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*Minireview*

# The regulation of plant growth and development in liquid culture

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**Plant liquid culture offers many benefits over solidified media. Growth and multiplication rate of shoots, roots, bulblets and somatic embryos is enhanced in liquid culture, as a consequence of better availability of water and nutrients resulting from a lower resistance to diffusion and closer contact between the explant and the medium. Morphogenic development, such as meristemoid production and somatic embryogenesis, is influenced by the physical environment provided by the liquid culture, and benefits from reduced gradients within the medium and dilution of exuded toxins. Chemical growth regulating factors, including plant hormones, growth retardants and other bioregulators are also discussed in terms of their influence on growth and development. Automation and scaling up for bioreactor production of propagules requires optimisation before becoming commercially viable. Considerable potential exists for future research into the molecular aspects regulating developmental pathways such as somatic embryogenesis, storage organ formation and meristemoid induction.**

*Abbreviations*: 2,4-D = 2,4-dichlorophenoxyacetic acid, ABA = abscisic acid, AOA = aminooxyacetic acid, AVG = aminoethoxyvinylglycine, B-9 = daminozide, BA = benzyladenine, BSAA = 3-(benzo[b]selenyl)acetic acid, IAA = indole-3-acetic acid, iP = isopentenyladenine, NAA = α-naphthaleneacetic acid.

## Introduction

Tissue culture is an important tool for research that has been widely used and applied to many disciplines of science. Explants *in vitro* will undergo two phenomena: growth and development. Growth is, in essence, the irreversible increase in shape and/or size and/or number of cells. Cell growth requires extensive modifications to the properties of the cell wall, which are mediated by auxins and expansins (Cosgrove 1999), while cell division requires replication of genetic material in addition to *de novo* wall synthesis. Development in plants refers to morphogenetic changes that determine cell fate. While both growth and development are genetically regulated events, morphogenesis involves redirection of growth to a completely new programme of metabolism, where both structure and function are likely to change.

Liquid culture of plants has provided many opportunities to improve plant propagation *in vitro*. In fact, many significant physiological discoveries have occurred in liquid culture: the demonstration of indefinite culture of an organ by White (1934), somatic embryogenesis in carrot callus suspension cultures confirming the theory of totipotency (Steward *et al*. 1958), and the first concrete evidence that roots synthesise and secrete cytokinins (Van Staden and Smith 1978).

Over the past decade, there has been a steady move toward optimising liquid culture systems for the purpose of automated micropropagation. This shift is a consequence of realizing the advantages that culture in liquid media has over culture on solidified media. Liquid culture is more cost effective as a gelling agent is not required. Since the explant is entirely covered by the medium, all cells have the opportunity to absorb nutrients and plant growth regulators directly from the medium. In addition, the medium can be changed automatically to replenish depleted nutrients, remove toxic metabolites, switch between stages of physiological development if bioreactors are used (Moorhouse *et al*. 1996), and reduce labour costs for subculturing (Maene and Debergh 1985, Seon *et al*. 2000).

If one defines liquid culture as growth of explants on non-solid medium, then various modifications to the basic system are possible. The simplest liquid culture system contains explants in a non-agitated (static) liquid medium. For example, androgenic pollen grains of wheat can form thousands of embryos in a static liquid culture (Jones and Petolino 1987, Liu *et al*. 2002). Modifications include aeration by bubbling air through the medium, use of a support on

which the explant rests (for example, cellulose substrate), shaking of the culture to maximise contact between medium and explants, partial immersion of explants where portions of the plant material are submersed, temporary immersion where explants undergo immersion and emersion in the medium for varying time periods, and the use of bioreactors, which may either be closed or open. These modifications have become necessary to alleviate the inherent problems associated with liquid culture, namely hyperhydricity (Ziv 1991a), aeration and shear stresses (Moorhouse *et al.* 1996).

Hvoslef-Eide *et al*. (2003) have reviewed some of the liquid culture systems used for plant propagation, especially scaling up and use of bioreactors for commercial production, and Ziv (1999) has described the use of bioreactors for the production of organogenic propagules such as bulblets. While an extensive review of all phenomena and implications reported in liquid culture is beyond the scope of this work, the manner in which liquid culture is able to regulate plant growth and development is discussed. The benefits that liquid culture has over the conventional gelled media are described in terms of growth rates and morphogenetic patterns, and the limitations are highlighted. Chemical factors specifically involved in coordinating growth and development are also reviewed.

## Liquid Culture: Prospects and Problems

### *Prospects*

*Growth rate*

In general, liquid cultures exhibit higher multiplication and proliferation rates than do conventional gelled cultures. Kim *et al*. (2003) reported that garlic shoots exhibited an increase in multiplication rate and fresh weight compared to shoot explants on solidified cultures. For potato micropropagation, explants grown in liquid media exhibited higher shoot growth rates than explants cultured on solidified media (Avila *et al*. 1996). Lorenzo *et al*. (1998) compared growth rates for sugarcane meristems cultured in both liquid and solid media, as well as in a temporary immersion system. Interestingly, there was no significant difference in the growth rate between liquid and solid media, while the temporary immersion system produced a growth rate twice that of the other two methods.

This improved rate of multiplication in liquid culture has been demonstrated for shoots of *Pinus radiata* (Smith *et al*. 1980), tea (Sandal *et al*. 2001), wild pear (Damiano *et al*. 2000) and increased rooting of *Calotropis gigantean* shoots compared to explants rooted on agar-solidified media (Roy *et al*. 2000). Root cultures of aspen showed a rapid increase in biomass when grown in a liquid-shake culture (Vinocur *et al*. 2000). However, increased growth rate in a liquid medium is not a universal phenomenon.

The use of suspension culture can lead to very high numbers of somatic embryos being formed, and thus has great potential for propagation and synthetic seed production. For coffee leaves under optimal growth conditions, one gram of embryogenic callus can produce $1.2 \times 10^5$ somatic embryos after just eight weeks in culture (Van Boxtel and Berthouly 1996). Gawel and Robacker (1990) reported that greater numbers of cotton somatic embryos are produced in liquid cultures than on gelled cultures.

This improved growth rate was suggested to be due to a number of factors, including better availability of nutrients (Singha 1982, cited by Avila *et al*. 1996), greater availability of water (Debergh 1983), a more gradual pH change during culture, a reduction in nutrient and endogenous hormone gradients (Gawel and Robacker 1990), removal of polarity, and a reduction in the effect of toxins.

Greater availability of nutrients and water is thought to result from lower resistance to diffusion and closer contact between the explant and culture medium (Singha 1982, cited by Avila *et al*. 1996). But Avila *et al*. (1996) found that the water content of micropropagated potato shoots was not significantly different between liquid and solid cultures. Increase in shoot fresh weight was the result of greater accumulation of carbohydrates and organic nitrogen, suggesting that nutrient assimilation is favoured in liquid culture (Avila *et al*. 1996). Whether increases in growth rate result from greater accumulation of carbohydrates, enhanced water uptake, or a combination of both, is dependent on the species in question, as well as the type of explant and specific culture conditions.

Agitation of a liquid culture allows even distribution of nutrients and growth regulating substances, thus no depletion zones (gradients) are formed around the explant, as is the case in gelled cultures. This is advantageous since the effect of exogenously applied growth regulators often varies with concentration; in agitated liquid cultures, the concentrations can be kept even. In addition, agitation of the culture leads to better aeration of the explants, and hence, improved growth rates (Jackson *et al*. 1991).

To obtain haploid wheat plants, microspores are cultured in a liquid medium in the presence of immature ovaries. The ovaries act as a ‘nurse culture’ and secrete factors required by the developing microspores to complete androgenic development into a haploid embryo (Hu and Kasha 1997). However, an extract prepared from immature ovaries does not stimulate androgenesis (Liu *et al*. 2002), suggesting that ovaries actively produce the critical factor/s in response to the physical environment of the liquid medium. Androgenesis will also benefit from the reduced resistance to diffusion, ensuring that all factors and regulators are distributed evenly throughout the medium.

Explants cultured on solid media will exhibit polarity in their response — cells located on the opposite side to those in direct contact with the medium have to assimilate nutrients and process regulatory signals by diffusion from neighboring cells. This can lead to a different signal being perceived at the peripherally located cells. This situation does not arise in liquid culture as the entire surface area of the explant is bathed in the medium and is therefore able to perceive chemical signals.

Another advantage of using liquid systems for culture is the reduction in the deleterious effect of toxins. Any metabolite that is released into the medium by the explant may have inhibitory or toxic effects on further growth and/or development. Unlike solidified media where released substances remain in close proximity to the explant, toxins rapidly

become diluted in liquid systems, thereby decreasing any potential inhibitory effect.

To achieve maximum multiplication for micropropagation, several variables need to be optimised. These include the nutrient composition of the medium, the physical growth conditions, the presence of plant hormones, and the type of liquid culture system to be used. At present, three liquid culture systems have been popularised for research: liquid-shake culture, temporary immersion and bioreactors.

*Liquid-shake*

Liquid-shake culture, as the name implies, consists of explants in a liquid culture that is rotated or shaken. Strictly speaking, liquid-shake culture also encompasses cell suspension cultures that are used for somatic embryogenesis and secondary metabolite production (Gao *et al*. 2000, Zhao *et al*. 2001). Liquid-shake culture offers improved aeration compared to static cultures, as the explants are in constant contact with the medium. Consequently, growth rate is often increased, as has been reported recently for various *Eucalyptus* species and hybrids (Whitehouse *et al*. 2002). If explants are submerged, leaf growth is inhibited and a cluster of buds, or meristemoids may be formed (Ziv 1991a).

*Temporary immersion*

Temporary immersion of explants allows excellent aeration whilst still providing nutrients and plant growth regulators when the explants become bathed in the medium. Another advantage of temporary immersion is the reduction in shearing force as the culture does not require shaking. Since bubbling air through the medium is not required, the problem of foaming is eliminated. However, the duration of immersion and intervals between successive immersion phases need to be optimised for each species.

The beneficial effects of temporary immersion may depend on the species in question, or the type of growth pattern of the explant *in vitro*. For example, *Hevea brasiliensis* calli showed significantly lower growth rates under temporary immersion regimes, compared to calli grown in agitated liquid culture and on semisolid media (Martre *et al*. 2001). On the other hand, shoots grown under temporary immersion regimes exhibit higher growth rates than those cultured in other liquid systems (Damiano *et al*. 2000, Kim *et al*. 2003).

Banana shoots propagated using four different liquid culture systems showed maximum multiplication under temporary immersion (Alvard *et al.* 1993). McAlister *et al*. (2002) and McAlister (2003) used a RITA® temporary immersion system to propagate *Eucalyptus* hybrids and obtained a four- to six-fold increase in multiplication rate in half the time compared to conventional methods. Somatic embryogenesis occurs best in liquid-shake cultures, whilst germination and micropropagation by organogenesis is optimum in temporary immersion systems (Alvard *et al.* 1993, Kosky *et al*. 2002).

*Bioreactors*

Examination of the literature has revealed that bioreactors are used in three particular instances: suspension cultures for regenerating somatic embryos (Kosky *et al*. 2002), suspension cultures used for production of secondary metabolites (Gao *et al*. 2000), and for the production of bulblets (Seon *et al*. 2000). Bioreactors offer several advantages over other liquid systems: they can be programmed to exchange media, supply bubbles and stir the culture, thereby minimising labour cost; bioreactors use large culture volumes, and hence large quantities of the desired end product can be obtained.

As with any other system, sequential optimisation of critical factors needs to be performed for the culture to be successful. Such factors include the type of bioreactor, method of aeration and circulation, environmental conditions, shearing forces, pH, media composition, plant growth regulators and osmoticum (Takahashi *et al*. 1992, Ziv 1995).

*Growth patterns*

Liquid culture has effectively been used to induce two types of growth patterns in explants: organogenic and embryogenic propagules. Organogenic propagules include bulblets, cormlets, meristemoids and microtubers. Embryogenic propagules, on the other hand, include embryos obtained from somatic cells (via somatic embryogenesis), cells of the gynaecium (via gynogenesis) and pollen grains (via androgenesis). Meristemoid production and somatic embryogenesis are discussed, as extensive work has been conducted in these particular areas.

Meristemoids

The term ‘meristemoid’ has been used to describe a cluster of buds or apical meristems that each have the potential to produce a normal shoot (White 1984, Ziv 1991a). However, this term was originally used to describe a single densely staining cell involved in ontogenic development of other cells such as stomata (Kannabiran 1975) or root primordia (Coleman and Greyson 1977). Other terms describing similar structures include ‘nodules’ (Ito *et al*. 1996), ‘nubbins’ (Krikorian and Kann 1986, cited by Ziv *et al*. 1994), ‘meristemmatic agglomerates’ (Arezki *et al*. 2000) ‘proliferating bud aggregates’ (Ziv 1989) and ‘protocorm-like bodies’ (Ziv 1999, Young *et al.* 2000).

Meristemoid production has not been reported for any species cultured on solidified media, and is thus unique to liquid cultures. A number of workers have reported successful meristemoid production in numerous ornamentals such as *Gladiolus* (Lipsky *et al*. 1997), *Crinum* (Fennell 2002), *Dierama* (L Madubanya pers. comm.), *Brodiaea* (Ziv 1999), daylily (Krikorian and Kann 1986, cited by Ziv *et al*. 1994), *Nerine* (Ziv *et al*. 1994), *Cryptanthus* (Arrabal *et al*. 2002) and *Ornithogalum* (Ziv and Lilien-Kipnis 1997). Success has also been achieved with banana and potato (Levin *et al*. 1997), as well as aspen (Vinocur *et al*. 2000) and *Pinus radiata* (Ziv *et al*. 1994).

Ziv (1991a) reported that meristemoid production in the fern *Nephrolepis axaltata* cv. Bostoniensis occurred regardless of whether the cultures were rotated or kept stationary. Leaf development was observed in older cultures when the biomass grew above the liquid medium, while constant submergence completely inhibited leaf development (Ziv 1991a). This suggests that it is the physical environment of the liquid medium that allows an explant to proliferate into a meristemoid. Since explants of the same nature and species

produced shoots on gelled cultures (Ziv 1991a), suppression of shoot growth may be critical for meristemoid formation.

If considered logically, then the inhibition of shoot growth results in more energy (carbohydrates) and building blocks being available for other uses. Thus, the nutrients absorbed from the culture medium are used to produce more apical domes, by redirecting the growth pattern from simple shoot growth to a developmental (morphogenic) pattern. How this is achieved in the liquid medium remains unknown. Suppression of leaf growth by a liquid environment may be species dependent. For example, the inhibition of *Philodendron* leaf development was only obtained by adding inhibitors of gibberellin biosynthesis, such as paclobutrazol and ancymidol (Ziv 1991a).

Somatic embryogenesis

Somatic embryogenesis is defined as a process in which a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell without vascular connection to the original tissue (Thorpe 1993). Somatic embryos are used as a model system in studying various aspects of embryogenesis (Von Arnold *et al*. 2002a). Perhaps the greatest importance of producing somatic embryos is the potential for large-scale vegetative propagation. This technique allows genetically uniform plants from a superior parent to be produced, as well as allowing propagation of plants that are considered difficult to propagate. Furthermore, the production of large amounts of embryogenic callus in liquid culture is beneficial for genetic transformation experiments (Van Boxtel and Berthouly 1996). This paper will not consider the developmental pathways of somatic embryogenesis, as this topic has recently been reviewed by Von Arnold *et al*. (2002a).

For somatic embryogenesis to occur in liquid culture, the correct type of suspension culture must be established. Undifferentiated callus often gives rise to large vacuolated cells in suspension, which die within two weeks (Gude and Dijkema 1997). In contrast, an embryogenic suspension can be created when the callus has been induced on a medium containing an auxin such as 2,4-D; these cells are generally smaller and have dense cytoplasmic contents (Wang *et al*. 2001). In the case of asparagus, a change in the type and ratio of the hormones triggers the formation of embryogenic callus (Reuther 1996). Globular callus clumps were observed when the hormone combination of IAA, BA and iP was replaced with kinetin and 2,4-D (Reuther 1996). In contrast, no plant growth regulators are required for somatic embryo production in *Ophiopogon japonicus* suspension cultures (Strandberg 1993).

In some species, suspension cultures are used to produce large quantities of embryogenic cells or cell clumps that are filtered and plated on a solid medium for somatic embryo production and subsequent plant regeneration (Wang *et al*. 1999, Moghaddam *et al*. 2000). In these cases, the role of the liquid phase of the culture is simply to allow cell growth and division (i.e. multiplication) while still maintaining the cells and cell clumps in an embryogenic state.

Jayasankar *et al*. (2003) compared somatic embryos derived from both solid and liquid media. These authors reported that embryos originating on a liquid medium had smaller cotyledons, a distinct suspensor and a flat-to-convex shoot apical meristem while those obtained from a solid medium had large cotyledons, a poorly developed suspensor and a relatively undeveloped concave apical meristem. Embryos from the solidified medium exhibited dormancy but those from the liquid medium were not dormant and gave high plant regeneration rates (Jayasankar *et al*. 2003).

It remains unknown what property of the liquid medium is able to maintain cell clumps in an embryogenic state. It is likely to be a combination of factors acting synergistically. For example, greater availability of metabolites and growth regulating substances (due to closer contact with the medium) that can be absorbed by all parts of the explant (a result of being bathed in the medium) in combination with reduced gradients of nutrients, all probably contribute to maintaining the embryogenic potential of the culture.

## *Problems*

*Physical consequences associated with liquid media*

One of the biggest disadvantages of liquid culture is that the entire explant, or portions thereof, are submersed constantly (in the case of suspension cultures, partial immersion and in liquid-shake culture) and are thus oxygen deficient (Alvard *et al*. 1993). This leads to abnormal leaf development, such as elongated leaves that are hyperhydric (Ziv 1991a). Hyperhydricity (vitrification) can be overcome by bubbling sterile air through the medium, although this solution is not universal (Alvard *et al.* 1993).

Other physical consequences include shearing forces that cause damage to cells and the break up of cell aggregates. This happens when air is bubbled through the medium and excessive agitation of a liquid-shake culture occurs, which then leads to foam build up (Abdullah *et al*. 2000). Aeration of liquid culture systems is a major problem: oxygen levels are low and carbon dioxide rapidly builds up, and may dissolve in the medium leading to a decrease in the pH.

Cells in agitated suspension cultures are subjected to a partial absence of horizontal polarity in that they are not physically attached to other cells. Thus, intercellular trafficking that would normally occur during metabolism *in vivo*, presumably does not occur. Since they are being rotated at a constant speed and are entirely and continuously bathed in the medium, only polarity in the direction of gravity can presumably be sensed.

*Overcoming hyperhydricity in liquid cultures*

Constant, partial and temporary immersion of explants can lead to regenerated structures becoming hyperhydric, and this is especially the case in woody plants such as *Eucalyptus* (Le Roux and Van Staden 1991, Whitehouse *et al*. 2002). It is generally thought that high concentrations of cytokinins (Taji *et al*. 1996) or auxins (Gaspar *et al*. 1995) lead to explants and regenerated structures becoming hyperhydric. Hyperhydricity of explants is characterised by a glassy, translucent or swollen appearance to the tissue and can often reduce multiplication rates, induce poor quality shoots and even necrosis of the tissues (Ziv 1991b). Some workers have suggested that hyperhydricity results from an inability of the organs to break down activated oxygen species and soluble reductants (Gaspar *et al*. 1995).

Methods employed to combat hyperhydricity include altering the carbohydrate concentrations (Zimmerman and Cobb 1989), changing the levels of gelling agents (Debergh *et al*. 1981), and adding Bacto-Peptone fractions (Sato *et al*. 1993) or agar hydrolysates (Nairn *et al*. 1995). Unfortunately, these supplements may lead to a decrease in the multiplication rate of the culture (Zimmerman *et al*. 1995).

In some cases, hyperhydricity can be overcome by bubbling sterile air through the medium, although this has not been effective in preventing vitrification of banana shoots (Alvard *et al.* 1993). An alternative may be bubble-free gaseous exchange (Moorhouse *et al*. 1996), but this has not yet been tested for banana micropropagation. It has been reported that substitution of BA with thidiazuron in tea liquid cultures eliminates hyperhydricity of shoots (Sandal *et al*. 2001). In contrast, most research suggests that polysaccharides, especially methylated and sulfated galactose derivatives, are involved in preventing hyperhydricity (Marga *et al*. 1997, Whitehouse *et al*. 2002). Commercially available media supplements such as EM2, M-Gel, iotacarrageenan and Gelcarin GP812 are thought to control hyperhydricity because they contain galactans and/or sulfate as part of their structure (Whitehouse *et al*. 2002).

*Factors regulating plant growth and development in liquid culture*

Since meristemoid production is unique to liquid cultures, the physical environment of the liquid must be the factor inducing the formation of these bud clusters. In addition to this physical effect imposed by the liquid, other factors act in concert to control growth and morphogenesis. These may be conveniently grouped into culture conditions, such as temperature, oxygen partial pressure, light regime and pH, and regulation by chemical compounds, including hormones, carbohydrates, growth retardants, as well as other molecules. The effects of physical culture conditions will not be discussed, as these have been dealt with by other workers (see Majada *et al*. 1992, Reuther *et al*. 1992). The five groups of plant hormones, and the growth retardants are considered below.

Auxins

Auxins are the most widely used of all plant hormones for general tissue culturing purposes. This is especially the case for liquid cultures, and only in a few cases are auxins not used (Strandberg 1993). Auxins are often used in conjunction with cytokinins, since these hormones have synergistic effects on both growth and development, and in many cases it cannot be determined if a particular effect is due to either the auxin, cytokinin, or both.

The synthetic auxin 2,4-D has been extensively used for inducing somatic embryogenesis in suspension cultures, while workers producing bulblets and cormlets in liquid-shake culture use NAA as the source of auxin (Takayama and Misawa 1983, Ziv 1989, Lim *et al*. 1998, Seon *et al*. 2000, Young *et al*. 2000, Kim *et al*. 2003). Meristemoid induction in liquid-shake cultures and bioreactors has been achieved with either IAA (Levin *et al*. 1997) or NAA (Ziv 1991a; Lipsky *et al*. 1997, Ziv and Lilien-Kipnis 1997).

For many species, somatic embryogenesis occurs as a two-step process — the formation of embryogenic callus on an auxin enriched medium (embryogenic induction) followed by culture (in either liquid or on solid medium) on a medium not containing auxin (embryo regeneration) (Strandberg 1993, Choudhary and Chin 1995, Van Boxtel and Berthouly 1996, Aberlenc-Bertossi *et al*. 1999, Kevers *et al*. 2000, Máthé *et al*. 2001).

In contrast, some researchers report that both induction and regeneration of somatic embryos occurs in the presence of an auxin (Bieniek *et al*. 1995, Büyükalaca and Mavituna 1995, Shimizu *et al*. 1997, Anbazhagan and Ganapathi 1999, Shibli and Ajlouni 2000, Barry-Etienne *et al*. 2002, Kosky *et al*. 2002). In the case of *Panax ginseng*, induction of embryogenic callus occurred on an auxin enriched medium while regeneration of somatic embryos took place on a cytokinin enriched medium (Kevers *et al*. 2000).

Embryogenic callus is sometimes induced on solid medium with auxins such as picloram (Saito and Nakano 2002), 2,4-D (Wang *et al*. 2001, Kosky *et al*. 2002), 2,4-D with BA (Zlenko *et al*. 2002), 2,4-D with kinetin (Shimizu *et al*. 1997), 2,4-D and NAA with kinetin (Shibli and Aljouni 2000), BSAA with kinetin (Kevers *et al*. 2002) although hormone-independent production of embryogenic callus has been reported for ginseng using $NH_4NO_3$ (Choi *et al*. 2003). In these cases, the induced embryogenic callus is transferred to an appropriate liquid culture system (e.g. liquid-shake or bioreactor) for regeneration of the embryos. As such, the role of the liquid culture is to provide the correct conditions for growth and maturation, that is to say, the culture is not used for inducing the embryogenic growth pattern but rather to foster development of the embryos.

Cytokinins

Cytokinins are essential plant growth regulators and are thus used extensively in tissue culture to direct growth and morphogenesis. In addition to their role in cell division, the ratio of cytokinin to auxin often determines if shoots, roots or callus will develop from a particular explant (Skoog and Miller 1957).

That cytokinins are crucial in producing and maintaining bud clusters or meristemoids in liquid culture, is evidenced by their almost universal use for this purpose. BA is most popular, and has been used to induce meristemoid clusters in *Gladiolus* (Ziv 1989, Lipsky *et al*. 1997), banana and potato (Levin *et al*. 1997), *Ornithogalum* (Ziv and Lilien-Kipnis 1997), lilies (Lim *et al*. 1998), *Crinum moorei* (Fennell 2002) and garlic (Kim *et al*. 2003). Takayama and Misawa (1983) used high concentrations of kinetin to produce bud clusters in *Lilium*. In some instances, sucrose and sorbitol promote meristemoid formation in conjunction with cytokinin (Mello *et al*. 2001). Cytokinins break apical dominance in members of the Liliaceae, Iridaceae and Amaryllidaceae, and consequently improve shoot multiplication (Hussey 1976). This response, may act in concert with the suppression of leaf elongation imposed by the liquid medium to induce meristemoid formation *in vitro*.

The requirement for cytokinins in somatic embryogenesis is less ubiquitous than the requirement for auxins, and depends on the stage of embryogenic development as well

as the species in question. In some species, the requirement for induction of embryogenic cells is absolute, while in others, cytokinins act to enhance the process by increasing the rate at which embryos regenerate. For the induction of embryogenic callus, iP has been used for coffee (Barry-Etienne *et al*. 2002), and BA for *Hevea brasiliensis* (Martre *et al*. 2001), black iris (Shibli and Ajlouni 2000) and dune reed (Wang *et al*. 2001). For embryo development and formation, kinetin has been used for cotton (Gawel and Robacker 1990), asparagus (Reuther 1996) and ginseng (Kevers *et al*. 2000, 2002).

In the case of oil palm, supplementation of the medium with BA resulted in somatic embryos having a complete embryonic axis (both root and shoot apices), whereas a unipolar root axis formed in the control (Aberlenc-Bertossi *et al*. 1999). It is thought that addition of cytokinins compensates for the detrimental effects of auxins on meristem development (Merkle 1995).

Gibberellins

In general, the gibberellins are little used in liquid culture, although Emons *et al*. (1993) report that adding gibberellic acid improves the maturation of maize somatic embryos in synchronised agitated suspension cultures if ABA is not effective. The development of plantlets from torpedo-stage embryos was enhanced in grape by using $GA_3$ in the liquid medium (Zlenko *et al*. 2002).

Ethylene

Ethylene accumulates in the headspace of culture vessels during the culture period (Bieniek *et al*. 1995) but little work has been conducted to investigate the regulatory effect of ethylene in liquid culture. Nissen (1994) reported that adding submicromolar concentrations of ethephon (an ethylene-releasing agent) and ACC (ethylene precursor) to carrot suspension cultures significantly increased somatic embryo production by increasing the concentrations of endogenous ethylene. Whether this resulted from ethylene dissolving in the liquid medium, with the effect being a consequence of both the presence of ethylene and the physical properties of the medium, or by another mechanism, was not discussed by the author. Embryo production was inhibited by AVG and AOA both of which block the synthesis of ACC, suggesting that ethylene is essential for somatic embryogenesis in carrot suspension cultures.

The effect of ethylene on somatic embryogenesis in other species has not been investigated, and it would be interesting to elucidate the role ethylene plays in this process. Does exogenously applied auxin stimulate ethylene production, thereby inducing somatic embryogenesis, or is it the auxin itself that results in a cell becoming embryogenic? Does ethylene stimulate embryogenesis in explants on solidified media, or is this phenomenon restricted to liquid culture?

Abscisic acid

ABA is well known for its role in stress responses, especially heat and drought, as well as regulating maturation of embryos (Karssen *et al*. 1983) and imposing dormancy in both seeds and buds (Hilhorst 1995). In liquid culture, ABA has been used in the later stages of embryo development for maturation in spruce (Moorhouse *et al*. 1996, Ingram and Mavituna 2000) as well as in maize, where it mimics the effects of sucrose (Emons *et al*. 1993). In garlic, ABA has been used to suppress shoot elongation and promote bulblet formation and growth in liquid-shake culture (Kim *et al*. 2003).

Other bioregulators and growth retardants

Embryo production in *Panax ginseng* was increased by supplementing the medium with polyamines or their precursors arginine and ornithine (Kevers *et al*. 2000). Supplementation with spermine and spermidine has also increased the frequency of somatic embryogenesis (Kevers *et al*. 2002). These authors suggest a shifting antagonistic-synergistic relationship between auxin and polyamines during different stages of somatic embryo production. Polyamines were detrimental in multiplication (maintenance) of embryogenic callus, while embryo initiation, regeneration and development were all enhanced by adding specific polyamines (Kevers *et al*. 2002). Hormone independent production of embryogenic callus has been achieved for *Panax ginseng* using $NH_4NO_3$, although somatic embryos were not actually produced (Choi *et al*. 2003). This situation was reversed when the callus was subcultured onto media containing hormones (Choi *et al*. 2003). $NH_4NO_3$ in combination with KCl improves somatic embryo formation in sweet potato (Bieniek *et al*. 1995). In contrast, $NH_4NO_3$ levels are reduced to enhance somatic embryogenesis in pepper (Büyükalaca and Mavituna 1995). Thus, it seems the effect of any exogenously applied bioregulator is often dependent on the species.

In *Narcissus triandrus* liquid cultures, supplementing the medium with jasmonic acid greatly increased both the induction and growth of bulblets (Santos and Salema 2000). Similar findings have been reported for garlic bulblets grown in temporary immersion systems (Kim *et al*. 2003).

In a number of cases, growth retardants are used in liquid cultures. The growth retardants chlorocholine chloride (CCC), B-9 and ABA have been found to be successful in increasing bulblet growth and formation in garlic (Kim *et al*. 2003). Ancymidol has been used to increase the mass of meristemoid clusters, as well as decreasing the proportion of necrotic areas in banana and potato cultures (Levin *et al*. 1997). Inhibition of shoot growth results in meristemoid formation in *Gladiolus* by paclobutrazol and daminozide (Ziv 1991a, Lipsky *et al*. 1997) and in aspen by ancymidol (Vinocur *et al*. 2000). In sugarcane, paclobutrazol supplementation results in significantly higher numbers of shoots per explant (Lorenzo *et al*. 1998, 2001).

Growth retardants act as gibberellin antagonists by blocking the biosynthetic pathway, consequently reducing shoot elongation and increasing shoot proliferation (Ziv 1989). This inhibition of leaf growth appears similar to that imposed by liquid medium on the fern *Nephrolepis* (Ziv 1991a) and may simply be a consequence of redirection of assimilates freely available in the medium.

### *Conclusions and Future Prospects*

The number of papers published in this field demonstrates the widespread use of liquid culture for research and poten-

tial commercial applications. Liquid cultured explants exhibit improved rates of growth (Kim *et al*. 2003) and multiplication (Sandal *et al*. 2001) compared to those on solidified media, because of the greater availability of water (Debergh 1983) and metabolites (Avila *et al*. 1996) made possible by decreased resistance to diffusion and closer contact between the explant and medium (Singha 1982, cited by Avila *et al*. 1996).

The induction of organogenic and embryogenic propagules is also a direct result of the physical conditions created by the aqueous environment. These growth patterns likely benefit from the decreased gradients in the medium, thus enabling better control by growth regulating factors. Furthermore, the entire surface of the explant can perceive chemical signals because it is bathed by the medium.

It must be borne in mind that these effects are only mediated in part by the medium. Consideration must be given to the culture conditions (light regime, temperature, pH and ventilation requirements) as well as to the various growth regulating substances (hormones, growth retardants and metabolites).

The production of meristemoids in liquid culture provides a rare opportunity to examine further this unique physiological effect that is regulated simply by the physical state of the medium. In addition, meristemoid production offers an alternative to somatic embryogenesis and microcuttings as a means of propagating plants *in vitro*.

Many workers have suggested that the greatest potential for liquid culture in the future is scaling-up for bioreactor production of the desired propagules (Aitken-Christie and Davies 1988, Bieniek *et al*. 1995, Choi *et al*. 2003). This could increase the production rate while substantially decreasing the operating cost, thus making it a commercially viable venture. For this to become a reality, however, much more fundamental research is required, in terms of optimising existing pilot studies, before this technology can be applied with success to economically important crops.

In future, liquid culture may be used as a tool in conjunction with molecular biology to investigate the regulation of several developmental pathways. For example, the inhibition of leaf growth by high concentrations of cytokinin or growth retardants results in meristemoid induction. Cells become reprogrammed as a consequence of changes in patterns of gene expression. Is there a 'master gene' at work and can we manipulate it? Similarly, meristemoids may develop either into shoots (Ziv 1989), storage organs (Ziv and Kahany 1995) or somatic embryos (Ziv 2002).

An important aspect of geophyte micropropagation is the induction and growth of a storage organ *in vitro*. Molecular approaches may shed light on how this process is directed since a number of factors, including high levels of sucrose (Lipsky *et al*. 1997) and temperature (De Bruyn and Ferreira 1992), stimulate storage organ formation.

The intriguing differences between somatic embryos initiated in liquid or solid media (Jayasankar *et al*. 2003) raises questions concerning the effect of the liquid medium on the growth and development of explants. A switch in the pattern of gene expression in Norway spruce, for example, leads to the development of the protoderm in somatic embryos (Von Arnold *et al*. 2002b) while in sunflower, the expression of $Ca^{2+}$ channel proteins appears to determine cell fate (Petitprez *et al*. 2002). Substantial variation exists for direct regeneration of somatic embryos from various tissues, suggesting this developmental process is genotype-dependent (Bolandi *et al*. 2000, cited by Petitprez *et al*. 2002).

Production and release of secondary metabolites in liquid culture generally occurs from differentiated tissues. What features of an organised system contribute to the induction of these often-complex biosynthetic pathways, and can they be manipulated to alter the quantity and timing of production of these valuable compounds?

An international symposium on liquid systems for the *in vitro* mass propagation of plants was held in Norway in 2002, and considering the potential for future research, this is likely to be the first of many. Although much work has been done, little is known about the environmental and genetic control of these various phenomena occurring in liquid culture. Liquid culture systems therefore offer exciting opportunities for research. Once the fundamental principles governing each system have been established, it may then be possible to exploit the technology for commercial purposes.

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