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Simple bioreactors for mass propagation of plants

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Abstract

Bioreactors provide a rapid and efficient plant propagation system for many agricultural and forestry species, utilizing liquid media to avoid intensive manual handling. Large-scale liquid cultures have been used for micropropagation through organogenesis or somatic embryogenesis pathways. Various types of bioreactors with gas-sparged mixing are suitable for the production of clusters of buds, meristems or protocorms. A simple glass bubble-column bioreactor for the proliferation of ornamental and vegetable crop species resulted in biomass increase of 3 to 6-fold in 3–4 weeks. An internal loop bioreactor was used for asparagus, celery and cucumber embryogenic cultures. However, as the biomass increased, the mixing and circulation were not optimal and growth was reduced. A disposable pre-sterilized plastic bioreactor (2–5-l volume) was used for the proliferation of meristematic clusters of several ornamental, vegetable and woody plant species. The plastic bioreactor induced minimal shearing and foaming, resulting in an increase in biomass as compared to the glass bubble-column bioreactor. A major issue related to the use of liquid media in bioreactors is hyperhydricity, that is, morphogenic malformation. Liquid cultures impose stress signals that are expressed in developmental aberrations. Submerged tissues exhibit oxidative stress, with elevated concentrations of reactive oxygen species associated with a change in antioxidant enzyme activity. These changes affect the anatomy and physiology of the plants and their survival. Malformation was controlled by adding growth retardants to decrease rapid proliferation. Growth retardants ancymidol or paclobutrazol reduced water uptake during cell proliferation, decreased vacuolation and intercellular spaces, shortened the stems and inhibited leaf expansion, inducing the formation of clusters. Using a two-stage bioreactor process, the medium was changed in the second stage to a medium lacking growth retardants to induce development of the meristematic clusters into buds or somatic embryos. Cluster biomass increased 10–15-fold during a period of 25–30 days depending on the species. Potato bud clusters cultured in 1.5 l of medium in a 2-l capacity bioreactor, increased during 10–30 days. Poplar *in vitro* roots regenerated buds in the presence of thidiazuron (TDZ); the biomass increased 12-fold in 30 days. Bioreactor-regenerated clusters were separated with a manual cutter, producing small propagule units that formed shoots and initiated roots. Clusters of buds or meristematic nodules with reduced shoots, as well as arrested leaf growth, had less distortion and were optimal for automated cutting and dispensing. In tuber-, bulb- and corm-producing plants, growth retardants and elevated sucrose concentrations in the media were found to enhance storage organ formation, providing a better propagule for transplanting or storage. Bioreactor-cultures have several advantages compared with agar-based cultures, with a better control of the contact of the plant tissue with the culture medium, and optimal nutrient and growth regulator supply, as well as aeration and medium circulation, the filtration of the medium and the scaling-up of the cultures. Micropropagation in bioreactors for optimal plant production will depend on a better understanding of plant responses to signals from the microenvironment and on specific culture manipulation to control the morphogenesis of plants in liquid cultures.

Abbreviations: ANC – ancymidol; APX – ascorbate peroxidase; BA – benzyl aminopurine; CAT – catalase; DW – dry weight; FW – fresh weight; 2iP-2 – isopentenyladenine; PAC –paclobutrazol; ROS – reactive oxygen species; TDZ – thidiazuron

Introduction

Micropropagation is currently applied to a large number of agricultural and forestry species, but is still costly due to intensive manual manipulation throughout the various culture phases. Automation and scaled-up liquid cultures for *in vitro* plant propagation are essential to overcome some of the limitations imposed by labour-intensive methods and high production costs. Progress in tissue culture automation depends on the use of liquid cultures in bioreactors that can provide rapid proliferation, mechanized tissue cutting and separation, and automated dispensing (Aitken-Christie et al., 1995; Ziv, 1995a).

Liquid cultures have been used for plant culture in both agitated vessels and in bioreactors, for somatic embryogenesis (Styer, 1985; Nadel et al., 1990; Preil, 1991; Scragg, 1992) and as a system for plant propagation through the organogenic pathway (Takayama, 1991; Ziv, 1992). The propagation aspects of several plant species in bioreactors and some of the problems associated with the operation of bioreactors were reviewed by Ziv (1995b, 2000) and Takayama and Akita (1998).

Propagation in bioreactors through the organogenic pathway has been achieved in banana, Boston fern, *Spathiphyllum*, strawberry, potato, poplar, coffee, *Gladiolus*, lilies, pineapple, orchids, *Ornithogalum, Nerine, Narcissus* and *Cyclamen* (Ziv, 2000).

In various types of bioreactors, the mechanical or gas-sparged mixing provided stirring and aeration (Scragg, 1992). Large-scale cultivation of plant cells, embryos or organs has made use of airlift or bubble-column bioreactors instead of stirred-tank bioreactors, due to their low shear properties. The main advantages of airlift bioreactors is their relatively simple construction, the lack of regions of high shear stress, reasonably high mass and heat transfer and reasonably high yields at low input rates (Denchev et al., 1992). However, bioreactor configuration must be determined according to mixing and aeration requirements of specific plants or tissues, as well as for minimization of shear stress.

In general, bioreactor-culture offers many advantages, including better control of the culture conditions; optimal supply of nutrients and growth regulators; renewal of the culture atmosphere; changing the medium during the culture period according to the developmental stage; filtration of the medium for exudates; contamination control; and production of clusters of buds or somatic embryos for automated handling of the propagules.

The use of liquid cultures in bioreactor for plant propagation imposes several problems such as leakage of endogenous growth factors, the need for an initial high concentration of the inoculum, hyperhydricity and malformation, foam development, shearing and oxidative stress (Ziv, 2000). The major disadvantage encountered is the problem of shoot malformation in liquid media (Ziv, 2000). Attempts to control hyperhydric deformities have focused on better aeration and intermittent plant submergence in the medium, using temporary immersion bioreactors (Teisson et al., 1996; Escalona et al., 1999). Levin (personal communication) used two disposable plastic bioreactors (Life Reactor, Osmotek) as an ebb and flow system for intermittent submergence in micropropagated *Spathiphyllum*. Growth retardants (Ziv, 1992, 2000) have been used to reduce leaf expansion, thus minimizing shoot malformation.

The present paper reviews the use of simple bioreactors for micropropagation and describes the advantages and limitation of bubble-column and airlift bioreactors used for plant proliferation.

Morphogenesis and growth in bioreactors

At present, commercial propagation of most plants is carried out through the organogenic pathway in agar-gelled cultures, even though the protocols are long and costly. The commercial use of bioreactors for unipolar structures such as protocorms, buds or shoots is limited to a small number of plants, mainly due to the hyperhydricity problem in the leaves and shoots. Controlling

shoot growth and providing culture conditions that reduced abnormal leaves and enhanced formation of bud or meristematic clusters in potato, *Gladiolus* and *Ornithogalum dubium*, by use of growth retardants, resulted in a high proliferation rate, and limited hyperhydricity (Takayama, 1991; Ziv and Hadar, 1991; Ilan et al., 1995; Levin et al., 1997; Takayama and Akita, 1998; Ziv et al., 1998; Ziv, 2000).

The development of spherical meristematic or bud clusters in liquid cultures provided a highly proliferating and rapidly growing system. The clusters were amenable to the control of the medium components, to mechanical separation, and to automated inoculation as an efficient delivery system to the final stage for plant growth (Levin et al., 1997; Ziv et al., 1998). In most species, cluster formation appears to be associated with the continuous submergence, circulation and agitation of the plant biomass in the medium, as well as with a balanced ratio of growth-promoting and growth-retarding regulators. The formation of condensed organized structures, in which the shoots are reduced to buds or meristematic tissue in liquid media, has been reported for several plant species. The clusters were made-up of densely-packed meristematic cells, actively dividing and forming new meristemoids on the outer surfaces. The meristemoids surrounded loosely-packed cells in the centre and exhibited some vascularization as was shown in liquid cultured poplar clusters (McCown et al., 1988). In banana, on the other hand, the clusters were made-up of condensed buds surrounding a central core with a cavity (Ziv et al., 1998). Protocorm-like clusters were also induced in liquid-cultured *Gladiolus* buds (Ziv, 1990) and in several species of the complex *Brodiaea* (Ilan et al., 1995) by addition of PAC or ANC (gibberellin biosynthesis inhibitors) to the medium. In *Phalaenopsis*, protocorm-like bodies were proliferated in bioreactors and regenerated on agar-based medium (Young et al., 2000). In potato and banana (Figure 1), bud clusters were induced by a balanced ratio between kinetin and ancymidol in the liquid medium (Ziv et al., 1998). In woody species, McCown et al. (1988) and Aitken-Christie et al. (1995) described nodules in poplar in liquid medium and in radiata-pine in agar-gelled medium, respectively, induced by a balanced ratio of growth regulators. Levin et al. (1997) worked with several ornamental species and described a several-fold increase of an organogenic biomass consisting of clusters, which proliferated in bioreactors. These were separated mechanically prior to dispensing to agar-gelled cultures for further growth. The production of clusters in *Philodendron* cultured in liquid media

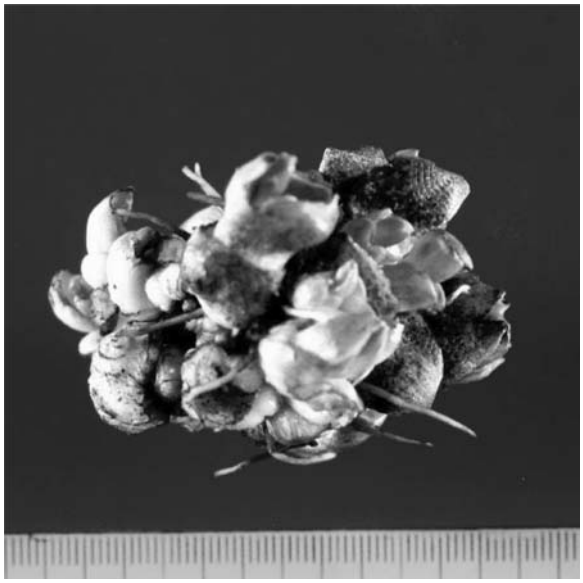

Figure 1. A cluster of banana buds from a bubble-glass-bioreactor (9.75 μmol ancymidol, 22.2 μmol BA and 11.4 μmol IAA).

required the presence of BA and an inductive treatment for 24–48 h with ancymidol. The short inductive treatment annulled a carry-over dwarfing effect of ancymidol on leaf and shoot development after transplanting of the clusters to agar-gelled medium for further plant growth (Ziv and Ariel, 1991). Growth regulators, promoting or retarding substances when supplied in a specific balanced ratio, apparently act as morphogenic signals and control the development of the spherical meristematic or bud clusters.

Types of bioreactors

Various types of simple bioreactors with mechanical or gas-sparged mixing were used in plant and cell cultures to provide stirring, circulation and aeration (Takayama, 1991; Scragg, 1992; Takayama and Akita, 1998, Ziv 2000). Mechanically-stirred bioreactors depend on impellers, including a helical ribbon impeller (Archambault, 1994), magnetic stirrers or vibrating perforated plates. Aeration, mixing and circulation in bubble-column or airlift bioreactors, is provided by air entering the vessel from the side or from the basal opening through a sparger. As the air bubbles rise they lift the plant biomass and provide the required oxygen (Styer, 1985; Merchuk, 1990, Cazzulino et al., 1991; Preil, 1991).

It has been shown that mixing by gas sparging in bubble-column or airlift bioreactors lacking impellers or blades, was far less damaging for clusters than mechanical stirring, since shearing stress was minimized (Ziv and Hadar, 1991; Ilan et al., 1995; Ziv and Shemesh, 1996). The use of plastic disposable airlift bioreactors for bud or meristematic clusters was found to increase proliferation and reduce shearing stress (Ziv et al., 1998).

The main advantage of airlift bioreactors is their relatively simple construction, the lack of regions with high shearing potential, reasonably high gas and nutrient transfer rates and relatively high yields at low input rates (Denchev et al., 1992). A bubble-free oxygen supply bioreactor with silicone tubing was found suitable for embryogenic cell suspensions and provided foam-free cultures (Luttman et al., 1994). For hairy root cultures an acoustic mist bioreactor was found to increase root biomass significantly (Chatterjee et al., 1997). Simple glass, polycarbonate cylinder (Figure 2), or plastic bioreactors were used for propagation of ornamental, vegetable and woody plant species. These included the foliage plants – Boston fern, *Philodendron* and *Spathiphyllum*; the ornamentals – *Gladiolus*, lilies, *Ornithogalum dubium*, *Narcissus, Nerine*, *Brodiaea;,* and the vegetable crops – cucumber, asparagus and potato.

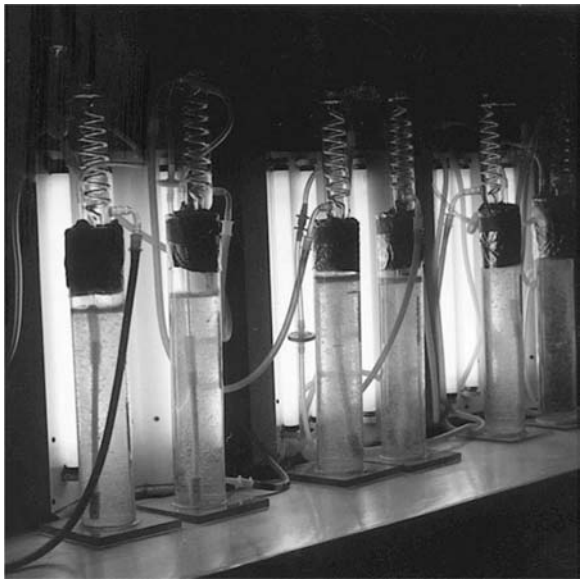

Figure 2. A polycarbonate internal-loop bioreactor used for asparagus embryogenic clusters.

Poplar root cultures regenerating buds, and banana were also propagated in bioreactors. In banana, the type of culture and the volume of the medium affected biomass growth (Table 1). Increasing the concentration of ancymidol decreased biomass growth, increased bud or meristem number per cluster and decreased leaf elongation and expansion (Table 2).

Temporary immersion – or ebb and flow bioreactors – were described as a better aeration system through the periodic immersion and exposure to the gaseous phase (Etienne et al., 1997). Temporary immersion was used for banana, rubber and coffee (Teisson and Alvard, 1995; Teisson et al., 1996; Etienne et al., 1997), for tea (Akula et al., 2000) and on a much larger scale for pineapple and sugarcane (Lorenzo et al., 1998; Escalona et al., 1999). Temporary immersion was found to enhance potato microtuber growth in a rotating bioreactor (Yu et al., 2000). The periodic immersion cycle adapted to each species was found to decrease hyperhydricity, but not eliminate it altogether.

The gaseous atmosphere

The culture vessel gas composition is influenced by the volume of the vessel, the volume of the medium and the ventilation. In bioreactors, control of the gaseous phase depends on the gas flow and can be easily manipulated to provide the required concentrations of O_2, CO_2 and C_2H_4. In airlift or bubble-column bioreactors, the air supplied is used for both mixing and aeration. The importance of aeration and the gaseous phase were shown in potatoes cultured in airlift bioreactors. Induction of tubers was inhibited under continuously submerged conditions. Microtubers developed only after the shoots elongated and reached the gaseous phase. A two-phase culture, substituting the growth medium with a tuber-induction (9% (w/v) sucrose) medium, enhanced tuber formation from shoots which developed above the medium and were exposed to the gaseous phase (Akita and Takayama, 1994).

Oxygen concentrations in liquid cultures depend on the presence of O_2 in the gas phase above and in the air bubbles inside the medium, as well as in the dissolved O_2 in the medium. Air is sparged through a sparger located at the base of the bioreactor. Oxygen requirements may vary from one species to another, and concentration of O_2 in liquid cultures in bioreactors can be regulated by agitation or stirring and through aeration, gas flow and air bubble size.

Increasing O_2 concentrations, in the sparging air, from 21 to 80% (v/v), in bioreactor cultures of

Table 1. The effect of culture type and medium volume on banana bud-cluster proliferation in the presence of 2.5 ppm ancymidol after 30 days

Culture vessel capacity (ml)	Medium (volume ml)	FW (g)		Growth value[a]	No. buds per cluster
		Initial	Final		
Erlenmeyer 250	50	6.5 ± 0.7	17.5 ± 1.8	1.46	5.6 ± 0.6
Erlenmeyer 500	100	10.8 ± 1.2	57.2 ± 3.1	4.29	6.7 ± 0.8
Glass bioreactor 1000	500	17.6 ± 2.8	123.5 ± 2.1	6.01	12.0 ± 1.8
Disposable bioreactor 2500	1250	21.6 ± 2.8	269.4 ± 4.8	11.4	15.1 ± 2.1

[a]Growth value: $(FW_{final}-FW_{initial})/FW_{initial}$.

Table 2. The effect of ancymidol on banana-cluster proliferation in disposable plastic bioreactors after 26 days culture

Ancymidol (μmol)	ΔGrowth[a] (FW g)	Buds or meristems per cluster	Leaf tissue (% FW)
0	172 ± 16	12 ± 0.8	89
3.95	165 ± 12.2	18 ± 2.1	86
9.75	111 ± 9.7	26 ± 3.1	58
19.50	80 ± 5.3	48 ± 3.9	17

[a]Initial inoculum 30 g l^{-1} medium.

Boston fern clusters, enhanced growth values (final FW−initial FW/initial FW) from 0.61 to 0.92 (Ziv and Hadar, 1991). Reducing O_2 concentrations to 10% (v/v) affected cell differentiation in bioreactor cultures of carrot embryogenic tissue. Under these conditions embryo production was severely inhibited (Jay et al., 1992).

There are reports that high aeration rates, rather than excessive oxygen concentrations, inhibit growth and that reduced growth could be due to depletion of CO_2 or to the removal of various culture volatiles, including CO_2 (Hegarty et al., 1986). CO_2 enrichment in an illuminated bioreactor culture of *Brodiaea* clusters did not affect biomass growth. Increasing CO_2 from 0.3 to 1% (v/v) gave a similar growth value, 4.2 and 5.3, respectively, under the two CO_2 concentrations and 135 μmol $m^{-1}s^{-1}$ light intensity (Ilan et al., 1995). In *Cyclamen persicum* high CO_2 concentrations correlated with increased production of pro-embryogenic masses (Hvoslef-Eide and Munster, 1998).

Mineral nutrients consumption

In bioreactors, in which either humidified air or condensers are used to prevent dehydration, the concentration of the nutrients in the medium is affected mainly by absorption rate and by cell lysis (Archambault et al., 1995). Differentiation and proliferation of micropropagated fern, *Gladiolus*, and *Nerine* nodular clusters in bioreactors was better on half-strength, rather than on the full-strength, Murashige and Skoog (1962)-minerals (Ziv, unpublished). This was also true for *Lilium* bulblets differentiating on bulb scales, which were cultured in bioreactors (Takayama, 1991). A drop in pH to 4.5 and lower values and the subsequent increase to pH 5.5 were attributed to the initial utilization of ammonium and to the uptake of nitrate at a later stage.

The composition of minerals, monitored during the culture of *Brodiaea* in liquid media changed, with phosphate, ammonia, nitrate and potassium depleting faster and prior to the depletion of Ca^{++} and Mg^{++} (Ilan et al., 1995). In general, biomass growth is limited by the availability of phosphate, nitrogen and carbohydrates, and to a lesser extent by the availability of calcium, magnesium and other ions.

Carbohydrate supply and utilization

Sucrose, and to a lesser extent glucose, fructose, or sorbitol, are the most commonly used carbohydrates *in vitro*. In general, sucrose is removed rather rapidly from the medium and after 10–15 days the sucrose can be completely depleted or reduced to 5–10 g l^{-1} from an initial concentration of 30 g l^{-1} in both agar-gelled and liquid cultures. At the same time glucose and fructose that appear in the medium due to sucrose hydrolysis increases in the presence of invertase in the culture medium, and can reach concentrations of 5–10 g l^{-1}. In embryonic suspension cultures of celery, the addition of mannitol reduced cell lysis and enhanced somatic embryogenesis. When 40 g l^{-1} mannitol was added, a higher number of embryos was produced and the frequency of singulated normal embryos was increased (Nadel et al., 1990).

The biomass of Boston fern meristematic clusters in a bubble-column bioreactor was increased with the increase in sucrose concentrations from 7.5 to 30 g l^{-1}, while higher concentrations caused a decrease in cluster growth. Elevated sucrose concentrations in the medium caused a decrease in the clusters size and leaf chlorophyll content (Ziv and Hadar, 1991). *Gladiolus* clusters cultured in the presence of growth retardants had a higher concentration of starch – 845 as compared to 585 mg g^{-1} DW in the control (Ziv, 1992). Potato microtubers grew at a faster rate in a rotating bioreactor when the medium was replaced frequently and the number of tubers >1 g increased 4-fold when 8% (w/v) sucrose was used (Yu et al., 2000).

Growth regulator effects

The availability of growth regulators in bioreactor cultures can be more effective in controlling the proliferation and regeneration potential than in agar cultures, due to the direct contact of plant cells and aggregates with the medium.

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

an elevated concentration of phosphate enhanced bulblet growth in *Nerine* (Vishnevetsky et al., 2000). Excised aspen roots cultivated in liquid medium in bioreactors in the presence of thidiazuron (TDZ) regenerated buds on the entire root surface, as compared to BA-treated root explants, in which the buds regenerated only in close proximity to the site of initiating lateral roots. Roots cultured in the presence of ancymidol developed clusters that were separated mechanically and developed normal shoots on a rooting medium (Vinocur et al., 2000).

Since one of the major problems in liquid cultured plants is malformation of shoots and hyperhydricity, the induction of meristematic or bud clusters with arrested leaf growth (McCown et al., 1988; Ziv, 1991; Ziv and Shemesh, 1996) was one of the solutions to reduce hyperhydricity. The use of relatively high cytokinin concentrations or growth retardants, which inhibit gibberellin biosynthesis, was the most effective method to reduce shoot and leaf growth and to promote the formation of meristematic clusters (Ziv, 1990). In *Spathiphyllum,* addition of PAC decreased biomass growth (Figure 3). The removal of PAC after subculture, enhanced growth in cultures treated with the highest concentrations of growth retardant (Figure 4). However, even after the removal of PAC on subculture, shoot development was inhibited in particular in cultures that were treated with high concentrations of the retardant (Figure 5) (Shalev N. M.Sc. Thesis in Hebrew).

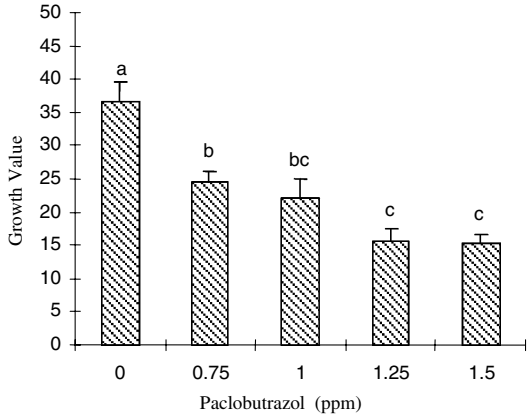

Figure 3. The effect of paclobutrazol on the growth of *Spathiphyllum* in liquid culture.

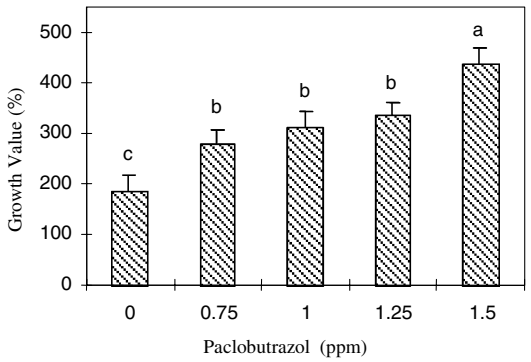

Figure 4. Clusters growth of *Spathiphyllum* after subculture to a medium lacking paclobutrazol following a pre-treatment with the growth retardant.

Shearing and oxidative stress

In many plants cultivated in bioreactors, the continuous aeration, mixing and circulation cause shearing damage and cell wall breakdown. The cell debris adheres to the vessel and causes foaming that prevents adequate liquid circulation and oxygen supply. Foaming was reduced when half-strength MS medium minerals were used (Ziv, 1995b) and by lowering the concentration of calcium in the medium (Takayama, 1991). Disposable plastic bioreactors with a volume of 2 and 5-l capacity used for organogenic micropropagation were found to provide good circulation with reduced cell shearing, cell damage and foaming. Meristematic and bud clusters are less shear-sensitive than large, vacuolated cells (Ziv et al., 1998).

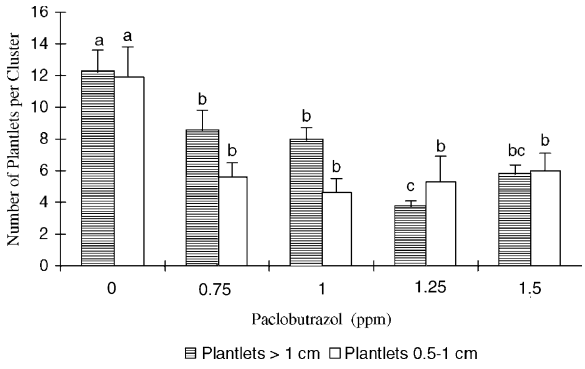

Figure 5. Shoot and bud growth of *Spathiphyllum* clusters in paclobutrazol-free medium after a pre-treatment with the growth retardant.

The excessive accumulation of water in plant tissue (the most characteristic symptom of hyperhydricity) can result in oxygen depletion in the cells, induce oxidative stress, production of reactive oxygen species (ROS) and cause injury to the plant tissue. Several developmental processes in tissue cultured plants can be affected by ROS leading to recalcitrance and loss of morphogenetic competence (Benson, 2000). ROS such as H_2O_2 and hydroxyl free-radicals react instantaneously with almost any substrates (Levine, 1999).

In *Narcissus* liquid cultures, antioxidant enzyme activities were found to correlate with hyperhydric shoots and leaf section explant malformation (Chen and Ziv, 2001). In ancymidol (ANC) treated hyperhydric *Narcissus* shoots, ascorbate peroxidase (APX) and catalase (CAT) activities were significantly greater than in their non-treated, non-hyperhydric counterparts. In the ANC-treated hyperhydric *Narcissus* leaf sections, APX and CAT activities were significantly less than in the non-hyperhydric ones cultured in medium lacking ANC, especially during the period of meristematic centre formation.

The formation of meristematic centres on ANC-treated hyperhydric leaf sections during the 3rd and 4th weeks in culture, could have resulted from lower H_2O_2 concentrations than in non-treated ones. Lower concentrations of H_2O_2 in ANC-treated hyperhydric leaf sections may cause a lower hydroxyl free-radical accumulation and therefore ANC-treated leaf sections may have the ability to develop meristematic centres.

Conclusion

The understanding of the signals and mechanisms that control organogenesis and somatic embryogenesis in liquid media will greatly advance the use of bioreactors for commercial micropropagation. The immediate microenvironment, both chemical and physical factors, is the major factor involved in the control of normal morphogenesis of plants. The physiological status of the plant tissue will affect cell interaction with the environmental signals and will determine developmental events, proliferation and growth. Further basic and applied research can provide the information necessary for an efficient and economic use of bioreactors for plant propagation.

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