor blade in a plastic Petri dish containing 400 µl Extraction Buffer for 30–60 s. After incubation in Extraction Buffer for 1–2 min at the room temperature the sample was filtered through a 50 µm filter.
- Staining Solution 1.6 ml was added to the test tube and sample was incubated for 30–60 s. Samples were analysed in flow cytometer in the blue fluorescence channel.

The histograms obtained after nuclear DNA analysis contained a single dominant peak corresponding to nuclei in G₁ phase of the cell cycle with 2C DNA content. Very few nuclei were found having 4C DNA content. The distribution of nuclear DNA content obtained after the analysis of nuclei isolated from meristem-derived clone was similar to those of adventitious regeneration-derived clones (Figure 3A–D). It can be stated that all tested clones are characterized by significant genetic stability and polyploid cells occurred in tissues individually without a significant tendency towards polyploidization. These findings suggest that no changes in ploidy levels occurred during regeneration process (Gajdošová et al., 2006).

Considering the results of flow cytometry analyses, it seems highly probable that the stability of ploidy level under *in vitro* conditions is a more general phenomenon in *Vaccinium* spp. plants.

3. CONCLUSION

In conclusion we can state, that an efficient cloning protocol was developed for *V. vitis-idaea* L. cultivars, which enables large-scale propagation of high quality, true to type plants for need of practice. Our results, as well as results of other authors, prevailingly confirmed that *in vitro* regeneration ability is highly genotype depending, therefore small protocol modifications may be necessary in different cultivars.

Acknowledgements. The work was supported by Slovak Grant Agency VEGA, project no. 2/5128/25 and COST 863 Action.

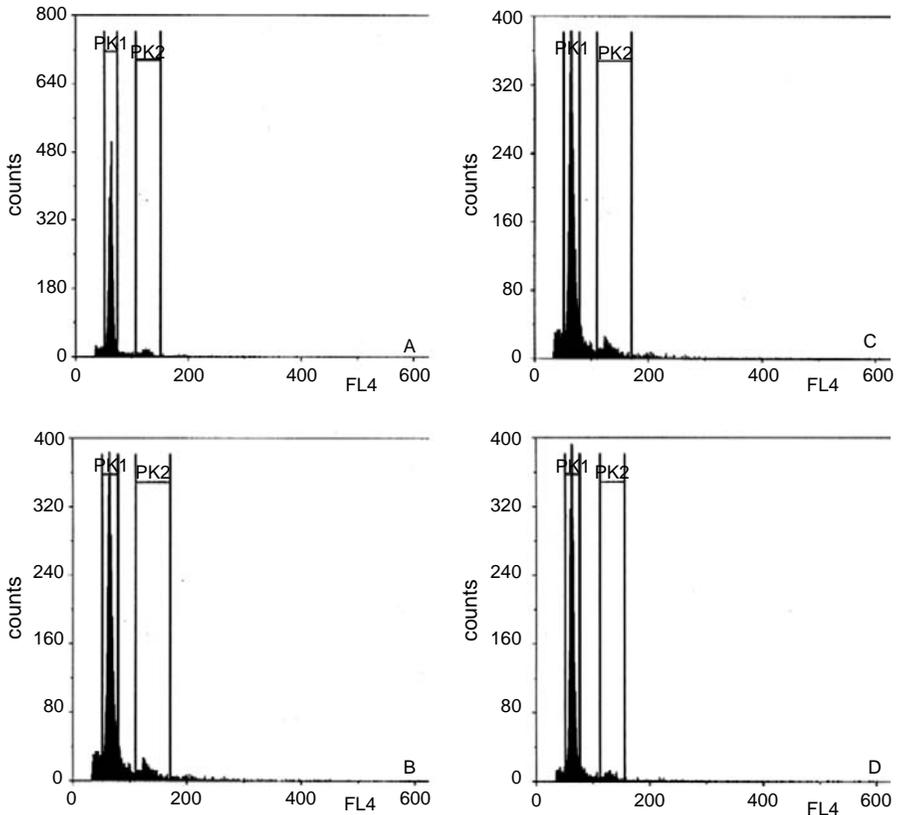


Figure 3. Flow cytometry histograms of lingonberry cv. 'Red Pearl' A – meristem-derived clone, B–D – adventitious regeneration-derived clones.

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CHAPTER 43

MICROPROPAGATION OF BAMBOO SPECIES THROUGH AXILLARY SHOOT PROLIFERATION

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1. INTRODUCTION

Bamboos are woody perennial evergreen monocotyledonous of considerable economic, social and ecological importance. Woody bamboos belong to the tribe Bambuseae that comprises 9 to 10 sub tribes, containing between 59 and 111 genera and at least 1447 species (Ohrnberger, 1999). The diversity observed within this group of plants is huge, ranging from very small members up to tall stately individuals measuring over 30 m tall.

Conventionally, the propagation of bamboos is done with seeds, clump divisions, and rhizome and culm cuttings. However, gregarious flowering, low seed viability, high costs, problems facing long-distance transportation of vegetative propagules, and poor efficiency of plant production, compelled development of alternative propagation methods (Gielis et al., 2001). *In vitro* micropropagation constitutes a feasible alternative to mass-propagate individuals in this plant group. Somatic embryogenesis (Lin et al., 2004 and references therein) and propagation using axillary buds (Jiménez et al., 2006; Ramanayake et al., 2006 and references therein) have effectively been used to multiply bamboos *in vitro*. The latter procedure has been implemented successfully in several bamboo species producing high multiplication rates. According to Gielis and Oprins (2002), this method will be of choice for mass scale propagation of bamboos because the regenerated plants are genetically uniform.

Since the diversity of bamboos is so vast, it is difficult to present a unique step-by-step protocol for micropropagation of all plants classified within this group. Gielis and Oprins (2002) indicate that they have succeeded in developing a common micropropagation protocol for at least 60 temperate and tropical bamboos through axillary branching, but commercial interests have made the procedure elusive for the use among the scientific community. The protocol described in this chapter is based

on our experience with *Guadua angustifolia* and *Dendrocalamus giganteus*, two giant members of this tribe. However, with the aim of producing a general protocol that could be followed up for propagation of other bamboo species, additional information, reported for other species, is also included.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Growing Condition of Mother Plants

Both, adult field- and small greenhouse-grown plants are source of explants for *in vitro* propagation of bamboo. The latter, when vegetatively derived from selected adult plants with known characteristics, are the most adequate because they can be managed to reduce contamination, particularly of endogenous nature. This is especially critical in tropical conditions.

Water greenhouse-grown plants only to the base and keep them in excellent growing and sanitary conditions (adequate fertilization and pest management). Spray aerial parts of the plants regularly with a combination of a bactericide and a fungicide (e.g., 2 g l⁻¹ Agrimycin [Pfizer] and 2 g l⁻¹ benomyl [Jiménez et al., 2006]). If the plants do not have new actively growing shoots (about 2–3 mm in diameter, with well-distinguishable buds), prune them severely about 2–4 weeks in advance to explant excision (depending on the species). This will induce growth of new shoots (Figure 1A).

2.1.2. Explant Excision and Sterilization

Lateral and healthy actively growing branches, about 2–3 mm in diameter, seem to be the most suitable source of nodal segments. According to our experience, thinner branches are more vulnerable to the sterilization procedure, while in thicker branches browning and necrosis occur at a higher rate. In some cases, plantlets obtained from *in vitro*-germinated seeds were effective as the starting material (Saxena, 1990; Chambers et al., 1991; Arya et al., 1999). However, the large variability observed in these plants makes them less suitable for the clonal propagation of elite individuals.

For explant excision, cut individual nodes, having a conspicuous bud underneath the leaf bracts, leaving at least 2 cm of internode at each side of the bud and keeping in mind the polarity of the section. Use sharp pruning shears previously cleaned with 0.5% sodium hypochlorite (NaOCl) or 70% ethanol (Figure 1B). Carefully remove the leaf bracts manually or with fine forceps, clearing the bud completely (Figure 1C). Discard explants without a visible bud. Wash isolated node sections in running tap water for 15 min.

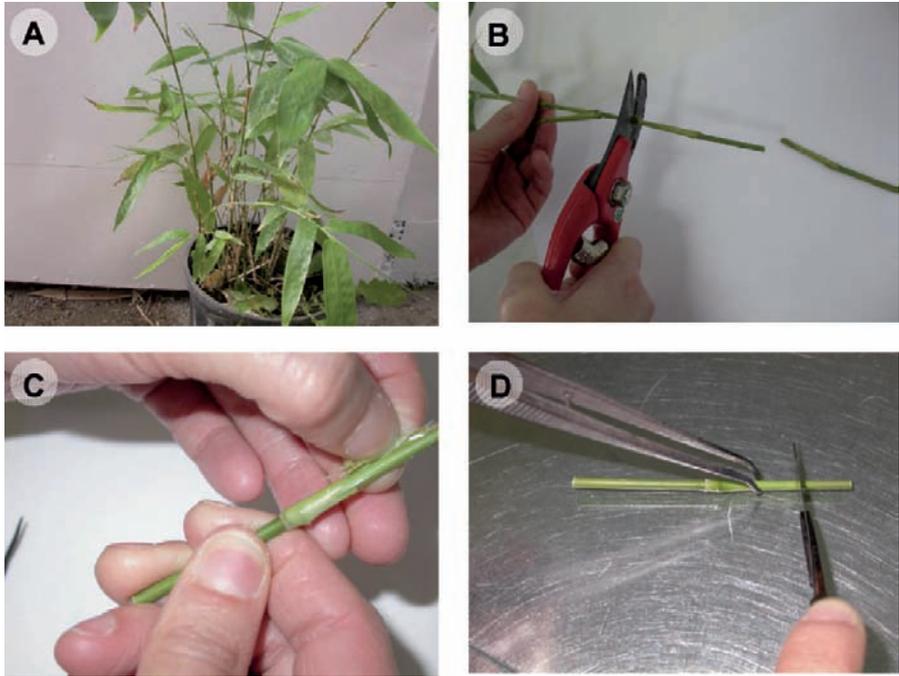


Figure 1. Excision of bamboo nodes. A) Adult-derived plants growing in the greenhouse with new shoots produced after severe pruning, B) Dissecting the cuttings with disinfected pruning shears, C) Eliminating the bracts, D) Eliminating the damaged ends after sterilization.

Obtaining contaminant-free explants has been difficult in some cases (Das & Pal, 2005b; Jiménez et al., 2006; Ramanayake et al., 2006). Pre-sterilization treatments have reduced significantly initial contamination in several cases. For a first pre-sterilization step, stir the nodes in 0.05% alkaline detergent Extran® MA 01 (Merck, Darmstadt, Germany) for 10 min. Subsequently, stir the nodes for 10 min in a mixture of bactericide Agrimycin (Pfizer) and fungicide benomyl, 2 g l⁻¹ of each (Jiménez et al., 2006).

For the sterilization, stir the nodes in an aqueous solution of NaOCl (1.5%), added with one drop of Tween 80 for each 100 ml solution, for 10 min. Afterwards, wash the explants three times with sterile distilled water in a laminar flow cabinet. Finally, remove the damaged borders of the explants with a flamed sharp blade or flamed pruning shears, reducing their size to 2–3 cm length (Figure 1D).

Sterilization with NaOCl can be substituted by stirring the explants in mercuric chloride (0.1–0.3% w/v) for 5–10 min (Nadgir et al., 1984; Ramanayake & Yakandawala, 1997; Bag et al., 2000; Marulanda et al., 2002). Sequential use of both products has also been employed (Das & Pal, 2005a). However, NaOCl should be preferred over mercuric chloride because the former is a less hazardous compound.

2.2. Culture Medium

2.2.1. Medium Composition

Murashige & Skoog (MS) mineral salts and vitamins (Murashige & Skoog, 1962), as described in Table 1, work for most of genera and species evaluated up to date. Plant growth regulators (PGRs) composition varies according to the culture step (bud break, development of lateral shoots or of roots) and on the species under study, and is referred in Tables 3 to 5. Gelrite is preferred over agar as gelling agent because it forms a more transparent gel than agar, facilitating detection of contamination in earlier stages.

Table 1. Formulation of culture medium frequently used for bamboo micro-propagation through axillary bud proliferation.

Component	Stock solution	Amount to add per liter	Final concentration in medium
MS macronutrients	10X	100 ml	1X
MS micronutrients	100X	10 ml	1X
NaFeEDTA*	7.34 mg ml ⁻¹	5 ml	36.7 mg l ⁻¹
Thiamine-HCl	0.1 mg ml ⁻¹	1 ml	0.1 mg l ⁻¹
Nicotinic acid	0.1 mg ml ⁻¹	5 ml	0.5 mg l ⁻¹
Pyridoxine	0.1 mg ml ⁻¹	5 ml	0.5 mg l ⁻¹
Myo-Inositol	10 mg ml ⁻¹	10 ml	100 mg l ⁻¹
Glycine	2 mg ml ⁻¹	1 ml	2 mg l ⁻¹
PGR(s)	variable	variable	variable
Sucrose		30 g	3%
Gelrite		2 g	0.2%

* Sodium iron (III) ethylenediaminetetraacetic acid.

2.2.2. Medium Preparation

To prepare one liter medium follow the next steps. Scale for smaller or larger volumes. Add appropriate amount of stock solutions of all ingredients to ~400 ml distilled water as indicated in Table 1 (filter-sterilized PGRs can also be added after autoclaving). To address serious contamination problem (especially latent endogenous contamination), addition of 2 ml l⁻¹ Plant Preservative Mixture[®] (PPM, Plant Cell Technology, Washington DC, USA) (Jiménez et al., 2006) or 1 g l⁻¹ benomyl (Ramanayake and Yakandawala, 1997) prior to autoclaving might be useful. Add sucrose and stir well until it dissolves completely, raise volume up to one liter, adjust pH to 5.8 with KOH or HCl, and add gelrite. Heat it in the microwave for approximately 7 min for each liter of medium (1300 W) and stir well with a glass stick. Continue heating at 2–3 min interval, stir intermittently. Dispense the medium into the culture vessels only after it has boiled at least twice. Autoclave at 121°C and 1.05 kg cm⁻² for the corresponding time specified in Table 2. Alternatively, medium can be autoclaved in larger volumes and then be poured into sterile vessels in the laminar flow cabinet.

Table 2. Minimum autoclaving time for plant tissue culture media (adapted from Burger 1988)

Volume of medium per vessel (ml)	Minimum autoclaving time (min)
25	20
50	25
100	28
250	31
500	36

2.3. Bud Break and Development of Lateral Shoots

Insert nodal segments vertically into the gelled culture medium in the upright position up to the nodal region (Figure 2A). N⁶-benzylaminopurine (BAP), at a concentration of 3 mg l⁻¹, has given the best results in our work with *Guadua angustifolia*. Table 3 could be referred to look for adequate composition of PGRs for other species.

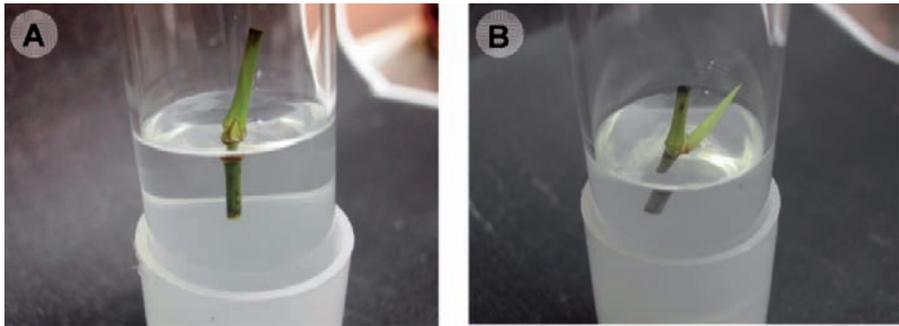


Figure 2. Growth of the bamboo nodal segments. A) Nodal segment inserted upright in the culture medium, B) Nodal explants with a growing bud.

Initially, place cultures in the dark at 26°C for 2 weeks to reduce oxidation and necrosis. Thereafter, transfer them to light, 16-h photoperiod regime, where axillary buds start growing (Figure 2B). The light intensity does not seem to be a critical point for growth of bamboo axillary buds, since there are references in the literature with successful bud development under light intensities ranging from 10 to 140 $\mu\text{mol m}^{-2} \text{s}^{-1}$, provided by fluorescent tubes.

Subculture every 3–4 weeks to fresh culture medium with the same composition until a well-developed axis forms (Figure 3A). Depending on the size of the explants, larger vessels might be necessary. Keep in mind autoclaving time for larger medium volumes (Table 2).

Table 3. PGR combinations that produced highest bud break in different bamboo species.

Compound(s)	Concentration	Species	Reference
BAP + kinetin	2.5 mg l ⁻¹ + 1 mg l ⁻¹	<i>Bambusa balcooa</i>	Das & Pal (2005b)
BAP + kinetin + coconut water	2 mg l ⁻¹ + 1 mg l ⁻¹ + 8%	<i>Bambusa tulda</i>	Das & Pal (2005a)
BAP	1 mg l ⁻¹	<i>Bambusa ventricosa</i>	Huang & Huang (1995)
BAP	2 mg l ⁻¹	<i>Bambusa vulgaris</i>	Ramanayake et al. (2006)
BAP + kinetin	2 mg l ⁻¹ + 0.1 mg l ⁻¹	<i>Dendrocalamus giganteus</i>	Ramanayake & Yakandawala (1997)
BAP + kinetin	2.7 mg l ⁻¹ + 0.65 mg l ⁻¹	<i>Dendrocalamus longispathus</i>	Saxena & Bhojwani (1993)
BAP + kinetin + coconut water	0.2 mg l ⁻¹ + 0.5 mg l ⁻¹ + 10%	<i>Dendrocalamus strictus</i> <i>Bambusa arundinacea</i> <i>Bambusa vulgaris</i>	Nadgir et al. (1984)
BAP	1 mg l ⁻¹	<i>Guadua angustifolia</i>	Marulanda et al. (2002)
BAP	3 mg l ⁻¹	<i>Guadua angustifolia</i>	Jiménez et al. (2006)

Release of brown-colored exudates (probably phenolic compounds) seems to be a problem during establishment of bamboo nodal explants in certain cases. More frequent subcultures (every 1–2 weeks) generally suffice to avoid a detrimental effect of these exudates. Liquid culture medium is helpful to alleviate these problems (Das & Pal, 2005a,b).

To induce growth of lateral shoots at the base of the growing axis (Figure 3B), transfer well-developed axes, about 6–8 cm long, to proliferation medium (Table 4) contained in larger vessels. It is important that the developing shoots can grow without contacting the vessel.

Sometimes the medium used for bud break also worked for shoot proliferation (Saxena & Bhojwani, 1993; Das & Pal, 2005b).

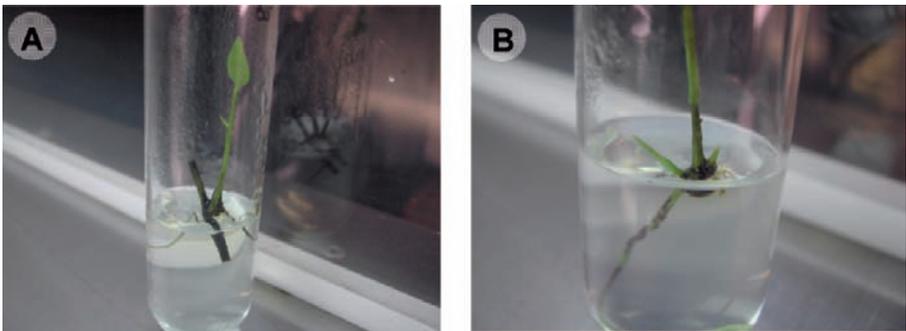


Figure 3. Growth of the bamboo nodal segments. A) Developing axis, B) Development of lateral shoots.

Table 4. PGR combinations that produced highest development of lateral shoots in different bamboo species.

Compound(s)	Concentration	Species	Reference
BAP	0.45 mg l ⁻¹	<i>Bambusa bambos</i> var. <i>gigantea</i>	Kapoor & Rao (2006)
Thidiazuron	0.01–6 mg l ⁻¹	<i>Bambusa edulis</i>	Lin & Chang (1998)
IBA*	3 mg l ⁻¹	<i>Bambusa tulda</i>	Das & Pal (2005a)
BAP + kinetin	1.8 mg l ⁻¹ + 0.86 mg l ⁻¹	<i>Bambusa tulda</i>	Saxena (1990)
BAP	4 mg l ⁻¹	<i>Bambusa vulgaris</i>	Ramanayake et al. (2006)
BAP	3 mg l ⁻¹	<i>Dendrocalamus asper</i>	Arya et al. (1999, 2002)
BAP + kinetin + coconut water	6 mg l ⁻¹ + 0.1 mg l ⁻¹ + 8%	<i>Dendrocalamus giganteus</i>	Ramanayake & Yakandawala (1997)
BAP	6 mg l ⁻¹	<i>Dendrocalamus giganteus</i>	Ramanayake et al. (2001)
BAP	1 mg l ⁻¹	<i>Dendrocalamus hamiltonii</i>	Chambers et al. (1991)
BAP + kinetin + coconut water	0.5 mg l ⁻¹ + 0.5 mg l ⁻¹ + 20%	<i>Dendrocalamus strictus</i>	Ravikumar et al. (1998)
BAP	0.5 mg l ⁻¹	<i>Dendrocalamus strictus</i>	Shirgurkar et al. (1996)
Thidiazuron	0.5 mg l ⁻¹	<i>Dendrocalamus strictus</i>	Singh et al. (2001)
BAP	5 mg l ⁻¹	<i>Guadua angustifolia</i>	Jiménez et al. (2006)
BAP + NAA**	1 mg l ⁻¹ + 0.5 mg l ⁻¹	<i>Pseudoxynthera stocksii</i>	Sanjaya et al. (2005)
BAP	1.13 mg l ⁻¹	<i>Thamnocalamus spathiflorus</i>	Bag et al. (2000)

* indolebutyric acid, ** naphthaleneacetic acid.

2.4. Plant Multiplication

To multiply bamboo shoots *in vitro*, place large shoot clumps (8 to 10 shoots) on a sterile surface in the laminar flow cabinet. Carefully divide in groups of 3–5 axes, with at least one actively growing shoot in each one (Figure 4A, red line). Trim off the roots before subculture in those genotypes that spontaneously form them (discussed below). Place the individual shoot clumps (Figure 4B) on fresh medium containing the combination of PGRs that best induced development of lateral shoots (Table 4). Alternatively, dissect single-node explants when growing axes are 7 to 10 cm long, and insert them into the fresh medium, similarly to the original explants placed *in vitro* (Figure 4A, blue lines). Nevertheless, the first method should be preferred since it has produced higher multiplication rates when both methodologies were compared (Jiménez et al., 2006).

In vitro rhizome formation might be an alternative for the *in vitro* multiplication of developing shoots. This event has occurred spontaneously in *Dendrocalamus strictus* during the rooting phase (Shirgurkar et al., 1996). *In vitro* rhizome formation has been also reported in *Pseudoxynthera stocksii* with the addition of 0.1 mg l⁻¹ BAP and 1 mg l⁻¹ IBA (Sanjaya et al., 2005), and in *Bambusa bambos* var. *gigantea* with 9.3 mg l⁻¹ NAA, 0.45 mg l⁻¹ BAP, 0.035 mg l⁻¹ gibberellic acid (GA₃) and 5% sucrose (Kapoor & Rao, 2006).

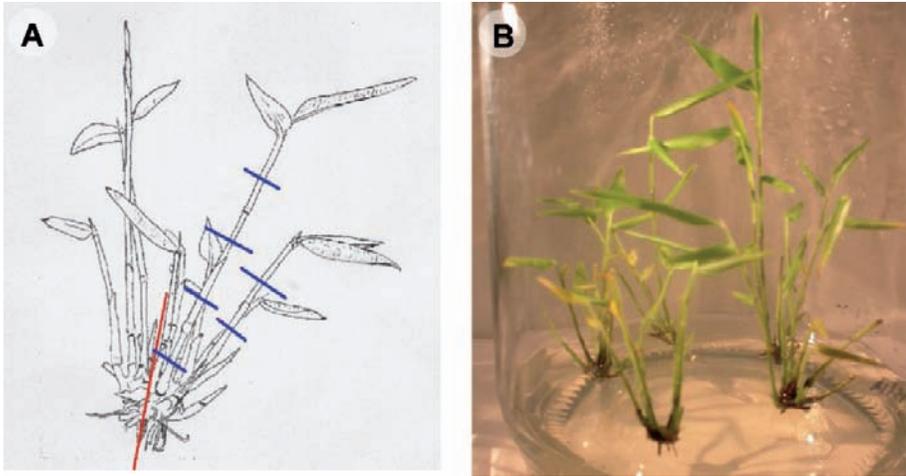


Figure 4. Multiplication of the developed shoot clumps. A) Scheme of a shoot clump with possible cutting sites (the red line indicates site of shoot clump division, while blue lines are for multiplication through single nodes), B) Newly transferred small shoot clumps after division of the large ones.

If PPM or a similar compound was included into the culture medium to deal with contamination problems (see Section 2.2.2), start eliminating this compound from the culture medium in part of the culture vessels at each subculture. This would allow expression of endogenous latent contamination and therefore allows selection of contaminant-free shoot clumps. PPM inhibits growth of microorganisms, but at the same time masks endogenous contamination.

2.5. Rooting

For root induction, transfer well developed actively growing shoot clumps to the rooting medium (see Table 5). If gelled culture medium does not induce rooting, transfer large shoot clumps into large-volume vessels containing a relatively thin film of liquid medium (1 cm in depth), which avoids the necessity of shaking the cultures. In other cases, rooting can be induced *ex vitro* by dipping the shoots in 200 mg l⁻¹ IBA and planting them in polyethylene bags containing sterilized soil and vermiculite (1:1) (Ravikumar et al., 1998).

In *Bambusa vulgaris* 'Striata', a conditioning treatment with 0.1 mg l⁻¹ thidiazuron and exposure to continuous light (48 μmol m⁻² s⁻¹) for two to three subculture cycles of 2 weeks each, before applying the PGR combination indicated in Table 5 for this species, considerably enhanced rooting (Ramanayake et al., 2006). Rooting has also occurred spontaneously in the same medium used for shoot and plant multiplication in *Dendrocalamus strictus* (Shirgurkar et al., 1996) and *Guadua angustifolia* (Jiménez et al., 2006).

Table 5. PGR combinations that induced root development *in vitro* in different bamboo species.

Compound(s)	Concentration	Species	Reference
Thidiazuron + 2,4-D*	0.01 mg l ⁻¹ + 0.5 mg l ⁻¹	<i>Bambusa edulis</i>	Lin & Chang (1998)
IAA** + coumarin	0.0175 mg l ⁻¹ + 0.1 mg l ⁻¹	<i>Bambusa tulda</i>	Saxena (1990)
IBA	0.2 mg l ⁻¹	<i>Bambusa tulda</i> and <i>Bambusa balcooa</i>	Das & Pal (2005a)
NAA + BAP	1 mg l ⁻¹ + 0.1 mg l ⁻¹	<i>Bambusa ventricosa</i>	Huang & Huang (1995)
IBA	3 mg l ⁻¹	<i>Bambusa vulgaris</i>	Ramanayake et al. (2006)
IBA + NAA	10 mg l ⁻¹ + 3 mg l ⁻¹	<i>Dendrocalamus asper</i>	Arya et al. (1999, 2002)
IBA + coumarin	3 mg l ⁻¹ + 10 mg l ⁻¹	<i>Dendrocalamus giganteus</i>	Ramanayake & Yakandawala (1997)
IAA + IBA + coumarin	0.175 mg l ⁻¹ + 0.2 mg l ⁻¹ + 10 mg l ⁻¹	<i>Dendrocalamus longispathus</i>	Saxena & Bhojwani (1993)
IBA	1 mg l ⁻¹	<i>Dendrocalamus strictus</i>	Singh et al. (2001)
IBA + BAP	1 mg l ⁻¹ + 0.1 mg l ⁻¹	<i>Pseudoxytenanthera stocksii</i>	Sanjaya et al. (2005)
IBA	30.5 mg l ⁻¹	<i>Thamnocalamus spathiflorus</i>	Bag et al. (2000)

* 2,4-dichlorophenoxyacetic acid, ** indole-3-acetic acid.

2.6. Hardening and Acclimatization

To simplify and shorten the *in vitro* process, acclimatize the rooted plantlets directly under greenhouse conditions, without a hardening phase. This has been successfully accomplished by Lin and Chang (1998) and Jiménez et al. (2006). For routine acclimatization, carefully remove gelrite from roots and, eventually, rhizomes. Transfer the well-rooted plantlets to small pots (10 cm in diameter) containing moist potting substrate (see Table 6).

Place the plants under mist irrigation and dim light by using a 50% saran shade cloth for the first weeks. Later on, transfer them to standard greenhouse conditions, with normal watering. Discard contaminated plantlets, even if they have a well-developed root system. Their survival rate is very low.

In case of low plant survival, include a hardening phase. For hardening, transfer the rooted clumps to 200–400-ml glass bottles with enough autoclaved potting substrate (like peat moss or vermiculite) to cover the roots completely. Keep the potting substrate moist by adding 50–100 ml of hormone and sucrose-free MS medium (Saxena, 1990; Saxena & Bhojwani, 1993). After 2 weeks of growth in aseptic conditions, remove the caps. After seven additional days of culture in these conditions, acclimatize the plants as described above.

Table 6. Composition of potting substrates employed for acclimatization of bamboo plantlets originated from axillary shoots.

<i>Species</i>	<i>Substrates</i>	<i>Proportion</i>	<i>Reference</i>
<i>Bambusa bambos</i> var. <i>gigantea</i>	Soil, sand, manure	1:1:1	Kapoor & Rao (2006)
<i>Bambusa edulis</i>	Soil		Lin & Chang (1998)
<i>Bambusa tulda</i>	Soil, soilrite, organic manure	1:1:1	Saxena (1990)
<i>Bambusa tulda</i>	Sand, soil, vermiculite	1:1:1	Das & Pal (2005a)
<i>Bambusa ventricosa</i>	Peat, perlite, vermiculite	1:1:1	Huang & Huang (1995)
<i>Bambusa vulgaris</i>	Soil, coir dust	1:1	Ramanayake et al. (2006)
<i>Dendrocalamus asper</i>	Soil		Arya et al. (1999)
<i>Dendrocalamus strictus</i>	Sterilized soil, vermiculite	1:1	Ravikumar et al. (1998)
<i>Dendrocalamus strictus</i>	Soil		Chaturvedi et al. (1993)
<i>Dendrocalamus strictus</i>	Sand, soil	1:1	Shirgurkar et al. (1996)
<i>Dendrocalamus strictus</i>	Sand, garden soil	1:1	Singh et al. (2001)
<i>Guadua angustifolia</i>	Soil, sand, rice hulls	1:1:1	Jiménez et al. (2006)
<i>Guadua angustifolia</i>	Compost		Marulanda et al. (2002)
<i>Pseudoxytenanthera stocksii</i>	Compost, sand, soil	5:4:1	Sanjaya et al. (2005)

3. CONCLUSION

This chapter presents an easy-to-follow core micropropagation protocol for bamboo plants through axillary shoot proliferation. This approach has an advantage over somatic embryogenesis of circumventing callus production, which is more prompt to suffer genetic or epigenetic changes.

It is important to consider the possibility of having to change the PGR composition of the culture media if adequate results are not obtained. Tables 3 to 5 illustrate PGR combinations that produced best results in several bamboo species. The procedure described has the potential of generating many thousands of plants per year from selected superior bamboo genotypes to recover cleared natural stands or to start new plantations. However, it is advisable to start new cultures from greenhouse-grown plants at least every 1–2 years, to reduce the risk of genetic variation arisen *in vitro*.

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CHAPTER 44

IN VITRO CULTURE OF TREE PEONY THROUGH AXILLARY BUDDING

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1. INTRODUCTION

Peonies are long living perennials belonging to the family *Paeoniaceae*, which is one of the world's most ancient flowering plant group once included in the *Ranunculaceae*. These plants have proved to be useful for medicinal use and they have also been attractive ornamental flowers for over 3.000 years in China and at least for years 500 in Europe. The genus *Paeonia* is native to Morocco and Spain across the mountainous regions of Europe and the Mediterranean, through the Caucasus to central Asia, and into China and Japan and even the western United States. It consists of a relatively small number of species (about 33 species, according to Rogers, 1995) and it is taxonomically very complex. There are two basic types of peonies: herbaceous and tree. The former ones are the most common and, as their name implies, these plants present an annual thermoperiodic life cycle where a perennial crown serves for the accumulation of the storage products and plant renewal (Barzilay et al., 2002). Tree peonies are flowering shrubs which grow from 1 to 6 feet tall and are divided into two main groups: the hybrid and the non hybrid group. The latter ones are varieties of *P. suffruticosa*, comprising more than 500 cultivars.

P. suffruticosa cultivars are used as outside ornamentals and as pot plants. Their cultivation under warmer climates, where the flowering process is accelerated, provides an excellent early market niche as cut flowers. The reproduction, under a commercial point of view, is mainly done by two primary methods of vegetative propagation: tuft division and grafting. In division methods, a large plant is simply divided into small plants, each bearing its own roots. In grafting methods, an herbaceous rootstock is used to nourish a tree peony scion until the tree peony

produces its own sustaining roots. The choice of the propagation method is related to the degree of branching of plants which can present a different number of divisible bushes. Taking into consideration that these conventional propagation methods lead to a high cost of the plants due to technical and time-consuming difficulties (Aoki & Inoue, 1992), the peony culture could benefit greatly from micropropagation. A reliable micropropagation system might overcome these difficulties and it could also be used for the multiplication of virus-free stock material or new cultivars.

In vitro culture of peony for industrial applications has for long time been faced by the difficulties such as serious explant contamination, browning and establishment of an ideal medium. Table 1 summarizes the relatively scarce information available on tissue culture of *P. suffruticosa*. The *in vitro* culture of *P. suffruticosa* has been applied principally for plant breeding (haploid embryogenesis, Zenkteler et al., 1975) and for the production of secondary metabolites (Yamamoto, 1988). The propagation system has also been provided at disposal for the horticultural market. Different explant sources have been tested with variable degree of success (Meyer, 1976 a, and b; Li et al., 1984; Buchheim & Meyer, 1992; Bouza et al., 1994a) but the results are sometimes limited to a particular cultivar. Callus could be induced from zygotic embryos (Demoise & Partanen, 1969; Wang & van Staden, 2001), flower buds (Meyer, 1976), stems (Gildow & Mitchell, 1977) and roots (Meyer, 1976b; Gildow & Mitchell, 1977) but neither organogenesis nor embryogenesis was achieved. Results on plantlet regeneration were referred when axillary buds (Li et al., 1984; Bouza et al., 1994a; Beruto et al., 2004), leaves and petioles (Li et al., 1984), filaments and petals (Beruto et al., 2004) and stems (Harris & Mantell, 1991) were cultured. By culturing axillary buds, Li et al. (1984) estimated that rather high multiplication rates could be achieved, but this parameter was strictly dependent on cultivars (generally speaking, the dwarf types are more difficult to be *in vitro* propagated) and sometimes on shoots derived from callus. The objective of this paper is to describe a detailed budding protocol for *in vitro* propagation of tree peony, *P. suffruticosa*, through axillary budding.

2. EXPERIMENTAL PROTOCOL

Procedures described here have been developed for different genotypes of *P. suffruticosa* and, consequently, these techniques should be, in principle, applicable to other tree peony cultivars. Taking into consideration that a prerequisite to apply a micropropagation protocol is that no somaclonal variations occur and that changes on ploidy number was found in callus cultures of *P. suffruticosa* (Buchheim & Meyer, 1992), in this study we have considered the *in vitro* propagation through axillary bud stimulation which, generally, has proven to be the most applied and reliable system for true-to-type *in vitro* propagation. Moreover, our choice is supported by the observation that for many cultivars shoot development from adventitious regeneration is still an important constraint to the application of a propagation protocol for the tree peony (Wang and van Staden, 2001; Beruto et al., 2004). The protocol developed in our laboratory was applied to seventeen Chinese cultivars, two Japanese cultivars ('Orange' and 'Red') and an old traditional cultivar from our region ('Old Pink') (Figure 1).



Figure 1. Tree peony cultivars tested to develop the micropropagation protocol in our laboratory. a) Bai Yu; b) Feng Dan Bai; c) Shi Yuan Bai; d) White Pearl; e) Xue Lian; f) Yao Huang; g) Huang Hu; h) Orange Yellow; i) Golden Palace; j) Hu Hong; k) Da Jin Fen; l) Fish Scale Pink; m) Zhuang Yuan Hong; n) Zi Zy Qiao; o) First Red; p) Red Diamond; q) Zhu Sha Lei; r) Orange; s) Red; t) Old Pink .

However, a part from the genotype, many other factors have been shown to be of considerable importance for successful micropropagation of tree peony. Among others, the physiological status of the shoots, the medium formulation and the type and concentrations of cytokinins. BA has been found to be a cytokinin effective in inducing organogenesis from shoots of *P. suffuticosa* which can be used alone (Bouza et al., 1994a) or in combination with kinetin (Yulong et al., 1984), 2iP (Harris & Mantell, 1991) and NAA (Černá et al., 2001). Generally, hormone concentration must be carefully tested in order to avoid shoot hyperhydricity and a different performance can be scored according to the genotype. The number of subcultures can influence the multiplication efficiency and the quality of the shoots (Bouza et al., 1994a) and, once again, this could support the reaction of tree peony shoots to the hormonal supply. Tree peony shoots have been cultured on both MS (Murashige & Skoog, 1962) and WPM (Woody Plant Medium of Lloyd & McCown, 1980) with Ca^{2+} in double strength (Bouza et al., 1994a; Černá et al., 2001; Beruto et al., 2004). Apical and leaf necroses leading also to the shoot death can occur if calcium concentration

is let to the normally used concentration (3 mM) and if GA₃ is supplied for different subsequent subcultures. Root development for the micropropagated shoots has been achieved either by applying a quick dip of auxin solutions or by culturing them continuously on a rooting medium. Moreover, environmental conditions (darkness and low temperatures) have been found to be critical factors for rooting of tree peonies.

Taking into consideration the examined various parameters influencing the efficiency of *in vitro* tree peony shoot production, we have developed a protocol for micropropagation through axillary branching. The scheme of the protocol is illustrated in Figure 2 and compositions of culture media used are listed in Table 2.

Axillary buds are collected from stock plants. Plantlets can be obtained in about 2 months after the initiation of the culture. Peony shoots are cultured by axillary branching over a year of culture. After rooting, shoots can be acclimatized under *in vivo* conditions and a satisfactory growth development till flowering can be scored (Figure 2).

2.1. Stage 1: Initiation Phase

Axillary buds of an adult shrub of *P. suffruticosa* are collected at the beginning of sprouting in the early spring. Bud scales are discarded and buds are successively sterilized. The ease with which the initial explants can generate a successful culture varies among cultivars and depends on the sterilization procedure applied. Generally, in our laboratory we have found that a dip in a HgCl₂ solution (0.5% w/v) for 3 min followed by a solution of NaOCl (1% available chlorine) for 15 min can provide satisfactory results which can be further improved if the explants are submitted to an additional dip in PPM (Plant Preservative Mixture, Plant Cell Technology, IncTM). Generally speaking the incidence of the contamination is scored already at the end of the initiation phase (8 weeks of culture), but further lost can be scored also after the first subculture (16 weeks of culture) (Figure 3).

The developmental stage of the collected buds can influence the further *in vitro* culture. Our experiments considered the evaluation of the culture of buds at five different developmental stages: stage 1 = bud just emerged; stages 2–5 = buds with progressively expanded leaves (Figure 4). A significant linear correlation ($p < 0.01$) was found between the developmental stage of the bud and its ability to start the culture; just emerged buds, with unexpanded leaves, gave the lowest success rate (43%) compared to buds with expanded leaves (64%).

A negligible percentage (<10%) of necrosis can be observed in this phase of culture. However, in our experiments no cultivars were lost due to this problem and no significant differences in the incidence of this phenomenon were scored among the different cultivars.

Table 1. Summary of in vitro studies conducted on *P. suffruticosa*. Basal media: LS= Linsmaier & Skoog (1965); MS = Murashige & Skoog (1962) with modifications (Mod.); SH = Schenk & Hildebrandt (1972); LB = Liau & Boll (1970); WPM = Woody Plant Medium of Lloyd & McCown B (1980). Supplements: 2,4-D = 2,4-dichlorophenoxyacetic acid; 2-iP = 6- γ - γ -(dimethylallylamino)-purine; BA = benzylamino purine; CM = coconut milk; GA3 = gibberellic acid; IAA = indole-3-acetic acid; Kin = kinetin; NAA = naphthaleneacetic acid; PIC = Picloram, 4-amino-3,5,6-trichloropicolinic acid; TDZ = thidiazuron.

Inoculum	Medium (mg L ⁻¹)	Growth response	Reference
Zygotic Embryo	Steeves (1995) + CM (150) + 2,4-D (0.2)	Callus	Demoise & Partanen (1969)
Anther	Mod. MS + Kin (1) + IAA (1)	Embryos	Zenkter et al. (1975)
Flower Bud	Mod. MS + Kin (2.5) + NAA (2.5–10)	Callus, Roots	Meyer (1976b)
Zygotic Embryo	Mod. LS	Radicle Expansion	Meyer (1976a)
Axillary Vegetative Bud	Mod. MS + Kin (2.5) + NAA (2.5–10)	Callus, Roots	Meyer (1976b)
Root	Mod. MS + Kin (2.5) + NAA (2.5–10)	Callus, Roots	Meyer (1976b)
Stem	Mod. SH or LB + NAA (10) or 2,4-D (0.2–2)	Callus, Roots	Gildow & Mitchell (1977)
Axillary Vegetative Bud	Mod. MS + Kin (0.2-1) + BAP (0.5–1) + GA3 (0.1–0.5)	Callus, Shoot Meristems, Shoots	Li et al. (1984)
Leaf, Petiole	Mod. MS + BAP (2) + NAA (0.1–0.5)	Callus, Shoot Meristems, Plants	Li et al. (1984)
Axillary Vegetative Bud	Mod. MS + BA (1)	Shoots	Bouza et al. (1994a)
Zygotic Embryo, Young Leaves And Petioles	Mod. WPM + 2,4 D (0.5–1) + BA (0.5–1)	Callus	Wang & van Staden (2001)
Stem	MS + BA(1) + NAA (0.2)	Shoots	Černá et al. (2001)
Axillary Vegetative Bud	Mod. WPM + citric acid (75) + ascorbic acid (50)+ BA(1)	Shoots	Beruto et al. (2004)
Filaments	MS + 2iP(5) + PIC(1) or MS + TDZ (0.5) + 2,4D (2)	Callus	Beruto et al. (2004)
Petals	MS + TDZ (0.5)	Callus, Shoots	Beruto et al. (2004)

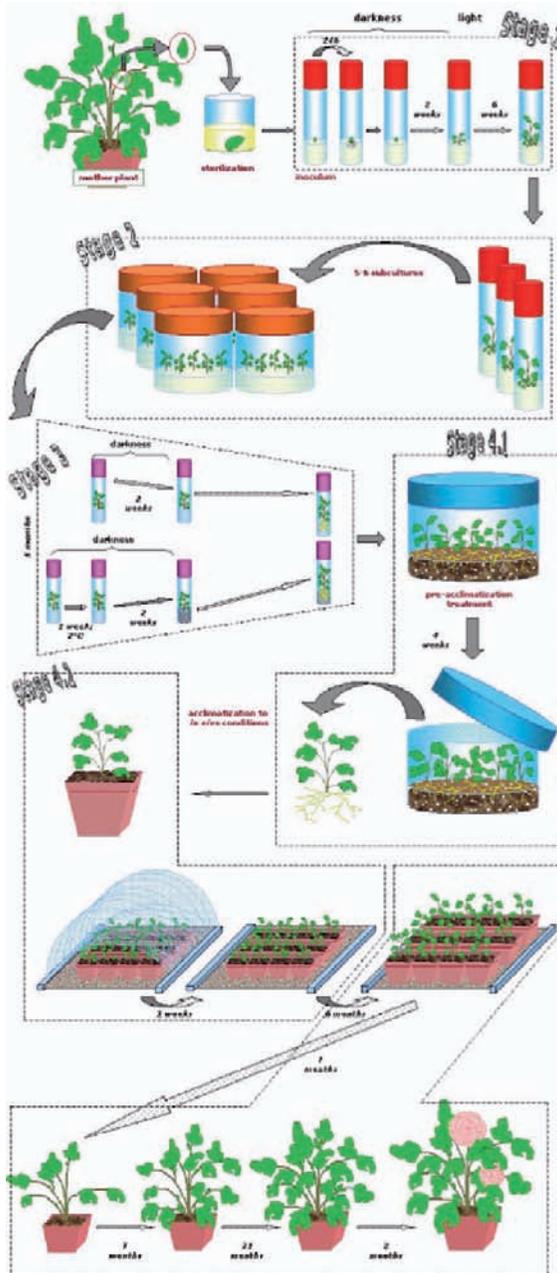


Figure 2. Scheme of the protocol to propagate tree peony through axillary budding (see text and table 2 for media).

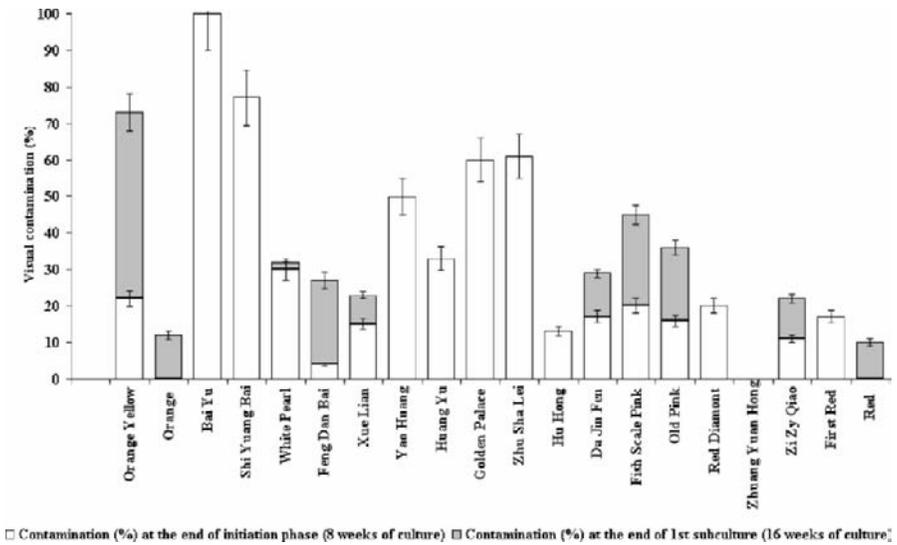


Figure 3. Visual contamination (%) scored at the end of the initiation phase and at the end of the first subculture (8 and 16 weeks of culture respectively).

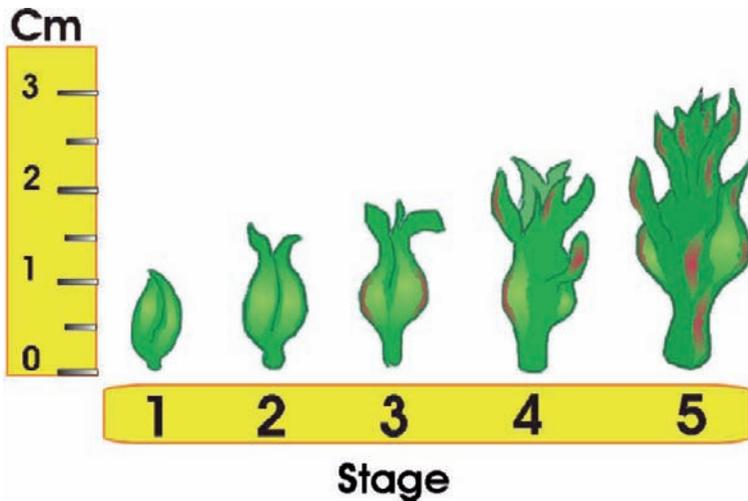


Figure 4. Developmental stage of the bud collected from stock mother plants. Stage 1 = bud just emerged; stages 2-5 = buds with progressively expanded leaves.

Table 2. Culture media used to micropropagate tree peony through axillary budding.

BASAL MEDIUM TO MICROPROPAGATE TREE PEONY (WPMP)		
Major elements (McCown Woody Plant medium, 1980)	(mg L⁻¹)	(mM)
CaCl ₂ ·2H ₂ O	95.56	0.65
Ca(NO ₃) ₂ ·4H ₂ O	554.95	2.35
KH ₂ PO ₄	170.00	1.25
K ₂ SO ₄	990.00	5.68
MgSO ₄ ·7H ₂ O	369.72	1.50
NH ₄ NO ₃	400.00	5.00
Iron and minor elements (Murashige & Skoog, 1962)	(mg L⁻¹)	(μM)
CoCl ₂ ·6H ₂ O	0.025	0.11
CuSO ₄ ·5H ₂ O	0.025	0.10
FeNaEDTA	36.70	0.10
H ₃ BO ₃	6.20	0.10
KI	0.83	5.00
MnSO ₄ ·H ₂ O	16.90	0.10
Na ₂ MoO ₄ ·2H ₂ O	0.25	1.03
ZnSO ₄ ·7H ₂ O	8.60	29.91
Organic components	(mg L⁻¹)	(μM)
Myo-Inositol	100.00	0.56
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	0.10	0.30
Citric acid	75.00	
Ascorbic acid	50.00	
	(g L⁻¹)	(mM)
Sucrose	30.00	87.64
Agar-agar	8.00	—

The method described below can be applied to most of the tree peonies cultivars but a different degree of success can be recorded according to the genotype and the physiological status of the mother plant.

PHASE:	INITIATION		MULTIPLICATION		ROOTING	
	WPMP1		WPMP2		WPMP3	
Additional requirement of Calcium	(mg L ⁻¹)	(mM)	(mg L ⁻¹)	(mM)	(mg L ⁻¹)	(mM)
CaCl ₂ ·2H ₂ O	441.00	3.00	–	–	–	–
Calcium gluconate monohydrate	–	–	1342.5	3.00	1342.5	3.00
Growth regulators	(mg L ⁻¹)	(μM)	(mg L ⁻¹)	(μM)	(mg L ⁻¹)	(μM)
BA	1.00	4.44	1.00	4.44	–	–
IBA	–	–	–	–	1–10	4.92–49.2

Preparation to make 1 L of medium: Weigh all the ingredients and dissolve in 300–400 mL of bi-distilled water (conductivity $2 \pm 0.5 \mu\text{S cm}^{-1}$). For microelements, vitamins and growth regulators stock solutions will be used and an adequate amount of milliliters will be added to the bi-distilled water. Adjust the volume of the solution to 500 mL. In the meanwhile, add 8 g of agar to 500 mL of bi-distilled water and heat for 10–12 min, taking care to avoid evaporation. Add the hot agar-solution to the previously prepared ingredient solution and mix for some seconds. The pH of all media are adjusted to 5.85 ± 0.01 while the media are liquid ($T = 76^\circ\text{C} \pm 1$). Subsequently media, poured into tubes and vessels, are autoclaved at 120°C , 101 kPa, 15 min.

2.1.1. Steps in the Procedure

1. Collect axillary buds from mother plant stock; buds with progressively expanded leaves will be more reactive to the *in vitro* culture (Figure 5A).
2. Remove the bud scales (Figure 5B) and sterilize by a dip in HgCl₂ solution (0.5% w/v; 3 min) followed by a solution of NaOCl (1% available chlorine; 15 min) and a rinse with sterile double distilled water. Before inoculum, buds are submitted to an additional dip in PPM (Plant Preservative Mixture, Plant Cell Technology, IncTM; 50% v/v; 30 min).
3. For shoot development, place buds on 25 × 150 mm test tubes closed by plastic cap (Bellco KaputsTM) and containing 10 mL of agar gelled WPMP1 medium (Table 2; Figure 5C). Incubate at $19 \pm 1^\circ\text{C}$ in darkness.
4. After 1 day, browning exudates from the explants are visible at different degree according to the cultivar (Figure 5D). At this time, explants need to be transferred without any cutting to a fresh WPMP1 medium and incubated again in darkness at the same temperature.
5. After 2 weeks of culture, explants are incubated at the same temperature under a 12 h photoperiod with light energy $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by fluorescent lamps (TLD 36W/33 Philips).

6. Initial explants start to develop in shoot cluster after 8 week of cultures. A reaction depending on genotype can be observed and the aptitude to multiplication can be enhanced after a further subculture on the WPMP2 medium (Figure 6).

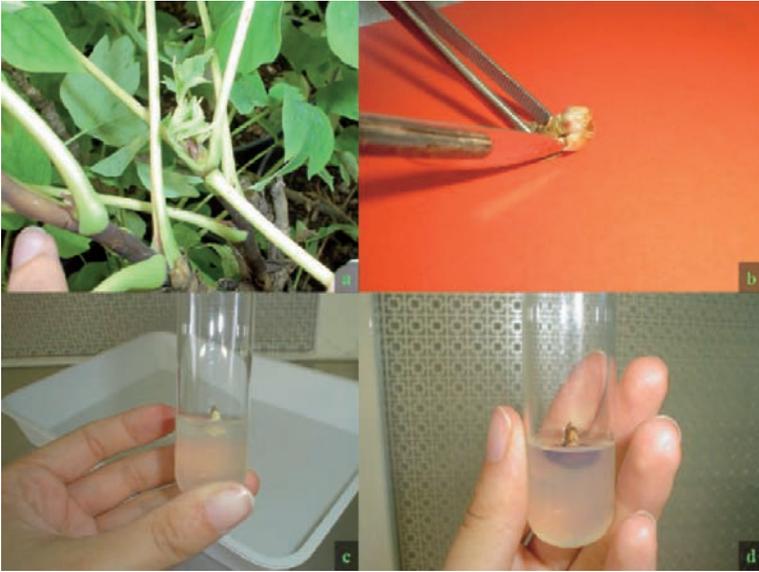


Figure 5. Micropropagation of tree peony: steps in the procedure of inoculation. a) collection of axillary buds from mother plant; b) Removing of bud scales before sterilization; c) Inoculation onto WPMP1 medium; d) Browning medium observed after 1 day of culture; at this time, the explants are transferred to the same fresh medium, without any further cutting.

2.2. Stage 2: Multiplication Phase

Accordingly to our protocol, initial explants start to develop in shoot clusters of axillary shoots and no adventitious shoots are observed. In our experiments, most cultivars (about 70%) already showed their aptitude to propagate at the end of the initiation stage, and this trend was reinforced during the subsequent subculture; only one cultivar presented a delayed response, showing multiplication after 16 weeks of culture. Two third of the tested cultivars reached 100% of propagating explants after 16 weeks in culture (Figure 6).

Plant growth regulators and calcium level in medium have been recognized as important factors controlling the *in vitro* multiplication of tree peony. In order to achieve higher multiplication rates, Bouza et al. (1994a) used the mineral salts of MS with 6 mM Ca^{2+} (instead of 3 mM). Other researchers confirmed signs of Ca-deficiency when MS and WPM media were used for *in vitro* culture tree peonies (Černá et al., 2001; Wang & van Staden, 2001).

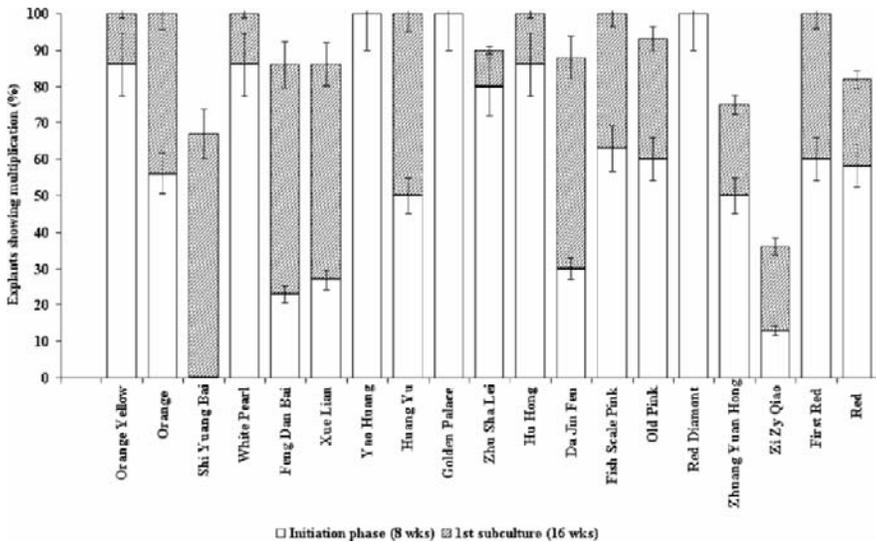


Figure 6. Percentage of explants showing multiplication after the initiation phase and at the end of the first subculture (8 and 16 weeks of culture respectively).

Generally, tree peony axillary shoots may also develop on growth regulator-free media but no multiplication is observed (Černá et al., 2001). Studies of the effect of different cytokinins on the induction of organogenesis showed that BA was the growth regulator effective to stimulate multiplication of tree peony shoots (Bouza et al., 1994a; Černá et al., 2001). A satisfactory multiplication has been achieved when BA has been used at 4 μM (1 mg L^{-1}) alone or in combination with NAA at low concentration (1.1 μM ; 0.2 mg L^{-1}). However, different percentages of hyperhydricity can be scored, particularly when the concentrations of these growth regulators are raised (Bouza et al., 1994a; Černá et al., 2001).

The experiments carried out in our laboratory showed that the propagation ratio can vary as a function of the cultivar and ranges between 2 and 5 (Figure 7). Moreover, the multiplication rate generally decreased after about 1 year of culture (Figure 8). Bouza et al. (1994a) demonstrated that explant age influences both multiplication rate (MR) and hyperhydricity and that a periodic treatment (all four passages) on medium containing a combination of BA (4 μM) and GA_3 (1–2 μM) could stimulate MR without causing necroses. In our experiments, we have found that different percentages of necrotic and hyperhydric shoots are scored as a function of the cultivars. Figure 9 shows the percentage of hyperhydricity scored along the subcultures carried out over about 1 year. ‘Red’, ‘Xue Lian’ and ‘White Pearl’ were found susceptible to hyperhydricity (Figure 9). At the end of the second subculture (24 weeks), the percentage of necrotic explants ranged between 0% (‘Old Pink’, ‘Huang Yu’ and ‘Shi Yuang Bai’) and 25% (‘Xue Lian’).

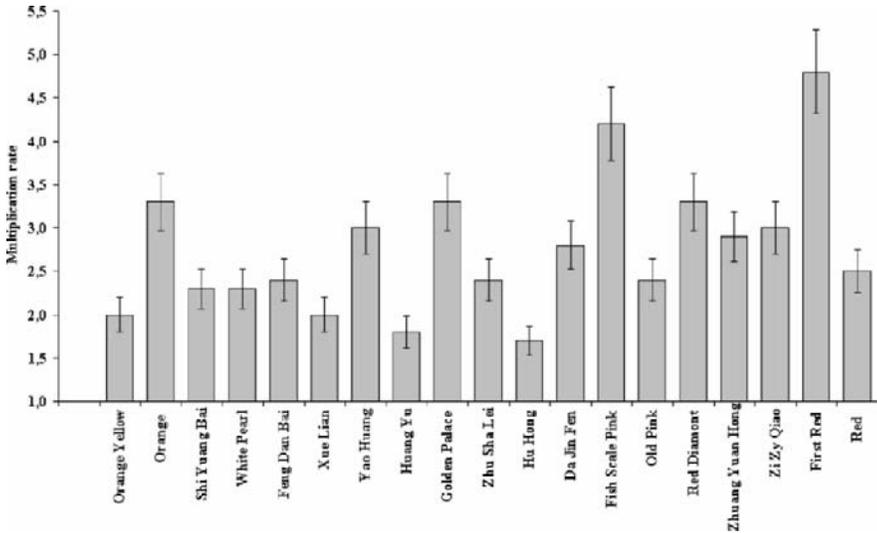


Figure 7. Multiplication rate of different tree peony cultivars grown on WPM2 and scored at the end of the first subculture (16 weeks of culture).

2.2.1. Steps in the Procedure

1. Isolate the *in vitro* shoots formed by transverse section and subsequently separate them (Figure 10A).
2. Place the *in vitro* shoots onto fresh WPMP2 medium (test tubes firstly, then vessel culture (Ø 7.5 cm; 320 mL) sealed with polycarbonate lid and a polyethylene film (Domopack™; 10 mL and 100 mL of medium respectively) and incubate at $19 \pm 1^\circ\text{C}$ under a 12 h photoperiod with $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ light provided by fluorescent lamps (TLD 36W/33 Philips) (Figure 10B).
3. After 1 day, browning exudates from the explants are released to medium; plantlets are transferred without any cutting to a fresh WPMP2 medium (tubes or vessels) and incubated further.
4. A new transfer of the plant material is made every 7 to 8 weeks to achieve further multiplication. The multiplication rate (MR) represents the mean number of usable shoots for the following subculture, acquired from one explant. The shoots to be utilized are the initial ones and their axillary shoots; hyperhydrous plantlets are discarded.

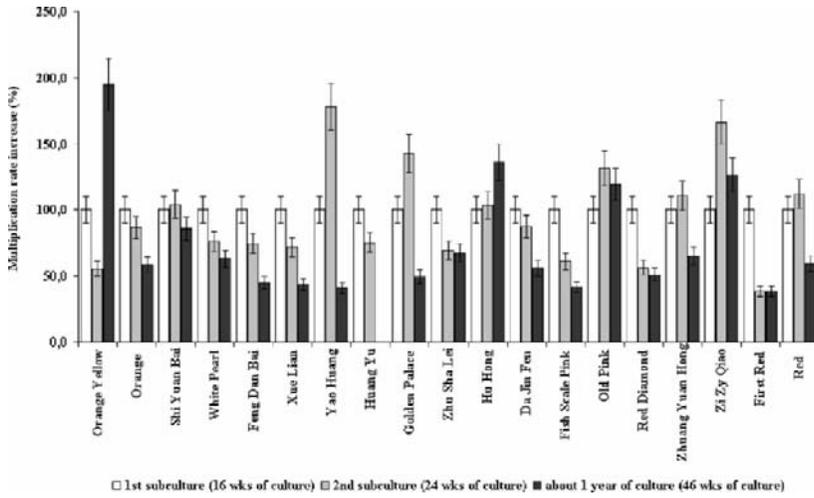


Figure 8. Histogram showing the influence of subcultures on multiplication rate of tree peony shoots grown on WPM2 medium (100% multiplication after the first subculture).

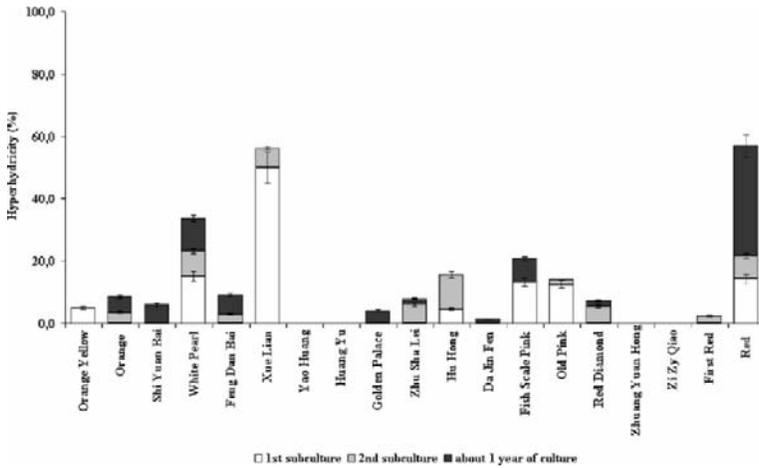


Figure 9. Histogram showing the influence of hyperhydricity for different tree peony cultivars grown on WPM2 medium for different subcultures.



Figure 10. Initial explants develop in shoot clusters on WPMP1 medium. a) *In vitro* shoots formed are isolated by transverse section and subsequently separated and subcultured on WPMP2 b).

2.3. Stage 3: Rooting Phase

In vitro rooting of tree peony has been widely studied with varying degrees of success. Different factors have been found to influence root induction and development. Different treatments have been applied such as the application of a quick dip of IBA at high concentrations (0.25–0.5 mM IBA) for 2–3 h (quick dip) followed by a transfer to an hormone-free medium (Yulong et al., 1984) and the continuous culture of microcuttings on a rooting medium containing 4.9 μM IBA (Harris & Mantell, 1991). In addition, environmental conditions (darkness during root induction and low temperatures), the physical status of the culture medium (liquid versus solid) and the quality of micropropagated plantlets have been found to be critical factors for rooting (Hosoki et al., 1989; Albers & Kunneman, 1992; Bouza et al., 1994b). Our experiments indicated that light quality did not affect the rooting efficiency of the tested cultivars but auxin concentration in the medium and the application into low temperatures improved the results.

When using the one-step protocol described here, the shoots are continuously cultured on agar gelled medium supplemented with IBA. Under these conditions, we found a significant linear correlation ($p < 0.01$) between the rooting capacity of microcuttings and the hormone concentration (Beruto et al., 2004). An improved rooting percentage was reached when IBA 4.92 or 49.20 μM was added to rooting medium (Figure 11) which also resulted faster production of rooted plantlets (Figure 12). Since the *in vitro* rooting ability of tree peony has been dependent on the degree of shoot development (Bouza et al., 1994b), a two-step method can also be envisaged to reach efficient rooting for a wide number of cultivars. In two-step protocol, a root induction phase can be performed in the dark by chilling the *in vitro* shoots (2°C for 7 days); the following root development can be performed by culturing the chilled shoots on hormone-free medium under the usual culture conditions. The chilling

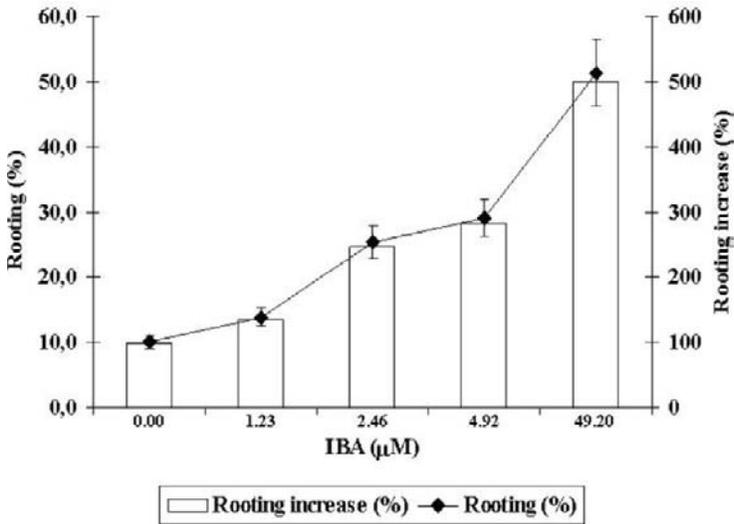


Figure 11. Rooting percentage of tree peony shoots grown in the presence of different IBA concentrations over a 5 months culture period. A rooting increase percentage is expressed compared to control (100%, no growth regulator).

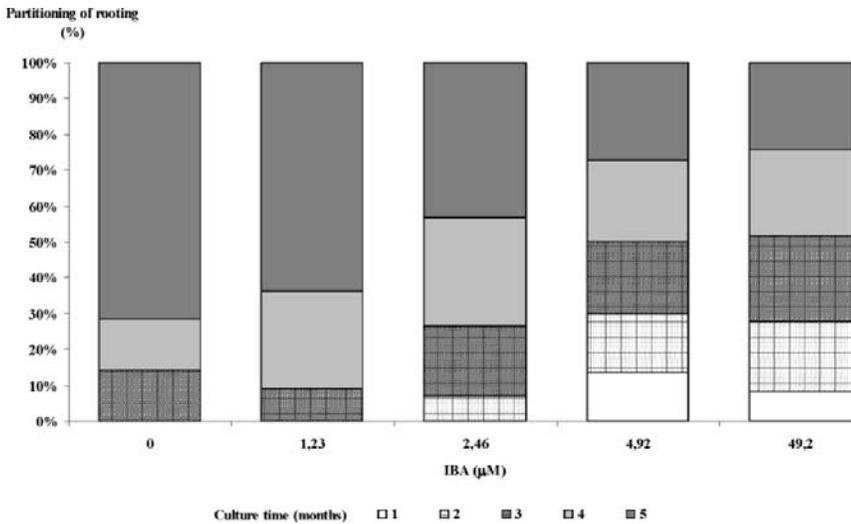


Figure 12. Partitioning of rooting of tree peony shoots in the presence of different IBA concentrations, at the end of the rooting experiments (5 months).

treatment has been found to partly reactivate shoot development of *in vitro* plants which, under the micropropagation cycle, can accumulate ABA and become dormant (Bouza et al., 1994b). For all rooting treatments the average number of roots is between 1 and 2 and the root number is enhanced when one step treatment is applied with higher IBA level (4.92 and 49.2 μM). According to the cultivar used, some roots can be formed from a basal callus originated when the highest IBA concentration is applied (49.2 μM); in this case the use of IBA at 4.92 μM has to be preferred.

2.3.1. Steps in the Procedure

One-step method

1. Microcuttings (2.5 ± 0.4 cm high) from the multiplication medium (WPM2) are placed onto fresh WPMP3 medium (25×150 mm test tubes closed by plastic caps (Bellco Kaputs™ and containing 10 mL of medium) and incubate at $19 \pm 1^\circ\text{C}$ in darkness.
2. After 2 weeks, cultures are transferred under 12-h photoperiod with $50 \mu\text{mol m}^{-2}\text{s}^{-1}$ light provided by fluorescent lamps (TLD 36W/33 Philips)] till the end of the rooting phase. Rooting percentage of about 50–60% can be achieved after 5 months of culture, although the non-rooted shoots can show new roots after transfer on hormone-free medium for two additional months (Beruto et al., 2004).

Two-step Method

1. Microcuttings (2.5 ± 0.4 cm high) from the multiplication medium (WPM2) are placed on basal WPMP medium without growth regulators (25×150 mm test tubes closed by plastic cap (Bellco Kaputs™ and containing 10 mL of medium) and incubated at 2°C in darkness.
2. After 7 days, shoots are cultivated at $19 \pm 1^\circ\text{C}$, on the same medium in darkness for additional 2 weeks.
3. Shoots are transferred on WPMP supplemented with activated charcoal (AC; 0.3% w/v; Figure 13) and cultivated as described at the step 2 of the one-step method. Rooting percentage, about 50%, achieved is at the same level as in the one step method.

2.4. Stage 4: Acclimatization Phase

As in other woody species, the development and survival of *in vitro* tree peony plants can be limited e.g. due to dormancy induced during the micropropagation process (Bouza et al., 1994b). Our protocol is based upon two-step procedures which allow gradual adaptation of the *in vitro* plants to *in vivo* conditions. The first step is still performed under the same conditions as the culture rooms applied during the micropropagation process. At the end of this step, plantlets increase in height (mean height being 7 to 8 cm which corresponds to a 4 times increase compared to the development at the end of the stage 3). The root development is also more

pronounced with 2 to 3 principal roots per plant (mean length being 8.5 ± 0.7 cm) and also development of secondary roots may be observed (2 to 4 secondary roots per plant with 2–3 cm mean length). The second step is carried out under greenhouse conditions where the plantlets start to receive the standard culture practices regarding nutrition, irrigation and pest control. This system allowed us to produce quality plants with a survival rate of $80 \pm 10\%$ (Beruto et al., 2004).



Figure 13. Rooting of tree peony shoots following a two-step procedure (see text).

2.4.1. Steps in the Procedure

Step 1

1. Fill glass vessels (500 mL) with a mixture (200 mL) of milled peat (Rekiva, cd 1A9 and perlite (# 3) (1:1) and sterilize at 120°C , 101 kPa for 40 min.
2. Add 30–40 mL of sterilized deionized water to the sterilized mixture in order to lightly moisten the substrate.
3. Take the rooted plantlets from stage 3, pay attention not to break the roots and wash the root system to get rid off the agar pieces.
4. Place the rooted shoots (about 3 cm in height) on the mixture, close the vessel with the corresponding polycarbonate lids and polyethylene film and incubate at the usual culture conditions as described above for the micro-propagation process.
5. The culture period of the first step of acclimatization is 4 weeks; along this period excessive humidity accumulation in the vessel should be avoided by removing the polyethylene film and progressively increasing the gas-exchange in the culture.
6. At the end of the first step, select the rooted plantlets (Figure 14A,B) ready to be transferred to greenhouse conditions (step 2).



Figure 14. Gradual acclimatization of rooted tree peony plantlets to *in vivo* conditions. Plantlets have been grown on vessels filled with a mixture of sterilized milled peat and perlite under the usual conditions in the culture room a) (step 1; see text). At the end of the culture period (4 weeks), plantlets show an improved development of leaves and roots b) and are transferred to peat-pot c) and d) under greenhouse conditions (step2; see text).

Step 2

1. Fill the peat pot (10 cm in diameter) with a mixture of milled peat (80%), pumice (15%), bentonite (2%) and slow release plant fertilizer (3%).
2. Transfer the plantlets into the pots and water widely (Figure 14C,D). Since this step, plantlets are grown under unheated greenhouse conditions.
3. Cover the plantlets with a white polypropylene film (17 g/m²) for 2 weeks. During this period, plantlets should be watered taking care to avoid excessive humidity.

2.5. Notes on the Following *In Vivo* Growth Phase

Scanty literature is available on the development of tree peony *in vitro* plants due to an arrest of shoot growth which even prevents the survival during the acclimatization phase (Bouza et al., 1994b).

The *ex vitro* plantlets obtained in the present work, showed a continuously developing root system and growth of leaves and shoots till complete flowering (Figure 15). After the acclimatization phase, plantlets have been grown under greenhouse (unheated), watered and fertilized according to the normal cultural practices advised for tree peony (Rogers, 1995). Once a month a 0.1% solution of Rovral (Iprodione 270 g L⁻¹) was applied to avoid fungal contamination and after about 1 year the developed plants were transferred to plastic pots (14 cm in diameter). About one year after the acclimatization, plants increased in size and they had a pronounced development of roots and leaves (Figure 15A1,A2,B1,B2,C1,C2).



Figure 15. Further development of tree peony in vitro shoots after the acclimatization phase. Growth after 6 (a1 & a2), 8 (b1 & b2), 13 (c1 & c2), 20 (d1) and 33 (e1) months after the transfer under in vivo conditions. Development of new axillary buds after 20 (d2) and 33 months (e2). Appearance of flower buds (f1; after 41 months from the acclimatization) and complete blooming 2 months later (f2).

At this stage, new axillary buds arised (Figure 15D1,D2,E1,E2) and during spring they developed into new branches with many new leaves. After dormancy in late fall, plantlets started to bloom; the flower buds reached a complete development and the flower production was true-to-type (Figure 15E1,E2).

3. CONCLUSIONS

Although tree peonies are not yet routinely micropropagated, the advances described above could move us closer to that goal. In fact, the applicability of the described micropropagation protocol has been tested for different genotypes, included the dwarf types which are more difficult to *in vitro* propagate. Using buds with expanded leaves as starting material for micropropagation was the key to successful establishment and a plant propagation ratio between 2 and 5 per cycle was achieved according to the genotype. Plantlets were rooted and successfully acclimatized under *in vivo* conditions. So far, scanty reports are available about the *in vivo* performance of the micropropagated tree peony plants. Our protocol provides *in vitro* shoots able to flower after about 3 years and half from their establishment under *in vivo* conditions. Taking into consideration that usually a 3 year old or older tree peony plant is capable of blooming the season after planting, our results are approaching to a commercial application. Further investigations will consider how to overcome the difficulties related to multiplication decrease over the number of subcultures and to provide quality assurance systems for the produced *in vitro* shoots.

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CHAPTER 45

MICROPROPAGATION OF PINEAPPLE, *ANANAS COMOSUS* (L.) MERR.

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1. INTRODUCTION

Pineapple, *Ananas comosus* L. Merr., ($2n = 50$), is a perennial tropical monocot herb of Bromeliaceae and is one of the commercially important tropical fruits constituting a major export item for some countries (Duval et al., 2001). It is closely related to the monotypic genus *Pseudoananas*, both having the characteristic syncarpous fruit but is distinguished from it by the presence of the terminal crown of reduced leaves (Pickergill, 1976). Pineapple is best suited to a mild tropical climate, with temperatures between 16 and 32°C and cannot withstand temperatures below 0°C. South Africa, Thailand, Phillipines, India, Mexico, Costa Rica, Nigeria, Kenya, Indonesia, Brazil, China and Hawaii in the US are the major producers of pineapple in the world. The plant is 90–100 cm in height with 70–80 leaves forming a dense rosette attached to a short, thick stem (Rangan, 1984).

The pineapple fruit is a good source of vitamins A, B and C besides several minerals. Manganese, found abundantly in pineapple fruits can prevent osteoporosis. Another unusual aspect of pineapple's chemistry is that, it has no starch reserves of its own (unlike other fruits). This means that the sugar content that makes up 15% of the fruit must be accumulated before it is harvested – otherwise, it does not have the resources to sweeten any further. Therefore it has to be picked when it is already ripe unlike a banana or a peach, which ripen after they are picked (Beauman, 2005). Interestingly, all parts of the plant are significantly useful. The fruit is used for canning and in the preparation of juice, jam, jelly and crystallized glaze fruit. Alcohol, calcium nitrate, citric acid and vinegar are other products for which pineapple juice is used. The leaves, stem and fruits of the pineapple plant contain *bromelain* which is a mixture of industrially important proteolytic enzymes. Biopolymers derived

from various natural plant resources such as protein and starch have been regarded as alternative materials to petroleum plastic because they are abundant, renewable, inexpensive and biodegradable (Liu et al., 2005). Lately, there has been a growing interest in the use of natural cellulosic fibres as the reinforcement for polymeric matrix. It has been reported that pineapple leaf fibre, which is rich in cellulose, but a waste once the fruits have been harvested, has the potential for polymer-reinforced composite (Arib et al., 2004).

Pineapple is propagated vegetatively by planting *crowns*, *slips*, *hapas* and *suckers*. *Crowns* are the preferred planting material in most pineapple growing countries since they have the potential to develop better root systems. Each crown can be sectioned and planted to increase planting material but this is often not enough and alternate avenues have to be sought. 'Natural flowering' is one of the important unsolved problems in pineapple cultivation and management (Pineapple News, 1996). Natural flowering occurs unexpectedly even in fields which have as near optimum planting and growing conditions as possible. In addition to yielding abundant planting material of uniform age, micropropagated plants have been known to mature and fruit all at one time. Perhaps the use of micropropagated (tissue cultured) pineapple plants as planting material would help to minimize the problem of 'natural flowering' which is sporadic and rampant in pineapple cultivation. The micropropagation protocol has now become fairly standardized for some important cultivars of pineapple and with minor changes can be applied to newly derived ones. Since all the plantlets are developed from one initial culture, most of them should flower and set fruit at the same time when transferred to the field.

Being a vegetatively propagated plant, conventional hybridization techniques for the generation of better varieties are many times too cumbersome and time consuming. Most existing pineapple varieties are products of sexual recombination and the heterozygosity level observed indicates their hybrid origin. In conventional breeding, clonal selection is tedious and requires several generations of backcrossing to develop a plant with desired traits. A study on somaclonal variation from *in vitro* produced population and selection of desirable variants has a great potential and application in breeding programmes. This can be better implemented by studying and selecting the numerous desirable traits in *in vitro* derived plants. For example, the report by Collins and Kerns (1946) addresses one such desirable trait – spinelessness in pineapple which can now be achieved. Success with *in vitro* studies in pineapple are restricted to micropropagation via dormant axillary buds from crowns (Soneji et al., 2002a) and multiple shoot induction in *in vitro* produced leaf bases of pineapple (Soneji et al., 2002b). Preparation of synthetic seeds is another option for pineapple preservation and micropropagation (Soneji et al., 2002c). Propagation in temporary immersion bioreactors (Espinosa et al., 2002) which claims to be an improvement over the existing micropropagation protocol, reports a better performance of such plantlets over those propagated by conventional methods of micropropagation.

2. EXPERIMENTAL PROTOCOL

2.1. *Explant Preparation*2.1.1. *Pineapple Varieties That Can Be Micropropagated*

Cultivated types of pineapple are called clones because they are vegetatively propagated and are self sterile but cross easily with plants outside their varietal group (Evans et al., 2002). There are many commercial clones (Table 1) classed in 4–5 groups including ‘Cayenne’, Spanish’, ‘Queen’ and ‘Pernambuco’ which may represent botanical varieties. The most popular variety for micropropagation in many parts of the world is cv. Smooth Cayenne Serrana (Daquinta and Benegas, 1997) but some others such as, cvs. Queen, Kew, Giant Kew and Mauritius are also widely cultivated in other regions.

Table 1. *Commercially sold pineapple varieties.*

<i>Leading type (cv)</i>	<i>Countries involved in cultivation</i>	<i>Remarks</i>
Smooth Cayenne	Thailand, Philippines, Hawaiian Islands	Most commonly available
Queen	South America, Australia, India	Smaller, little drier and less sweet than Smooth Cayenne
Red Spanish	Caribbean	Medium sized, purple-hued skin, light yellow flesh
Pernambuco		Medium sized
Sugarloaf		Large and heavy
Baby		Very sweet
MD ₂ (‘Extra Sweet’ or ‘Golden Ripe’)	Europe	Sold as fresh fruit

2.1.2. *Supply of Plant Material*

Explant – Dormant Axillary Buds from Crowns. Crowns from harvested ripe pineapple fruits can be used as source of dormant axillary buds for culture initiation. Conventionally, after removal of all the leaves the base of the crown is sectioned and each quarter is then planted to yield a single plant. Beneath each leaf of the crown is a 1–2 mm sized dormant bud (nearly 12–15 buds per crown) which remains dormant in this cultivation method. However, under *in vitro* conditions it is possible to scoop out this bud and culture it on defined medium so that it can be made to sprout and yield numerous tiny shoots. Each shoot can be subsequently rooted to obtain a complete plant. However of the 12 to 15 buds present in each crown, only the larger 8–10 buds in the middle portion of the crown have the potential to sprout in culture (Soneji et al., 2002a).

Explant – Leaf Bases of In Vitro Shoots. The basal ends of *in vitro* produced shoots isolated from stock cultures can also be used as explants after peeling them and placing them with their ventral sides in contact with the defined multiplication medium. The basal section can be cultured (one per tube) in the medium for the induction of multiple shoots. This process would take 6 weeks and the shoots thus obtained can be multiplied and maintained on the same medium. Elongation of shoots can be done on hormone-free medium and rooted on liquid rooting medium (Soneji et al., 2002b).

2.2. Disinfection of Plant Material

Dormant axillary buds (one at the base of each leaf of the crown) of pineapple can be carefully excised with minimum unavoidable surrounding tissue (Figure 1, step 1; Figure 2A) and collected in a 5 × 5 cm pre-sterilized damp (dampened with tap water) muslin cloth, tied into a bundle with a string and placed into a pre-sterilized conical flask and stoppered. The bundle containing the excised axillary buds can then be washed thoroughly with soap and tap water. Following this, surface sterilization of the buds should be carried out in a laminar air flow unit. The axillary buds can be sterilized in 0.1% mercuric chloride for 2 min and rinsed several times (≈ 4–5 washes) in sterile tap water (Figure 1, step 2). Each axillary bud (one per culture tube) can be placed vertically for sprouting with its basal end slightly embedded in the bud sprouting medium (Figure 2B).

2.3. Shoot Regeneration and Maintenance

Media. The surface sterilized dormant axillary buds of pineapple should be cultured on ‘bud sprouting media’ (BSM) comprised of: hormone-free N (Nitsch’s, 1951) or MS (Murashige & Skoog, 1962) basal medium, containing BAP (4.44 μM). The buds will sprout and give rise to 1–1.5 cm, 1–2 shoots (Figure 2C) after 4–6 weeks in culture (Figure 1, step-3). A single shoot should then be transferred to ‘shoot multiplication medium’ (SMM) – comprised of MS basal medium supplemented with NAA (9.67 μM), IBA (9.84 μM) and Kn (9.29 μM) for further proliferation (Figure 2D). In this medium each shoot can be made to proliferate to give rise to 10–20 shoots. SMM can be used also as a maintenance medium on which a tuft of 4–5 (3–4 mm) shoots can be subcultured and maintained as a cyclic proliferating stock culture leading to 50–60 shoots per culture (Figure 2D). SMM can also be used as liquid medium (without addition of phytagel) in flasks for further proliferation of shoots (Figure 2E). However, shoots produced in flasks tend to be elongated and can be directly rooted on rooting medium. A tuft of 4–5 (1–2 cm) shoots proliferated on SMM solid medium, can be subcultured on ‘hormone-free MS’ basal medium (HFMS) for shoot elongation. It is necessary for each shoot to attain a height of 4–5 cm before they can be isolated and induced to root. Shoot elongation would take 4–5 weeks on HFMS medium (Table 2).

Table 2. Nutrient media compositions used for pineapple micropropagation.

Medium	Macro-elements	Micro-elements	Carbon source (%)	Growth adjuvants (μM)
BSM	1	1	2	BAP 4.44
SMM	1	1	2	NAA 9.67 IBA 9.84 Kn 9.29
HFMS	1	1	2	–
RM	1	1	1	NAA 0.54 IBA 1.97

BSM: Nitsch's (1951) or MS (1962) macro- and microelements, vitamins;

SMM: MS (1962) macro- and microelements, vitamins;

HFMS: MS (1962) macro- and microelements, vitamins;

RM: White's (1954) macro- and microelements, vitamins;

Gelation of solid media was with gelrite (0.2%); Fe EDTA was added separately according to Murashige and Skoog (1962) medium and the pH was adjusted to 5.8 prior to autoclaving; NAA, naphthalene acetic acid; IBA, indole butyric acid; Kn, kinetin; BAP, benzyl amino purine.

2.4. Culture Vessels and Culture Conditions

All culture vessels (test tubes and flasks) used in the protocol should be made of Borosil glass, 25 × 150 mm size. Non-absorbent cotton should be used for plugging the tubes prior to autoclaving at 121°C, 1 kg/cm for 15 min. All cultures should be incubated at 25 ± 2°C in 16/8 h dark/illumination (12.1 $\mu\text{Mm}^{-2}\text{s}^{-1}$, Philips) cycle. Phytigel (Sigma) 0.2% is appropriate for solidifying culture media – BSM, SMM and HFMS. RM is devoid of phytigel and therefore the shoots (3–4 cm) have to be placed over filter paper bridges for support and for the induction of rooting. Carbon source used was sucrose (1–2%) as described in the text and Table 2. The pH of all media should be adjusted to 5.8 using 1N NaOH or 1N HCl, prior to autoclaving. Soilrite (Soilrite mix, TC is a mixture of 75% Irish peatmoss and 25% expanded Perlite, obtained from M/s Chougule Industries Ltd., Mumbai) or autoclaved fine soil may be used to harden the *in vitro* raised plantlets.

2.5. Rooting of Shoots, Recovery of Plantlets and Acclimatization

Each elongated shoot can then be isolated and subcultured on liquid 'rooting medium' (RM) comprised of White's (1954) basal medium supplemented with NAA (0.54 μM) and IBA (1.97 μM) and 1% sucrose (Table 2), for the induction of roots (Figure 2F). Plantlets with actively growing roots can be carefully removed from culture tubes and washed with tap water and transferred to paper cups containing either autoclaved soilrite or soil (Figure 2G). All plantlets were transferred to

soil in polythene bags after 4 weeks, and maintained in the green house for hardening. In 30–45 days, these plantlets can be transferred to the field for further growth, flowering and fruiting (Figure 2H). Fruiting begins at the end of 14 months from the time of field transfer and can be harvested in 4–5 weeks (Figure 2I,J).

- Step 1: Pineapple – mature fruits – crowns –
excise dormant axillary buds (1 at the base of each leaf)
- Step 2: Surface sterilization of dormant axillary buds (washing with soap and water – treat with 0.1% HgCl₂, 2 min – rinsing with autoclaved tap water, 4–5 times)
- Step 3: Culture buds in Medium I (BSM = hormone free Nitsch 1951/ Murashige & Skoog's 1962 solid basal medium + BAP)
↓
Buds sprout – (3–8 shoots produced in 4–6 weeks)
- Step 4: Culture in Medium II (SMM = Murashige & Skoog's 1962 solid basal medium + NAA+ IBA + Kinetin)
↓
Formation of multiple shoots (≈tuft of 10–20 tiny 2 mm sized shoots, 4 weeks)
- Step 5: Tuft of 4–5 shoots subcultured on Medium II (maintained as stock cultures)
↓
Proliferation – 50–60 shoots (in 2–3 months)
- Step 6: Subculture tuft of 4–5 shoots (1–2 cm) in Medium III (HFMS = hormone free Murashige & Skoog's 1962 basal medium) (shoot elongation, ≈ 4 cm, 4–5 weeks)
- Step 7: Isolate each shoot and subcultured for rooting in Medium IV (RM = White's 1954 liquid basal medium + NAA + IBA and 1% sucrose) over filter paper bridges
- Step 8: Acclimatization of rooted plantlets in soilrite/soil in the greenhouse, 30–45 days
- Step 9: Transfer the hardened plants to the field

Figure 1. Protocol for the micropropagation of pineapple.

2.6. Mass Multiplication in Bioreactors

A procedure for the mass propagation of pineapple (cv. 'Smooth Cayenne') plants using a temporary immersion bioreactor has been described by Escalona et al. (1999). This procedure is automated and involves three distinct phases: shooting, bud differentiation and elongation. The initial explant used in this protocol is shoots from previously established cultures in a 'shooting medium' formulated by Daquinta and Benegas (1997).

In this protocol the bioreactor system should consist of two 1000-ml vessels – one to act as a reservoir for the liquid medium (volume = 1000 ml) and the other (volume ranging from 25 to 100 ml) for growing the plants. The two vessels were connected by silicone and glass tubes and the airflow can be sterilized during passage through 0.2- μm hydrophobic filters (Escalona et al., 1999). Using an air compressor, sterile medium can be pressured to flow from the reservoir to completely immerse five explants (2–3 cm shoots) placed in the other vessel (plant vessel). The medium can be drained back into the first vessel by controlled reversion of air flow. The frequency and length of immersion can be electronically controlled. Exposure to the medium for 2 min every 3 h at 25°C under cool-white fluorescent tube (80 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) with a 16 hr photoperiod is necessary. Addition of paclobutrazol (3.3 μM) in the immersion medium promotes shoot growth and induces axillary bud proliferation but avoids unnecessary leaf development during the shoot-formation stage (Escalona et al., 1999). Shoot multiplication period ranged from 4–7 weeks. After this a change in medium composition to MS supplemented with BAP (2.2 μM) and GA₃ (2.9 μM) for 7 days (step 1) and later to MS supplemented with GA₃ (2.9 μM) – step 3, can achieve shoot elongation. All shoots produced by this method can be rooted in the green house *ex vitro* by transplantation onto a substrate consisting of compacted red ferralitic soil mixed in a 1:1 ratio with sugarcane mill bagasse. The temporary immersion system combines the advantages of solid and liquid medium and exhibit high multiplication rates.

2.7. Micropropagation via Synthetic Seeds

Synthetic seeds can be prepared using tiny shoots (2–5 mm), from *in vitro* grown stock cultures, as encapsulation propagules (Soneji et al., 2002c). Isolated shoots should be mixed in an alginate matrix [3% sodium alginate (Sigma) prepared in MS basal medium or SMM and autoclaved] for encapsulation. Each shoot thus coated with alginate can be picked up using a pair of forceps and gently dropped into an autoclaved solution of CaCl₂·2H₂O (1.36 g/150 ml). The coated buds should be allowed to remain in this solution for 30 min for complexation to occur. At the end of 30 min, the CaCl₂·2H₂O solution can be carefully decanted off and the encapsulated shoots washed 3–4 times with sterile tap water and blot dried on sterilized filter paper to remove the excess solution. Synthetic seeds are ready to be either cultured on solidified nutrient media for germination or stored in sealed Petri plates at 4°C in the refrigerator.

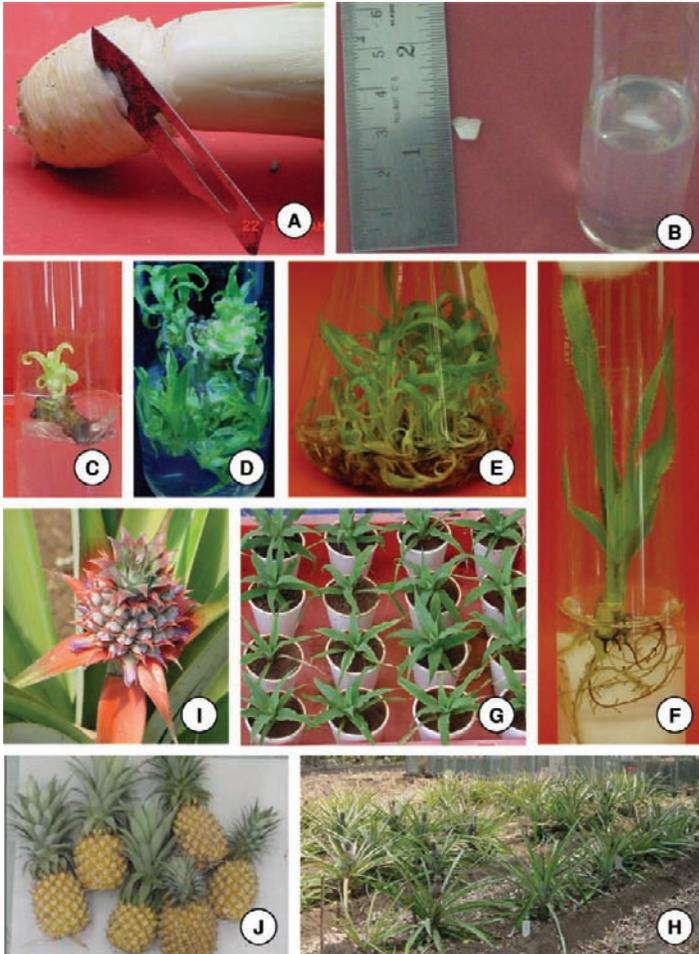


Figure 2. Pineapple, Serial Micropropagation: scooping of dormant axillary bud from crowns A), culture of bud on BSM solid medium B), sprouting of dormant axillary bud C), proliferation in solid SMM D), proliferation in SMM liquid medium E), rooting of isolated shoot in RM F), transfer to papercups with Soilrite G), field transfer of in vitro produced plants H), fruiting I), harvested fruits J).

2.8. Pretreatment and Encapsulation Matrix

Prior to encapsulation the shoots should be soaked in liquid White's (1954) basal medium supplemented with sucrose (1%), NAA (10.8 μM) and IBA (39.4 μM) and agitated at 100 rpm (on a gyratory shaker) for 48 h. The shoots can be blot dried on a filter paper, mixed in a gel of 3% sodium alginate (Sigma) prepared in MS basal medium or SMM and encapsulated using $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.36g / 150 ml) solution.

2.9. Storage and Synthetic Seeds

Synthetic seeds of pineapple can be stored in sealed petriplates at 4°C for a period of 2 months without sprouting. Whenever required, they can be cultured on HFMS medium to recover shoots and subsequently rooted plantlets for multiplication.

3. CONCLUSION

Micropropagation of pineapple *via* axillary buds can be achieved in many ways. A cyclic production of shoots leading to high frequency plant recovery on solidified media without loss of regenerative capacity and vigour is the most extraordinary part of the protocol described here (Soneji et al., 2002a). Firoozabady and Gutterson (2003) have contributed immensely towards the development of a cost effective micropropagation protocol for commercially important pineapple cv. Smooth Cayenne. Their protocol combines use of longitudinal sections of shoots or leaf bases as explants with a 5–10 min/h periodic immersion bioreactor (PIB) system which can generate 3000–4000 shoots from every initial shoot in less than 6 months. By the manipulation of media and explant sizes it has been also possible to improve micropropagation protocols to obtain 1 million *in vitro* plantlets after 9 months from a single bud (Dal Vesco et al., 2001). Gonzalez-Olmedo et al. (2005) have further improved the cultivation conditions by introducing photomixotrophism as an intermediate link of photoautotrophic growth during *ex vitro* acclimatization. Variations in the parameters of light effects such as photosynthetic photon flux density (PPF), sucrose concentration and CO₂ enrichment levels resulted in marked improvement in the protocol. Most of the existing protocols describe scale up of micropropagation methods for pineapple cv. Smooth Cayenne with some exceptions (Soneji et al., 2002a; Sriparaya et al., 2006) which leave immense scope for studies amongst other cultivars or selections. The micropropagation protocols described here can and have found application in the recovery of transgenic pineapple plants improved via transformation studies (Espinosa et al., 2002; Sriparaya et al., 2006).

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CHAPTER 46

DATE PALM *PHOENIX DACTYLIFERA* L. MICROPROPAGATION

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1. INTRODUCTION

Date palm, *Phoenix dactylifera* L., a monocotyledonous angiosperm belonging to the Palmaceae (Arecaceae) family, is native to the tropical or subtropical regions of Africa or Southern Asia. Thousands of years of selection have produced the 3,000 varieties currently cultivated around the world in regions where hot arid climatic conditions favored by date palm prevail (Zaid, 2002).

Date palm is mainly a diploid, $2n = 36$, dioecious tree species with separate male and female plants. For fruit setting, fertilization of the female flowers is required which often involves manual or mechanical pollination. The date fruit is a single, oblong, one-seeded berry, consisting of pericarp or fruit skin, fleshy mesocarp, and membranous endocarp around the seed. It is highly nutritious and rich in source of sugar, minerals, and vitamins and is considered the most important economical product of date palm. Because of the great diversity in fruit characteristics, industrial processing is offered an array of applications utilizing whole dates, date fruit preparations, products derived from date fruit, and by-products. In addition, all the vegetative plant parts of the date palm tree have integrated in traditional or industrial applications (Barreveld, 1993).

Access to modern agricultural practices and commercial knowledge has encouraged date production. According to FAOSTAT data (2006), world dates production has doubled in the last 15 years as it changed from 3,431,207 metric ton in 1990 to 6,924,975 metric ton in 2005. In a decreasing order, countries produced over 200 thousands metric ton of dates in 2005 were, Egypt, Saudi Arabia, Iran, United Arab Emirates, Pakistan, Algeria, Sudan, and Oman. Other producing countries with yields ranging from 20 thousands to 150 thousands metric ton of dates are Libya, China,

Tunisia, Morocco, Yemen and Mauritania. To a limited extent, dates are also produced in the United States of America, Mexico, and Spain among other countries.

Date palm may reach an age of over 100 years and a height of over 20 m. Only about 20 offshoots are produced during the first 10 to 15 years of the tree life. This puts limitations on the efficacy of the traditional propagation method involving separating and planting offshoots. Propagation by seeds is not applicable for cultivation of known cultivars because genetic traits including fruit characteristics are not maintained. Alternatively, micropropagation is gaining increased interest as it provides a rapid mass clonal propagation means.

Date palm micropropagation began a quarter of a century ago (Reuveni, 1979; Reynolds & Murashige, 1979; Tisserat, 1979) and has progressed relatively slowly due to its inherent slow growth nature and limited research resources available to the developing countries where date palm is mostly grown. Several review articles describing early work on plant regeneration through indirect somatic embryogenesis and indirect adventitious organogenesis (Benbadis, 1992; Omar et al., 1992; Tisserat, 1984). Reports demonstrating the ability to regenerate date palm through direct regeneration, without callus stage, are also available (Sudharsan et al., 1993). Micropropagation through direct regeneration is thought to reduce the potential for undesirable somaclonal variants among regenerants while somatic embryogenesis mediated by callus stage is highly efficient and more popular in research laboratories (Al-Khayri, 2005).

In recent years, several studies have examined various components of the culture medium including sucrose (Veramendi & Navarro, 1996), silver nitrate (Al-Khayri & Al-Bahrany, 2001, 2004a), biotin and thiamine (Al-Khayri, 2001), auxins and salt strength (Al-Khayri, 2003), and other tissue culture factors (Bekheet et al., 2001). Cell suspension cultures have been employed to study aspects related to physiology (Al-Khayri, 2002; Al-Khayri & Al-Bahrany, 2004b), and somatic embryogenesis (Fki et al., 2003; Zouine et al., 2005). Expected to revolutionize date palm propagation, development of synthetic seeds is gaining research interest (Bekheet et al., 2002). In addition to offering an effective propagation means, tissue culture proved applicable in genetic improvement of date palm through induced mutations and *in vitro* selection (El Hadrami et al., 2005; Jain, 2005).

The micropropagation protocol described herein is based on indirect somatic embryogenesis using apical shoot tip explant for callus induction and subsequent plant regeneration.

2. EXPERIMENTAL PROTOCOL

2.1. *Explant Sterilization and Excision*

2.1.1. *Separation of Offshoots from Mother Plant*

Select 2 to 3-year-old healthy offshoots from the desired cultivars. Note that offshoots separated in summer usually respond poorly to tissue culture procedure which is affected by the amount of phenolics exudates. Using a hatchet and a serrated knife, trim the leaves to about 30 cm above the shoot tip to facilitate handling (Figure 1A–C).



Figure 1. Procedures for shoot tip isolation from date palm offshoots. A) An offshoot after separation from mother tree. B) Trimming the leaves. C) The remaining stub after leaves trimming. D) Trimming the root region from the trunk stub with a chain saw. E) Separating the trimmed root region. F) Note the cut end of the trunk still consists of woody tissues. G) Removal of green older leaves bases and brown fibrous leaf sheath tissue. H) More trimming of the cut base of trunk stub with a chain saw. I) Cutting the side with knife to facilitate removal of surrounding tissues. J) Unrolling of the leaf sheath for removal. K) Gradual removal of white young leaves bases and surrounding white fibrous leaf sheath tissue. L) Removal of the last sheath with brown fibrous tissue. M) Cutting the shoot tip region; separation of the shoot tip region. N) Separation of shoot tip region. O) Note the compactness of the immature leaves bases surrounded with a single layer of leaf sheath to exclude disinfectant from reaching the shoot tip.

2.1.2. *Separation of Shoot Tip from Offshoots*

Remove outer leaves and surrounding fibrous tissues by cutting at the leaf base in an acropetal fashion until the shoot tip region is exposed. Careful manipulation is necessary to avoid fracturing the characteristically brittle shoot tip and to avoid damaging the axillary buds found at the base of leaf which also can be used as explants source. Continue trimming until the white tissue of the shoot tip region becomes 10-cm high, 4-cm in diameter, cylindrical-shape of white leaf fleshy tissue of young leaves and sheaths protecting the interior containing the shoot tip and the surrounded leaf primordia. Insert a knife about 2 cm away from the circumference of the cylindrical-shaped tip region and cut a circle around the base in a 45° angle. Alternatively, where a chain saw is available, follow the stepwise procedures described in Figure 1A–O. Date palm tissue is known to produce phenolics upon cutting causing browning and tissue deterioration; therefore, keep excised shoot tips in a cooled antioxidant solution (150 mg l⁻¹ each, ascorbic acid and citric acid).

2.1.3. *Surface Disinfection*

Place isolated shoot tip region and axillary buds tissues in a beaker or a jar containing a solution of 70% ethanol for 1 min, then replace with 1.6% w/v sodium hypochlorite (30% v/v commercial bleach) containing two drops of Tween 20 per 100 ml disinfection solution and shake or stir for 15 min. Under aseptic conditions, rinse the tissue with sterile distilled water three times and place in a chilled sterile antioxidant solution through explant manipulation process to minimize browning.

2.1.4. *Explants Dissection*

Three types of explants can be effectively isolated from offshoots including the axillary buds, leaf primordia, and shoot tip meristem. Section axillary buds into 2 to 4 longitudinal segments, and use them as explants. To isolate leaf primordia and shoot tip meristem explants, follow the steps described in Figure 2A–H. The shoot tip region consists of apical meristematic bud surrounded by a number of leaf primordia, encased by fleshy white tissue of leaf sheaths. In a Petri dish, use a scalpel and forceps to trim the ends exposed to disinfectant, and then remove the outer tissue by cutting longitudinally along the leaf axial and transversely underneath the base of the leaf at the point of attachment. Trim this outer tissue when it is about 5 cm long and 1 to 2 cm wide. Separate leaf primordia and cut each primordium into two transverse sections and use as explants. Section the remaining shoot tip meristematic tissue into 4 to 8 longitudinal sections and use as explants for both somatic embryogenesis and adventitious organogenesis protocols. In addition other explant sources have been utilized including inflorescence parts and leaf tissue (Bhaskaran & Smith, 1992; Fki, et al., 2003).

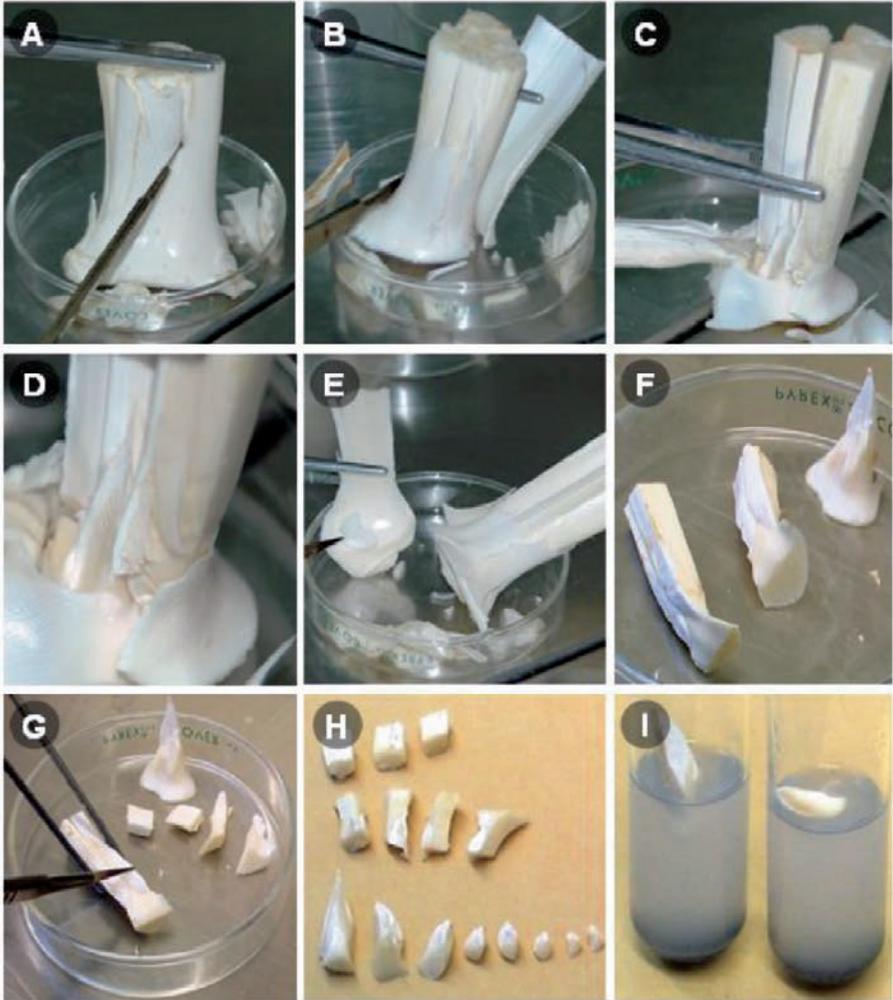


Figure 2. Date palm shoot tip region dissection stepwise procedures used to excise leaf primordia and tip explants for culture initiation. A) Make a longitudinal cut in the sheath surrounding the immature leaf petioles. B) Remove and discard the leaf petioles surrounding the tip in a whorl fashion until 4 or 5 are remaining. C) Remove the remaining petioles and keep for explanting. D) Exposing the leaf primordia and leaf tip meristematic region. E) Note the bright white region where an axillary bud has formed at the base of the immature interior leaf petioles. F) Selection of tissue with meristematic regions including immature petiole bases and shoot tip shown still surrounded by leaf primordial. G) Dissection of the immature axillary bud regions from the petiole. H) Explants isolated from shoot tip region ready for inoculation. I) Explants on culture initiation medium containing activated charcoal.

2.2. Culture Medium

2.2.1. Basal Medium Components

For medium used to induce adventitious organogenesis, the reader is referred to other protocols reviewed above since this protocol focuses on indirect somatic embryogenesis. In both cases, however, the basal medium commonly used for date palm tissue culture is based on MS salts formulation (Murashige & Skoog, 1962) with modifications by different authors to achieve genotype-specific and stage-dependent optimizations, by particularly manipulating the hormonal and vitamin content. Either commercially available prepackaged MS salt formulation or in-house prepared stock solutions can be used for medium preparation. Table 1 lists the components of MS medium and specifies other inorganic and organic additives.

2.2.2. Medium Preparation

Prepare the basal medium including all additives except plant growth regulators and activated charcoal which are added according to culture stage as shown in Table 1. Adjust medium to pH 5.7 with 1 M KOH and HCl, and dispense in 150 × 25-mm culture tubes (15 ml per tube), 125-ml culture flasks (30 ml per flask), or GA-7 magenta vessels (50 ml per vessel). These vessels are autoclaved for 15 min at 121°C and 1×10^5 Pa (1.1 Kg cm⁻²) and kept in a cool place ready for culturing. Culture tubes are more suitable for culture initiation and rooting stages but the other vessels are more appropriate for callus multiplication and regeneration stages.

2.3. Callus Induction and Plant Regeneration

2.3.1. Callus Induction and Multiplication

1. Inoculate explants on culture initiation medium prepared according to Table 1. Place explants vertically with the basal end slightly inserted, 1–2 mm deep, into the medium (Figure 2 I). Because of potential latent internal contamination, a persistent problem in date palm tissue culture, it is advisable to culture explants in individual vessels throughout the procedure to limit cross contamination.
2. Incubate cultures at $23 \pm 2^\circ\text{C}$ in complete darkness for 9 weeks, during which transfer at a 3-week interval. Note explants swelling and slight expansion, a useful criterion to determine viability of newly cultured explants (Figure 3A, B).
3. While avoiding cutting the tissue to minimize browning, transfer entire explants to callus induction medium (Table 1) and maintain in darkness for 3 weeks. Callus formation becomes clearly visible. Note that auxin was switched from 2,4-D to NAA to reduce potential mutations. However, it is possible to use the initiation medium (Table 1) throughout callus stages from initiation to maintenance.
4. Again, transfer entire explants to callus proliferation medium (Table 1) and maintain in darkness for 9 weeks. More callus growth occurs that can be easily separated from the original explants at the end of this stage.

Table 1. Formulation of culture medium used for date palm micropropagation based on modified MS salt augmented with culture stage-specific plant growth regulators.

Components	Chemical formula	Stock (g/L)	Medium (mg/L)	
<i>Major nutrients, 10 × stock, use 100 ml per L medium</i>				
Ammonium nitrate	NH ₄ NO ₃	16.5	1650	
Potassium nitrate	KNO ₃	19.0	1900	
Calcium chloride-2H ₂ O	CaCl ₂ ·2H ₂ O	4.4	440	
Magnesium sulfate-7H ₂ O	MgSO ₄ ·7H ₂ O	3.7	370	
Potassium orthophosphate	KH ₂ PO ₄	1.7	170	
Sodium phosphate	NaH ₂ PO ₄ ·2H ₂ O	1.7	170	
<i>Minor nutrients, 100 × stock, use 10 ml per L medium</i>				
Potassium iodide	KI	0.083	0.83	
Boric acid	H ₃ BO ₃	0.62	6.2	
Manganese sulfate-4H ₂ O	MnSO ₄ ·4H ₂ O	2.23	22.3	
Zinc sulfate-7H ₂ O	ZnSO ₄ ·7H ₂ O	0.86	8.6	
Sodium molybdate-2H ₂ O	Na ₂ MoO ₄ ·2H ₂ O	0.025	0.25	
Cupric sulfate-5H ₂ O	CuSO ₄ ·5H ₂ O	0.0025	0.025	
Cobalt chloride-6H ₂ O	CoCl ₂ ·6H ₂ O	0.0025	0.025	
<i>Iron-EDTA, 100 × stock, use 10 ml per L medium</i>				
Iron sulfate-7H ₂ O	FeSO ₄ ·7H ₂ O	2.78	27.8	
Ethylenediamine tetraacetic acid disodium	Na ₂ EDTA·2H ₂ O	3.73	37.3	
<i>Vitamins, 100 × stock, use 10 ml per L medium</i>				
<i>Myo</i> -Inositol		12.5	125	
Nicotinic acid		0.1	1	
Pyridoxine hydrochloride		0.1	1	
Thiamine hydrochloride		0.1	1	
Glycine		0.2	2	
Calcium pantothenate		0.1	1	
Biotin		0.1	1	
<i>Other additives</i>				
Glutamine			200	
Ascorbic acid			100	
Citric acid			100	
Sucrose			40000	
Agar			7000	
<i>Plant growth regulators and activated charcoal, add according to culture stage</i>				
Final concentrations (mg/L)				
Culture stage	2,4-D	2iP	NAA	Charcoal
Culture initiation	100	3	—	1500
Callus induction	—	30	10	1500
Embryogenic callus	—	6	10	1500
Callus multiplication	—	1.5	10	—
Embryogenesis	—	—	—	—
Rooting	—	—	0.2	—

- Isolate callus growth from original explants and transfer to callus multiplication (or maintenance) medium (Table 1) and maintain in darkness until desired amount of callus is obtained (Figure 3C). Callus maintained over 2 years has preserved embryogenic potential; however, lengthy maintenance periods ought to be avoided to minimize mutations and ensure practical embryogenic capacity.

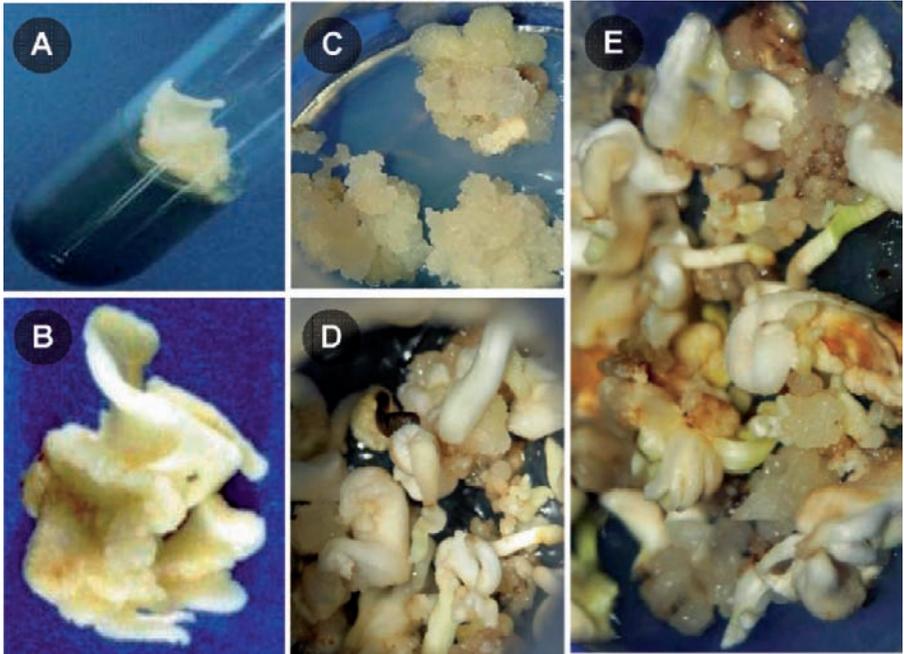


Figure 3. Date palm callus and regeneration of cultures. A) Explant cultured on initiation medium. B) Expansion of explant and callus induction. C) Callus multiplication culture. D) Various stages of somatic embryogenesis on hormone-free medium. E) Early somatic embryo germination.

2.3.2. Somatic Embryogenesis

- Transfer callus from callus multiplication cultures to a hormone-free medium (Table 1) to encourage somatic embryo development, maturation, and germination which normally requires a minimum of 9 to 12 weeks. Incubate cultures at $23 \pm 2^\circ\text{C}$ and 16-h photoperiods ($50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).
- After 4 to 8 weeks, globular embryos become visible and subsequently elongate forming bipolar-shape (Figure 3D). Mature somatic embryos are transferred individually to a fresh medium for germination and elongation (Figure 3E).

2.3.3. Cell Suspension Culture

1. Cell suspension culture is applicable for efficient mass micropropagation and provides a versatile tool for various *in vitro* studies. To establish cell suspension cultures inoculate 1 g of embryogenic callus into 150-ml culture flask containing 50 ml liquid medium. Use liquid medium for establishing and maintaining cell suspension culture (Table 1).
2. Incubate culture flasks on an orbital shaker set to 100 rpm at $23 \pm 2^\circ\text{C}$ and 16-h photoperiods ($50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) provided by cool-fluorescent lamps for somatic embryogenesis or in darkness for callus multiplication.
3. For the first 2 to 3 weeks, replace culture medium every 3 to 5 days to wash out phenolics accumulation. Subsequently, maintain cultures by bi-weekly decanting half of the liquid medium after the suspension has been allowed to settle to the bottom of the flask and adding an equal volume of fresh medium.
4. For somatic embryogenesis, use hormone-free liquid medium. However, the addition of certain compounds were found to stimulate embryo development in some cultivars including 1 mg l^{-1} 2,4-D combined with 300 mg l^{-1} , and $25 \mu\text{M}$ silver nitrate (AgNO_3) combined with $0.5 \mu\text{M}$ 2iP. Embryo maturation can be enhanced by the addition of 10% PEG or $10 \mu\text{M}$ ABA. The somatic embryos are germinated on a solid hormone-free medium. Partial desiccation of mature somatic embryos to 80% water content will improve germination.

2.4. Rooting

1. After 3 to 6 weeks on germination medium, the embryos form green shoot growth but root development often requires an additional step in which MS medium containing a rooting auxin such as NAA or IBA is used.
2. Transfer germinating embryos, with 2 to 3 cm long shoot, to rooting medium consisting of half-strength MS medium supplemented with NAA (Table 1) and continues to incubate in the light for a minimum of 9 weeks. Note root formation and elongation, shoot elongation, and development of complete plantlets (Figure 4A,B).

2.5. Hardening

1. When the plantlets are 5 to 10 cm long, gently, remove them from culture vessels and rinse under a slow stream of water to remove residual agar from roots (Figure 4C). Place plantlets upright in a beaker containing enough water to submerge the roots and cover with a transparent plastic bag for 3 to 6 days. To reduce *ex vitro* infestation, submerge plantlets in 500 mg l^{-1} Benlate for 15 min.
2. Plant regenerants individually in 5-cm plastic pots containing moistened potting mix consisting of equal portions of soil, peat moss, and vermiculite (Figure 4D). To prevent desiccation, keep plantlets moistened during the process of soil transfer.

3. Water the transplants with 100 mg l^{-1} N-P-K fertilizer (20-20-20) and keep potted plantlets in clear plastic enclosures under culture room conditions. Gradually reduce humidity by adjusting air flow in the plastic enclosures (Figure 4E). Upon noting any signs of wilting, mist with water and readjust opening as necessary.
4. After 1 month transfer plantlets to a cool shaded greenhouse for further growth, after 3 to 6 months transfer to a shade house and maintain for 12 to 24 months depending on cultivar, and then transplant in the field (Figure 5 A–C).

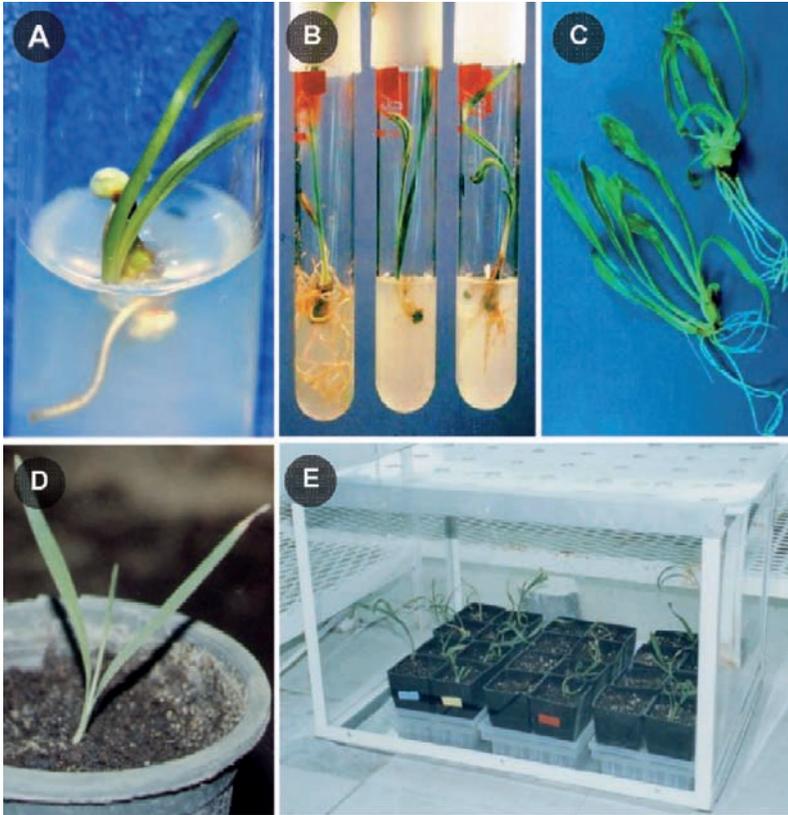


Figure 4. Rooting and complete date palm plantlets. A) Shoot development and root initiation. B) Shoot and root elongation, from left to right, with 0.2 mg l^{-1} NAA, no hormones, and 0.2 mg l^{-1} IBA, respectively. C) Complete plantlets after rinsing agar residues. D) Soil transfer of plantlets. E) Plantlets in an acclimatization chamber as an alternative to plastic bags or mist systems.

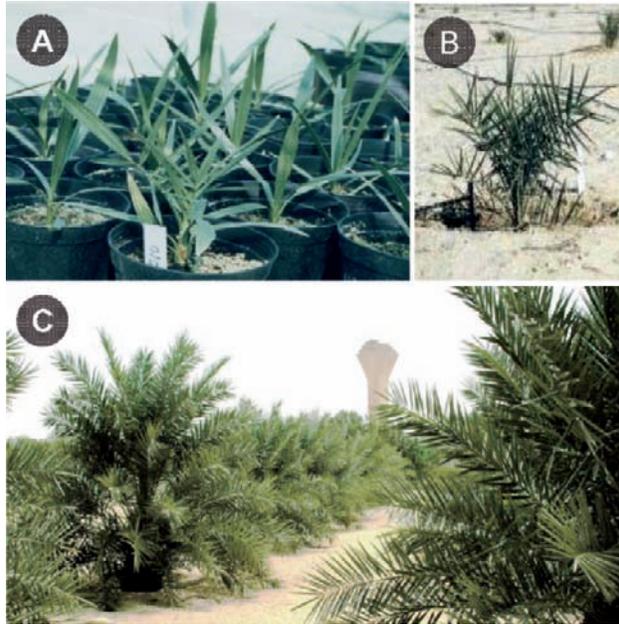


Figure 5. Field transfer of *in vitro*-derived date palm plants. A) Formation of a true leaf under greenhouse conditions. B) Plants after 1 year of transfer to field, C) The same field 1 after 4 years showing normal phenotype and vigorous growth.

2.6. Morphological and Molecular Analysis

The issue of genetic fidelity of date palm plantlets derived from tissue culture has been a controversial subject. Some reports have suggested limited occurrence of somaclonal variants among regenerants based on morphological characteristics and DNA profile, while others observed no variability. Such differences in results could be attributed to the mode of regeneration used, the length of the callus culture maintenance period, the growth regulators augmented to the culture medium, as well as genotypic differences.

Although changes in some of the morphological characteristics and growth parameters of vegetative and fruit stages are visually obvious, correct cultivars identification in date palm is usually not possible until fruit stage which manifests several years after field transplanting. Furthermore, performance characteristics, such as fertility, yield potential, fruit quality or disease susceptibility, are hidden in the juvenile stage. This makes morphological characterization impractical and does not allow either correct cultivar identification or early detection of off-type regenerants at juvenile stage.

Since morphological identification of off-types is unreliable particularly at the juvenile stage, molecular markers describe the internal make-up of a plant and identify the variance based on either the composition of DNA or protein products

expressed from certain regions of the DNA (Kunert et al., 2003). In addition to the commonly used Southern analysis, new PCR-based methods namely, randomly amplified polymorphic DNA (RAPD) and amplification fragment length polymorphism (AFLP) are gaining interest to assess variation among date palm regenerants (Saker et al., 2006).

2.6.1. DNA Isolation and Purification

Grind 1–2 g plant tissue, young leaves of offshoots or regenerants tissue, in liquid nitrogen with a mortar and pestle, then extract for 30 min at 65°C in 10 ml extraction buffer (0.1M Tris-HCl pH 8.0, 50 mM EDTA, 0.5M NaCl, 1% SDS, 150 µg/ml proteinase K and 3% CTAB). Centrifuge lysate for 10 min at 4°C, precipitate DNA with 0.6 vol of cold isopropanol, and then centrifuge to pellet the DNA. Resuspend DNA pellet in 1 ml TE buffer. For further purification of DNA, mix the partially purified DNA with CsCl, then mix in ethidium bromide (EtBr), incubate on ice for 30 min, and then centrifuge at $60,000 \times g$ for 16 h. Collect the DNA band, and remove EtBr and CsCl. Incubate DNA for 1 h at –20°C, centrifuge, and then resuspend pellet in TE buffer. Precipitate DNA by adding 0.1 vol 3 M sodium acetate and 2 vol ethanol. Quantification of DNA can be assessed using UV spectrophotometer at 260 nm.

2.6.2. Restriction Enzyme Digestion and Electrophoresis

Dilute 2 µg DNA in 25 µl TE buffer pH 8, add 10 µl digestion buffer (Pharmacia Biotech), 1 µl of selected restriction enzymes (10 units enzyme per µg DNA) such as EcoRI, BamHI, PstI, HindIII, HinfI, TaqI. Bring final volume to 50 µl with TE buffer and incubate at 37°C for 3 h. Add 5 × gel loading dye and incubate at 65°C for 20 min to terminate digestion. Allow the mixture to cool to room temperature and load samples along with 100 bp ladder DNA marker on 1.8% agarose gel in 0.5 × TBE and electrophoresis for further analysis.

2.6.3. Southern Analysis

Gel preparation and DNA transfer. After restriction digestion of genomic DNA is complete, load 2 µg samples on 1.2% agarose gel in 1 × TAE buffer and electrophorese. Incubate the gel in 200 mM HCl for 10 min to dephosphorylate the DNA, then in denaturation solution (1.5 M NaCl + 0.5 M NaOH) for 25 min, followed by incubation in neutralization solution (1.5 M NaCl + 0.5 M Tris-HCl, pH 7.5) twice for 20 min each at room temperature with gentle shaking and brief wash between different solutions. Transfer DNA to the blot membrane by placing the gel onto a nitrocellulose membrane and position in the capillary blotting assembly using 20 × SSC buffer and following standard procedures. Wash membrane briefly in 2 × SSC, dry between folds of Whatman No.1 filter paper, then bake at 80°C to fix the DNA to the membrane.

Probe labeling and hybridization. Southern procedures require homologous probes which are either randomly selected or specified according to the sequence of the gene of interest. Probes labeling can be performed using a radioactive or a non-radioactive

random prime labeling kit (Gene Images Southern Analysis Kit, Amersham) following the manufacturer's instructions. The procedures call for taking 100 ng aliquots of purified probe DNA, diluting to 25 μ l with TE buffer pH 8.0, and placing in an ice bath. Heating in a boiling water bath for 5 min to denature the DNA then immediately chill on ice. Place another tube on ice and sequentially add 10 μ l nucleotide mix, 5 μ l primer, 100 ng denatured DNA (25 μ l from the previous tube), 1 μ l of enzyme (Klenow fragment, 5 unit/reaction), then bring total volume to 50 μ l with distilled H₂O. Incubate the reaction mixture at 37°C for 1 h, boil for 5 min to stop the reaction, and immediately place on ice. Pour the labeled probes in the prepared hybridization tube containing the membrane and hybridization buffer (5 \times SSC with 0.1% SDS, 5% dextran sulphate, and 1:20 liquid block). Incubate overnight at 60°C in the hybridization oven.

Washing of blots and detection. Place membrane in a tray and wash with gentle agitation at room temperature firstly with 1 \times SSC containing 0.1% (w/v) SDS at 60°C for 15 min; secondly with 0.5 \times SSC containing 0.1% (w/v) SDS at 60°C for 15 min; thirdly with 100 ml of diluted (1:10 in buffer A) liquid blocking agent for 1 h. Incubate blot for 1 h at room temperature in a solution of the anti-fluorescein-AP conjugates (5000 fold dilution prepared in 0.5% BSA in buffer A). Remove unbound conjugates by washing three times in buffer A containing 0.3% Tween 20 for 10 min each wash. Drain buffer from blot and place on a sheet of saran wrap on a flat surface with the sample side up. Pipette the detection reagent on to the membrane (1 ml per blot), allow to spread uniformly cover the surface for 5 min. Drain excess detection reagent and transfer into a film cassette. In the dark room place a sheet of Hyperfilm MP on the sample side, close the cassette, wrap with a cloth, expose for 1 h at -80°C, and develop using an X-ray processor (Kodak X-Omat 5000 RA processor).

2.6.4. RAPD Analysis

Restriction digestion, electrophoresis, and blotting. Digest 2 μ g DNA samples with restriction enzymes, as described above, in the presence of 2 mM spermidine. Separate DNA restriction fragments by electrophoresis on 0.9% agarose gels at 40 V for 23 h in TAE buffer pH 8.3. Denature gel in 0.5 M NaOH, 1.5 M NaCl (30 min), neutralize in 0.5 M TRIS-HCl, pH 7.4, 3 M NaCl (30 min), and transfer DNA to Hybond N⁺ membranes (Amersham) by capillarity using 10 \times SSC.

Polymerase chain reaction (PCR). Using commercially-available or custom-designed primers (Operon Technologies Inc., Alameda, CA), PCR reaction can be conducted using Perkin-Elmer Gene Amp PCR Kit with AmpliTaq DNA polymerase. Amplification of DNA segments from at least 10⁵ to potentially as high as 10⁹ fold can be achieved under the specified conditions. Standardization may be required to achieve optimum amplification. Amplification procedures of date palm genomic DNA described for non-stringent or stringent conditions based on the reaction mixtures and PCR conditions as shown in Table 2. Thermocycler devices are commercially available from various vendors such as GeneAmp 2400 PCR system (Perkin Elmer; Amplitron II Thermolyne). To recover the PCR amplified products, increase the volume of the reaction mixture to 200 μ l and extract once with 25 phenol: 24 chloroform: 1 isoamyl

alcohol and twice with 24 chloroform: 1 isoamyl alcohol. Precipitate the DNA in 600 μ l ethanol with 0.3 M sodium acetate. Centrifuge and wash DNA pellet with 70% ethanol, air dry, and then dissolve DNA in 15 μ l TE buffer pH 7.4.

Table 2. Reaction mixture and thermocycler parameters used to amplify date palm genomic DNA under both non-stringent and stringent PCR conditions.

Reaction components	Reaction conditions			
	Non-stringent		Stringent	
	Concentration		Concentration	
Amplification buffer, 10 \times	1 \times		1 \times	
MgCl ₂	4.5 mM		2.5 mM	
Genomic DNA template	100 ng		100 ng	
dNTP's mix	200 μ M		200 μ M	
Primers, each	50 pM		50 pM	
Taq DNA polymerase	5 U		2.5 U	
Final volume with water	100 μ l		100 μ l	
Thermocycler program	Temp.	Time	Temp.	Time
Pre-denaturation	94°C	2 min	94°C	2 min
Denaturation	94°C	30 sec	94°C	1 min
Annealing	36°C	1 min	60°C	1 min
Extension	72°C	1 min	72°C	2 min
Cycles number	50	—	35	—
Post-extension	72°C	7 min	72°C	7 min

Probe labeling and hybridization. Label probes, described above, by random priming with [α -³²P]dCTP (3000 Ci/mmol, Amersham) using Megaprime Labeling Kit (Amersham) to a specific activity of 10⁹ counts/min/ μ g. Prehybridize filters for 2 h at 65°C in 0.5 M Na₂HPO₄ pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS. Remove unincorporated nucleotides by chromatography using Bio Spin P30 columns (Bio Rad). Heat denature recovered probe by placing in 100°C water bath for 10 min. Rinse filters at 65°C twice, 15 min each, in 125 mM Na₂HPO₄ pH 7.2 with 2% SDS, then twice, 20 min each, in 25 mM Na₂HPO₄ pH 7.2 with 1% SDS. At -80°C, autoradiograph for 24 – 48 h by using X-OMAT (LS or RP) film (Kodak) or Hyperfilm RP (Amersham). After hybridization, strip probes of the membranes, by washing three times, 20 min each, in 0.1 \times SSC with 0.1% SDS at 80°C. Observe film and analyze data after autoradiography and processing.

2.7. Flow Cytometry

Flow cytometry offers a simple, rapid, and accurate method for determining ploidy levels of DNA in plants by estimating nuclear DNA content based on appropriate external reference plant species (Srisawat et al., 2005). Although this technique has not been applied extensively in date palm, flow cytometric analysis conducted by Fki et al. (2003) showed no variability in the ploidy level of date palm regenerants following a micropropagation protocol based on somatic embryogenesis. Flow cytometry

was also used to assess ploidy level of fruits produced from hormone-treated unpollinated inflorescences (Ben Abdallah & Lepoivre, 2000). This technique provides a convenient method for identification of date palm cultivars and early detection of genetic variation among regenerants that may spontaneously arise by detecting changes in the genome size as a result of alteration in chromosome number or ploidy level.

2.7.1. Tissue Processing

Select date palm tissue to be tested such as callus, cell suspension, somatic embryos, or young leaf. Include proper external reference plant tissue such as soybean (2C = 2.5), tomato (2C = 1.96), or corn (2C = 5.72). With a razor blade, finely chop approximately 25 mg tissue in 1.0 ml extraction buffer (0.2 M Tris, 4 mM MgCl₂, 0.5 % w/v Triton X-100 and 3.0 % w/v polyvinylpyrrolidone). Add 50 µl of RNase and propidium iodide then filter through 42 µm nylon mesh.

2.7.2. Measurements and Device

Calculate the nuclear DNA content, according to the following equation:

2C nuclear DNA content (pg) = (2C peak mean of date palm sample)/(2C peak mean of reference).

The number of base pairs per haploid genome (Bp 1C-1 nuclei) is calculated based on the equivalent of 1 pg DNA = 965 Mega base pairs. Measure the PI's at 585 nm following the manufacturer's directions of a flow cytometer device equipped with 488 nm argon iron laser. An example is FACScalibur programmed with CellQuest software (Becton Dickinson Biosciences, San Jose, CA). Check the calibration of the device between samples.

2.7.3. Data Analysis

Include a minimum of replications per sample, and repeat the experiment at least once to maximize accuracy. Data are usually subjected to ANOVA analysis to assess significant differences in the DNA contents. Multiple mean comparison can be performed with LSD or Tukey test.

2.8. Cryopreservation of *In Vitro* Cultures

Cryopreservation, the storage of biological materials at ultra-cold temperatures, from -79°C to -196°C, while maintaining freeze-thaw damage to sublethal levels, is considered an excellent technique for preservation of plant germplasm. It is applicable to a wide variety of plant tissues including buds, seed, seed parts, twigs, as well as *in vitro* cultures such as callus, cell suspension, adventitious shoots, and somatic embryos. Studies related to the behavior of date palm *in vitro* cultures under cryopreservation conditions are relatively limited. Tisserat et al. (1985) found that date palm tissue survived cryopreservation for 3 months at -69°C in a cryoprotectant solution containing 10% PEG, 8% glycerol, and 10% DMSO. Mater (1987) found that freezing callus cultures at -250°C for 4 months did not affect somatic embryogenesis

potential. Similarly, immature somatic embryos continued normal growth and development after cryopreservation using plunge freezing method in liquid nitrogen, -196°C , when pretreated with a cryoprotectant mixture of 10% glycerol and 10% sucrose followed by partial drying (Mycock et al., 1997).

2.8.1. Tissue Pretreatment

Culture target tissues such as callus, cell suspension, adventitious buds, or somatic embryos, in a pretreatment liquid MS medium supplemented with 0.5 M sucrose. Incubate cultures at $24 \pm 3^{\circ}\text{C}$ in complete darkness on a gyratory shaker set at 100 rpm for 48 h. Allow tissue to settle to the bottom of the culture flask and decant the liquid medium in preparation for cryopreservation.

2.8.2. Cryoprotectant Treatment

Prepare cryoprotectant solution by supplementing MS medium with 0.5 M sucrose, 10% DMSO, and 2 M glycerol. Cool the solution to 4°C and mix in plant materials and gently shake on an orbital shaker for 1 h. In the case of cell suspensions or callus tissue, place 1-ml aliquots into 2-ml cryopreservation ampoules (cryovials), cap, and label. Use appropriate vial size for the sample of interest. Gradually cool cryovials by refrigerating at 4°C for 2 h, then at -20°C for 2 h, and finally submerge in liquid nitrogen for storage, or subsequently place in an ultra-freezer where available.

2.8.3. Thawing and Incubation

To thaw the cryopreserved date palm *in vitro* tissues, transfer ampoules to a water bath set at 40°C until samples are completely thawed. Transfer thawed cell suspension, callus, or embryos to a semisolid medium containing 7 g/L agar dispensed in Petri dishes (25 ml culture medium per plate). Seal dishes with a double layer of Parafilm and incubate at $24 \pm 3^{\circ}\text{C}$.

2.8.4. Viability Assessment

To assess the effectiveness of cryopreservation treatments, various parameters can be observed including cell colonies re-growth and amount of resultant microcalli, in case of cell suspensions. Callus growth and somatic embryo germination are other indicative parameters. Callus masses recovered from the cryopreserved cells can be transferred to a hormone-free regeneration medium to examine their capacity to develop somatic embryos.

3. CONCLUSION

This chapter has highlighted recent research progress related to date palm tissue culture and described a reproducible micropropagation protocol based on somatic embryogenesis. The availability of reproducible regeneration systems for date palm has paved the way to several biotechnological applications during the current decade including somaclonal variation selection, cryopreservation, synthetic seeds, as well as cell and protoplast cultures. Furthermore, molecular approaches have been utilized

in cultivar identification and assessment of genetic fidelity of regenerants. At the present, researchers are striving to develop a genetic transformation system to enable improving essential characteristics.

Tissue culture has proved effectiveness for date palm propagation; however, current tissue culture protocols utilize high concentrations of 2,4-D as the main auxin for callus induction and proliferation. Because this compound is known to induce mutations, it may be advisable to develop tissue culture protocols utilizing auxins other than 2,4-D or use lower levels of 2,4-D than currently used. Although somaclonal variants can be useful, variants are extremely undesirable for clonal mass production. To eliminate mutants, it is essential to peruse a routine reliable method suitable for early detection of somaclonal variants.

Furthermore, current protocols utilize mainly the shoot tip as explant source, thus sacrificing the tree. This restricts the applicability of tissue culture to cultivars characteristically produce low numbers of offshoots. Although, other explants such as leaf and inflorescent tissue have been reported as explants source, they have not gained popularity because of their limited success. More research is needed to optimize conditions to demonstrate the use of alternative explants in a wide range of genotypes.

Another main challenge for mass production of date palm using tissue culture is to attain an expedited micropropagation protocols since available procedures require over a year in most cultivars. Moreover, some date palm cultivars still remain recalcitrant to current *in vitro* protocols. Responsive cultivars often produce somatic embryos at asynchronous nature. This characteristic is considered undesirable particularly for the purpose of commercial date palm micropropagation and in the case of synthetic seeds production. Synchronization of somatic embryogenesis merits further investigations on the effect of ABA and PEG.

The future appears promising for date palm biotechnology particularly in commercial production. The relatively high sale price of *in vitro* date palm has not met the consumer expectations which may be reduced by research in automation, bioreactor, and synthetic seed technologies. Albeit research progress, achievements and commercial applicability of date palm micropropagation, further research is necessary to maximize the benefits of biotechnology.

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CHAPTER 47

LIGHT-EMITTING DIODES AS AN EFFECTIVE LIGHTING SOURCE FOR *IN VITRO* BANANA CULTURE

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1. INTRODUCTION

Plantains and bananas (*Musa* spp.) are important staple crops in tropical and sub-tropical countries. They are an excellent source of carbohydrates, minerals and vitamins. Their trade also creates a considerable income as a cash crop. *In vitro* propagated plants are an increasing material of choice because of disease control, uniformity and rapid multiplication of elite genotypes. However, growers face higher cost of production and pay up to five times more than for suckers, which restricts the potential use of *in vitro* culture (Robinson, 1996).

Light-emitting diodes (LEDs) have been demonstrated as an artificial flexible lighting source, which has a significant effect on the biological processes. Numerous studies have shown the effects of LEDs on plants such as elongation, axillary shoot formation, leaf anatomy, and rhizogenesis (Hoenecke et al., 1992; Robin et al., 1994a,b; Tennessen et al., 1994; Tripathy & Brown, 1995; Okamoto et al., 1996; Tanaka et al., 1998) as well as on animals such as cellular proliferation, collagen synthesis, growth factor metabolism in cells, cell growth enhancement, and cancer treatment (Whelan et al., 2001; Eels et al., 2003; Schmidt et al., 2004). The LEDs have a wide range of applications, such as a radiation source for plant production, investigations on animal nerve system and cell growth, and other applications for fishery and creative nature photographing, etc.

Light-emitting diodes as a radiation source for plants have attracted a considerable interest in the recent years because of its vast commercial potential (Nhut, 2005). Bula et al. (1991) described the characteristic features of gallium aluminum

arsenide chip based light-emitting diodes. These features are far better than the commonly used radiation source-fluorescent, metal halide, high pressure sodium and incandescent irradiation sources. Because of their wavelength specificities and narrow bandwidths, LEDs have been used in photobiology research such as chlorophyll synthesis (Tripathy & Brown, 1995), photosynthesis (Tennessen et al., 1994), algal photobioreactor (Lee & Palsson, 1994), and morphogenesis (Hoenecke et al., 1992; Robin et al., 1994a,b; Nhut et al., 2005, 2006).

LEDs are a promising electric light source even for space-based plant growth chambers and bioregenerative advanced life support system because of their small mass and volume, solid state construction, safety and longevity. The new super bright blue LEDs can evaluate the effectiveness of a total LED irradiation system involving both the super bright blue and red LEDs on *in vitro* plantlet growth. The high photon level of blue and red LEDs and their wavelength specificity have certainly given the new system with an added advantage. In these systems, clarifying the optimum blue to red LED ratio for plantlet growth is the most important; it is the key to detail evaluation the demand of the blue to red LED ratio (Nhut, 2002). Tanaka et al. (1998) suggested to use LEDs for improving the quality of plant mother stock for shoot multiplication because of better photosynthetic ability of plantlets cultured under LEDs as compared to the ability under PGF (plant growth fluorescent) lamps. The growth and development of some horticultural plants were remarkably improved by using novel film culture systems processing thermally stable, high light transmittant and gas-permeable film material (Tanaka et al., 1998).

This chapter describes a procedure using LEDs as a lighting source for banana plant tissue culture as a case study. With further improvements depending on specific plant species, the whole irradiation system using super bright red and blue LEDs could become a major light source for a wide range of plant culture systems.

2. EXPERIMENTAL PROTOCOL

2.1. Plant Material

Use banana (*Musa paradisiaca*) shoots (3–3.5 cm long) with three leaves as explants. These shoots are obtained and proliferated on MS medium containing 1 mg/l BA, 30 g/l sucrose and 8 g/l agar.

2.2. Preparation of Culture Systems

Use the “Culture Pack” (CP 7.5 × 7.5 × 10.5 cm, the outer size of stainless frame) made of Neoflon[®] PFA film (Tanaka et al., 1991). The medium substrate is rock wool (RW; four-joined block, two by two, of Grodan[®] Rock wool Multiblock[™] AO18/30, Grodania A/S, and Denmark). The “Culture Pack” – rock wool system (CP-RW) consists of the CP and RW.

Pour 100 ml liquid medium in CP-RW system, and RW is previously sterilized by dry oven (150°C, 1 h) and place in the CP. They are first autoclaved (35 min at 121°C) and then sterile liquid medium is poured in the vessel. Insert 16 shoots in small holes, made in each Multiblock. The opening of the CP is then heat-sealed.

The “Miracle Pack” is the practical model of CP. The medium substrate was RW (twenty five joined-blocks, five by five) (Tanaka et al., 1988). The culture system using MP with RW is named as “Miracle Pack” – rock wool (MP·RW) system. The Neoflon[®] PFA film bag was placed inside the skeleton frame of the MP (made of polycarbonate) followed by heat sterilized rock wool. Pour 160 ml sterile liquid medium in the vessel. Insert 25 shoots, one-by-one into 25 small holes made on a RW. The opening of MP is covered by a lid (Nhut et al., 2003).

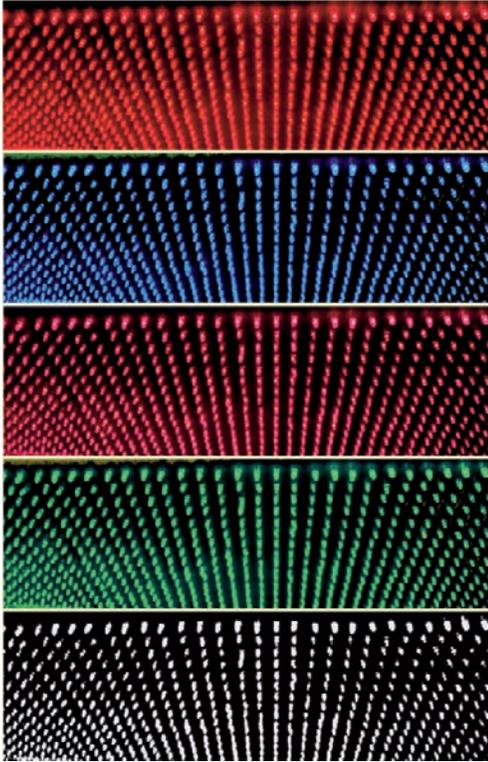


Figure 1. Red, blue, red + blue, green and white LEDs for studying the growth and development of horticultural plants.

2.3. Basal Culture Media and Conditions

The basal MS medium (Murashige & Skoog, 1962) is used for the whole procedure. The media containing 8 g·l⁻¹ Difco Bacto agar (Difco Laboratories, USA) or 2.4 g·l⁻¹ Gellan gum (Gelrite[®], Merche & Co., Inc., USA) can also be tested for specific objectives. The medium pH is adjusted to 5.8 by HCl 1N and KOH 1N prior to autoclaving at 100 kPa for 17 min.

Cultures were placed at 25 ± 1°C under 16 h photoperiod. Adjust light intensity to 45 μmol·m⁻²·s⁻¹ when the plant growth fluorescent lamps (PGF, Homo-Lux, National Electric Co. Ltd., Tokyo, Japan) are used as control.

2.4. LED Irradiation Devices

The apparatus used in LED experiments is an aluminum box named “LED PACK” (25 × 31 × 25 cm). As a light source, the LED board with many pieces of LEDs is mounted on the ceiling of the box. Direct current (DC) supply and electrical circuit are provided on the back-side of the LED PACK (Figure 2).



Figure 2. Plantlets cultured in LED PACK.

Use a novel culture vessel equipped with blue and red LED lighting source, named the UniPACK. It was developed by Okamoto et al. (1996) and consists of MP with RW substrate, LED CAP light source, having 9 blue LEDs and 36 red LEDs. The UniPACK is also placed under CO₂ enrichment condition in all the experiments (Figure 3).

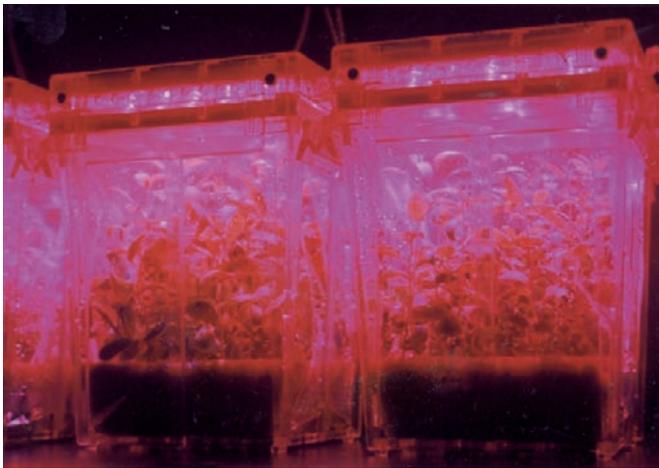


Figure 3. Plantlets cultured in UniPACK.

Use the spectral energy distribution of red and blue LEDs described by Tanaka et al. (1998). The red LED (Figure 1) has a peak emission at 660 nm with a spectral bandwidth of 25 nm and the blue LED has a peak emission at 450 nm. The emission spectrums of red and blue LEDs coincide with the maximum absorptions of chlorophylls a and b.

2.5. Data Collection

The numbers of leaves, plant height, shoot fresh weight, number of roots, and root length and root fresh weight of plantlets are recorded after 1 to 3 months of culture depending on the plant material.

Chlorophyll measurement. Chlorophyll content in the third leaf from the top downwards of the sampled plantlets was measured as SPAD value by a chlorophyll meter (SPAD-502, Minolta Co., Ltd., Japan). Fresh weight of shoots (stems and leaves) and roots of the plantlets were determined separately.

Determination of dry weight. Shoot and root are dried in an oven at 105°C for 30 min and then kept at 60°C for 48 h to constant weight.

Measurement of photosynthetic rate. the Portable Photosynthetic System (LI-COR Inc., USA) can be used for measuring the photosynthetic rate in plantlets after 3 months of culture.

2.6. *In Vitro* Growth of Banana Plantlets Cultured in Various Culture Systems on Sugar-containing and Sugar-free Media without CO₂ Enrichment

Excise shoots from banana shoot masses derived from shoot-tip culture on sugar-containing MS agar medium containing 5 mg·l⁻¹ 6-benzylaminopurine (BA) with or without 3 mg·l⁻¹ sucrose.

Shoots can be cultured on different substrates, RW, Oasis (Smithers-Oasis, USA), 8 g·l⁻¹ agar, 8 g·l⁻¹ Difco Bacto agar (Difco Laboratories, USA), and 2.4 g·l⁻¹ Gellan gum (Gelrite®, Merch & Co., Inc., USA) using 70 ml MS medium supplemented with 0.02 mg·l⁻¹ IBA in two different culture systems, BO (Bottle) and CP. Grow plantlets in two culture systems under PGF for 30 days. Figures 4, 5, and 6 show *in vitro* growths of banana plantlets cultured in various culture systems on sugar-containing medium without CO₂ enrichment after 30 days of culture.

Using the CP system for *Musa paradisiaca* cv. 'Nam Dinh' (Figure 5), Nhut (2002) demonstrated that the plant height and the number of leaves of plantlets cultured in CP·RW, CP with Oasis (CP·Oasis) and CP with Gellan gum (CP·Gel) were greater than plantlets cultured in CP containing Agar (CP·AG) or CP supplemented with Difco Bacto agar (CP·D·AG) (Figure 4).

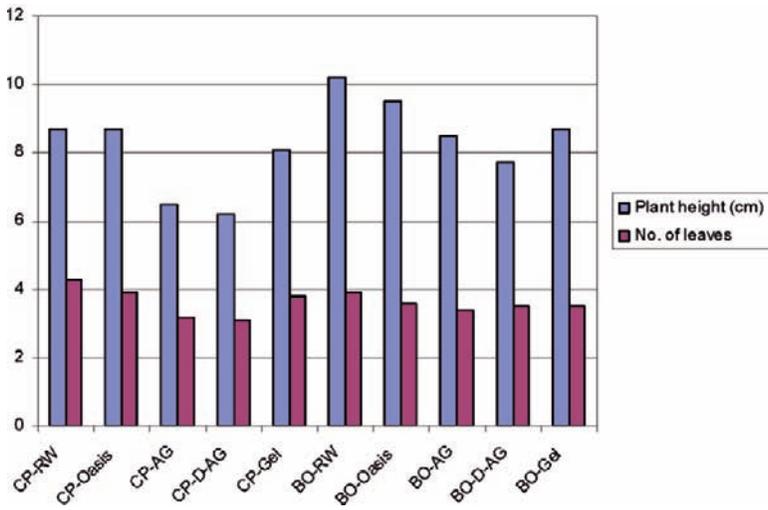


Figure 4. Plant height and number of leaves of banana (*Musa paradisiaca* cv. 'Nam Dinh') plantlets in various culture systems on sugar-containing medium.

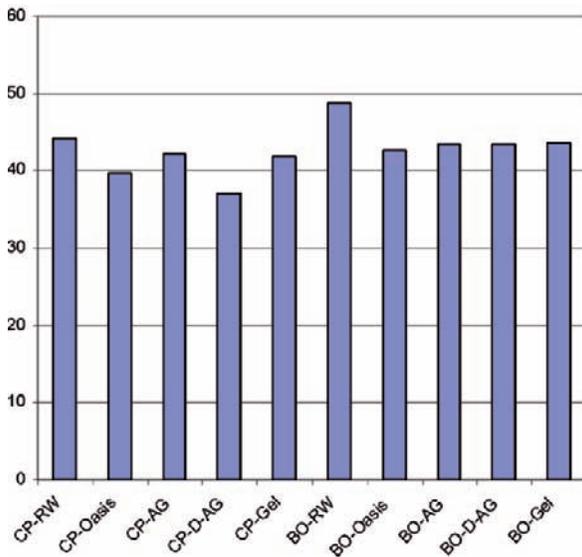


Figure 5. SPAD value of leaves of banana cv. 'Nam Dinh' plantlets in various culture systems on sugar-containing medium.

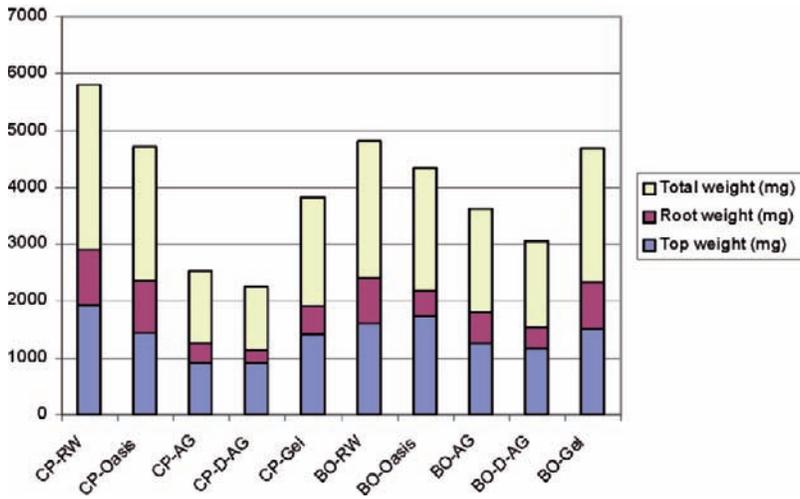


Figure 6. Fresh weight of banana cv. 'Nam Dinh' plantlets in various culture systems on sugar-containing medium.

In the BO systems (Figure 7), the significant differences in number of leaves cannot be observed although greater differences could be observed in plant height.

The shoot fresh weight of plantlets cultured in BO-RW, BO-Oasis or BO-Gel was higher than that in BO-Agar and BO-D-AG. The root fresh weight of plantlets in BO-RW and BO-Gel was higher than that of plantlets cultured in BO-Oasis, BO-Agar or BO-D-AG. It was shown that the *in vitro* growth of banana plantlets, which were cultured in the CP-RW and BO-RW systems with sugar-containing medium without CO₂ enrichment, grew well and that value was higher than that of the others. The RW showed to be the best substrate in the rooting stage of banana



Figure 7. Banana cv. 'Nam Dinh' plantlets in different BO culture systems.

plantlets. The CP-RW system has been demonstrated to be useful for the growth and development of banana plantlets (Nhut et al., 2001) (Figure 8).

On sugar-free media, in the CP system, the banana cv. 'Nam Dinh' plantlets cultured on RW and Oasis were higher than that of plantlets cultured on solid medium with agar, Difco Bacto agar or Gellan gum. The number of leaves when cultured in CP-RW, CP-Oasis or CP-Gel was larger than that of plantlets cultured in CP-Agar and CP-D-AG (Figure 9). The SPAD value of leaves in CP-D-AG was lowest (Figure 10).



Figure 8. Banana cv. 'Nam Dinh' plantlets in different CP culture systems.

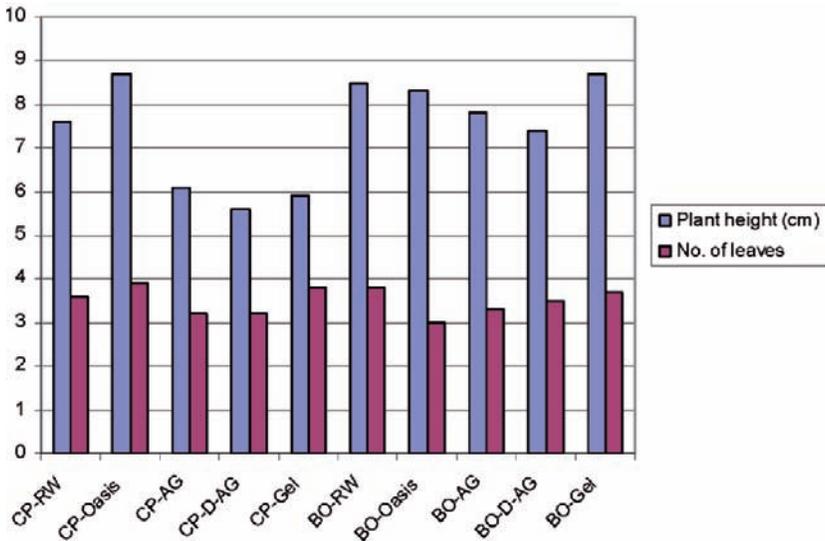


Figure 9. Plant height and number of leaves of banana cv. 'Nam Dinh' plantlets in various culture systems on sugar-free medium.

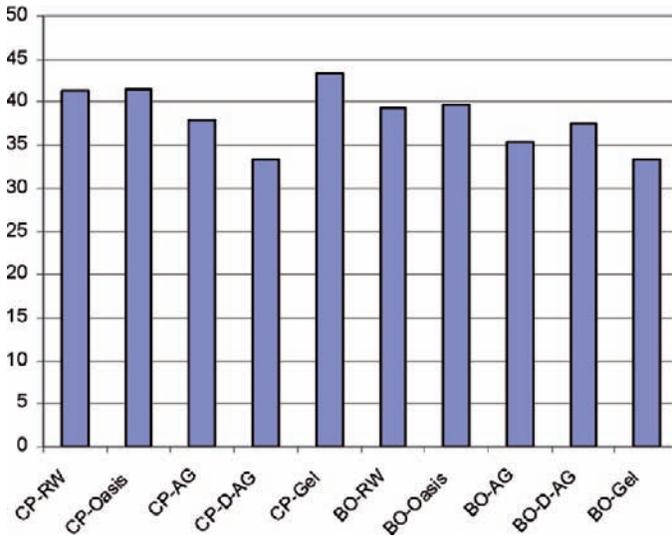


Figure 10. SPAD value of leaves of banana cv. 'Nam Dinh' plantlets in various culture systems on sugar-free medium.

The vigorous *in vitro* growth of banana plantlets cultured in the CP-RW and BO-RW systems on sugar-free medium with CO₂ enrichment was demonstrated (Nhut et al., 2001). From the fresh weight, CP-RW is better than BO-RW, and might be the optimal systems for *in vitro* culture of banana using sugar-free and CO₂ enrichment (Figure 11).

2.7. Effect of LED Irradiation Levels on the Growth of Banana Plantlets on Sugar-free Medium in the CP-RW System under CO₂ Enrichment

Culture the banana plantlets in CP-RW systems under 90% red + 10% blue LED at 45, 60 or 75 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 30 days by using LED PACK 3. Examine the plantlet growth, number of leaves and roots under all the LED irradiation levels.

The number of banana cv. 'Nam Dinh' leaves was greater when plantlets were grown under the light, 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The plantlets grown under light, 75 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, were lowest and the stem diameter under 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ was biggest. The roots under 45 and 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ were longer as compared to those under 75 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The SPAD value of leaves was highest under 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The highest shoot fresh weight was also obtained under 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The banana plantlets cultured in the CP-RW system under 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with sugar-free medium with CO₂ enrichment, grew well *in vitro*, suggesting that banana plantlets also grew well under 90% red + 10% blue LED at 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and that can be used for the micropropagation of banana plantlets (Nhut et al., 2001).

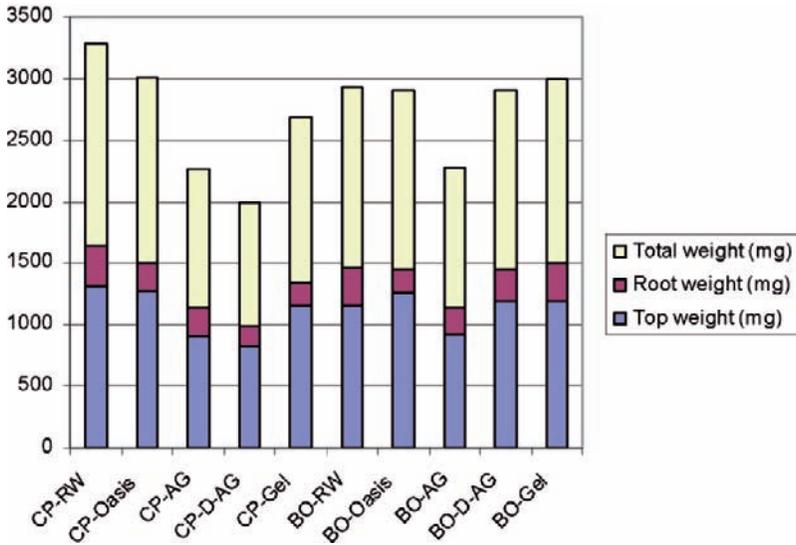


Figure 11. Fresh weight of banana cv. 'Nam Dinh' plantlets in various culture systems on sugar-free medium.

2.8. Subsequent Growth of *In Vitro* Banana Plantlets Cultured under Different Blue to Red LED Ratios after Transferring to Soil

Subsequently grow banana plantlets previously cultured in the CP-RW system under 80% red + 20% blue LED and PGF *in vitro* for further investigation.

Transfer plantlets cultured in CP-RW system under 80% red + 20% blue LED and PGF *in vitro* for four weeks to soilless mixture, in plastic pots (5 × 5 cm) and place in an temperature-controlled chamber at 25°C for 30 days (Figure 12).

The results for banana cv. 'Nam Dinh' are shown in Figure 13. The plantlets under 80% red + 20% blue LED was higher than those under PGF. The number of leaves was higher under PGF while SPAD value of leaves cultured fewer than 80% red + 20% blue LED and PGF was equal. The highest top and root fresh weight of plantlets was obtained under 80% red + 20% blue LED (6092.2 mg, compared to 5427.1 mg under PGF). Subsequent growth of *in vitro* banana plantlets cultured under LED *in vitro* was enhanced after transferring to soil as compared to the growth of plantlets under PGF (Nhut et al., 2001).

2.9. Subsequent Growth of Banana Plantlets Cultured under Different LED Irradiation Levels *In Vitro* after Transferring to Soil

Transfer banana plantlets cultured in the CP-RW system under different LED irradiation levels (i.e. 45, 60 or 75 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) (90% red + 10% blue LED) for 30

days *in vitro* to soil and observe to investigate the optimal irradiation levels for the growth. Effects of irradiation levels *in vitro* on the subsequent growth of banana cv. 'Nam Dinh' are shown in Figures 14, 15, and 16.

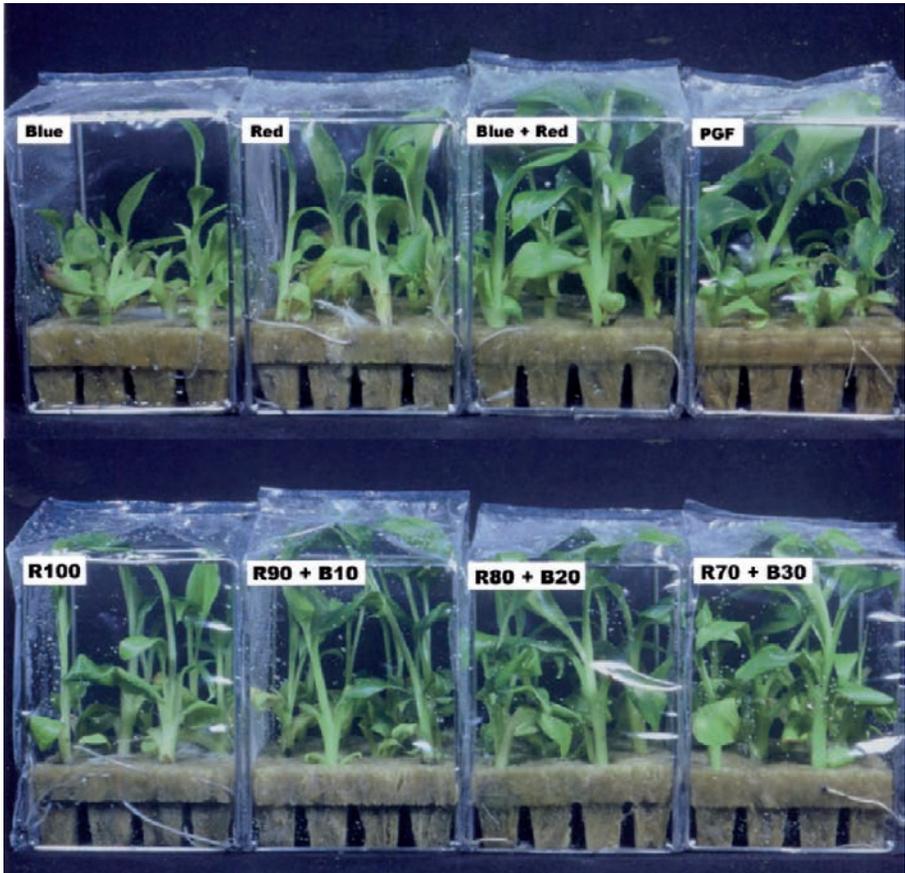


Figure 12. Banana cv. 'Nam Dinh' plantlets in various culture systems under different blue to red LED ratios.

The numbers of leaves, plant height, stem and leaf diameter and plantlet root length under $60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity were highest. The number of roots was higher under $75 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ LED than that under the other irradiation levels. The SPAD value of leaves was highest under $60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ LED. The shoot and root fresh weight of plantlets cultured was highest under $60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ LED. Under similar conditions, the shoot and root dry weight of plantlets was also optimal.

These results showed that subsequent growth of banana plantlets cultured under $60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ LED *in vitro* was enhanced after transferring to soil, indicating the effectiveness of a total radiation system by using LEDs for micropropagation of banana and subsequent acclimatization in *ex vitro* conditions.

D.T. NHUT, N.T. DON AND M. TANAKA

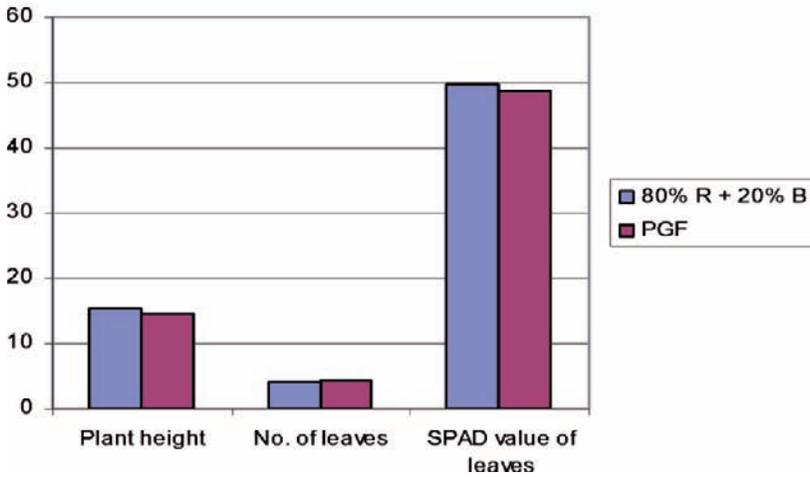


Figure 13. Subsequent growth of *in vitro* banana cv. 'Nam Dinh' plantlets under LEDs and PGF after transferring to soil.

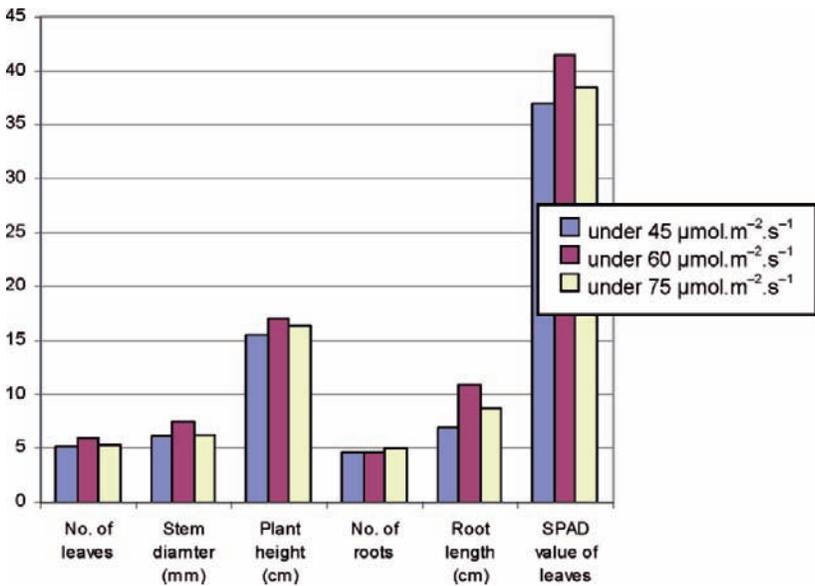


Figure 14. Subsequent growth of banana cv. 'Nam Dinh' plantlets cultured under different LED irradiation levels after transferring to soil.

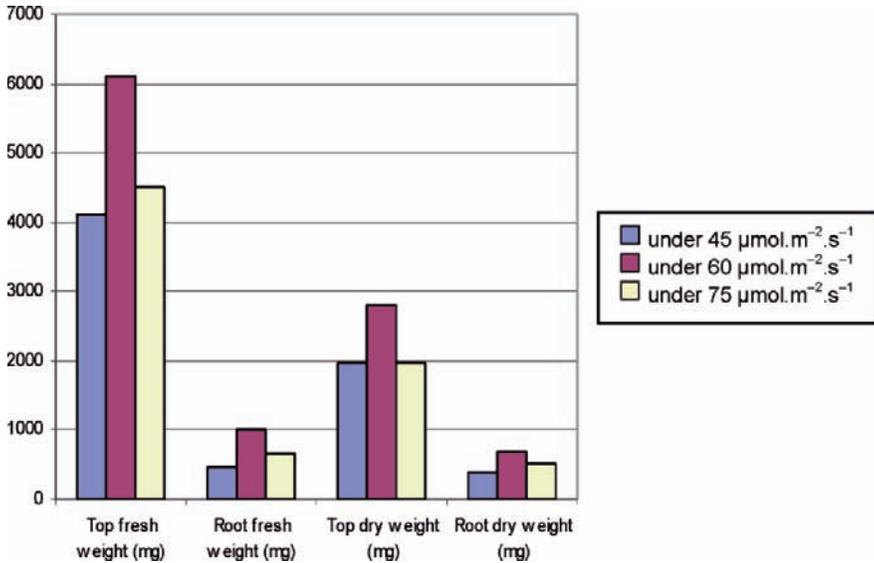


Figure 15. Banana plantlet weight under different LED irradiation levels after transferring to soil.

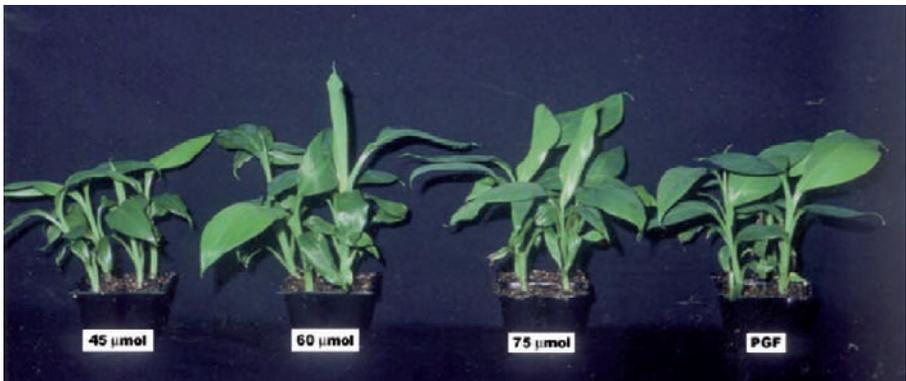


Figure 16. Subsequent growth of banana plantlets cultured under different LED irradiation levels *in vitro* after transferring to soil.

3. CONCLUSION

Film culture and total irradiation system by using super bright red and blue LEDs can be applied to micropropagation of banana with well-adapting plantlets in acclimatization.

The response of plantlets cultured under different blue to red LED ratio and LED irradiation levels is considered for commercial application. The response depended on the plant species and to clarify the optimum blue to red LED ratio and LED irradiation level for plantlet growth is the most important finding. This work is the key to evaluating in details the demand of the blue to red LED ratio.

Results of each specific investigation would indicate the optimal *in vitro* physiological responses of the *in vitro* banana plantlets and the responses after acclimatization. From the results and a lot of attractive feature of LEDs, it is reasonable to expect that the total LED irradiation system will be a major light source for a wide range of micropropagation systems in the future with further improvement to be made.

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CHAPTER 48

IN VITRO MUTAGENESIS IN BANANA (*MUSA* SPP.) USING GAMMA IRRADIATION

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1. INTRODUCTION

Bananas and plantains have a major contribution towards world's food production (FAO, 2006) and are an important staple food for millions of people inhabiting humid and sub-humid tropics (INIBAP, 1992). These are amongst world's leading fruit crops, with an annual global production of about 88 million metric tons from an area of approximately 10 million ha. Although several banana varieties are under cultivation, the global market is dominated by the Cavendish types owing to their higher yields and short duration. Most of the existing cultivars need to be improved for one or other agronomically important characters (dwarfness, duration, yield, quality of fruit etc.). Banana production is threatened by several bacterial, fungal, viral diseases and other pests and building-up of genetic resistance towards these biotic factors is urgently needed. There is also a greater need to develop tolerant genotypes for salinity, drought, cold and unfavourable soils. The genetic improvement of bananas is hampered because of several important reasons such as inherent polyploidy, parthenocarpic fruit development, low levels of female fertility and raising asexual progeny in sufficient numbers to recombine desirable characters. Further, long life cycle and low *in vivo* rate of propagation are the main reasons behind unsuccessful attempts of conventionally mutagenizing the vegetative plant parts such as suckers (Kulkarni et al., 1997).

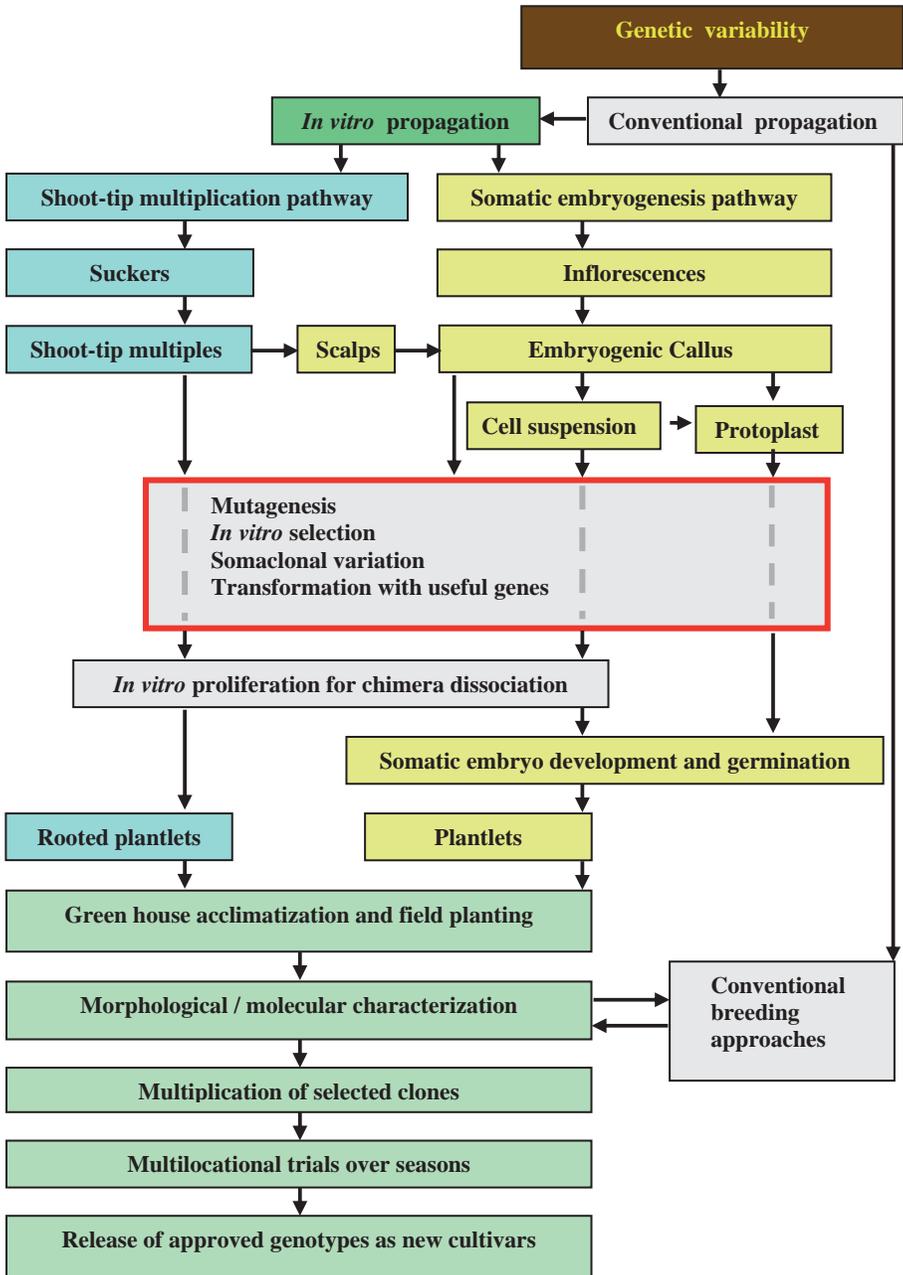


Figure 1. Schematic representation of a generalized banana improvement programme using biotechnological approaches.

A typical banana improvement programme (Figure 1) would primarily depend on the availability of elite and diverse germplasm (genetic variability or the gene-pool). It is obvious that in the case of banana, it would be essential to generate additional genetic variability facilitating the selection for desirable traits. In this regard, use of cellular and molecular techniques viz., plant regeneration via tissue/cell cultures, *in vitro* mutagenesis, genetic transformation and molecular markers has greater relevance (Jain & Swennen, 2004; Smith et al., 2005). Mutation induction and selection of desired traits in combination with *in vitro* techniques offer several advantages over conventional methods such as mutagenizing the plant parts of minute size, handling large number of samples in short time span, rapid production of large populations to separate chimeras, uniform mutagen treatment and facilitating *in vitro* selection (van Harten, 1998). For the induction of mutations, both chemical as well as physical mutagens have been employed, and amongst the physical mutagens, gamma rays have higher penetrance and energy than any other ionizing radiations, and unlike neutrons, do not result in secondary radioactivity. Gamma rays have also practically proved to be better as evidenced by the release of more than 55% of the total released mutant varieties (Maluszynski et al., 1995). The present article describes a generalized protocol for *in vitro* mutagenesis in banana (*Musa* spp.) using gamma irradiation of shoot-tip multiples (shoot-tip or multiple shoot cultures) and cell suspension cultures.

2. ESTABLISHMENT OF *IN VITRO* CULTURES

In banana, *in vitro* regeneration can occur through two main pathways: shoot-tip culture (direct organogenesis or shoot morphogenesis; Vuylsteke, 1998) and somatic embryogenesis (indirect morphogenesis via callus and cell suspensions). Shoot apices containing shoot apical meristems give rise to plantlets following the inhibition of apical dominance. Indirect morphogenesis involves the initial culturing of male flower buds or *in vitro* proliferating shoot-meristems, to produce callus (an undifferentiated mass of cells) and somatic embryogenesis (Strosse et al., 2003).

The first step in conducting *in vitro* mutagenesis experiments is to establish the profusely proliferating tissue/cell cultures with high frequency regeneration potential. These can be used for inducing the mutations using ionizing radiations such as gamma rays. Details of all the culture media compositions are provided in Table 1.

2.1. Shoot-tip Culture Pathway

2.1.1. Initiation of Aseptic Shoot-tip Cultures

The shoot apices either from side-suckers (Figure 2A) or male inflorescences are used as the source materials for the establishment of *in vitro* shoot-tip cultures. The suckers collected from the field growing plants are thoroughly washed with tap water to remove adhering soil, after removing leafy top and the roots. These are trimmed to a size of approximately 3–4 cm in length and 2 cm in diameter, by removing several sheathing leaves and excision of a part of the corm tissue.

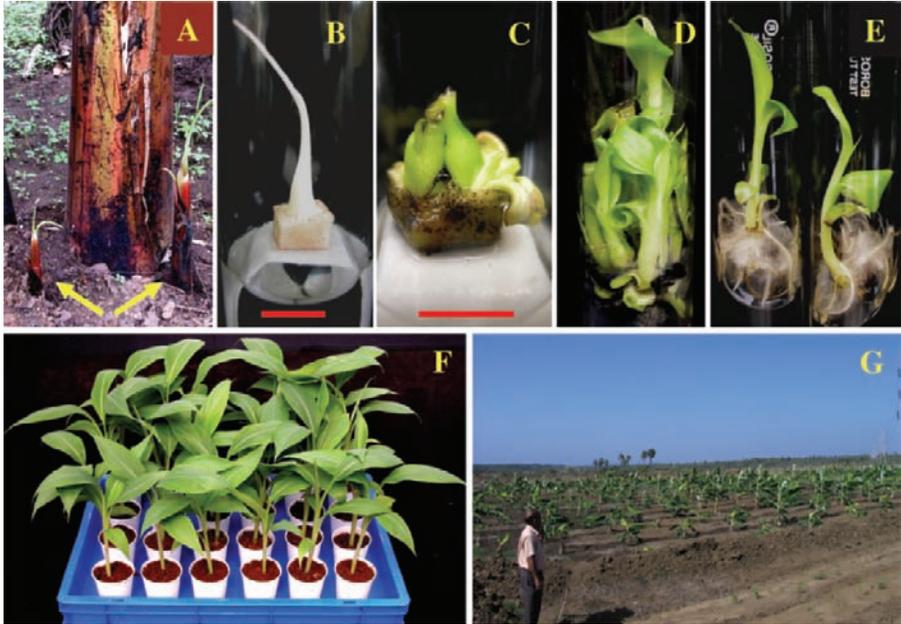


Figure 2. *In vitro* regeneration of banana via shoot-tip multiplication pathway. A) Sword suckers of a field growing banana plant. B) Shoot-tip from sword sucker cultured on a filter paper bridge, bar = 10 mm. C) Swelling of the shoot-tip after 10 days of initiation, bar = 10 mm. D) Shoot-tip multiples *in vitro*. E) Profusely rooted shoots (complete plantlets). F) Greenhouse hardened plantlets. G) Field planting of the tissue culture derived plants.

The shoot apices are transferred to a 250 ml capacity conical flask and washed with a standard liquid detergent followed by several washes with distilled water. For surface sterilization, the explants are sequentially treated with commercial chlorine bleach (5% w/v, 15 min), 70% ethanol (8 min) and 0.1% HgCl₂ (7 min), under aseptic conditions. Each of these treatments is followed by minimum of 4 rinses with sterile distilled water, and excision of few sheathing leaves and some corm tissue. Finally, the shoot apices (trimmed to a size of 1.0–1.5 cm, with minimum basal corm tissue) are cultured on a filter paper support (Figure 2B) on SM1 liquid medium.

2.1.2. Proliferation of Multiple Shoots and Rooting of Individual Shoots

After 10 days of initiation, the cultured shoot tips are sectioned vertically and sub-cultured on SM2 medium (Figure 2C). Multiple shoots (Figure 2D) formed need to be maintained as stock cultures by regular subculturing at an interval of about 4 weeks, on SM2 medium. The stock cultures are freshly initiated as and when necessary. Individual shoots can be separated from the shoot-tip multiples and rooted by culturing on SM3 or SM4 medium.

Table 1. Media compositions for banana shoot-tip and callus/cell cultures.

Code	Culture phase	Medium composition	Growth conditions
½MS	Culture of irradiated shoots	MS (half strength), Suc (1%), pH 5.7, Gelrite (0.2%)	Light
SM1	Shoot-tip culturing	MS + BAP (5) + AH (50), Suc (3%), pH 5.7, liquid	Light
SM2	Shoot-tip multiplication	MS + BAP (2) + AH (30), Suc (3%), pH 5.7, Gelrite (0.2%)	Light
SM3	Rooting	MS + Suc (1%), pH 5.7, Gelrite (0.2%)	Light
SM4	Rooting	MS + NAA (1), Suc (1%), pH 5.7, Gelrite (0.2%)	Light
BM1	Callus induction	MS + 2,4D (4) + IAA (1) + NAA (1) + Biot (1), Suc (3%), pH 5.8, Gelrite (0.2%)	Dark
BM1B	CLM formation	MS + BAP (10), Suc (3%), pH 5.7, Gelrite (0.2%)	Dark
BM2L	EC maintenance	MS + 2,4D (1) + Biot (1) + ME (100), Suc (4.5%), pH 5.3, Liquid	Light
BM2G	EC maintenance	MS + 2,4D (1) + Biot (1) + ME (100), Suc (4.5%), pH 5.3, Gelrite (0.2%)	Light
BM2Z	Callus induction and maintenance	MS + 2,4-D(1) + Zeatin (0.2), Suc (3%), pH 5.7, Gelrite (0.2%)	Dark
BM3	Embryo development	SH + MS Vitamins + Pic (1) + Glut (100) + ME (100), Suc (4.5%), pH 5.8, Gelrite (0.2%)	Light
BM4	Conversion or plantlet development	MS alone, Suc (3%), pH 5.8, Gelrite (0.2%)	Light
BM5	Conversion or plantlet development	MS + BAP (0.5), Suc (3%), pH 5.8, Gelrite (0.2%)	Light

Unless indicated, figures in parentheses are mg/l.

2,4-D (2,4-dichlorophenoxyacetic acid), AH (Adenine hemisulfate), BAP (6-Benzylamino-purine), Biot (D-Biotin), Glut (L-Glutamine), IAA (3-Indoleacetic acid), IBA (Indole-3-butyric acid), ME (Malt Extract), MS (Murashige & Skoog, 1962), NAA (α -Naphthaleneacetic acid), Pic (Picloram), SH (Schenk & Hilderbrandt, 1972), Suc (Sucrose).

2.1.3. Greenhouse Hardening of Plantlets

After 3–4 weeks, plantlets with well developed root-system (Figure 2E) are carefully removed from the culture vessel and gently washed in running tap water to remove the entire gelled medium. If needed, a paint brush with smooth bristles can be used to remove adhering gel pieces. The plantlets shall then be transferred to perforated polythene bags, portrays or suitable containers filled with autoclaved mixture of soil and a suitable commercial hardening mixture such as Soilrite, Cocopeat etc. (Figure 2F). The plantlets are maintained in the greenhouse for 2–4 months (depending on the growth), under natural light with relative humidity of 90–100% and an ambient temperature of 25°C. Approximately 40–60 cm tall plants can be field planted for evaluation.

2.2. Somatic Embryogenesis Pathway

The development of embryogenic cell suspensions (ECS) capable of producing embryos and plants is achieved using different types of explants. In majority of the reports, the immature male flowers and/or shoot-tip derived scalps are a choice system for developing ECS cultures. In both these methods, at least a few hundred explants are required to be cultured and maintained for several months to be able to obtain good quality embryogenic callus. Although somatic embryogenesis in banana is now a well-established method (Ganapathi et al., 2002; Strosse et al., 2003), the initiation of a “genotype-independent” embryogenic cell culture is still far from routine. This is mainly due to the low embryogenic potential, the long gestation periods required for initiating an embryogenic cell suspension, the risk of somaclonal variation (in case of long culture periods) and contamination problems. The detailed protocols for obtaining embryogenic callus, suitable for initiation of superior quality ECS, are given below.

2.2.1. Initiation of Embryogenic Callus Cultures through Immature Male Inflorescence Culture

The male inflorescences are collected from field growing plants after complete bunch emergence (Figure 3A) and washed well with distilled water. These are trimmed to 10 cm in length (Figure 3B), and can be kept until sterilization under a laminar flow in a beaker with a few drops of sterile water and covered with aluminium foil. These are surface-sterilized in 70% ethyl alcohol for 2 min and the immature male flower buds are isolated using a binocular dissection microscope. The immature flowers are isolated from position 16 to 1 (1 being the innermost flower bud cluster, closest to the floral tip). The innermost whorl at the tip can be cultured as such. The excision and isolation should be done with a sharp and pointed surgical blade. While culturing, it must be observed that the cut surface is in contact with the callus induction medium (BM1, Figure 3C) in 9 cm Petri dishes and incubated in total darkness under standard tissue culture conditions.

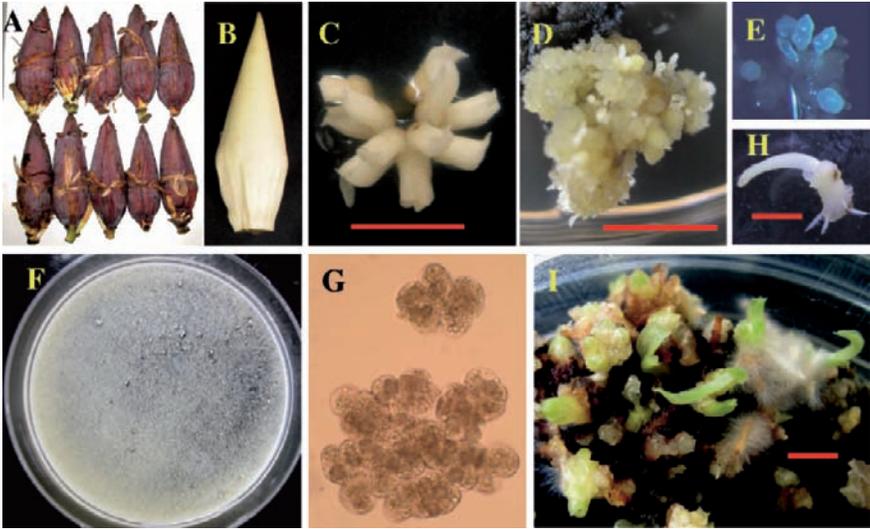


Figure 3. *In vitro* regeneration of banana via somatic embryogenesis. A) Male inflorescences collected from field grown plants, each about 25–30 cm in length. B) Male inflorescence, aseptically reduced to 10 cm length. C) Immature male flower buds cultured on BM1 medium, bar = 4 mm. D) Embryogenic callus with somatic embryos on the surface, bar = 4 mm. E) Developing somatic embryos. F) Embryogenic Cell Suspension in BM2G medium. G) Embryogenic cell clusters. H) Fully developed somatic embryo, bar = 5 mm. I) Somatic embryo conversion on BM3 medium.

In the span of initial 12 weeks (induction phase), the cultures shall be examined for the contamination and the morphogenetic response every alternate week. Thereafter these shall be observed weekly for any embryogenic response from the inoculated tissues. The explants curl up and show callus formation during initial 4 weeks. Subsequently, most of the explants would turn black but still may exhibit some meristematic globules on the surface. This is followed by the development of calli consisting of individual embryos or compact callus formation (Figure 3D,E). The compact callus is generally not suitable for initiating ECS. Good embryogenic callus (EC) can be observed after 3–5 months of culture initiation, which can subsequently be maintained on BM2G medium.

The conversion of the somatic embryos into plantlets (Figure 3H) can be achieved on BM3 medium (approximately 1–2 monthly passages) followed by transfer onto BM4 medium till fully grown plantlets with well developed shoot and roots are obtained (4–6 weeks).

2.2.2. *Initiation of Embryogenic Callus Cultures through Scalp Cultures*

In this method, highly proliferating shoot cultures (initiated from shoot-tips) are used as the starting explants (Dhed'a et al., 1991; Schoofs, 1997). The shoots are cut 0.5 cm above the apical meristem and cultured on BM1B medium and observed for the highly proliferating cluster of tiny shoots at the base of the explant. These cultures can be subcultured on the same medium till cauliflower like meristem (CLMs) cultures are obtained. These can be subcultured and maintained on the same medium. Good quality scalps (defined as a clump of white, compact, cauliflower-like proliferating meristems) are selected from these cultures using a dissection microscope. The scalps usually contain a high proportion of meristematic domes, are isolated from the clusters and cultured on BM2Z medium for embryogenic callus development. Swelling of the explants, development of corm tissues and dedifferentiation of leafy portions into watery callus is commonly observed during the initial few weeks. This is followed by the development of heterogeneous globules and formation of a friable embryogenic callus bearing many translucent proembryos.

A friable embryogenic callus mass bearing many translucent proembryos (Figure 3D,E), obtained either from immature male inflorescence or scalp cultures, is ideal for initiating ECS.

2.2.3. *Initiation of Embryogenic Cell Suspension Cultures*

Cell suspensions can be obtained by culturing about 100 mg of embryogenic calli with proembryos in 25 ml capacity conical flask containing 8 ml of BM2L medium (Cote et al., 1996; Ganapathi et al., 2001) in case of male flowers and BM2Z medium in case for scalps. These are maintained on a gyratory shaker at 80 rpm under a photoperiod of 16 h light at $45 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ and a temperature of $26 \pm 1^\circ\text{C}$. A good cell suspension culture containing actively dividing and densely packed cytoplasmic cells can be recovered after 3–4 months (Figure 3F,G). During this period the suspensions are subcultured every 10 days by replacing 4 ml of used medium with the fresh BM2L medium. Hereafter, the suspensions are cultured in 125 ml capacity conical flasks containing 25 ml BM2L medium. At each subculture, 0.5 ml packed cell volume (PCV) + 4.5 ml of the spent medium are added to 25 ml of BM2L medium. In order to control release and oxidation of the phenolic compounds, ascorbic acid at 10 mg/l is often added to the BM2L or BM2G media.

For embryo development, 0.5 ml of PCV of cell suspension is aspirated onto glass fibre filters and is transferred to BM3 medium. The embryo development can be observed within approximately 3–6 weeks. The globular stage embryos are visible initially. The conversion of the somatic embryos into plantlets (Figure 3I) can be achieved on BM4 medium (1–2 monthly passages). If required these shall be followed by transfer onto BM5 medium till fully grown plantlets with well developed shoot and roots are obtained (4–6 weeks).

2.2.4. *Greenhouse Hardening of Emblings*

The somatic embryogenesis derived plantlets (emblings) are hardened (Figure 2F) essentially in the same way as described above in Section 2.1.3 in the case of shoot-tip derived plantlets. These can further be field planted for evaluation (Figure 2G).

3. RADIOSENSITIVITY ESTIMATION, MUTAGENESIS AND CHIMERA SEPARATION

The success of the *in vitro* mutagenesis programme largely depends upon induction of high frequency of mutations and selection of the desirable mutants. Determining the radiosensitivity followed by irradiation at selected doses can help in increasing frequency of the mutations (IAEA, 1977; Karmarkar et al., 2001). Further, optimization of *in vitro* regeneration system and the efficient screening of the mutagenized populations would lead to higher probability of recovering desired mutations. The radiosensitivity estimation, mutation induction and subsequent chimera dissociation in the *in vitro* cultures can be carried out as described in the following sections.

3.1. Shoot-tip Multiples

3.1.1. Radiosensitivity Estimation

The individual shoots are separated from *in vitro* shoot-tip multiples and the leafy portion is excised leaving behind 1–2 cm long piece containing a shoot-meristem inside, without any leaf lamina. These are cultured on 5 ml of $\frac{1}{2}$ MS medium, in culture tubes and exposed to Gamma (γ) rays ranging from 0 to 100 Gy. Following irradiation, the shoot-tips are immediately transferred to fresh medium (20 ml/tube). Although the lethal doses of irradiation would kill the meristematic region, surrounding leafy tissue may continue to grow further, which leads to an overestimation of the number of living shoots and underestimation of the dead shoots. This way, lethal dose 50% (LD_{50}) cannot be precisely estimated. Hence, in order to circumvent this problem, the emerging leaves are trimmed 4 weeks after irradiation and re-cultured on the fresh $\frac{1}{2}$ MS medium. The number of surviving shoots is then recorded after another 4 weeks and the LD_{50} and lethal dose (LD_{100}) are determined.

In a case study by Kulkarni et al. (1997), radiosensitivity of individual shoots of six *Musa* genotypes viz; Basrai (BAS, AAA), Karibale Monthan (KRM, ABB), Lal Kela (LAK, AAA), Nanjangud Rasthali (NJR (AAB), Shreemanti (SHR, AAA) and a Wild Diploid (WDK, BB) was determined. Individual *in vitro* shoots isolated from the cluster of multiples were subjected to different doses (0 to 80 Gy, step of 10 Gy and 100 Gy) of gamma irradiation and survival percentage of shoots were recorded. The linear regression coefficients of dose (Gy) on the % survival of shoots were worked out (Figure 4). The LD_{50} ranged from 30 Gy (NJR, AAB) to 52 Gy (LAK, AAA), with an average of about 40 Gy. In general, the irradiation dose of about 70 Gy is completely lethal; however, a dose of 100 Gy can be included as the lethal control.

3.1.2. Gamma Irradiation and Chimera Dissociation

For the purpose of induction of mutations, the fresh and actively proliferating shoot-tip multiples (each with 4-6 shoots) are utilized. The selected cultures are labeled/numbered properly and then irradiated at predetermined doses in a suitable gamma-irradiator. Immediately after irradiation, the single shoots are separated and subcultured (M_1V_0) onto fresh medium.

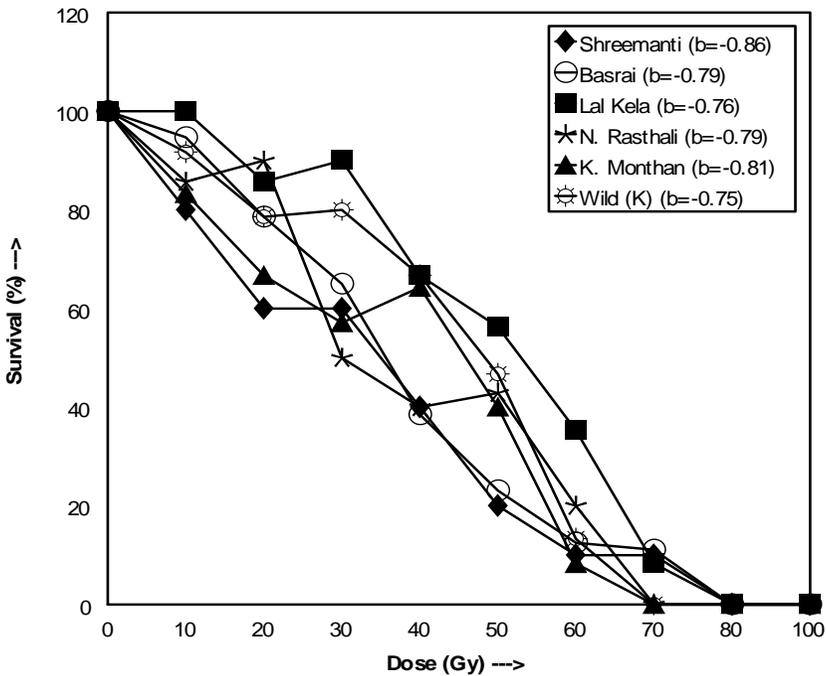


Figure 4. Effect of gamma irradiation on survival (%) of *in vitro* individual shoots of banana.

In order to dissociate the chimeras, the irradiated shoot-tip multiples are subcultured for at least 4–8 cycles ($M_1V_0 - M_1V_{4-8}$) on the same proliferation medium. As a trend, the shoot multiplication ratio during subcultures decreases with increasing dose of irradiation. However, owing to several growth controlling factors, smooth curves are difficult to be obtained.

Upon adequate subculturing the individual shoots are separated and transferred for the induction of rooting (M_1V_{5-9}). The rooted plantlets are weaned in the greenhouse and subsequently field planted for evaluation.

An important constraint in evaluating the mutant populations in the case of a vegetatively propagated crop like banana is to correctly judge at which stage complete chimera separation could be achieved. Unless this is known, there is always a risk that the selected mutant may be chimeric and thus will not produce true-to-type progeny. On the other hand, excessive multiplication *in vitro* (with a fear of improper chimera separation) may result in the generation of a large plant population, unmanageable for field evaluation. Several factors such as the basic proliferation rates of the non-irradiated cultures and the decreasing proliferation rates due to irradiation can affect chimera dissociation. It becomes difficult to decide the “number of subcultures” for dissociating the chimeras subsequent to irradiation of the shoot-tip multiples.

In an earlier study, Kulkarni et al. (1997) subcultured the irradiated populations for four cycles (including rooting). Subsequently it was decided to test the adequacy of subculturing up to M_1V_4 for chimera dissociation, by studying the variability at the field level (Kulkarni et al., unpublished results). An experiment was hence designed to test the 5 populations derived from 5 suckers (originating from a single selected mutant plant) for existence of inter-population variability. Detection of significant differences between all the 5 populations indicated that the “selected initial plant” was possibly chimeric at the time of field testing, and thus additional *in vitro* multiplication cycles were necessary for proper chimera separation. It was also possible that the populations differed because of the somaclonal variations during *in vitro* passages. The differential expression could as well be an integral effect of these and other unknown causes. In view of this, it is suggested that the subculturing shall continue till M_1V_{5-7} (Novak et al., 1990; Rao et al., 1998) or even more as the case may be.

3.2. Cell Suspensions

The induction and establishment of ECS in a specific banana genotype of interest is still not a routine practice, and thus, only a few reports are available on gamma-irradiation of ECS (Kulkarni et al., 2004a; Roux et al., 2004). The following procedure is suggested for mutagenizing the banana cell suspensions using gamma irradiation.

3.2.1. Radiosensitivity Estimation

About 0.5–1.0 ml aliquot of the ECS containing about 25–50 mg fresh weight cells in the exponential (G_1) growth phase are transferred to Eppendorf type plastic tubes of 1.5 ml capacity and centrifuged at 5000 rpm for 5 min (Kulkarni et al., 2004a). Alternatively, these cells can also be irradiated in the sterile Petri dishes (Roux et al., 2004).

Supernatant medium is discarded and the tube with pelleted cells is exposed to ^{60}Co gamma rays (0–100 Gy, in steps of 10 Gy). Each treatment shall contain minimum 3 replications. Immediately after irradiation, the cell pellets are transferred to 20 ml of fresh liquid medium in 100 ml capacity conical flasks. The growth of the cells during the subcultures is determined by recording fresh and dry weights in 1 ml aliquot. The cells are plated (as described above) for embryo development and plantlet regeneration (as given in the earlier section). The plantlet regeneration data is compared with the non-irradiated control cells and the LD_{50} is estimated.

Kulkarni et al. (2004a) observed that the growth of cells reduced with increasing dose up to 30 Gy, and a dose of 40 Gy and beyond was observed to be completely lethal (Figure 5). Accumulation of cell weight (with each successive subculture) was maximum in control, and reduced with increasing dose. The gain in fresh and dry weights of the cells with increasing irradiation dose was very gradual during first two subculture cycles, but became evident in the 3rd subculture and was maximum in the 4th one.

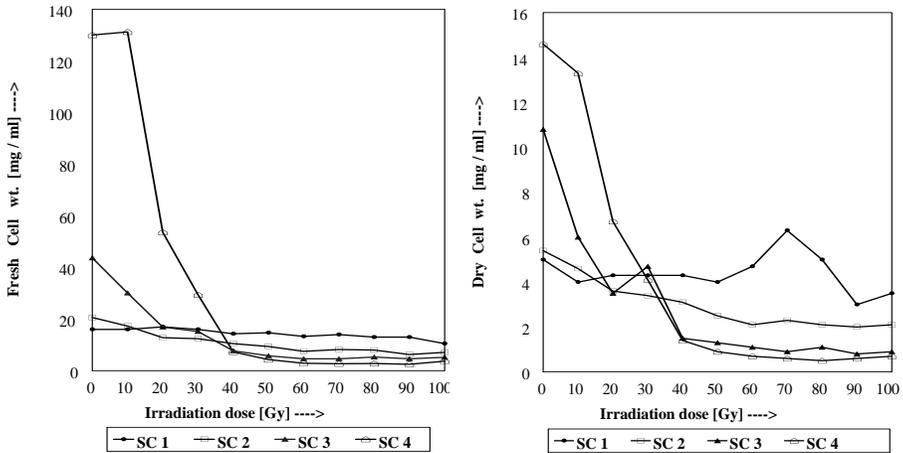


Figure 5. Effect of gamma irradiation on the cell suspension cultures of banana cv. Grand Nain.

The liquid medium can turn dark purple/brown and the degree of darkening depends on the dose. Frequent replenishment of the darkened medium can reduce this problem. Previous reports indicate that the gamma-irradiation at approximately 70 Gy was completely lethal to the shoot-tip multiples. Owing to the higher hydration levels, the ECS were more radiosensitive and the irradiation dose of about 40 Gy was found to be lethal (Kulkarni et al., 2004a). Surprisingly, Roux et al. (2004) reported that the ECS of cv. Williams and Three Handy Planty grew even at a relatively very high dose of 250 Gy (the dose about 6 times higher than Kulkarni et al., 2004a). This discrepancy may be due to several factors related to genotype, physiological status and water content of the cell line which needs to be investigated with different banana genotypes.

3.2.2. Gamma Irradiation and Chimera Dissociation

It has been shown that the somatic embryos arising from banana cell suspensions most “probably” have a single cell origin (Roux et al., 2004). Thus, the main advantage of using the ECS for mutagenesis would be either in obtaining non-chimeric populations or the quick dissociation of the chimeric sectors if any (Roux et al., 2001).

The cells in the synchronized growth state shall preferably be used for irradiation. The established ECS (4–6 days after subculture, i.e. in exponential phase) are ideal for irradiation purpose. It would be appropriate to sieve the ECS using 1000 μm pore size mesh to obtain a fine ECS, before irradiation. The cell suspensions shall essentially be mutagenized using pre-determined doses (see the “NOTES” section below) of gamma-irradiation. The mutagenized and control cells are optionally multiplied for 3 or 4 weekly subcultures for dissociation of the chimeras. The mutagenized as well as the control ECS shall be plated for embryo development and conversion to plantlets, as described in Section 2.2.3.

3.3. Observations to be Recorded

3.3.1. Shoot-tip Cultures

For tissue culture experiments, data are scored on various traits such as number of shoots (total number of scorable shoots per culture), shoot length (average length of the shoots in the culture, cm), number of roots (total number of scorable roots per shoot), root length (length of the longest root, cm), multiplication ratio (estimated by dividing “number of cultures after subculturing” by “number of cultures before subculturing” and shoot survival (proportion of shoots resuming growth upon gamma irradiation, %). If deemed necessary, the post-irradiation *in vitro* rooting response can also be determined by culturing the surviving shoots on SM4 medium and the observations with respect to number of days for first root initiation, number of roots, root length and shoot length are recorded.

3.3.2. Cell Suspension Cultures

Weight gain (final fresh weight/initial fresh weight of the cells), settled cell volume (can be measured by transferring known volume of cell suspension into sterile graduated tube and allowing to settle for 10 min), number of somatic embryos (total number of the globular, torpedo or advanced stage somatic embryos obtained per ml of cells plated), number of plantlets regenerated (total number of plantlets obtained per ml of cells plated).

3.3.3. Plant Survival during Hardening

The proportion of surviving plants (%) for “shoot-tip multiplication” and “ECS” derived plants in the greenhouse is recorded.

4. FIELD PLANTING AND EVALUATION

It would be desirable to evaluate maximum number of plants in the field; however, banana field trials cannot be compared with the field crops requiring high planting densities. It is also difficult to recommend any particular population size for field trial. However, at least about 500 plants are field evaluated. It is strongly suggested that only the standard cultivation practices shall be adopted based on the local conditions.

The greenhouse hardened non-irradiated (control) and irradiated populations are field transferred for evaluation and screening for variants. The sources of variation could be the individual effects or a combined effect of genetic causes, physiological effect of irradiation, physiological effect of *in vitro* culturing and somaclonal variation.

4.1. Observations to be Recorded

As a guideline, the standard descriptors are to be referred for data scoring (INIBAP, 1996); however, data can also be recorded on any other traits of specific interest as well (Kulkarni et al., 2002). At least 2 border rows of a phenotypically matching banana genotype/s shall essentially be incorporated from all sides of the experimental block. Data are scored as described above (excluding the border rows).

4.2. Field Evaluation

In the first field trial, in principle, each greenhouse hardened plant coming from mutagenesis experiment is treated as separate genotype. Thus, a replicated field evaluation experiment is not possible at this stage, and the data are scored on each and every plant from mutagenized populations. Non-irradiated plants (derived from shoot-tip multiples/ECS) of same age are included as standard control.

The shoot-tips from preferably all of the side-suckers from the “selected variant/s” are appropriately labelled and cultured *in vitro* for maintenance and multiplication. The regeneration of plantlets and greenhouse hardening of these populations can be done by following the above discussed procedures.

In the second and subsequent evaluations, the populations of individual suckers are maintained separately to obtain sufficient number of plants for subsequent field evaluation/s. At this stage, a field trial can be laid down either for individual plant selection (like first field trial) or with appropriate number of replications. The stability of the selected variants can be confirmed by conducting multilocational trials over successive generations.

5. NOTES

- Sword suckers and flower buds are collected from a disease-free area and a reliable source. The materials certified/indexed as disease-free are ideal for initiating *in vitro* cultures.
- It is known that the *in vitro* response of different banana accessions differs considerably (Kulkarni et al., 2004b) resulting in the genotypic interference. In general, the genotypes with AAA genome respond better for proliferation as compared to the ones with B genome.
- As a thumb rule, the subculturing of the shoot-tip multiples shall not exceed the proliferation beyond 1→1000.
- Delayed subculturing of shoot-tip multiples results in cultures containing single or unusually elongated shoots (apical dominance) and with lower than normal proliferation rates. Such cultures are not ideal for mutagenesis because this would reduce the chances of chimera dissociation.
- The apical dominance can be minimized by timely subculturing the shoot-tip multiples.
- The embryogenic calli and proembryos can be maintained on BM2G medium by regular subculturing once in 4 weeks. These also can be used for mutagenesis *in vitro*, however, owing to their lower regeneration potential (than embryogenic cell cultures) and also the associated problem of chimera formation, their use for mutagenesis is not recommended. However, these can be tried if one fails to obtain embryogenic cell suspensions.
- Subculture of the cell suspensions is done generally by taking 10% of the cell mass replenished with fresh BM2L medium. Periodic checking for the embryogenic status (very high proportion of embryogenic cells/clumps that are endowed

with isodiametric cells with dense cytoplasm studded with starch grains) and their potential to develop into somatic embryos and plantlets is equally important.

- Knowledge about the LD50 dose helps in finalizing the actual irradiation-doses for mutation induction. For example, if the LD50 is observed to be 30 Gy, the *in vitro* cultures can be mutagenized by gamma-irradiation at 20, 30 and 40 Gy. The doses above this range would possibly result in abnormal/detrimental variations and the doses in lower range will result in production of excessively large populations with low chances of recovering a desirable mutant.
- It is well known that the irradiation-damaged cells can lose competence for proliferation and hence either are suppressed or lost in competition with normal ones. For this reason, no shoot-apical regions shall be discarded during subculture of the mutagenized shoot-tip multiples.
- The dose rate (Gy/min) at the time of irradiation shall be recorded before beginning with irradiation experiments.
- The cells for irradiation as well as the non-irradiated control shall be derived from same cell line.
- The non-irradiated control populations are maintained in parallel with the irradiated populations. Each dose is replicated at least 3 times.
- At the time of each subculture of irradiated and control cells, an aliquot of 1 ml cells are plated for checking embryo development and plantlet regeneration.
- The number of cultures increases exponentially with each subculture cycle warranting advance-planning and availability of the resources.

6. CONCLUSIONS AND PROSPECTS

Efficient *in vitro* regeneration system via direct organogenesis and somatic embryogenesis is critical for studies on genetic manipulation using either mutagenesis or genetic transformation. In order to be applicable, *in vitro* mutagenesis needs to be extended to diverse banana cultivars. Estimation of radiosensitivity is important in determining the actual irradiation doses. Shoot-tip multiples and cell suspensions can be effectively used for mutagenesis; however both have their own advantages and limitations. Shoot-tip cultures are easy to establish but pose a serious problem for chimera separation. On the other hand, the cell suspensions have been reported to be ideal for overcoming chimerism but still are not routine to establish and regenerate. So far, there has been no published data in case of the mutant-populations arising from mutagenesis of cell suspensions. Although there could be comparatively less probability of having chimeric genotypes for field-testing, this needs to be validated with experimental evidences.

The ability to culture and manipulate a large number of totipotent cells provides a greater opportunity for *in vitro* selection of useful mutations at cellular level. It is possible to select for cell lines resistant to biotic stress for example disease resistance and tolerant to abiotic stress viz. aluminum tolerance, salt, drought and frost tolerance. In banana, studies have been conducted on *in vitro* selection following mutagenesis, mainly for fungal disease resistance. A complete operating strategy should involve *in vitro* mutagenesis and selection, *ex vitro* confirmation of

tolerance, precise field evaluation of existence, stability, and usefulness. The field of mutagenesis and *in vitro* selection has scope for the generation of novel banana genotypes with enhanced stress tolerance to sustain growth and productivity under varied environmental regimes.

The procedures described in this article on the establishment of shoot-tip multiples and embryogenic callus and cell suspension cultures and their use in radiation mutagenesis can be useful guidelines to researchers intending to undertake *in vitro* mutagenesis experiments aimed at banana improvement.

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