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Bioreactor Technology for Plant Micropropagation

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I. INTRODUCTION

In vitro propagation is based on enhanced axillary bud proliferation and on the ability of differentiated, often mature plant cells, to redifferentiate, and develop new meristematic centers that are capable of regenerating fully normal plants. Regeneration is potentiated through two morphogenic pathways: organogenesis—the formation of unipolar organs, and somatic embryogenesis—the production of bipolar structures, somatic embryos with a root and a shoot meristem (Steward et al. 1970; Ammirato 1985).

All plant somatic cells once isolated and cultured in vitro are capable of expressing totipotency. The injured cells in the outer layers of the isolated explant evolve ethylene that induces the inner layers of cells to undergo dedifferentiation. After the loss of coordinated control, which is ensued by cell division, the formation of new gradients of endogenous phytohormones in the dedifferentiated cells enhance cell divisions in response to the various growth regulators added to the medium. Cell division can take place in an unorganized pattern with callus formation, or in an organized pattern with the formation of meristematic centers directly in the explant tissues. Redifferentiation in the callus tissue takes place when new physiological gradients are formed in the non-organized parenchymatous tissue along with the formation of meristemoids (in vitro meristems), which can further differentiate into organized structures. In several plant species, meristematic centers form directly on the explant with very little or no callus formation and can develop into either shoots or somatic embryos. Plant regeneration can follow after the initial stages of controlled redifferentiation through either the organogenic or embryogenic pathways (Ammirato 1983, 1985; Ziv 1999).

Plant regeneration in vitro is dependent on the manipulation of the inorganic and organic constituents in the medium, as well as the type of explant and the species. In most plants, successful regeneration from the callus or directly from the explants takes place after a series of subcultures in various media, in a sequence which is often specific to the species, variety, or the newly introduced genotype. The determining factors are the combination of the concentration in relation to medium-volume and the composition of growth promoting and retarding regulators in the medium, the physiological status and competence of the cells and their capability for morphogenetic expression (Christianson 1985, 1987).

Micropropagation (in vitro propagation of axillary and/or adventitious buds as well as somatic embryos) is presently used as an advanced biotechnological system for the production of identical pathogen-free

plants for agriculture and forestry (George 1996). The technique, however, is still costly due to intensive hand manipulation of the various culture phases and is not used commercially for all plant species. In addition, in some plants, the initial stage of establishment and response is slow and the survival of the plants in the final stage ex vitro is often poor, which further reduces the micropropagation production potential (Ziv 1995a; Aitken-Christie et al. 1995).

Efficient commercial micropropagation depends on rapid and extensive proliferation along with the use of large-scale cultures for the multiplication phase. Furthermore, normal plant development during the acclimatization and hardening stage is mandatory to ensure a high percent of survival after transplanting to the greenhouse (Preece and Sutter 1991; Ziv 1991a,b, 1995b). Mechanization and automation of the micropropagation process can greatly contribute to overcoming the limitation imposed by existing conventional labor-intensive methods. Considerable attention has been directed toward automation of the repeated cutting, separation, subculture, and transfer of buds, shoots, or plantlets during the multiplication and transplanting phases (Levin et al. 1988; Aitken-Christie 1991, Aitken-Christie et al. 1995; Vasil 1994). Progress in tissue culture automation will depend on the use of liquid cultures in bioreactors, which will allow fast proliferation, mechanized cutting, separation, and automated dispensing (Alper et al. 1993; Sakamoto et al. 1995). These techniques were reported for some plants and were shown to reduce hand manipulation and thus reduce in vitro plant production costs (Levin et al. 1988; Ziv 1990b, 1992a,b, 1995b; Ziv and Hadar 1991; Vasil 1994; Aitken-Christie et al. 1995). The various propagation aspects of several plant species in bioreactors and some of the problems associated with the operation of bioreactors were recently reviewed by Takayama and Akita (1998). One major problem addressed was microbial contamination as affected by both the introduced plant material and the operation procedures of large-scale bioreactors.

Liquid media have been used for plant cells, somatic embryos, and organ cultures in both agitated flasks or various types of bioreactors (Smart and Fowler 1984; Ammirato and Styer 1985; Stuart et al. 1987; Chen et al. 1987; Preil 1991; Paque et al. 1992; Scragg 1992; Attree et al. 1994; Ziv 1995b; Archambault et al. 1995; Tautorus and Dunstan 1995). Although the use of bioreactors has been directed mainly for cell suspension cultures and secondary metabolites production, research directed at improving bioreactors for somatic embryogenesis has been reported for several plant species (Styer 1985; Preil et al. 1988; Nadel et al. 1990; Scragg 1990, 1992; Hale et al. 1992; Archambault et al. 1994; Tautorus et al. 1994; Attree et al. 1994; Ziv et al. 1994; Takayama and Akita 1998;

Hvoslef-Eide and Munster 1998). The cultivation in liquid media using a temporary immersion system with different frequencies of immersion was reported to improve plant quality and multiplication rates of banana, coffee, and rubber (Alvard et al. 1993; Teisson and Alvard 1995; Etienne et al. 1997). Bioreactors were used also for the cultivation of hairy roots mainly as a system for secondary metabolite production (Rodriguez-Mendiola et al. 1991; Flores 1995; Weathers et al. 1989). The use of a simplified acoustic window mist bioreactor for transformed roots and for carnation shoots was reported to improve biomass growth (Chatterjee et al. 1997).

Information on the use of bioreactors as a system for plant propagation (Table 1.1) through the organogenic pathway, although limited to a small number of plant species, is presently being applied to several ornamental and some vegetable and fruit crop plants (Takayama et al. 1991; Takahashi et al. 1992; Ziv 1992a,b; Akita and Takayama 1994; Ilan et al. 1995; Ziv and Shemesh 1996; Ziv et al. 1998).

However, liquid cultures confer several problems associated with abnormal plant development, a phenomenon described as hyperhydricity, previously termed "vitrification" (Debergh et al. 1992), which causes poor plant development in vitro and later in ex vitro. Attempts were carried out to overcome hyperhydricity in liquid cultures by the use of growth retardants, inhibitors of gibberellin biosynthesis, which decreased hyperhydricity, reduced shoot elongation, and induced bud or meristem cluster formation (Ziv 1990a,b; Ziv and Ariel 1991; Ziv 1992b; Ziv and Shemesh 1996; Ziv et al. 1998). The clusters were shown to be an alternative propagation system for bioreactor cultures, providing a biomass with limited leaf elongation. Recently micropropagation of pineapple was scaled up using clusters in a temporary immersion system (ebb and flow) in bioreactors (C. G. Borroto 1998, pers. commun.). Bioreactors are presently being used for commercial micropropagation in the USA, Japan, Taiwan, Korea, Cuba, Costa Rica, Holland, Spain, Belgium, and France for ornamental and bulbous plants, pineapple, potato, and forest trees. The cost per propagule unit of foliage plants was estimated to be reduced from 17 to 6–7 cents (US) (R. Levin, pers. commun.).

A recent review by Takayama and Akita (1998) describes several bioreactor techniques using plant propagules for large-scale propagation of potato, gladiolus, lilies, strawberry, *Hyacinth*, *Amaryllis*, and several *Araceae*. The present paper reviews various aspects of large-scale liquid culture in bioreactors for plant propagation, emphasizing the physical and chemical growth factors that control development and proliferation in liquid media for efficient plant production.

Table 1.1. Plants propagated in bioreactor cultures.

Species	Response	References
Amaryllis hippeastrum	Buds, plants, bulblets	Takayama and Akita 1998
Ananas comosus	Shoot clusters	Escalona et al. 1999
Apium graveolens	Somatic embryos	Nadel et al. 1990
Araceae species	Shoots, plants	Takayama and Akita 1998
Brodiaea complex	Bud clusters, corms	Ilan et al. 1995
Coffea arabica	Shoot clusters, plants	Alvard et al. 1993; Teisson and Alvard 1995
Cyclamen persicum	Callus, somatic embryos	Hvoslef-Eide and Munster 1998
Daucus carota	Callus, somatic embryo	Jay et al. 1994; Archambault et al. 1995
Dianthus caryophyllus	Shoots, plants	Chatterjee et al. 1997
Eschcholtzia californica	Somatic embryos	Archambault et al. 1994
Euphorbia pulcherrima	Somatic embryos	Preil 1991; Luttman et al. 1994
Fragaria ananasa	Shoots, plants	Takayama and Akita 1998
Gladiolus grandiflorum	Bud clusters, plants, corms	Ziv 1990; Ziv et al. 1998
	Shoots, plants, corms	Takayama and Akita 1998
Hevea brasiliensis	Buds, plants	Alvard and Teisson 1993; Teisson and Alvard 1995
Hyacinthus orientalis	Bulblets, plants	Takayama and Akita 1998
Lilium spp.	Plants, bulblets	Takayama 1991
	Bulblets, plants	Takayama and Akita 1998
Medicago sativa	Callus, somatic embryos	Stuart et al. 1985, 1987; Chen et al. 1987; Stuart et al. 1987; McDonald and Jackman 1989; Denchev et al. 1992
Musa spp.	Buds, plants	Alvard and Teisson 1993; Teisson and Alvard 1995
	Buds clusters, plants	Ziv et al. 1998
Nephrolepis exaltata	Buds, plants	Levin et al. 1988; Ziv and Hadar 1991; Ziv et al. 1998
Nerine sarniensis	Proembryogenic clusters, somatic embryos, bulblets	Lilien-Kipnis et al. 1994 Ziv et al. 1994
Ornithogalum dubium	Shoot clusters, plants, bulblets	Ziv and Lilien-Kipnis 1997
Populus tremula	Bud clusters, shoots, plants	McCown et al. 1988; Carmi et al. 1997
Picea glauca	Somatic embryos	Attree et al. 1994
Picea glauca-engelmannii	Somatic embryos	Tautorus et al. 1994
Picea marianna	Somatic embryos	Tautorus et al. 1994
Solanum tuberosum	Plants, tubers	Akita and Takayama 1994
	Bud clusters, plants, tubers	Levin et al. 1997a; Ziv et al. 1998; Ziv and Shemesh 1996
	Shoots, plants, tubers	Takayama and Akita 1998

II. PLANT DEVELOPMENTAL PATHWAYS IN BIOREACTORS

A. Somatic Embryogenesis

The use of liquid cultures for the cultivation of somatic cells in recapitulating embryogeny was reported by Steward et al. and Reinert as early as 1958 in carrot (*Daucus carrota* L.). The underlying concept for cell totipotency and the ability to renew growth and express morphogenesis was based on the assumption that isolation and bathing of the cells in a specific medium will simulate the zygotic embryo conditions in the ovary. The bathing liquid medium that contains various nutrients and growth regulators was assumed to be a source for similar stimuli that were present in the zygotic embryos' immediate environment. In addition, the segregation of embryogenic from non-embryogenic cells amplified the expression of cell totipotency (Steward et al. 1970). Somatic embryogenesis was achieved by the initial use of an induction medium containing an auxin, usually 2,4-dichlorophenoxyacetic acid (2,4-D), and coconut water; the latter was mainly a source of cytokinins, inositol, and reduced nitrogen compounds. Once the pro-embryonic clusters formed, the removal or lowering of the auxin concentration initiated a sequence of events similar to zygotic embryogeny (Halperin 1967; Ammirato 1985). Somatic embryogenesis was observed at first mainly in members of the Umbelliferae cultured in liquid media (Steward et al. 1970) and has since been reported in gymnosperms and angiosperms, including several ornamentals, and vegetable and field crops, as well as perennial woody plants.

The embryogenic pathway, as opposed to the organogenic pathway, can be a more efficient and productive system for large-scale clonal propagation. Somatic embryogenesis may result in less variation through chimerism. However, somaclonal variation will be less likely to take place in direct regeneration, but might be more pronounced in somatic embryos developing from continuously cultured callus tissue. Since the embryo contains both a root and an apical shoot meristem, the rooting stage required in conventional in vitro bud or shoot propagation technology is obviated. Somatic embryos are small and can be adequately handled in scaled-up procedures. They are amenable to sorting and separation by image analysis, dispensing by automated systems, and can be encapsulated and either stored or planted directly, with the aid of mechanized systems (Ammirato and Styer 1985; Cazzulino et al. 1991; Cervelli and Senaratna 1995; Sakamoto et al. 1995).

Somatic embryogenesis in liquid shake or bioreactor cultures was reported in carrot (Ammirato and Styer 1985; Jay et al. 1992, 1994; Archambault et al. 1995), in caraway (Ammirato 1983), poinsettia (Preil 1991),

alfalfa (Stuart et al. 1985, 1987; Denchev et al. 1992; Kuklin et al. 1994), celery (Nadel et al. 1990; Saranga and Janick 1991), in *Eschcholtzia californica* (Archambault et al. 1994), in *Nerine* (Ziv et al. 1994), in *Octea catharinensis* (Moura Costa 1992), in sweet potato (Harrell et al. 1994), in rubber (Etienne et al. 1997), and in spruce (Tautorus and Dunstan 1995). However, the ultimate goal of production of synthetic seeds through successful encapsulation of somatic embryos from liquid shake or bioreactor cultures was reported only for carrot (Kitto and Janick 1985; Redenbaugh et al. 1991; Sakamoto et al. 1995), alfalfa (Senaratna 1992; Fujii et al. 1992), celery (Kim and Janick 1989; Onishi et al. 1992, 1994), and white spruce (Attree et al. 1994) and is currently under continuous investigation for several other species.

B. Organogenic Pathway

The propagation of most plants is presently carried out commercially through the organogenic pathway in agar-gelled cultures, even though the protocols are long and costly. The advantages for the mechanization of the process are mainly for achieving a reduction in hand manipulation and labor costs. The process has to be scaled up using liquid cultures in bioreactors to amend it to automation (Levin et al. 1988; Kurata 1995). The information on the use of bioreactors for unipolar structures such as protocorms, buds, or shoots is limited, mainly because of the problems of hyperhydricity of the leaves and shoots (Debergh et al. 1992). One of the first attempts in using liquid cultures for micropropagation of buds was reported for orchids that formed protocorms with minimal shoot elongation during the liquid culture stage (Morel 1974). The protocorms could be separated and induced to form new plants after subculture to agar-gelled medium. Several other ornamental plants also have been propagated through the organogenic pathway in liquid shake cultures and bioreactors. By controlling shoot growth and providing culture conditions that reduced abnormal leaf growth and enhanced the formation of bud or meristematic clusters, as was shown for potato, gladiolus, and *Ornithogalum dubium* (Fig. 1.1A, B, C), a high proliferation rate was achieved without the phenomenon of hyperhydricity (Levin et al. 1988; Ziv 1990a, 1991a, 1992a; Ziv and Hadar 1991; Ziv and Ariel 1991; Takayama 1991; Takayama et al. 1991; Takayama and Akita 1994; Ilan et al. 1995; Ziv et al. 1998).

C. Bud or Meristem Clusters

The development of spherical meristematic or bud clusters in liquid cultures was shown to provide a highly proliferative and rapid growing

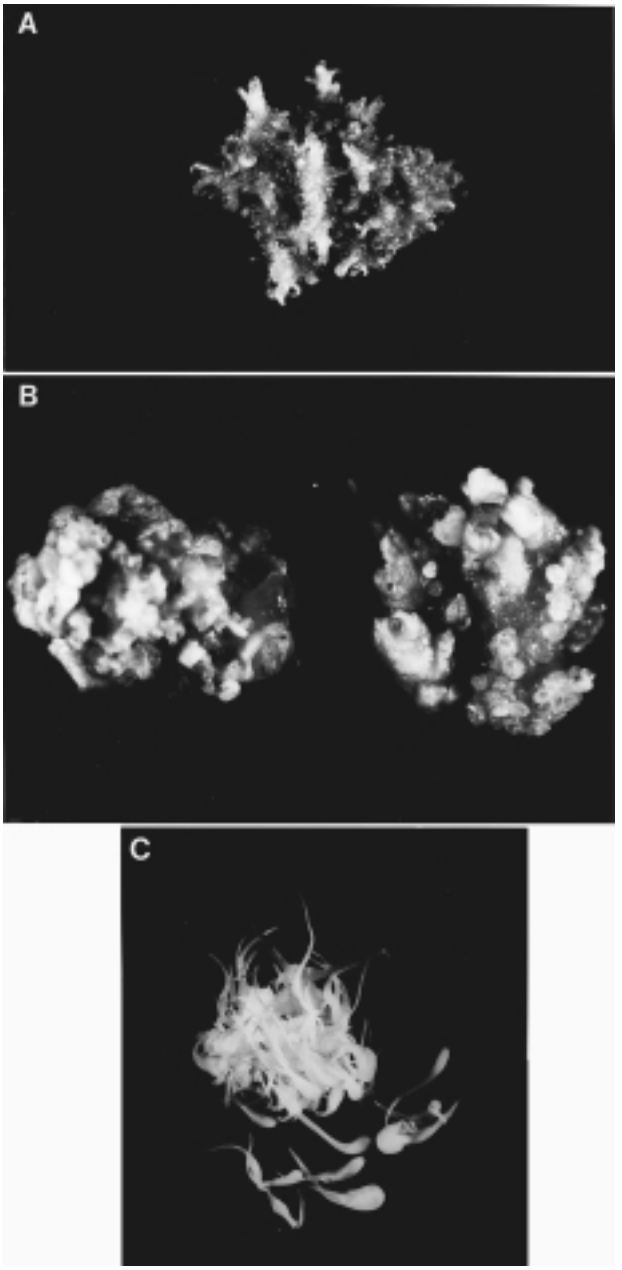

Fig. 1.1. Liquid cultured bud clusters of potato (A), meristematic clusters of gladiolus (B), and bulblet formation in *Ornithogalum dubium* bud clusters (C).

system amenable to automated inoculation, control of the medium components, mechanical separation, and efficient delivery to the final stage for plant growth and development (Levin et al. 1988; Ziv 1991b; Ziv et al. 1998). Cluster formation appears to be associated in most species with the continuous submergence, circulation, and agitation of the plant biomass in the medium, as well as with a balanced ratio of growth-promoting and growth-retarding regulators. Clusters can form from axillary buds and/or adventitious buds as well as from meristemoids in pro-embryogenic callus that later differentiate to somatic embryos (Fig. 1.1A, 1.2A, B).

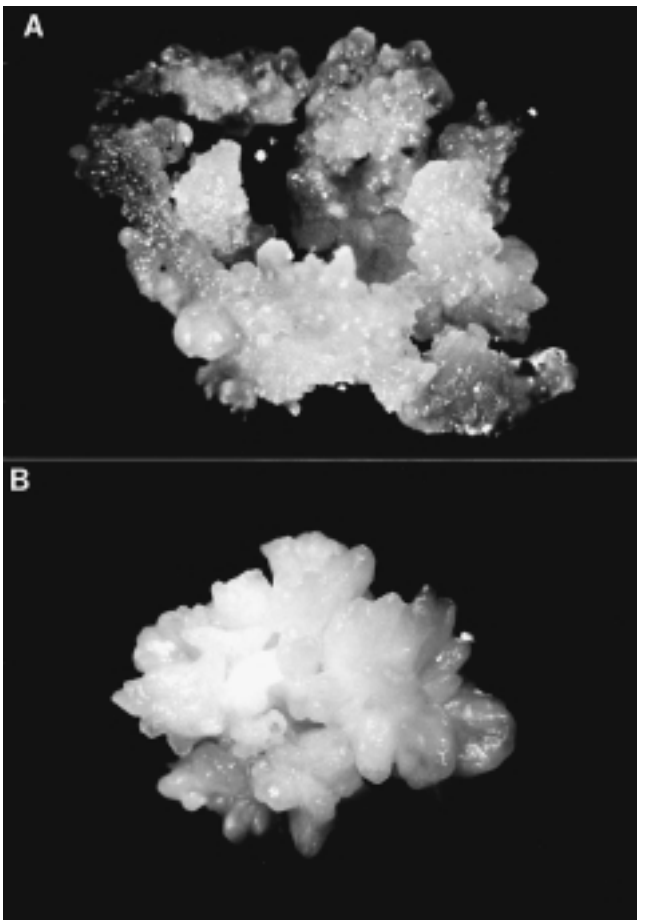

Fig. 1.2. Nerine proembryogenic clusters in a 2,4-D medium (A) and after 2,4-D removal with the addition of 2iP showing somatic embryos (B).

The formation of condensed organized structures in which the shoots are reduced to buds or meristematic tissue was reported in several plant species. The clusters were made up of densely packed meristematic cells, actively dividing and forming new meristematic centers on the outer surface. The meristemoids surrounded loosely packed cells in the center and exhibited some vascularization, as was shown in liquid-cultured poplar clusters (McCown et al. 1988). In banana, on the other hand, the clusters were made up of condensed buds surrounding a central core with a cavity (Ziv et al. 1998).

Halperin in 1967 described the formation of pro-embryogenic masses in carrot root explants which were induced by the addition of 2,4-D to the liquid medium, resembling orchids protocorm-like bodies that formed when isolated buds were cultured in liquid medium in shake cultures (Morel 1974). Protocorm-like clusters also were induced in liquid-cultured gladiolus buds (Ziv 1989, 1990a) and in several species of the complex *Brodiaea* (Ilan et al. 1995) by the addition of paclobutrazol or ancymidol (gibberellin biosynthesis inhibitors) to the medium. In chicory, nodules were induced on leaves by IBA and BA in the medium (Pieron et al. 1992). In potato and banana, bud clusters were induced by a balanced ratio between kinetin and ancymidol in the liquid medium (Ziv et al. 1998). In woody species, McCown et al. (1988) and Aitken-Christie et al. (1988) described nodules in poplar in liquid medium and in radiata-pine in agar-gelled medium respectively, induced by the use of a balanced ratio of growth regulators. Levin et al. (1988), working with several ornamental species, described a several-fold increase of an organogenic biomass of clusters which proliferated in bioreactors and were separated mechanically prior to dispensing to agar-gelled cultures for further growth. The production of clusters in *Philodendron* cultured in liquid medium required the presence of benzylaminopurine (BA) and an inductive treatment for 24–48 h with ancymidol. The inductive treatment prevented a carry-over dwarfing effect of ancymidol on leaf and shoot development after transplanting the clusters to agar-gelled medium for further plant growth (Ziv and Ariel 1991). Gladiolus clusters cultured in a disposable 2-L bioreactor proliferated and made up to 60% of the vessel volume after 4–5 weeks. The aggregates which reached 0.5–1.5 cm in diameter tended to sink to the bottom of the bioreactor as the biomass increased and could not be resuspended unless the rate of aeration was increased. An increase from 0.5 to 1.5 vvm (volume air/volume medium per minute) provided the required aeration for recirculation of the cluster biomass (Ziv et al. 1998). Potato and banana bud clusters proliferated in bubble and dis-

posable plastic bioreactors at a faster rate than on agar-solidified medium (Levin et al. 1997b)

Growth regulators, when given in a specific balanced ratio of promoting and retarding substances, apparently act as morphogenic signals and control the development of the spherical meristematic or bud clusters. The spherical meristematic or bud clusters were described by various terms: pro-embryogenic mass (PEM) in carrot (Halperin 1967), protocorms in orchids (Morel 1974), nodules in poplar (McCown et al. 1988) and radiata-pine (Aitken-Christie et al. 1988; Carmi et al. 1997), nubbins in daylilly (Krikorian and Kann 1981), and meristematic or bud clusters in gladiolus, *Brodiaea*, *Nerine sarniensis*, fern, *Philodendron*, potato, banana, and *Ornithogalum dubium* (Ziv 1989; Ziv and Hadar 1991; Ziv and Ariel 1991; Ziv et al. 1994; Ilan et al. 1995; Ziv and Shemesh 1996; Ziv et al. 1998; Ziv and Lilien-Kipnis 1997). It is suggested that the term *cluster* should be used, as it is more appropriate for describing these proliferative, usually rounded structures, which develop from buds or other proliferative tissue in liquid culture, through either the organogenic or somatic embryogenesis pathways.

D. Anomalous Plant Morphogenesis

Scaling-up in bioreactors requires the use of liquid instead of agar-gelled media during the proliferation and biomass production stages. The culture of plants in liquid medium is known to cause anomalous morphogenesis, resulting in plant hyperhydricity (Debergh et al. 1992). The plants that develop in liquid media are fragile, have a glassy appearance, with succulent leaves or shoots and a poor root system (Paque and Boxus 1987; Werker and Leshem 1987; Ziv 1991a,c, 1995a). The leaves are the organs affected most severely in liquid cultures. They develop an unorganized mesophyll tissue that is made up mainly of spongy parenchyma tissue with large intercellular spaces (Werker and Leshem 1987; Gaspar et al. 1987), a deformed vascular tissue, and an abnormal epidermis. The epidermal tissue in hyperhydric leaves lacks a well-developed cuticle and possesses malfunctioning guard cells which cannot respond to closure signals (Sutter 1985; Ziv et al. 1987; Ziv 1991a; Ziv 1995a). Hyperhydricity affects plant survival after transplanting and causes loss of the in vitro developed leaves, or even whole plants, which often wilt and die. The two major processes carried out by the leaves, photosynthesis and transpiration, are not fully functional in hyperhydric leaves and thus cause the poor performance of the transplanted plants ex vitro (Preece and Sutter 1991; Ziv 1991c, 1995a; Ziv and Ariel 1994).

In many plants propagated in vitro, the anomalous morphology and anatomical aberration often observed in plants result from deviation from the normal course of morphogenic events that are manifested in plants in vivo (Ammirato 1985; Ziv 1999). Some of the deformation, such as hyperhydric, malformed leaves and shoots, abnormal embryos, recurrent embryogenesis, and several other disorders (Ziv 1999), are apparently the result of interruption or faulty timing of the signals involved in the normal sequence of organizational events known to exist in vivo. These are problems that are manifested more severely in liquid medium and await further studies in order to understand and to help control plant morphogenic events in bioreactor cultures.

III. PLANT CELL AND TISSUE GROWTH IN BIOREACTORS

The advantages provided by aerated liquid cultures in bioreactors include better contact between the plant biomass and the medium, no restrictions of gas exchange, the control of the composition of both the medium and the gaseous atmosphere, and the ability to manipulate the plant biomass in relation to the medium volume (Cazzulino et al. 1991; Heyerdahl et al. 1995; Leathers et al. 1995). The need for efficient circulation and mixing of the plant biomass, especially for cluster and embryogenic tissue, is essential to prevent sedimentation and allow optimal growth (Scragg 1992; Doran 1993).

Various types of bioreactors with mechanical or gas-sparged mixing were used for plant cell cultures, to provide stirring, circulation, and aeration (Margaritis and Wallace 1984; Takayama 1991; Scragg 1992; Doran 1993; Takayama and Akita 1994). Mechanically stirred bioreactors depend on impellers, including a helical ribbon impeller (Archambault et al. 1994), magnetic stirrers, or vibrating perforated plates (Styer 1985; Preil 1991; Cazzulino et al. 1991). Aeration, mixing, and circulation in bubble column or airlift bioreactors is provided by air entering the vessel from a side or bottom opening through a sparger; as the air bubbles rise, they lift the plant biomass and provide the oxygen gas required (Merchuk 1990). Mechanically stirred bioreactors are used for effective mixing, aeration, dispersion of air bubbles, and prevention of large cell aggregates formation in cell suspension cultures (Denchev et al. 1992; Scragg 1992; Doran 1993).

In recent years, large-scale cultivation of plant cells, embryos, or organs has made use of airlift or bubble column bioreactors and, to a lesser extent, of mechanically stirred tank bioreactors, due to the former

lower shearing force properties. Plant cells are relatively large in size, have sensitive cell walls which are highly susceptible to shearing forces, and are easily damaged. Furthermore, since plant cells, unlike microbial cultures, do not require high O_2, the use of bubble column (Fig. 1.3A) or airlift bioreactors was found to be adequate and advantageous for plant tissue cultured in liquid media (Margaritis and Wallace 1984; Smart and Fowler 1984; Merchuk 1990). In addition, it was shown that mixing by gas sparging in bubble column or airlift bioreactors lacking impellers or blades is far less damaging for clusters than mechanical stirring, since they were shown to have a lower shearing stress (Ziv and Hadar 1991; Ilan et al. 1995; Ziv and Shemesh 1996).

The main advantage of airlift bioreactors is their relatively simple construction, the lack of regions of high shear, reasonably high mass and heat transfer, and relatively high yields at low input rates (Kawase 1989; Denchev et al. 1992). A bubble free oxygen supply bioreactor with silicone tubing was found suitable for embryogenic cell suspensions and provided foam-free cultures (Luttman et al. 1994). For hairy root culture, an acoustic mist bioreactor was found to increase root biomass significantly (Chatterjee et al. 1997).

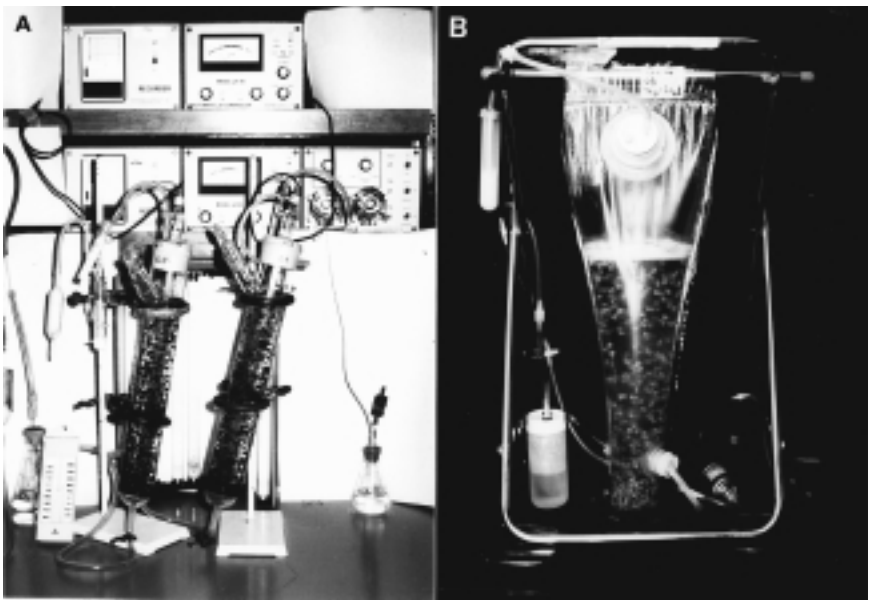

Fig. 1.3. Glass bubble column bioreactors with gladiolus clusters (A) and a plastic disposable bioreactor with *Lilium* clusters (B)

For efficient large-scale cultures of both somatic embryos and organogenic plant tissue, the bioreactor configuration and volume must be determined according to the mixing and aeration requirements of the specific plant or tissue propagated, as well as for minimizing the intensity of the shear stress (Doran 1993; Hvoslef-Eide and Munster 1998).

One major problem encountered in large-scale liquid cultures is contamination. Fungi, bacteria, yeast, and insects are a source of serious contaminations, causing heavy losses of plant material in commercial laboratories. In scaled-up liquid cultures, the losses are even greater, as the source of contamination due to manipulation of the bioreactor apparatus is dependent on the various stages of preparing and maintaining the equipment. In several laboratories, the operation area is kept sterile by a positive pressure airflow, which decreases contamination risks. In addition, constant screening of the plant tissue for contaminants and continuous indexing is another safeguard (Cassells 1991).

IV. PHYSICAL AND CHEMICAL FACTORS IN LIQUID CULTURES

In order to control plant morphogenesis and biomass growth in bioreactors, various culture conditions must be manipulated, i.e., the gaseous atmosphere, oxygen supply and CO_2 exchange, pH, minerals, carbohydrates, growth regulators, and the liquid medium rheology and cell density (Heyerdahl et al. 1995).

A. The Gaseous Atmosphere

The atmosphere of the culture vessel is made up mainly of nitrogen (78%), oxygen (21%), and carbon dioxide (0.036%). The culture vessel gas composition is influenced by the volume of the vessel and the extent of ventilation. Plants evolve CO_2 and consume O_2 during respiration, while during photosynthesis CO_2 is used and O_2 is produced. During the dark period, CO_2 levels were found to increase in cultures, and if photoautotrophic conditions prevail, its level decreased in the light (Fujiwara et al. 1987). Ethylene, ethanol, acetaldehyde, and other hydrocarbons are additional components of the gaseous atmosphere in vitro.

Most of the effects of CO_2, O_2, and C_2H_4 on plant growth in vitro were reported for agar-gelled or cell suspension cultures (Buddendorf-Joosten and Woltering 1994). In bioreactors, the control of the gaseous phase depends on the gas flow and can be easily manipulated to provide the required levels of O_2, CO_2, and C_2H_4. In airlift or bubble column

bioreactors, the air supplied is used for both mixing and aeration (Scragg 1992; Doran 1993). The importance of aeration and the gaseous phase were shown in potato cultured in airlift bioreactors. Induction of tubers was inhibited under continuously submerged conditions. Microtubers developed only after the shoots elongated and reached the gaseous phase at the top of the bioreactor. Enrichment with O_2 and manipulation of the hormonal and osmotic conditions had no effect and could not explain the phenomenon. A two-phase culture substituting the growth medium with a tuber induction with 9% sucrose in the medium enhanced tuber formation from shoots that developed above the medium and were exposed to the gaseous phase (Akita and Takayama 1994).These results emphasize the importance of the gaseous phase in the bioreactors for specific developmental phases.

1. Oxygen Level. Oxygen levels in liquid cultures depend on the presence of O_2 in the gas phase above and in the air bubbles inside the medium, as well as in the dissolved O_2 in the medium. Air is released through a sparger located at the base of the bioreactor. The available oxygen for plant cells in liquid cultures determined by oxygen transfer coefficient (k_La values) is the part that dissolves in water. Its depletion as a function of the metabolic activity of the growing cell biomass can affect the culture yield. Plant cells have a lower metabolic rate than microbial cells and a slow doubling time and therefore require a lower O_2 supply. In general, high aeration rates appear to reduce the biomass growth (Cazzulino et al. 1991).

The requirements for O_2 may vary from one species to another, and must be supplied continuously to provide adequate aeration, since it affects metabolic activity and energy supply as well as anaerobic conditions. The level of O_2 in liquid cultures in bioreactors can be regulated by agitation or stirring and through aeration, gas flow, and air bubble size. The use of a porous irrigation tube as a sparger generated fine bubbles, high k_La values, low mechanical stress and provided a high growth rate (Takayama and Akita 1998). Growth of poinsettia cell suspension in bioreactors was inhibited when the level of O_2 dropped below 10%. When the level of O_2 was elevated to 80%, cell number increased to 4.9 $\times$ 10^5/ml as compared to 3.1 $\times$ 10^5/ml at 40% O_2 (Preil et al. 1988). Somatic embryo development in alfalfa and poinsettia suspension cultures was enhanced at 78% and 60% O_2 levels, respectively (Stuart et al. 1987; Preil 1991). High aeration rates were found to inhibit cell growth in cell suspensions cultured in airlift bioreactors. This result was explained to be due to an effect of “stripping” of the volatiles produced by the plant cells, which are apparently necessary for cell growth (Smart

and Fowler 1984). Increasing O_2 levels from 21% to 80%, in bioreactor cultures of Boston fern clusters, enhanced growth values (final FW – initial FW/initial FW) from 0.61 to 0.92. Reducing O_2 levels to 10% (v/v) affected cell differentiation in bioreactor cultures of carrot embryogenic tissue. Under these conditions embryo production was severely inhibited (Jay et al. 1992). The best production of somatic embryos from embryogenic cultures of *Eschcholtzia californica* and carrot in a helical ribbon impeller bioreactor was achieved under 20% oxygen concentration. When O_2 was low (5–10%), it inhibited biomass and somatic embryo production, while high O_2 (60%) favored undifferentiated biomass production (Archambault et al. 1994, 1995). Additional studies are required to provide information on optimal dissolved O_2 requirements in large-scale liquid cultures.

2. CO_2 Effects. The effects of CO_2 were reported mainly for plants in agar-gelled cultures or for cell suspension cultures used for secondary metabolite production (Scragg 1992; Buddendorf-Joosten and Woltering 1994). The reports on the effects of CO_2 enrichment in sugar-free agar-gelled cultures suggest beneficial promotion of plant growth during plant acclimatization and transplanting ex vivo (Kozai et al. 1992). The contribution of CO_2 supply during the proliferation and multiplication stage in media supplied with sucrose in bioreactors is debatable. It is implicit that if photoautotrophic conditions do not prevail, CO_2 enrichment beyond the 0.36% in the air supply is unnecessary. There are reports that high aeration rates rather than excessive oxygen inhibit growth and that reduced growth could be due to depletion of CO_2 or to the removal of various culture volatiles including CO_2 (Hegarty et al. 1986; Kim et al. 1991). The requirement for CO_2 was not related to photosynthesis but to some other metabolic pathways involved in amino acid biosynthesis. In poinsettia, the growth of embryos in Erlenmeyer flasks was considerably higher than in bioreactors and it was suggested that the growth difference was related to the differences in the gaseous atmosphere in the headspace (Preil 1991). CO_2 enrichment in an illuminated bioreactor culture of *Brodiaea* clusters did not affect biomass growth. An increase from 0.3% to 1% gave a similar growth value under the two CO_2 levels and supply of 135 $\mu mols \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon-flux (Ilan et al. 1995). In *Cyclamen persicum* Mill, high CO_2 levels correlated with increased production of pro-embryogenic masses (Hvoslef-Eide and Munster 1998).

3. Ethylene. Ethylene level in the headspace in liquid cultures in flasks differs from that in continuously aerated bioreactor cultures. Most of the

reports on ethylene relate to agar-gelled or cell suspension cultures (Buddendorf-Joosten and Woltering 1994). In various plant species cultured in bioreactors, the rate of aeration will affect the level of ethylene. High rates of aeration, which are often required at high biomass densities, can cause "stripping" of volatiles that are apparently important for some plants grown in culture. In clusters of *Brodiaea* cultured in liquid medium, ethylene had no effect on growth, although its level was reduced in the presence of silver thiosulfate (an inhibitor of ethylene action). The level of ethylene was reduced from 0.38 to 0.12 ml/L in highly aerated bioreactor cultures without affecting biomass growth, which was similar under both levels (Ilan et al. 1995).

B. Mineral Nutrients Consumption

Revised MS medium (Murashige and Skoog 1962) or media with various partial modifications in the inorganic and organic constituents of MS are used for most plant species in agar-gelled or liquid cultures in vitro. The availability of mineral nutrients depends on the type of culture, whether agar-gelled or liquid, the type and size of the plant biomass, and the physical properties of the culture. Factors such as pH, temperature, light, aeration, the concentration of minerals, the medium volume, and the viscosity of the medium will determine the rate of absorption of the various nutritional constituents (Williams 1992; Debergh et al. 1994). Plant cells growing in liquid cultures are better exposed to the medium components and the uptake and consumption are faster.

Agar-gelled and liquid shaken cultures often dehydrate by water evaporation to the headspace and out of the vessel and such a water loss concentrates the medium. The content of a certain component in the medium is a product of its concentration and the medium volume. The effects of the medium volume and the initial strength on potato were discussed by Kozai et al. (1995). In bioreactors, in which either humidified air or condensers are used to prevent dehydration, the level of the nutrients in the medium is affected mainly by the absorption rate and by cell lysis (Archambault et al. 1994). Differentiation and proliferation of micropropagated fern, gladiolus, and *Nerine* nodular clusters in bioreactors was better on half-strength than on full-strength MS minerals (M. Ziv, unpubl.). This was also true for *Lilium* bulblets differentiating on bulb scales that were cultured in bioreactors (Takayama 1991). A drop in pH to 4.5 and lower values and the subsequent increase to pH 5.5 was attributed to the initial utilization of ammonium and to the uptake at a later stage of nitrate. In several species the depletion of NH_4^+ is the first limiting factor of biomass growth and somatic embryos development.

Increasing the concentration of NH_4^+ to 15 mM resulted in maximum somatic embryo production in carrot cultures in a helical ribbon impeller bioreactor (Archambault et al. 1995).

A thorough and detailed study of nutrient uptake in *Eschcholtzia californica* embryonic cultures was achieved in a helical ribbon impeller bioreactor. Following a lag phase of about 140 h, first NH_4^+ and then NO_3^-, PO_4^-, K^+ and SO_4^- uptake was observed to coincide with biomass increase and somatic embryo production. After 300 h, PO_4^- was exhausted, while after 600 h NH_4^+ was depleted and Mg^{++} and Ca^{++} composition continued to deplete after about 800 h. The K^+ and Na^+ ions were not depleted at that stage (Archambault et al. 1994). The composition of minerals, monitored during the culture of *Begonia* and *Brodiaea* in liquid media changed, with phosphate, ammonia, nitrate, and potassium depleting faster and prior to the depletion of Ca^{++} and Mg^{++} (Tormala et al. 1987; Ilan et al. 1995). In somatic embryos of spruce cultured in bioreactors, 80% of the ammonium was consumed by the growing biomass (Tautorus et al. 1994). In general, biomass growth is limited by the availability of phosphate, nitrogen, and carbohydrates and to a lesser extent by the availability of calcium, magnesium, and other ions.

C. Carbohydrate Supply and Utilization

Cultured plants require a constant supply of carbohydrates as their source of energy. Sucrose and to a lesser extent glucose, fructose, or sorbitol are the most commonly used carbohydrates in vitro. In general, sucrose is removed rather rapidly from the medium and after 10–15 days the sucrose can be completely depleted or reduced to 5–10 g/L from an initial level of 30 g/L in both agar-gelled and liquid cultures. At the same time, glucose and fructose that appear in the medium due to sucrose hydrolysis increase in the presence of invertase in the culture medium, and can reach levels of 5–10 g/L. Cell suspensions of *Catharanthus roseus* cultured in a column airlift bioreactor showed a lag phase of 5 days, during which there was a total hydrolysis of the sucrose to glucose and fructose (Smart and Fowler 1984). In suspension cultures of alfalfa, sucrose also was hydrolyzed during the first 5 days. Most of the sugars uptake occurs after day 5 and glucose is taken up preferentially over fructose (McDonald and Jackman 1989). A higher yield of alfalfa embryos was obtained when 30 g/L maltose combined with NH_4^+ was used instead of 30 g/L sucrose that was used in combination with various nitrogen sources (Stuart et al. 1987). In embryonic suspension cultures of celery, the addition of mannitol reduced cell lysis and enhanced somatic embryogenesis. When 40 g/L mannitol was added, a

higher number of embryos was produced and the frequency of singulated normal embryos was increased (Nadel et al. 1989). In embryogenic cultures of *Eschcholtzia californica* grown in a helical ribbon impeller bioreactor, sucrose uptake started after 100 h and the sucrose was depleted after 600–800 h in culture (Archambault et al. 1994).

In *Picea* species cultured in shaken flasks in a medium with 30 mM glucose, the carbohydrates depleted after 10–14 days (Lulsdorf et al. 1993). In mechanically stirred bioreactors, a level of 60 mM sucrose resulted in the highest cell biomass and somatic embryo number. The effects of various carbohydrates on the growth of spruce somatic embryos revealed that the response was species dependent (Tautorus et al. 1994).

The biomass of fern meristematic clusters in a bubble column bioreactor was increased with the increase in sucrose concentrations from 7.5 g/L to 30 g/L, while higher levels caused a decrease in cluster growth. Elevated sucrose concentrations caused a decrease also in the chlorophyll content of the clusters and leaves (Ziv and Hadar 1991).

Increasing sucrose concentrations from 30 g/L to 60 g/L in bioreactor cultured gladiolus clusters decreased the biomass FW by more than 50%. When 30 g/L or 60 g/L were combined with paclobutrazol, a further decrease in FW was observed. On the other hand, 60 g/L sucrose induced a higher DW increment, which could be attributed to an osmotic effect and the subsequent water status of the clusters. Gladiolus clusters cultured in the presence of growth retardants had a higher level of starch, 845 as compared to 585 mg/g DW in the control (Ziv 1992b). A higher number of bulblets were produced in bioreactor-cultured bulb scales of *Lilium* at 30 g/L than at higher sucrose levels. However, larger size microbulbs were produced at 90 g/L than at 30 g/L sucrose in the medium (Takayama 1991).

D. pH Effects

The initial pH in most plant cell cultures ranges between 5.5–5.9. Since most media are not buffered, changes during autoclaving and during the biomass growth in culture occurs. A rapid drop in pH to 4.0–4.5 took place within 24–48 h in cell suspension, organ, and embryogenic cultures (Stuart et al. 1987; Preil 1991; Ziv and Hadar 1991; Lulsdorf et al. 1993; Jay et al. 1994). These changes were related to an initial ammonium uptake and acidification due to cell lysis. However, the pH increased after a few days and reached a stable level around pH 5.0–5.5, which was related to the uptake of nitrates. In spruce species cultured in liquid medium, the pH levels were shown to increase to 6.5–6.8 after 14 days in culture (Tautorus and Dunstan 1995).

In embryogenic cultures of poinsettia, various initial pH levels stabilized after 20 days in batch bioreactor cultures, between pH 5.3–5.7. Oxygen levels affected pH changes by 0.2 pH units (Preil 1991). In fern cultured in an airlift bubble column bioreactor, the pH dropped to 4.2 after 24 h and subsequently increased to pH 5.4. Keeping a constant pH by titration with KOH did not affect the growth response (Ziv and Hadar 1991). In alfalfa, the development of somatic embryos was affected by the pH. A higher rate of embryo production was observed at a constant pH of 5.5 than at a non-buffered medium or at lower pH levels (Stuart et al. 1987).

Carrot cell differentiation in a controlled bioreactor was affected by the pH; the highest rate of embryo production was observed at a pH of 4.3. However, embryo development was arrested before the embryos reached the torpedo stage and continued only at pH 5.8. The changes in carrot embryo development were associated with sugar uptake and ammonium depletion and can be attributed to enzyme and metabolic activity at an optimal pH (Jay et al. 1994). It appears that pH requirements are species and developmental stage dependent.

E. Growth Regulator Effects

The use of growth regulators in liquid cultures can be more effective in controlling the proliferation and regeneration potential than in agar-gelled medium due to the direct contact of plant cells and aggregates with the medium. The limited information available on regulators seems to indicate that similar levels of growth regulators were used in both agar-gelled and liquid cultures even though availability appears to be better in liquid medium.

Somatic embryogenesis in many species was first induced in an auxin-containing medium that promoted rapid cell division. Expression of the embryogenic potential was achieved in auxin-free media, or with low levels of auxin in the medium (Halperin 1967; Steward et al. 1970). Further embryo conversion and maturation into plants was promoted by the addition of abscisic acid (ABA), which was found to control abnormal embryo and plant growth (Ammirato and Styer 1985).

Bulblets development from scales in *Lilium* cultured in bioreactors was higher in the presence of BA than kinetin. However, high BA had an inhibiting effect on further bulblet growth (Takayama et al. 1991). High kinetin also stimulated bulblets differentiation, but inhibited further growth of the bulblets. However, the addition of 0.1 mg/L naphthalenacetic acid (NAA) was found to further enhance kinetin activity, which was, however, inhibited again in the presence of high (90 g/L) sucrose levels (Takayama 1991; Takayama et al. 1991).

In embryogenic cultures of *Nerine*, auxin and cytokinins were used to induce proembryogenic clusters. Embryogenic expression was achieved, however, only after a short exposure to 2-iso-pentenyladenine (2iP) and further subculture in a growth regulator-free medium (Lilien-Kipnis et al. 1992, 1994).

Since one of the major problems in liquid-cultured plants is malformation of shoots, hyperhydricity, the induction of meristematic or bud clusters with arrested leaf growth (McCown et al. 1988; Ziv 1991b; Ziv and Shemesh 1996) was one of the solutions to hyperhydricity. The use of relatively high cytokinin levels and growth retardants which inhibit gibberellin biosynthesis was the most effective method to reduce shoot and leaf growth and promote the formation of meristematic clusters (Ziv 1990a,b).

The information on abscisic acid is limited and was found effectual mainly in the later stages of somatic embryo development, promoting normal embryo growth and maturation in carrot and alfalfa embryogenic cultures (Ammirato and Styer 1985; Denchev et al. 1990). Growth of callus and leaf development in bulblets regenerated on bulb scales of *Lilium* in bioreactor cultures was inhibited by abscisic acid, thus providing singulated propagules for easier handling and storage (Takayama et al. 1991). Ethylene effects appear to be species dependent (see IVA3). Information on the effects of growth regulators in bioreactor cultures is inadequate and further research is needed to optimize culture conditions.

F. Temperature Effects

The control of the temperature in the liquid medium inside the bioreactor can be easily manipulated by a heating element in the vessel or by circulating water in an enveloping jacket outside the vessel. There is, however, limited information on the effects of temperature in bioreactor cultures, which is usually kept constant at 25°C, with small day and night fluctuations. Temperature effects on potato tuber formation in an airlift bioreactor were studied by Akita and Takayama (1994). A higher number and larger-size tubers developed at 25°C than at 17°C; the lower temperature caused a decrease in tuber size. Ziv and Shemesh (1996), working with potato internode explants in liquid cultures, found that tuber formation was best at a 16 h photoperiod and 18/15°C day and night temperature. When bulblets of *Nerine* were cultured in liquid medium, the FW increase was higher at 25°C than at 17°C. However, when subcultured from liquid to an agar-gelled bulb induction medium, rooting was better at 17°C (J. Vishinevetzky, M. Ziv unpublished).

V. CELL AND AGGREGATE DENSITY, FOAMING AND MEDIUM RHEOLOGY IN BIOREACTORS

Growth and proliferation of the biomass in bioreactors depends on airflow supply for the aeration and mixing, and for the prevention of the plant biomass sedimentation. In many plants cultivated in bioreactors, continuous aeration, mixing, and circulation cause shearing damage, cell wall breakdown, and accumulation of cell debris, which is made up mainly of polysaccharides. Cell debris accumulation results in foaming, adhesion of cells and aggregates to the culture vessel walls, and the development of a "crust" at the upper part of the bioreactor vessel. This layer prevents adequate circulation, causing additional cell debris formation and a demand for higher aeration rates that intensify the clogging problem (Scragg 1992). As the biomass increases and the cultures become viscous, higher rates of aeration are required to allow for oxygen supply and circulation. Medium viscosity and foaming were reduced by the use of half the concentration of MS minerals (Ziv 1992a) and by lowering the level of calcium in the medium (Takayama et al. 1991). In bioreactor cultures in which the plant biomass used was of an aggregate or cluster nature, the foaming and viscosity were not as severe as that observed in cell suspension cultures (Ziv 1991b). Surprisingly, in cluster cultures in bioreactors, even when the mixing was poor and the clusters sedimented, biomass growth was observed, suggesting that perhaps the continuous circulation accompanied by aeration was secondary in importance to oxygen supply (Ziv 1995b).

Shearing stress and cell wall damage were greatly reduced in a Vibramix bubble-free oxygenated bioreactor, in which silicone tubing was used for air supply (Preil 1991; Luttman et al. 1994). Embryogenic cells cultured in a helical ribbon impeller bioreactor produced poor-quality embryos when the mixing speed was increased from 60 to 100 rpm. This was attributed to shearing stress and cell damage (Archambault et al. 1994).

The introduction of polyethylene glycol 6000 changed the rheology of the medium in alfalfa liquid cultures and improved somatic embryo development beyond the globular stage while it was arrested in a less viscous medium (Denchev et al. 1990).

Disposable plastic presterilized bioreactors (Osmotek 'LifeReactor'™, Fig. 1.3B), with a volume capacity of 2 and 5 L, were used for the micropropagation of various plant species through bud or meristematic clusters. The plastic bioreactors were found to provide good circulation with reduced shearing damage and foaming (Ziv et al. 1998). The problems of cell damage, foaming, and culture viscosity can be better controlled by

developing bioreactors with an optimal shape suitable for micropropagation through meristematic or bud clusters. Clusters that are compact and consist mainly of small meristematic cells are apparently less shear sensitive than highly vacuolated cells in parenchymatous cell suspension cultures.

VI. SUMMARY AND CONCLUSIONS

The application of liquid cultures for micropropagation in bioreactors using the organogenic or the embryogenic pathway is becoming a more efficient alternative system for scale-up and automation in vitro (Aitken-Christie et al. 1995). The successful exploitation of bioreactors as a commercial micropropagation system will depend on careful studies of plant morphogenesis in liquid media and the understanding of the control mechanisms of organ and embryo development from meristematic or bud clusters. The chemical and physical environment, in relation to biomass growth and controlled regeneration, should be further investigated. The level of carbohydrates and specifically the levels and ratios of growth-promoting and -retarding regulators will need to be further studied in more detail.

A major aspect which will have to be addressed is the problem of contamination in large-scale liquid cultures, which can cause severe losses (Leifert and Waites 1992). Attempts to control contamination in liquid cultures of foliage plants were reported by Levin et al. (1997a), who used continuous filtration systems to control bacterial growth in the medium.

Harvest and quality assessment of bioreactor propagules is a major aspect in automation and was addressed by computerized image analysis (machine vision) to classify organogenic and embryogenic plant material (Harell et al. 1994; Kurata 1995). These are factors of major importance to the success of scaled-up automated *in vitro* propagation.

In addition, the understanding of the effects of aeration, mixing, consumption, and depletion of the various components present in the medium will provide information for establishing semi-continuous or continuous culture systems and thus provide optimal conditions for biomass growth, differentiation, and eventual production of quality plants.

Implementation of scale-up and mechanization are mandatory for the expansion of commercial micropropagation. New technologies in robotics and machine vision systems for cutting, sorting, and dispensing have been established (Aitkin-Christie et al. 1995). However, these technologies have actually increased the cost of plant micropropagation.

Bioreactor cultures are being established in several commercial laboratories for ferns, spathiphylum, philodendron, banana, potato, lilies, poinsettia, sugar-cane, and some forest tree species such as eucalyptus, poplar, and early stages of conifer somatic embryos. The technique was estimated to render a 35% reduction in propagule unit costs when propagated through the organogenic pathway (R. Levin, pers. commun.). Using somatic embryogenesis as the propagation pathway in a semi-automated system (Cervelli and Senaratna 1995) reduced production costs by 24%. In conclusion, simple bioreactors combined with mechanized cutting, sorting, and delivery systems are the solution for low-cost micropropagation.

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