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INVITED REVIEW:

SECONDARY METABOLISM OF HAIRY ROOT CULTURES IN BIOREACTORS

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Summary

In vitro cultures are being considered as an alternative to agricultural processes for producing valuable secondary metabolites. Most efforts that use differentiated cultures instead of cell suspension cultures have focused on transformed (hairy) roots. Bioreactors used to culture hairy roots can be roughly divided into three types: liquid-phase, gas-phase, or hybrid reactors that are a combination of both. The growth and productivity of hairy root cultures are reviewed with an emphasis on successful bioreactors and important culture considerations. The latter include strain selection, production of product in relation to growth phase, media composition, the gas regime, use of elicitors, the role of light, and apparent product loss. Together with genetic engineering and process optimization, proper reactor design plays a key role in the development of successful large-scale production of secondary metabolites from plant cultures.

Key words: mist, aeroponics; bioreactor; hairy roots; light; artemisinin; productivity.

Introduction

Plant-derived chemicals are valuable sources for a variety of pharmaceuticals, flavors, dyes, oils, and resins (Balandrin et al., 1985; Parr, 1988). Many of these commercially valuable phytochemicals are secondary metabolites that are not essential to plant growth, are produced in small amounts, and often accumulate in specialized tissues, e.g. trichomes. These compounds usually have very complicated structures and/or exhibit chirality. Consequently, in many cases organic synthesis is not cost effective, and extraction from field-grown plants has been the major method used to economically produce these important secondary metabolites (Balandrin et al., 1985; DiCosmo and Misawa, 1995). Depending on the plant species, traditional agricultural methods often require months to years to obtain a crop (Kieran et al., 1997). Furthermore, the levels of secondary metabolites are affected by many factors, including pathogens and climate changes.

Plant cell suspension culture has been considered an alternative to agricultural processes for producing valuable secondary metabolites. Although there are some commercialized, industrial-scale plant cell cultures (Tabata et al., 1982; Curtin, 1983; Furuya et al., 1984; Kieran et al., 1997), the biggest challenge of producing secondary metabolites from plant cell suspension cultures is that secondary metabolites are usually produced by specialized cells and/or at distinct developmental stages (Balandrin et al., 1985). Some compounds are not synthesized if the cells remain undifferentiated (Berlin et al., 1985). Therefore, undifferentiated plant cell cultures often lose, partially or totally, their biosynthetic ability to accumulate secondary products (Rokem and Goldberg, 1985; Charlwood and Charlwood, 1991). Although there are some reports of co-cultured differentiated tissues (e.g. shoots+roots) being used to produce secondary metabolites (Subroto et al., 1996; Mahagamasekera and Doran, 1998), most efforts focus on transformed (hairy) roots and that is the focus here.

Hairy roots, the result of genetic transformation by Agrobacterium rhizogenes, have attractive properties for secondary metabolite production. They often grow as fast as or faster than plant cell cultures (Charlwood and Charlwood, 1991; Flores et al., 1999) and do not require hormones in the medium. The greatest advantage of hairy roots is that hairy root cultures often exhibit about the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Banerjee et al., 1998; Kittipongpatana et al., 1998). Many valuable secondary metabolites are synthesized in roots in vivo, and often synthesis is linked to root differentiation (Robins et al., 1991; Flores et al., 1999). Even in cases where secondary metabolites accumulate only in the aerial part of an intact plant, hairy root cultures have been shown to accumulate the metabolites. For example, lawsone normally accumulates only in the aerial part of the plant, but hairy roots of Lawsonia inermis grown in half- or full-strength MS medium (Murashige and Skoog, 1962) can produce lawsone under dark conditions (Bakkali et al., 1997). Although artemisinin was thought to accumulate only in the aerial part of Artemisia annua plant (Wallaart et al., 1999), several laboratories have shown that hairy roots can produce artemisinin (Weathers et al., 1994; Jaziri et al., 1995; Liu et al., 1999). Genetic stability is another characteristic of hairy roots. For example, cultures of Hyoscyamus muticus hairy roots

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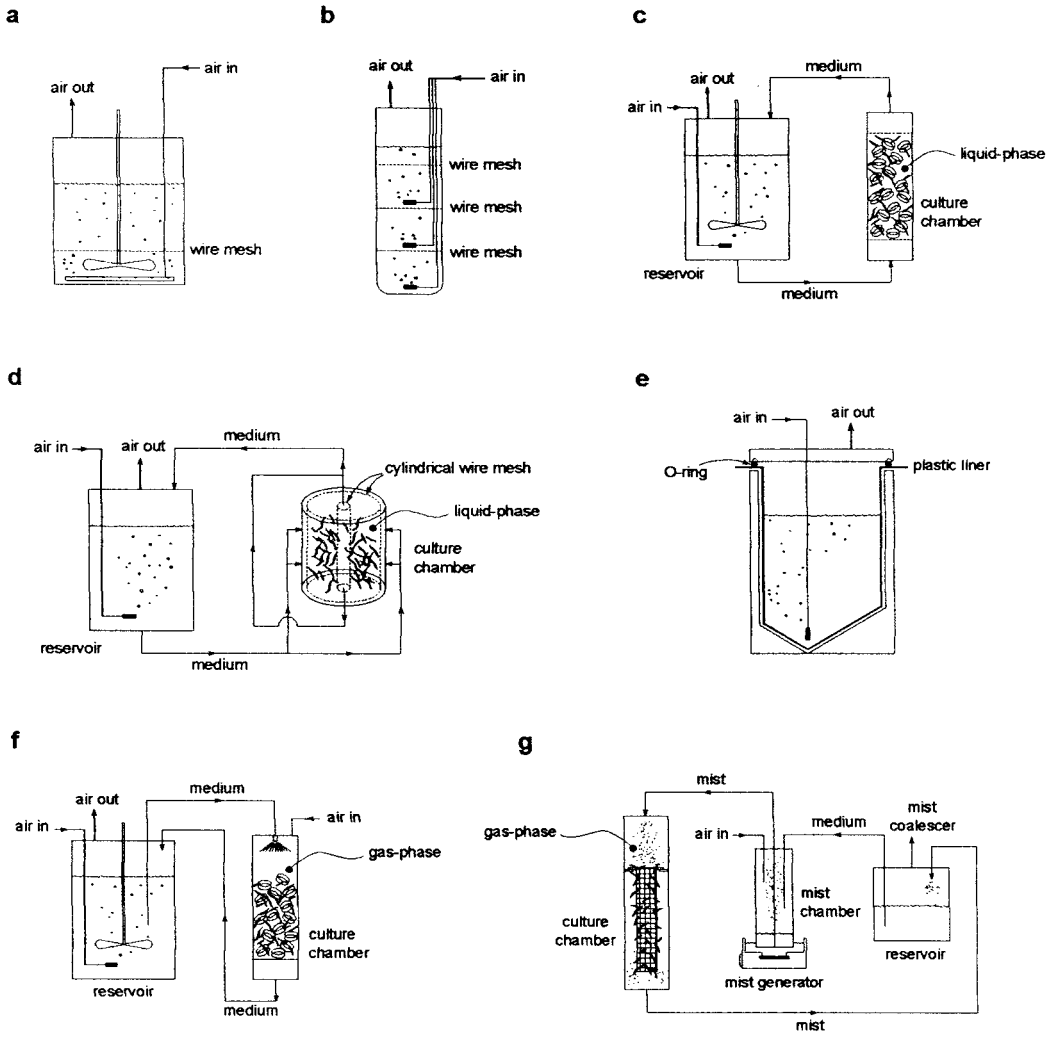

FIG. 1. Some promising bioreactor designs used for hairy root culture. a, Isolated impeller stirred tank reactor; b, segmented bubble column reactor; c, submerged convective flow reactor; d, radial flow reactor; e, plastic-lined vessel; f, trickle bed reactor; g, nutrient mist reactor.

showed equal or higher levels of hyoscyamine synthesis compared to the roots of a whole plant (Flores and Filner, 1985) and have maintained the same biosynthetic capacity for more than 15 years (Flores et al., 1999) even after cultures are shifted back and forth between the more productive differentiated (roots) and the less productive undifferentiated (cells) state (Flores, 1987). Indeed, Flores et al. (1999) emphasized that hairy roots provide a good experimental system for studying root-specific pathways. However, due to their special morphological characteristics, the difficulty in culturing hairy roots in bioreactors and the need for developing bioreactors suitable for their culture have long been recognized (Wilson et al., 1987).

Two recent reviews on hairy roots by Shanks and Morgan (1999) and Giri and Narasu (2000) briefly discussed the importance of the use of bioreactors for hairy root cultures along with other topics, including metabolic engineering and bioremediation. An earlier review by Wysokinska and Chmiel (1997) focused more in depth on cultivation methods and reactor systems suitable for hairy roots. Here, we provide an update that adds some new perspectives on the prospects and challenges of producing secondary metabolites from hairy roots in bioreactors.

Bioreactors for Hairy Root Cultures

Based on the continuous phase, reactors used to culture hairy roots can be roughly divided into three types: liquid-phase, gas-phase, or hybrid reactors that are a combination of both. Simple schematics of these reactors are depicted in Fig. 1 and a summary of some reported productivities is given in Table 1.

Liquid-phase reactors. In liquid-phase reactors, roots are submerged in the medium, and the term 'submerged reactors' is also used. Stirred tank (Wilson et al., 1987; Taya et al., 1989; Buitelaar et al., 1991), bubble column (Tescione et al., 1997; Buitelaar et al., 1991; Rodriguez-Mendiola et al., 1991), air-lift (Taya et al., 1989), liquid-impelled loop (Buitelaar et al., 1991), and submerged convective flow (Carvalho and Curtis, 1998) reactors

TABLE 1
COMPARATIVE GROWTH AND SECONDARY METABOLITE PRODUCTION IN BIOREACTORS

Reactor type	Working volume (liter)	Root culture	Culture period (d)	Inoculum	Final density (g DW l^{-1})	Biomass productivity (g DW l^{-1} d^{-1})	Secondary metabolite productivity[a]	Reference
Isolated impeller – batch	12	Datura stramonium	35	n.a.	8.4	0.24	Hyoscyamine, 66.5 mg l^{-1}	Hilton and Rhodes (1990)
Isolated impeller – continuous	12	Datura stramonium	33	n.a.	20.4	0.62	Hyoscyamine, 211.2 mg l^{-1}	Hilton and Rhodes (1990)
Isolated impeller – batch	25	Atropa belladonna	30	60 g FW l^{-1}	60.2	1.81	Atropine, 325.1 mg l^{-1}	Lee et al. (1999)
Segmented bubble column	2.5	Atropa belladonna	43	0.06 g DW l^{-1}	9.9	0.23	Atropine, 14 mg l^{-1}	Kwok and Doran (1995)
Bubble column	2	Hyoscyamus muticus	30	0.16 g DW l^{-1}	14.3	0.47	n.a.	Carvalho and Curtis (1998)
Bubble column	1.5	Artemisia annua	38	0.34 g DW l^{-1}	15.3	0.39	Artemisinin, 0.025 mg l^{-1}	Kim et al. (2001)
Bubble column→convective flow	2	Hyoscyamus muticus	11 + 19 = 30	0.16 g DW l^{-1}	25.3	0.83	n.a.	Carvalho and Curtis (1998)
Radial flow	0.39	Beta vulgaris	11.7	77 g FW l^{-1}	51	3.73	n.a.	Kino-Oka et al. (1999)
Submerged→droplet phase	500	Datura stramonium	21 + 40 = 61	n.a.	8	0.13	n.a.	Wilson (1997)
Bubble column→trickle bed	14	Hyoscyamus muticus	15 + 10 = 25	0.16 g DW l^{-1}	20	0.79	n.a.	Ramakrishnan et al. (1994)
Bubble column→nutrient mist	1.5	Artemisia annua	21 + 17 = 38	0.33 g DW l^{-1}	14.4	0.37	Artemisinin, 0.031 mg l^{-1}	Kim et al. (2001)
Modified inner loop nutrient mist	2.3	Artemisia annua	25	0.2 g DW l^{-1}[b]	13.6[b]	0.53	n.a.	Liu et al. (1999)
Erlenmeyer flask	1	Artemisia annua	10	10 g FW l^{-1}	13.4	1.24	Artemisinin, 0.088 mg l^{-1}	Xie et al. (2000)

When inoculum level was not available, the biomass productivity was calculated from the final biomass. When fresh weight inoculum level was available, initial dry weight was considered as 10% of the fresh weight.
DW, dry weight; FW, fresh weight; n.a., not available.
[a] Secondary metabolite level was at harvest.
[b] Calculated based on the medium volume.

belong to this category. In early studies of stirred tank reactors, the impeller damaged the roots, resulting in callus formation and ultimately poor biomass production (Wilson et al., 1987; Hilton et al., 1988). The problem was solved by using a steel cage or mesh to isolate roots from the impeller (Fig. 1a) (Kondo et al., 1989; Hilton and Rhodes, 1990). Instead of mechanical agitation, bubble column reactors, air-lift, and liquid-impelled loop reactors sparge gas through the reactor to both mix and supply oxygen to the roots.

In liquid-phase culture environments, including shake flasks, it has been shown many times that oxygen deficiency due to mass transport limitation is a growth-limiting factor (Yu and Doran, 1994; Yu et al., 1997). Kanokwaree and Doran (1997) demonstrated oxygen limitations in shake flasks using different medium volumes. The problem increases as the scale of the reactor increases. In addition, sparged air bubbles can be captured, enlarged, and remain entrapped in root clumps, resulting in gas flow channeling around clumps and total localized depletion of oxygen (Flores and Curtis, 1992; Singh and Curtis, 1994). It has been suggested that liquid–solid oxygen transfer rather than gas–liquid oxygen transfer is the limiting step (Kino-Oka et al., 1996; Tescione et al., 1997; Kanokwaree and Doran, 1998). Direct evidence for oxygen deficiency in shake flasks and bubble column reactors has been found by Weathers et al. (1999), who measured mRNA transcripts of alcohol dehydrogenase, an indicator of oxygen deprivation. They showed that in both liquid systems, oxygen was limiting.

Methods for improving the supply of oxygen to the roots in reactors have been proposed. Kwok and Doran (1995) introduced gas at multiple points into a bubble column reactor that was divided into three segments with wire mesh, with each segment containing a sparger (Fig. 1b). Although 9.9 g dry weight per liter (g DW l^{-1}) of Atropa belladonna hairy roots were harvested after 43 d, bulk mixing was poor even as early as day 14. Kanokwaree and Doran (1998) later investigated the use of a microporous polypropylene membrane tubing that was inserted into a gas-driven reactor along with a sparger to provide a supplementary oxygen to the root bed. Keeping the total air flow rate constant and using the sparger and membrane tubing at a flow rate ratio of 1:2 resulted in 32% more biomass yield compared to the use of the sparger alone. Although these results showed that site-directed aeration in root clumps can reduce the oxygen transport limitation, the sparger cannot be eliminated, because it promotes bulk mixing.

A convective flow reactor developed by Carvalho and Curtis (1998) (Fig. 1c) consisted of a stirred tank, a peristaltic pump, and a tubular culture chamber. In the stirred tank the medium was oxygenated, and a positive displacement pump recirculated the liquid between the stirred tank and the tubular reactor. After 30 d of culture, 550 g fresh weight per liter (FW l^{-1}) (23 g DW l^{-1}) of Hyoscyamus muticus hairy roots were harvested; this is more than a 79% increase in biomass compared to bubble column reactors. Although the convective flow reactor showed improved performance compared to a bubble column, it may not be a realistic large-scale system due to the pressure required to circulate the culture medium at a high enough velocity to overcome the flow resistance of the root bed (Carvalho and Curtis, 1998). Reactors similar to the convective flow reactor, however, are effective research tools. For example, Williams and Doran (1999) used a packed bed recirculation reactor to minimize the liquid–solid hydrodynamic boundary layer at the root surface and determine the critical oxygen level of hairy roots of Atropa belladonna. Kino-Oka et al. (1999) cultured red beet hairy

roots in a single column reactor with superficial velocities ranging from 2 to 28 m h^{-1}. Although the increased superficial velocity enhanced the root elongation rate, the viability of root tips was reduced due to damage from shear stress. Kino-Oka et al. (1999) found that under the conditions used in their study, a superficial velocity of 15 m h^{-1} resulted in optimum growth. Based on the specific cross-sectional area (a ratio of cross-sectional area to volume in the growth chamber) a radial flow reactor was subsequently constructed (Fig. 1d). In this reactor the air-saturated medium entered through the ports on the sidewall of the reactor and exited through the ports at the center of the top and bottom plates. The radial flow reactor was operated at 15 m h^{-1} of superficial velocity (the value of liquid flow rate per an arithmetic average of lateral areas of the inner and outer stainless-steel mesh), and after 12 d, 51 g DW l^{-1} of roots were harvested, a substantial increase in biomass compared to the results obtained by Carvalho and Curtis (1998).

Recently, a plastic-lined vessel (Fig. 1e) was used for plant cell suspension cultures (Hsiao et al., 1999). It has been reported that the same reactor is also being tested with hairy root cultures (Shanks and Morgan, 1999). Plastic bags may be the future for low-cost bioreactors for hairy root cultures.

Rotating drum (Kondo et al., 1989) and ebb-and-flow reactors (Taya et al., 1989; McKelvey et al., 1993) alternate cycles of liquid- and gas-phase. An initial problem observed with the rotating drum reactor was that in the early stage of culture roots did not attach well to the vessel wall. Consequently, as the roots were rotated above the culture medium, they detached from the wall and were damaged, resulting in low biomass accumulation. This problem was overcome by immobilizing the roots on polyurethane foam (Kondo et al., 1989).

Gas-phase reactors. Trickle bed (Fig. 1f) (Taya et al., 1989; Flores and Curtis, 1992; McKelvey et al., 1993), droplet phase (Wilson, 1997), liquid-dispersed (Williams and Doran, 2000), and nutrient mist reactors (Fig. 1g) (DiIorio et al., 1992a, b; Whitney, 1992; Buer et al., 1996; Woo et al., 1996; Liu et al., 1999; Weathers et al., 1999) are gas-phase reactors. In gas-phase reactors roots are exposed to air or other gas mixtures. Nutrients (medium) are usually delivered to roots as droplets. However, there is considerable variation in the size of droplets. For mist reactors using ultrasonic transducers, the droplet sizes are usually micron scale (0.5–30 μm; Weathers et al., 1999). For trickle bed or other gas-phase reactors using spray nozzles, the size of the droplets may be much larger (Wilson, 1997). Since the continuous phase is gas, the roots must be immobilized in the reactor. DiIorio et al. (1992a), Liu et al. (1999), and Woo et al. (1996) used horizontal sheets of mesh, and at inoculation, roots were spread onto mesh. Alternatively, Williams and Doran (2000) used a vertical structure. Chatterjee et al. (1997) cultured roots on packing rings made of nylon mesh in a shake flask for a week so that roots became anchored to the mesh rings. The rings were then used as inoculum for the mist reactor. Intalox metal process packing elements have also been used to immobilize roots (Ramakrishnan et al., 1994).

As a root bed entraps air in liquid-phase reactors, the root bed in gas-phase reactors can entrain the liquid medium, and this leads both to liquid channeling and to hold-up. The liquid hold-up in the reactors can be divided into a dynamic part (free-draining) and a stagnant part (non-free-draining) (Ramakrishnan and Curtis, 1994). The latter is of primary interest because the stagnant hold-up is the volume of medium entrapped in the root bed and can be three to four times the root bed's own weight (Williams and Doran, 2000). The trapped liquid can have different nutrient levels than the bulk fluid (Williams and Doran, 2000) or make the roots within effectively submerged and depleted of oxygen (Singh and Curtis, 1994). This phenomenon is less likely to happen in mist reactors, because less medium enters the culture chamber compared to trickle bed reactors. The medium flow rate is 1.1 ml min^{-1} for a 1.5 liter mist reactor (Kim, 2001) compared to Williams and Doran's (2000) 130 ml min^{-1} for a 4.4 liter reactor. In mist reactors, the capability of mist to penetrate into a densely packed root bed (50% packing density) has been demonstrated (Wyslouzil et al., 1997), and in fact mist capture efficiency can be a limiting factor in achieving a high root density (Kim, 2001).

Hybrid reactors. The disadvantage of gas-phase reactors is that there is no way to uniformly distribute the roots in the growth chamber without manual loading. The solution proposed by Ramakrishnan et al. (1994) was to initially run the reactor as a bubble column in order to suspend, distribute, and attach roots to the packing rings in the reactor. After 2 wk growth, root clumps were dense, and the reactor was switched to a trickle bed operation, thus exposing roots to a gas environment. In the same study roots were also mechanically chopped in a blender and then pumped as a slurry into the reactor, eliminating the manual labor of cutting and inoculation. A 14 liter reactor was run using these procedures for 4 wk and about 20 g DW l^{-1} packing density of Hyoscyamus muticus was obtained throughout the reactor at harvest (Ramakrishnan et al., 1994).

Wilson (1997) cultured Datura stramonium hairy roots in a 500 liter hybrid reactor that used submerged culture for 21 d followed by a droplet-phase for 40 d. It is noteworthy that in this experiment the root inoculation step was completed mechanically and required no manual labor. In a seed vessel placed on the top of the 500 liter reactor, the roots were grown as inoculum for the large-scale reactor. A helical screw with a blade located in the bottom of the seed vessel was used to cut and transfer the roots to the reactor. The main reactor contained assemblies of wire chains and bars to immobilize the roots. The distance between each assembly line (the assembly ran from top to bottom) was determined by effective volume, i.e., the volume that can be filled by roots from a single inoculation point. This parameter depends on the species and must be determined empirically. At the end of a run a total of 39.8 kg fresh weight of hairy roots was harvested, yielding 79.6 g FW l^{-1} packing density. This is not the highest packing density obtained for hairy roots; however, it demonstrates that with appropriate design, large-scale hairy root culture is possible.

Recently the effect of root hairs has been studied by removing the hairs chemically (Bordonaro and Curtis, 2000) or by using root-hair mutants (Shiao and Doran, 2000). In liquid-phase reactors, root hairs increase the flow resistance and the thickness of the liquid–solid hydrodynamic boundary layer. As a result, root hairs lower the oxygen availability to the roots (Bordonaro and Curtis, 2000) and increase critical oxygen tension (Shiao and Doran, 2000). Ramakrishnan and Curtis (1994) showed that roots with fewer hairs entrained less liquid than roots with more hairs. Therefore, in trickle bed reactors profuse root hairs may hinder the liquid drainage (Williams and Doran, 1999). In contrast, aerosol models suggest that root hairs are beneficial for growth in the mist reactor because they enhance mist capture (Wyslouzil et al., 1997), and may improve reactor performance.

Secondary Metabolites

Plants produce secondary metabolites for specific purposes including defense against pathogens and predators, protection from UV light damage, attraction of pollinators, and for other reasons yet to be discovered. Synthesis of secondary metabolites in vivo is often triggered by environmental factors, and thus, it is not surprising that the production of secondary metabolites in hairy roots is also greatly affected by culture conditions. Most of the studies on manipulating secondary metabolite levels in hairy roots are performed in shake flasks, and rarely in bioreactors. In this section, we summarize some of the conditions that have been shown to affect and improve secondary metabolite production. As always, optimization must be empirically determined for each clone and each culture condition.

Strain selection. Selection of good hairy root lines is a prerequisite, and plants producing high levels of secondary metabolites usually generate high-producing cell lines. Different strains of Agrobacterium rhizogenes can also affect transformation. For example, Banerjee et al. (1998) showed that in contrast to LBA 9402, the A4 strain of Agrobacterium rhizogenes was more effective at inducing hairy roots from Valeriana wallichii DC, and had a higher growth rate. Both transformed lines produced more valepotriate than non-transformed roots. Although the LBA 9402-induced hairy root line produced more valepotriate than the A4-induced line, this is not necessarily the case for all transformed plant species.

Relationship to growth phase. To effectively produce secondary metabolites on a large scale, it is important to understand when in the growth phase a specific product is formed. This information can then be used to develop an effective production strategy. For example, if a product is mainly formed during exponential growth, one might try to use a fed-batch culture strategy aimed at keeping the culture actively growing, thereby maximizing production of the secondary metabolite.

Production of secondary metabolites in vitro can occur at almost any stage of culture growth. For example, nicotine concentration in hairy roots of Nicotiana rustica grown in shake flasks remains constant throughout the growth phase, and production is, therefore, growth-related (Wilson et al., 1987). The production of tabersonine and ajmalicine by Catharanthus roseus hairy root cultures grown in the dark is also at maximum during the exponential phase (Bhadra and Shanks, 1997). Tabersonine derivatives, lochnericine and hörhammericine, are also growth-associated and show linear relationships between the total yields (not specific yields) and biomass production (Bhadra et al., 1998). On the other hand, the ajmalicine derivative, serpentine, was not growth-associated and exhibited the highest specific yield during the stationary phase (Bhadra and Shanks, 1997). In another example, specific production of hyoscyamine in Datura stramonium hairy roots also increased significantly during the stationary phase (Wilson et al., 1987). In contrast, the specific solasodine content in Solanum aviculare hairy root cultures showed a linear relationship with the growth index (biomass increase divided by initial biomass; Kittipongpatana et al., 1998), and since the inoculum amount was about the same in all shake flasks, the growth index can be directly translated as biomass increase.

Medium composition. Nutrients in the culture medium can be easily manipulated and have a great impact on secondary metabolite production. For example, ammonium ion altered the secretion pattern of products from hairy root cultures of Lithospermum erythrorhizon. In ammonium-free MS medium, L. erythrorhizon normally accumulates shikonin on the surface of roots and in border cells. When ammonium ion is present, however, hydroxyechinifurn B (Fukui et al., 1998) and the potent antifungal agent, rhizonone (Fukui et al., 1999) were secreted into the medium instead. Although an investigation of the natural existence of rhizonone in normal plants has not been conducted yet, it is suspected that rhizonone is an alternative defense compound produced in lieu of shikonin (Fukui et al., 1999). Phosphate also has been shown to affect secondary metabolite levels (Pannuri et al., 1993; Taya et al., 1994a). For example, Dunlop and Curtis (1991) showed that phosphate limitation along with elicitation increased sesquiterpene production in Hyoscyamus muticus. Carbon is also crucial in affecting not only growth, but also metabolite production. For example, by using fructose instead of sucrose, catharanthine production was doubled in C. roseus (Jung et al., 1992). Growth, however, decreased by about 40%, so a two-stage culture system was used. Roots were first grown in sucrose and then shifted into a fructose medium to enhance secondary metabolism.

Although not required for growth, exogenous addition of phytohormones affects secondary metabolite production. For example, the content of total alkaloids was increased in Hyoscyamus albus when roots were grown in indole-3-acetic acid (IAA) and kinetin in the dark (Sauerwein et al., 1992). Similarly, we have observed more than a 10-fold increase in artemisinin when A. annua hairy roots are grown in a cocktail of four phytohormones; growth, however, decreased by more than 75% (unpublished data). Clearly it is important to optimize culture medium constituents and to establish a suitable reactor protocol (e.g. one- versus two-stage culture) to optimize overall productivity.

Gases. Gases, mainly CO_2, O_2, and C_2H_4, also affect secondary metabolite production. Not many studies have been performed on hairy root cultures, however, compared to the number of studies done using plant cell suspension cultures. Most studies using roots have focused on the role that oxygen plays in growth and secondary metabolism. For example, Williams and Doran (1999) varied the dissolved oxygen tension for Atropa belladonna hairy root cultures in shake flasks and found that atropine production as well as specific growth rate were maximum at around 150% air saturation. Similarly, we have observed that more artemisinin is produced in Artemisia annua hairy roots grown in nutrient mist reactors, where, in contrast to bubble column reactors, oxygen is not limited (Kim et al., 2001). The link between oxygen availability and artemisinin biosynthesis, however, has not been conclusively demonstrated. Oxygen can also affect release of products into the culture medium. Taya et al. (1992) showed that when beet root cultures are not shaken for 2 d, oxygen becomes limited and betalains are released into the medium; cultures continued to grow provided that shaking resumed after 2 d. Altogether, these results show that the role of oxygen in secondary metabolism requires more investigation.

CO_2, an end product of respiration, can build up in root cultures and, if inadequate O_2 is available, leads to acidification of the cytoplasm and the cascade of events resulting from O_2 deficit stress (Buchanan et al., 2000). CO_2, however, can stimulate root growth as shown by DiIorio et al. (1992b) in studies with hairy roots. Furthermore, other aspects of root growth and morphology may be affected, such as border cell production (Zhao et al., 2000). Different plant species have different optimal CO_2 concentrations at which maximum growth occurs (DiIorio et al., 1992b). The response in Beta

vulgaris hairy roots seems to result from a shortening of the lag phase of the culture and not an increased growth rate (Weathers et al., 1997b). Not all hairy root species show an increase in growth yield in response to increased CO_2. For example, when single A. annua hairy roots were grown in mists enriched with CO_2, they did not grow faster than roots grown in air, but they appeared much healthier (Wyslouzil et al., 2000). It is not surprising that CO_2 affects root growth since CO_2 fixation is known to occur via phosphoenol pyruvate carboxylase leading mainly to organic acids (Farmer, 1996).

Although little is known about the role of ethylene in hairy root growth and associated secondary metabolite production, Sung and Huang (2000) recently showed that headspace ethylene accumulation, caused by adding an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, or an ethylene-releasing compound, 2-chloroethylphosphonic acid (ethephon), lowered the production of both biomass and L-DOPA (L-3,4-dihydroxyphenylalanine) by Stizolobium hassjoo hairy roots. When the ethylene inhibitor $CoCl_2$ was added to the culture, ethylene accumulation decreased and biomass and L-DOPA production were improved. Clearly, the role of both ethylene and CO_2 in hairy root growth and secondary metabolite production needs more attention.

Elicitors. Elicitors can be used to induce secondary metabolite production. Pitta-Alvarez et al. (2000) tested the effects of different biotic (salicylic acid, yeast extract) and abiotic ($CaCl_2$, $AgNO_3$, $CdCl_2$) elicitors on accumulation and release of scopolamine and hyoscyamine in Brugmansia candida hairy root cultures. Although salicylic acid, $AgNO_3$, and yeast extract all increased accumulation of both alkaloids, salicylic acid also affected release of both alkaloids significantly. On the other hand, $AgNO_3$ and yeast extract preferentially increased the release of scopolamine. $CdCl_2$ also increased the release of both alkaloids but inhibited growth. $CaCl_2$ had little affect on both alkaloid production and release (Pitta-Alvarez et al., 2000). In another study, Zabetakis et al. (1999) used methyl jasmonate, fungal elicitor (yeast cell wall), and oligogalacturonides in Datura stramonium hairy root cultures. Methyl jasmonate was the most effective and increased tropane alkaloid (littorine, hyoscyamine, scopolamine) synthesis. Rijhwani and Shanks (1998b) conducted transient studies after jasmonic acid and pectinase treatment and demonstrated that an optimum elicitor dosage and optimum harvesting time are required to enhance the production of certain metabolites. They also showed that elicitors can be used to study metabolite pathways. Indeed, Singh et al. (1998) showed that when Hyoscyamus muticus hairy roots were elicited with methyl jasmonate, sesquiterpenes produced early in the lubimin pathway were favored, whereas fungal elicitors favored production of the end product, lubimin. Although methyl jasmonate is a potent elicitor of many secondary metabolites, root growth is often inhibited, as shown by Yu et al. (2000) in studies with Panax ginseng hairy roots. Consequently, elicitors are often best added after growth is complete. A two-stage culture strategy usually serves this purpose.

Light. Light plays a role in both growth and secondary metabolite production. Many hairy root lines when exposed to light, turn green, and develop mature chloroplasts fully capable of photosynthesis (Flores et al., 1993). Green roots have metabolic capabilities distinct from their non-green counterparts. For example, Ipomoea aquatica hairy roots grown in the light produce twice as much biomass and four times as much peroxidase as roots grown in the dark (Taya et al., 1994b).

Roots do not have to turn green, however, to show profound alterations in secondary metabolism in response to light. For example, increasing light intensity to about 200 $\mu mol\, m^{-2}\, s^{-1}$ doubles the growth yield of A. annua hairy roots. In contrast, light inhibited the growth of Tagetes patula hairy roots and significantly altered the type of thiophenes produced compared to dark-grown cultures (Mukundan and Hjortsø, 1991). Sauerwein et al. (1992) also found that the alkaloid content of both normal and hairy roots of Hyoscyamus albus was greater in roots grown in the light compared to roots grown in the dark. Bhadra et al. (1998) studied the effect of light on growth as well as indole alkaloid accumulations of Catharanthus roseus hairy root cultures. The total yield of alkaloids was significantly different between light-adapted and dark-grown roots. More interestingly, they demonstrated that production of some alkaloids during a specific growth phase shifts when light conditions are altered. Light-adapted roots show growth-associated production of serpentine, and non-growth-associated production of tabersonine. When roots are shifted from dark to light, production of ajmalicine shifts from being growth-associated to non-growth-associated. Taken together these responses are not surprising since considerable evidence now shows that many of the enzymes in the terpenoid and other alkaloid pathways are regulated by light.

Delivery of light into reactors is problematic for hairy roots, because the cultures are generally immobilized. The biomass is not continuously moving past the light source as are cells in suspension cultures. Furthermore, hairy roots form mats or clumps with packing densities exceeding 50% (v/v), and light penetration into dense immobilized root beds presents a challenge. Interestingly, the roots themselves may assist in the delivery of signal-inducing levels of light into reactors since roots seems to have light guiding properties (Mandoli and Briggs, 1982, 1983). Indeed, when light from a He Ne laser was directed into the end of an A. annua hairy root and the output measured from an optical fiber inserted at the opposite end, we observed that about 0.0002% of the light was transmitted a distance of at least 2 cm down the interior of the root (Weathers and Swartzlander, unpublished). The roots, thus, seem to act as leaky optical fibers.

Other factors affecting production. Besides affecting root growth, other culture conditions such as temperature, subculture cycle, shear, and medium pH also affect secondary metabolite production. Subculture cycle was shown to affect growth and production of indole alkaloids in Catharanthus roseus hairy roots (Rijhwani and Shanks, 1998a). Culture temperature also affected hyoscyamine production in Datura stramonium hairy roots grown in stirred tank reactors (Hilton and Rhodes, 1990). Nuutila et al. (1994) observed that shear levels affected growth as well as the alkaloid content of Catharanthus roseus hairy roots grown in three types of liquid-phase bioreactors.

Changes in medium pH can change the permeability of cell membranes, resulting in the release of products into the culture medium (Saenz-Carbonell et al., 1993). Indeed, Taya et al. (1992) and Mukundan et al. (1998) both used pH shift to release products normally stored in vacuoles, and that they attributed to cell damage, probably at the vacuolar membrane. For example, betalains normally accumulate in roots of Beta vulgaris, but are released into the medium after 10 min of exposure to pH 2 followed by a return to regular medium at pH 5.5 (Mukundan et al., 1998). Up to 50% of the total pigment was released at the time of the exposure, and roots continued to grow and accumulate betalains afterwards. When roots

were exposed to pH 2 for >10 min, they failed to grow, suggesting that low pH caused the lysis of mature pigment-containing cells. The short pH exposure was beneficial for continuous production of betalains, especially since the pigment in the roots was maximized during exponential phase.

Reactor operation also affects secondary metabolite production. Continuous instead of batch culture stimulated product release and an increase in the rate of production of both hyoscyamine and nicotine in Datura stramonium (Hilton and Rhodes, 1990) and Nicotiana rustica hairy roots (Wilson et al., 1987), respectively.

Simplifying optimization. Statistical methods are very important to use in determining optimum culture conditions. In many cases there may be simple synergistic effects. For example, Dunlop and Curtis (1991) showed that solavetivone production was increased when phosphate was limited and fungal elicitors were added to Hyoscyamus muticus hairy roots. Optimization, however, is often far more complex. For example, to determine what interactions may occur between elicitor dosage, culture age, and exposure time, Bhagwath and Hjortsø (2000) used a factorial design to determine the best elicitation method (elicitor dosage, culture age, and the exposure time) to yield the highest desired product. Similarly, Weathers et al. (1997a) used a fractional factorial design to analyze four factors at three levels to optimize A. annua hairy root growth. Experimental design can minimize the number of experiments required to optimize culture growth and secondary metabolite production. For example, biomass production in A. annua showed statistically significant interactions between nitrate and sucrose (Weathers et al., 1997a); the response to one factor (e.g. sucrose) depended on the level of a second factor (nitrate), whereas other factors were less interactive. The resulting analysis showed that one combination of the four factors tested provided statistically greater amounts of biomass than all of the other conditions tested (Weathers et al., 1997a).

The use of six-well plates has also proved useful in optimization studies. Srinivasan et al. (1997) used six-well plates to optimize plant cell suspension culture processes. Each plate provides six replicate experiments with only minimal medium and space used. They can also be used to optimize root cultures. Indeed a considerable amount of information can be obtained just on root growth using a single root as inoculum in each well (Table 2). For example, using only two six-well plates, a comparative analysis of the growth of A. annua hairy roots in two medium formulations showed that there were statistically significant differences for seven of eight factors (Table 2).

Apparent product loss. Desired products may unfortunately be converted to other metabolites or degraded. For example, when $\leq 10\,mg\,l^{-1}$ of exogenous solasodine was added to Solanum aviculare hairy root cultures, net steroidal alkaloid production dropped to zero. When $>10\,mg\,l^{-1}$ ($25\,mg\,l^{-1}$) of exogenous solasodine was added to the medium, net steroidal alkaloid production was negative and suggested that exogenous solasodine was removed from the medium and converted or degraded (Yu et al., 1996).

Many secondary metabolites are toxic even to the plant producing them, and hence, require some sequestering, often by conjugation of the compound to a sugar moiety (Wink, 1997). They are often released via enzymatic hydrolysis when, through damage to the cell, the contents of subcellular compartments mix, as in the hydrolysis of allylisothiocyanate from sinigrine via myrosinase in roots of horseradish (Wink, 1997). Conjugated compounds may not be readily detected unless special steps are taken.

TABLE 2

GROWTH OF ARTEMISIA ANNUA HAIRY ROOTS (CLONE YUT16) IN SIX-WELL PLATES

Root characteristic	B5 medium	MS medium
Number of laterals	9.5 a	5.8 b
Length of primary root (cm)	4.7 a	3.0 b
Length of laterals (cm)	1.6 a	0.6 b
Total length (laterals+primary) (cm)	19.6 a	7.1 b
Total lateral length (cm)	14.9 a	4.1 b
Root growth unit (cm per root tips)	1.6 a	0.7 b
Fresh weight (g)	0.1 a	0.01 b
Roots attached to well?	No	No

A single 2.5 cm root was inoculated into each well in an uncoated six-well polystyrene plate and grown for 14 d in their respective medium on an orbital shaker and in $60\,\mu E\,m^{-2}\,s^{-1}$ cool white fluorescent light.

Letters after data indicate statistical differences at a 95% confidence level after Student's t-test between numbers within a row.

On the other hand, enzymes, especially peroxidases, are suspected to be involved in the degradation of many secondary metabolites (Stepan-Sarkissian et al., 1987; van der Heijden et al., 1990). Calcium, a cofactor of some types of plant peroxidases, affects their activity (Welinder, 1992; Xu and van Huystee, 1993; Kvaratskhelia et al., 1997). In an attempt to reduce the activity of Ca^{2+}-dependent peroxidases, Piñol et al. (1999) reduced the calcium concentration of B5 medium and observed that peroxidase activity decreased without any apparent effect on Datura stramonium hairy root growth. However, hyoscyamine production decreased and this was concurrent with decreased levels of mRNA for putrescine:SAM N-methyltransferase, an enzyme involved in diverting putrescine to tropane alkaloid synthesis. In a similar effort, A. annua hairy roots were grown in half calcium B5 medium to decrease peroxidase activity. Peroxidases degrade the endoperoxide bridge found in artemisinin, a sesquiterpene produced by A. annua (Iskra, Wobbe and Weathers, unpublished). Although peroxidase activity decreased, so did the levels of artemisinin. Peroxidases are likely such essential enzymes that control of product degradation by controlling peroxidases is impractical.

Conclusion

Hairy root cultures have shown promising biosynthetic capability toward production of secondary metabolites. At the same time they provide many challenges for large-scale culture. Because bioreactors may provide totally different culturing conditions than shake flasks, the results of yield improvement studies from shake flasks may not be directly transferable. Therefore, more studies on improvement of secondary metabolite production in bioreactors are needed.

Many secondary metabolites are constituents of very complicated biosynthetic networks. Their synthesis is triggered and regulated by nutrient availability and environmental factors, and they may be converted to or conjugated with other compounds. In many cases, the mechanism or enzymes responsible for synthesis of specific secondary metabolites are not fully understood. For example, the

indole alkaloid pathway of Catharanthus roseus hairy root cultures has been actively studied but many questions regarding regulation and interaction with other pathways still remain (Shanks et al., 1998; Morgan and Shanks, 1999, 2000). In Artemisia annua hairy roots, artemisinin (a sesquiterpene) and stigmasterol (a derivative of triterpenes) are derived from the common precursors of isopentenyl diphosphate and farnesyl pyrophosphate, and the stigamsterol production rate is 200 times greater than that of artemisinin (Xie et al., 2000). Ultimately, metabolic engineering of key regulatory enzymes may enable control of nutrient flux to a desired compound or allow us to selectively control a desired pathway. This type of genetic engineering, together with proper reactor design, is essential to the success of large-scale production of secondary metabolites.

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References

Bakkali, A. T.; Jaziri, M.; Foriers, A.; Vander Heyden, Y.; Vanhaelen, M.; Homès, J. Lawsone accumulation in normal and transformed cultures of henna, Lawsonia inermis. Plant Cell Tiss. Organ Cult. 51:83–87; 1997.

Balandrin, M. F.; Klocke, J. A.; Wurtele, E. S.; Bollinger, W. H. Natural plant chemicals: sources of industrial and medicinal materials. Science 228:1154–1160; 1985.

Banerjee, S.; Rahman, L.; Uniyal, G. C.; Ahuja, P. S. Enhanced production of valepotriates by Agrobacterium rhizogenes induced hairy root cultures of Valeriana wallichii DC. Plant Sci. 131:203–208; 1998.

Berlin, J.; Beier, H.; Fecker, L.; Forche, E.; Noé, W.; Sasse, F.; Schiel, O.; Wray, V. Conventional and new approaches to increase the alkaloid production of plant cell cultures. In: Neumann, K. H., Barz, W., Reinhard, E., eds. Primary and secondary metabolism of plant cell cultures. Berlin: Springer-Verlag; 1985:272–280.

Bhadra, R.; Morgan, J. A.; Shanks, J. V. Transient studies of light-adapted cultures of hairy roots of Catharanthus roseus: growth and indole alkaloid accumulation. Biotechnol. Bioeng. 60:670–678; 1998.

Bhadra, R.; Shanks, J. V. Transient studies of nutrient uptake, growth, and indole alkaloid accumulation in heterotrophic cultures of hairy roots of Catharanthus roseus. Biotechnol. Bioeng. 55:527–534; 1997.

Bhagwath, S. G.; Hjortsø, M. A. Statistical analysis of elicitation strategies for thiarubrine A production in hairy root cultures of Ambrosia artemisiifolia. J. Biotechnol. 80:159–167; 2000.

Bordonaro, J. L.; Curtis, W. R. Inhibitory role of root hairs on transport within root culture bioreactors. Biotechnol. Bioeng. 70:176–186; 2000.

Buchanan, R. B.; Gruissem, W.; Jones, R. L. Biochemistry and molecular biology of plants. Rockville: American Society of Plant Physiologists; 2000:1177–1189.

Buer, C. S.; Correll, M. J.; Smith, T. C.; Towler, M. J.; Weathers, P. J.; Nadler, M.; Seaman, J.; Walcerz, D. Development of a nontoxic acoustic window nutrient-mist bioreactor and relevant growth data. In Vitro Cell. Dev. Biol. Plant 32:299–304; 1996.

Buitelaar, R.; Langenhoff, A. A. M.; Heidstra, R.; Tramper, J. Growth and thiophene production by hairy root cultures of Tagetes patula in various two-liquid-phase bioreactors. Enzyme Microb. Technol. 13:487–494; 1991.

Carvalho, E. B.; Curtis, W. R. Characterization of fluid-flow resistance in root cultures with a convective flow tubular bioreactor. Biotechnol. Bioeng. 60:375–384; 1998.

Charlwood, B. V.; Charlwood, K. A. Terpenoid production in plant cell culture. In: Harborne, J. B., Tomas-Barberan, F. A., eds. Ecological chemistry and biochemistry of plant terpenoids. Oxford: Clarendon Press; 1991:95–132.

Chatterjee, C.; Correll, M. J.; Weathers, P. J.; Wyslouzil, B. E.; Walcerz, D. B. Simplified acoustic window mist bioreactor. Biotechnol. Techniques 11:155–158; 1997.

Curtin, M. E. Harvesting profitable products from plant tissue culture. Bio/Technology 1:649–657; 1983.

DiCosmo, F.; Misawa, M. Plant cell and tissue culture: alternatives for metabolite production. Biotechnol. Adv. 13:425–453; 1995.

DiIorio, A. A.; Cheetham, R. D.; Weathers, P. J. Growth of transformed roots in a nutrient mist bioreactor: reactor performance and evaluation. Appl. Microbiol. Biotechnol. 37:457–462; 1992a.

DiIorio, A. A.; Cheetham, R. D.; Weathers, P. J. Carbon dioxide improves the growth of hairy roots cultured on solid medium and in nutrient mists. Appl. Microbiol. Biotechnol. 37:463–467; 1992b.

Dunlop, D. S.; Curtis, W. R. Synergistic response of plant hairy root cultures to phosphate limitation and fungal elicitation. Biotechnol. Prog. 7:434–438; 1991.

Farmer, A. M. Carbon uptake by root systems. In: Waisel, Y., Eshel, A., Kafkafi, U., eds. Plant roots the hidden half. 2nd edn. New York: Marcel Dekker; 1996:679–687.

Flores, H. E. Use of plant cell and organ cultures in the production of biological chemicals. In: Mumma, R. O., Lebaron, H., Honeycutt, R. C., Duesing, J. H., eds. Applications of biotechnology to agricultural chemistry. Am. Chem. Soc. Symp. Series vol. 334. Washington DC: American Chemical Society; 1987:67–86.

Flores, H. E.; Curtis, W. R. Approaches to understanding and manipulating the biosynthetic potential of plant roots. Ann. N. Y. Acad. Sci. 665:188–209; 1992.

Flores, H. E.; Filner, P. Metabolic relationships of putrescine, GABA, and alkaloids in cell and root cultures of Solanaceae. In: Neumann, K. H., Barz, W., Reinhard, E., eds. Primary and secondary metabolism of plant cell cultures. Berlin: Springer-Verlag; 1985:174–186.

Flores, H. E.; Vivanco, J. M.; Loyola-Vargas, V. M. Radicle biochemistry: the biology of root-specific metabolism. Trends Plant Sci. 4:220–226; 1999.

Flores, H. E.; Yao-Rem, D.; Cuello, J. L.; Maldonado-Mendoza, I. E.; Loyola-Vargas, V. M. Green roots: photosynthesis and photoautotrophy in an underground plant organ. Plant Physiol. 101:363–371; 1993.

Fukui, H.; Hasan, A. F. M. F.; Kyo, M. Formation and secretion of a new unique quinone by hairy root cultures of Lithospermum erythrorhizon. Phytochemistry 51:511–515; 1999.

Fukui, H.; Hasan, A. F. M. F.; Ueoka, T.; Kyo, M. Formation and secretion of a new brown benzoquinone by hairy root cultures of Lithospermum erythrorhizon. Phytochemistry 47:1037–1039; 1998.

Furuya, T.; Yoshikawa, T.; Orihara, Y.; Oda, H. Studies of the culture conditions for Panax ginseng cells in jar fermentors. J. Nat. Prod. 47:70–75; 1984.

Giri, A.; Narasu, M. L. Transgenic hairy roots: recent trends and applications. Biotechnol. Adv. 18:1–22; 2000.

Hilton, M. G.; Rhodes, M. J. C. Growth and hyoscyamine production of hairy root cultures of Datura stramonium in a modified stirred tank reactor. Appl. Microbiol. Biotechnol. 33:132–138; 1990.

Hilton, M. G.; Wilson, P. D. G.; Robins, R. J.; Rhodes, M. J. C. Transformed root cultures – fermentation aspects. In: Robins, R. J., Rhodes, M. J. C., eds. Manipulating secondary metabolism in culture. Cambridge: Cambridge University Press; 1988:239–245.

Hsiao, T. Y.; Bacani, F. T.; Carvalho, E. B.; Curtis, W. R. Development of a low capital investment reactor system: application for plant cell suspension culture. Biotechnol. Prog. 15:114–122; 1999.

Jaziri, M.; Shimomura, K.; Yoshimatsu, K.; Fauconnier, M.-L.; Marlier, M.; Homes, J. Establishment of normal and transformed root cultures of Artemisia annua L. for artemisinin production. J. Plant Physiol. 145:175–177; 1995.

Jung, K. H.; Kwak, S. S.; Kim, S. W.; Lee, H.; Choi, C. Y.; Lin, J. R. Improvement of the catharanthine productivity in hairy root cultures of Catharanthus roseus by using monosaccharides as a carbon source. Biotechnol. Lett. 14:695–700; 1992.

Kanokwaree, K.; Doran, P. M. The extent to which external oxygen transfer limits growth in shake flask culture of hairy roots. Biotechnol. Bioeng. 55:521–526; 1997.

Kanokwaree, K.; Doran, P. M. Application of membrane tubing aeration and

perfluorocarbon to improve oxygen delivery to hairy root cultures. Biotechnol. Prog. 14:479–486; 1998.
Kieran, P. M.; MacLoughlin, P. F.; Malone, D. M. Plant cell suspension cultures: some engineering considerations. J. Biotechnol. 59:39–52; 1997.
Kim, Y. Assessment of bioreactors for transformed root cultures. PhD thesis, Worcester Polytechnic Institute, Worcester, MA; 2001.
Kim, Y.; Wyslouzil, B. E.; Weathers, P. J. A comparative study of mist and bubble column reactors in the in vitro production of artemisinin. Plant Cell. Rep. 20:451–455; 2001.
Kino-Oka, M.; Hitaka, Y.; Taya, M.; Tone, S. High-density culture of red beet hairy roots by considering medium flow conditions in a bioreactor. Chem. Eng. Sci. 54:3179–3186; 1999.
Kino-Oka, M.; Tsutsumi, S.; Tone, S. Oxygen transfer in bioreactor for culture of plant hairy roots. J. Chem. Eng. Japan 29:531–534; 1996.
Kittipongpatana, N.; Hock, R. S.; Porter, J. R. Production of solasodine by hairy root, callus, and cell suspension cultures of Solanum aviculare Forst. Plant Cell Tiss. Organ Cult. 52:133–143; 1998.
Kondo, O.; Honda, H.; Taya, M.; Kobayashi, T. Comparison of growth properties of carrot hairy root in various bioreactors. Appl. Microbiol. Biotechnol. 32:291–294; 1989.
Kvaratskhelia, M. C.; Winkel, C.; Naldrett, M. T.; Thorneley, R. N. F. Purification and characterization of a novel class III peroxidase from tea leaves. Plant Physiol. 114:1237–1245; 1997.
Kwok, K. H.; Doran, P. M. Kinetic and stoichiometric analysis of hairy roots in a segmented bubble column reactor. Biotechnol. Prog. 11:429–435; 1995.
Lee, K. T.; Suzuki, T.; Yamakawa, T.; Kodama, T.; Igarashi, Y.; Shimomura, K. Production of tropane alkaloids by transformed root cultures of Atropa belladonna in stirred bioreactors with stainless steel net. Plant Cell Rep. 18:567–571; 1999.
Liu, C. Z.; Wang, Y. C.; Zhao, B.; Guo, C.; Ouyang, F.; Ye, H. C.; Li, G. F. Development of a nutrient mist bioreactor for growth of hairy roots. In Vitro Cell. Dev. Biol. Plant 35:271–274; 1999.
Mahagamasekera, M. G. P.; Doran, P. M. Intergeneric co-culture of genetically transformed organs for the production of scopolamine. Phytochemistry 47:17–25; 1998.
Mandoli, D. F.; Briggs, W. R. Optical properties of etiolated plant tissues. Proc. Natl Acad. Sci. 79:2902–2906; 1982.
Mandoli, D. F.; Briggs, W. R. Physiology and optics of plant tissues. What's New in Plant Physiology 14:13–16; 1983.
McKelvey, S. A.; Gehrig, J. A.; Hollar, K. A.; Curtis, W. R. Growth of plant root cultures in liquid- and gas-dispersed reactor environments. Biotechnol. Prog. 9:317–322; 1993.
Morgan, J. A.; Shanks, J. V. Inhibitor studies of tabersonine metabolism in Catharanthus roseus hairy roots. Phytochemistry 51:61–68; 1999.
Morgan, J. A.; Shanks, J. V. Determination of metabolic rate-limitations by precursor feeding in Catharanthus roseus hairy root cultures. J. Biotechnol. 79:137–145; 2000.
Mukundan, U.; Bhide, V.; Singh, G.; Curtis, W. R. pH-mediated release of betalains from transformed root cultures of Beta vulgaris L. Appl. Microbiol. Biotechnol. 50:241–245; 1998.
Mukundan, U.; Hjortsø, M. Effect of light on growth and thiophene accumulation in transformed roots of Tagetes patula. J. Plant Physiol. 138:252–255; 1991.
Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473–497; 1962.
Nuutila, A. M.; Toivonen, L.; Kauppinen, V. Bioreactor studies on hairy root cultures of Catharanthus roseus: comparison of three bioreactor types. Biotechnol. Techniques 8:61–66; 1994.
Pannuri, S.; Reddy, G. R.; McNeill, D.; Curtis, W. R. Interpreting the role of phosphorus and growth rate in enhanced fungal induction of sesquiterpenes from Hyoscyamus muticus root cultures. Appl. Microbiol. Biotechnol. 38:550–555; 1993.
Parr, A. J. Secondary products from plant cell culture. In: Mizrahi, A., ed. Advances in biotechnology progress, vol. 9. Biotechnology in agriculture. New York: Alan R. Liss; 1988:1–34.
Piñol, M. T.; Palazón, J.; Cusidó, R. M.; Ribó, M. Influence of calcium ion-concentration in the medium on tropane alkaloid accumulation in Datura stramonium hairy roots. Plant Sci. 141:41–49; 1999.
Pitta-Alvarez, S. I.; Spollansky, T. C.; Giulietti, A. M. The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of Brugmansia candida. Enzyme Microb. Technol. 26:252–258; 2000.
Ramakrishnan, D.; Curtis, W. R. Fluid dynamic studies on plant root cultures for application to bioreactor design. In: Ryu, D. D. Y., Furusaki, S., eds. Studies in plant science. 4. Advances in plant biotechnology: production of secondary metabolites. Amsterdam: Elsevier; 1994:281–305.
Ramakrishnan, D.; Salim, J.; Curtis, W. R. Inoculation and tissue distribution in pilot-scale plant root culture bioreactors. Biotechnol. Techniques 8:639–644; 1994.
Rijhwani, S. K.; Shanks, J. V. Effect of subculture cycle on growth and indole alkaloid production by Catharanthus roseus hairy root cultures. Enzyme Microb. Technol. 22:606–611; 1998a.
Rijhwani, S. K.; Shanks, J. V. Effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by Catharanthus roseus hairy root cultures. Biotechnol. Prog. 14:442–449; 1998b.
Robins, R. J.; Bent, E. G.; Rhodes, M. J. C. Studies on the biosynthesis of tropane alkaloids by Datura stramonium L. transformed root cultures. 3. The relationship between morphological integrity and alkaloid biosynthesis. Planta 185:385–390; 1991.
Rodriguez-Mendiola, M. A.; Stafford, A.; Cresswell, R.; Arias-Castro, C. Bioreactors for growth of plant roots. Enzyme Microb. Technol. 13:697–702; 1991.
Rokem, J. S.; Goldberg, I. Secondary metabolites from plant cell suspension cultures: methods for yield improvement. Advances in biotechnological processes 4. New York: Alan R. Liss; 1985:241–274.
Saenz-Carbonell, L. A.; Maldonado-Mendoza, I. E.; Moreno-Valenzula, O.; Ciau-Uitz, R.; Lopez-Meyer, M.; Loyola-Vargas, V. M. Effect of the medium pH on the release of secondary metabolites from roots of Datura stramonium, Catharanthus roseus, and Tagetes patula cultured in vitro. Appl. Biochem. Biotechnol. 38:257–267; 1993.
Sauerwein, M.; Wink, M.; Shimomura, K. Influence of light and phytohormones on alkaloid production in transformed root cultures of Hyoscyamus albus. J. Plant Physiol. 140:147–152; 1992.
Shanks, J. V.; Bhadra, R.; Morgan, J.; Rijhwani, S.; Vani, S. Quantification of metabolites in the indole alkaloid pathways of Catharanthus roseus: implications for metabolic engineering. Biotechnol. Bioeng. 58:333–338; 1998.
Shanks, J. V.; Morgan, J. Plant 'hairy root' culture. Curr. Opin. Biotechnol. 10:151–155; 1999.
Shiao, T.-L.; Doran, P. M. Root hairiness: effect on fluid flow and oxygen transfer in hairy root cultures. J. Biotechnol. 83:199–210; 2000.
Singh, G.; Curtis, W. R. Reactor design for plant root culture. In: Shargool, P. D., Ngo, T. T., eds. Biotechnological applications plant cultures. CRC series of current topics in plant molecular biology. Boca Raton, FL:CRC Press; 1994:185–206.
Singh, G.; Gavrielli, J.; Oakey, J. S.; Curtis, W. R. Interaction of methyl jasmonate, wounding, and fungal elicitation during sesquiterpene induction in Hyoscyamus muticus in root cultures. Plant Cell Rep. 17:391–395; 1998.
Srinivasan, V.; Roberts, S. C.; Shuler, M. L. Combined use of sex-well polystyrene plates and thin layer chromatography for rapid development of optimal plant cell culture processes: application to taxane produciton by Taxus sp. Plant Cell Rep. 16:600–604; 1997.
Stepan-Sarkissian, G.; Grey, D.; Stafford, A.; Fowler, M. W. Some characteristics of peroxidase in plant cell cultures. In: Neijssel, O. M., van der Ruyben, R. R., eds. Proc. 4th Eur. Congr. on Biotechnology. Amsterdam:Elsevier Scientific; 1987:342–344.
Subroto, S. E.; Kwok, K. H.; Hamill, J. D.; Doran, P. M. Co-culture of genetically transformed roots and shoots for synthesis, translocation, and biotransformation of secondary metabolites. Biotechnol. Bioeng. 49:481–494; 1996.
Sung, L.-S.; Huang, S.-Y. Headspace ethylene accumulation on Stizolobium hassjoo hairy root culture producing L-3,4-dihydroxyphenylalanine. Biotechnol. Lett. 22:875–878; 2000.
Tabata, N.; Yoshikawa, N.; Tsukada, M.; Fukui, H. Localization and regulation of shikonin formation in the cultured cells of Lithospermum erythrorhizon. Proc. 5th Int. Congr. Plant Tissue Culture. Tokyo: Maruzen; 1982:335–336.
Taya, M.; Mine, K.; Kino-Oka, M.; Tone, S.; Ichi, T. Production and release of pigments by culture of transformed hairy root of red beet. J. Ferment. Technol. 73:31–36; 1992.

Taya, M.; Sato, H.; Masahiro, K.; Tone, S. Characterization of pak-bung green hairy roots cultivated under light irradiation. J. Ferment. Bioeng. 78:42–48; 1994b.

Taya, M.; Yakura, K.; Kino-Oka, M.; Tone, S. Influence of medium constituents on enhancement of pigment production by batch cultures of red beet hairy roots. J. Ferment. Bioeng. 77:215–217; 1994a.

Taya, M.; Yoyoma, A.; Kondo, O.; Kobayashi, T.; Matsui, C. Growth characteristics of plant hairy roots and their cultures in bioreactors. J. Chem. Eng. Japan 22:84–89; 1989.

Tescione, L. D.; Ramakrishnan, D.; Curtis, W. R. The role of liquid mixing and gas-phase dispersion in a submerged, sparged root reactor. Enzyme Microb. Technol. 20:207–213; 1997.

van der Heijden, R.; van der Graaf, G. M.; Pennings, E. J. M.; Verpoorte, R. Formation and degradation of some aspidospermatan indole alkaloids in a suspension culture of Tabernaemontana divaricata. Plant Physiol. Biochem. 28:351–358; 1990.

Wallaart, T. E.; Pras, N.; Quax, W. J. Isolation and identification of dihydroartemisinic acid hydroperoxide from Artemisia annua: a novel biosynthetic precursor of artemisinin. J. Nat. Prod. 62:1160–1162; 1999.

Weathers, P. J.; Cheetham, R. D.; Follansbee, E.; Teoh, T. Artemisinin production by transformed roots of Artemisia annua. Biotechnol. Lett. 16:1281–1286; 1994.

Weathers, P. J.; Hemmavanh, D.; Walcerz, D. B.; Cheetham, R. D.; Smith, T. C. Interactive effects of nitrate and phosphate salts, sucrose, and inoculum culture age on growth and sesquiterpene production in Artemisia annua hairy root cultures. In Vitro Cell. Dev. Biol. Plant 33:306–312; 1997a.

Weathers, P.; Whipple, M.; Wyslouzil, B. Laboratory-scale studies of nutrient mist reactors for culturing hairy roots. In: Doran, P. M., ed. Hairy roots: culture and applications. Amsterdam: Harwood Academic; 1997b:191–200.

Weathers, P. J.; Wyslouzil, B. E.; Wobbe, K. K.; Kim, Y. J.; Yigit, E. The biological response of hairy roots to O_2 levels in bioreactors. In Vitro Cell. Dev. Biol. Plant 35:286–289; 1999.

Welinder, K. G. Superfamily of plant, fungal, and bacterial peroxidases. Curr. Biol. 2:388–393; 1992.

Whitney, P. Novel bioreactors for the growth of roots transformed by Agrobacterium rhizogenes. Enzyme Microb. Technol. 14:13–17; 1992.

Williams, G. R. C.; Doran, P. M. Investigation of liquid–solid hydrodynamic boundary layers and oxygen requirements in hairy root cultures. Biotechnol. Bioeng. 64:729–740; 1999.

Williams, G. R. C.; Doran, P. M. Hairy root culture in a liquid-dispersed bioreactor: characterization of spatial heterogeneity. Biotechnol. Prog. 16:391–401; 2000.

Wilson, P. D. G. The pilot-scale cultivation of transformed roots. In: Doran, P. M., ed. Hairy roots: culture and applications. Amsterdam: Harwood Academic; 1997:179–190.

Wilson, P. D. G.; Hilton, M. G.; Robins, R. J.; Rhodes, M. J. C. Fermentation studies of transformed root cultures. In: Moody, G. W., Baker, P. B., eds. International conference on bioreactors and biotransformations. London: Elsevier; 1987:38–51.

Wink, M. Compartmentation of secondary metabolites and xenobiotics in plant vacuoles. In: Leigh, R. A., Sanders, D., eds. Advances in botanical research, vol. 25. The plant vacuole. San Diego: Academic Press; 1997:141–169.

Woo, S. H.; Park, J. M.; Yang, J. Root culture using a mist culture system and estimation of scale-up feasibility. J. Chem. Tech. Biotechnol. 66:355–362; 1996.

Wyslouzil, B. E.; Waterbury, R. G.; Weathers, P. J. The growth of single roots of Artemisia annua in nutrient mist reactors. Biotechnol. Bioeng. 72:143–150; 2000.

Wyslouzil, B. E.; Whipple, M.; Chatterjee, C.; Walcerz, D. B.; Weathers, P. J.; Hart, D. P. Mist deposition onto hairy root cultures: aerosol modeling and experiments. Biotechnol. Prog. 13:185–194; 1997.

Wysokinska, H.; Chmiel, A. Transformed root cultures for biotechnology. Acta Biotechnol. 17:131–159; 1997.

Xie, D.; Wang, L.; Ye, H.; Li, G. Isolation and production of artemisinin and stigmasterol in hairy root cultures of Artemisia annua. Plant Cell Tiss. Organ Cult. 63:161–166; 2000.

Xu, Y.; van Huystee, R. B. Association of calcium and calmodulin to peroxidase secretion and activation. J. Plant Physiol. 141:141–146; 1993.

Yu, K.; Gao, W.; Son, S.; Paek, K. Improvement of ginsenoside production by jasmonic acid and some other elicitors in hairy root culture of ginseng (Panax ginseng C.A. Meyer). In Vitro Cell. Dev. Biol. Plant 36:424–428; 2000.

Yu, S.; Doran, P. M. Oxygen requirements and mass transfer in hairy-root culture. Biotechnol. Bioeng. 44:880–887; 1994.

Yu, S.; Kwok, K. H.; Doran, P. M. Effect of sucrose, exogeneous product concentration, and other culture conditions on growth and steroidal alkaloid production by Solanum aviculare hairy roots. Enzyme Microb. Technol. 18:238–243; 1996.

Yu, S.; Mahagamasekera, M. G. P.; Williams, G. R. C.; Kanokwaree, K.; Doran, P. M. Oxygen effects in hairy root culture. In: Doran, P. M., ed. Hairy roots: culture and applications. Amsterdam: Harwood Academic; 1997:139–150.

Zabetakis, I.; Edwards, R.; O'Hagan, D. Elicitation of tropane alkaloid biosynthesis in transformed root cultures of Datura stramonium. Phytochemistry 50:53–56; 1999.

Zhao, X.; Misaghi, I. J.; Hawes, M. C. Stimulation of border cell production in response to increased carbon dioxide levels. Plant Physiol. 122:181–188; 2000.