Chapter 8

Cost-effective mass cloning of plants in liquid media using a novel growtek bioreactor

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Abstract: A low-cost Growtek bioreactor has been designed, patented and commercialised. It has unique features such as a floating and rotating explant-holder with perforated explant support and a side tube for medium changing, culture feeding and for content monitoring. The bioreactor can be operated both in static and agitated modes. Extensive performance studies have been conducted using representatives of trees (*Santalum album*), commercial ornamentals (*Dendranthema grandiflora*), monocotyledonous horticultural species (*Ananas comosus*), tuber crops (*Solanum tuberosum*) and a medicinal plant (*Catharanthus roseus*). In comparison to propagation in agar-gelled media as well as in liquid media using other culture vessels, this bioreactor exhibited 1.2 – 23.3 times shoot production, minimised root injuries by 32 – 48 %, reduced contamination by 12 – 18 % and reduced incubation time by 16- 42 %. Thousands of *Ananas comosus* plantlets raised in this bioreactor have been field tested. Additionally, it was found to be effective for hairy root culture of *C. roseus*.

Key words: *Chrysanthemum, Catharanthus,* cost-effectiveness, Growtek bioreactor, liquid medium, mass cloning, pineapple, potato, *Santalum*

Abbreviations: BAP- 6-benzylaminopurine; GA- gibberellic acid; GI- growth rate; IAA- indole-3-acetic acid; IBA- indole-3-butyric acid; MS- Murashige and Skoog' s (1962) medium; NAA- naphthalene acetic acid

1. Introduction

The industrial production of tissue cultured plants has largely been dominated by herbaceous ornamental species and a few vegetable, fruit or plantation crops (banana, oil palm etc.). The success with woody and semi-woody plants has been rare (Smith, 1997; Gupta et al., 1993). The high cost

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of production (~ US$ 0.10 – 0.15 per unit) owing to the labour-intensive nature (labour cost may be 50-85 % of production cost), prejudicing economic viability, was the single most important reason that discouraged *in vitro* industrial propagation of many species (Vasil, 1994; Goldstein, 1999). Plant tissue culture was practised initially with agar-gelled media. It was soon realized that agar was one of the costliest ingredients in the medium, though not a nutrient. Many gelling and non-gelling matrices were tested in order to achieve cost-effectiveness, by substituting agar (Sorvari, 1986; Henderson and Kinnersley, 1988; Bhattacharya et al., 1994). Subsequently, the use of liquid media, scale-up in bioreactors (Preil, 1991; Takayama, 1991; Das et al., 1999) and induction of automated production were some of the alternatives explored for the minimization of cost of production through improvement in propagation efficiencies (Tisserat, 1991; Smith and Spomer, 1995; Hvoslef-Eide and Melby, 2000; Dey, 2001). The prospects for temporary immersion have also been discussed (Etienne et al., 1997; Jimenez et al., 1999). The other aspects of cost minimisation are the use of low-cost culture vessels, prevention of contamination, improved quality of plantlets and their enhanced field survival. The successful adaptations of these alternatives may also enhance the scope for commercial exploitation of somatic embryogenesis, plant secondary metabolite production (Curtis and Emery, 1993; Hunter and Kilby, 1999) and heterologously-expressed healthcare products of human origin (Doran, 2000; Meyer et al., 2002). The recent attempts at the production of such new generation products as plantibodies (Peeters et al., 2001; Stoger et al., 2002) are indicators of further need for developing the most cost-effective bio-processes based on plant cell and tissue culture in liquid media. The use of liquid media in these cases will offer benefits of increased nutrient uptake, greater availability of dissolved oxygen, easier dispensing, automated scale up and process control, periodic sampling and more productivity.

Our laboratory has been working for more than a decade on cost-minimisation aspects through novel bio-process (Indian patent application No. 197/Cal/2001), product (Bhattacharya et al., 1994) and equipment development. This article describes the performance of the novel Growtek bioreactor for mass cloning of several commercially-important plants.

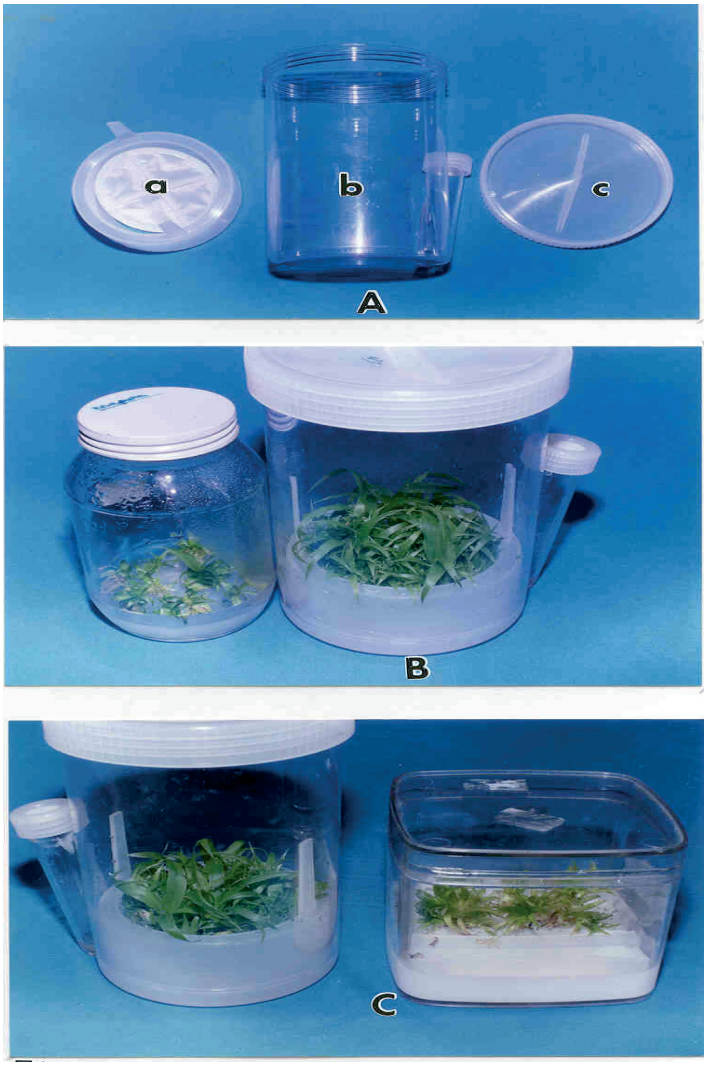

Figure 1: (A): Growtek bioreactor with floating explant-holder (a) main vessel with side tube (b) inside of the lid (c) shown separately; (B): pineapple shoot cluster production in glass jars and Growtek; (C): pineapple shoot cluster propagation in Life Guard and Growtek.

2. Materials and methods

2.1 Culture vessels

Growtek bioreactor (Indian patent No. 183604/2000), Life Guard culture box (107 X 107 X 96 mm h; Sigma Cat. No. C8062) with a Life Raft membrane raft (Sigma M7413), Magenta GA-7 (77 X 77 X 97 mm h; Sigma V8505, Phytacon (140 X 140 mm h; Sigma), Erlenmeyer flasks (250 ml) and locally available glass jars with metallic threaded caps (76 X 114 mm h) were used. Embryogenic calli of *Santalum album* were raised in borosilicate culture tubes without a rim (32 X 200 mm h).

The Growtek bioreactor (Figure 1 A) has unique features including a floating, rotating, non-absorbing explant-holder with perforated (a) explant-support matrix; a side–tube with silicon rubber septum for changing media and online monitoring of the medium environment (pH, dissolved oxygen, temperature etc.), a lid with a central and downwardly projected slope (c) for minimising condensate accumulation inside; and a polycarbonate body (b) with perfect transparency that will be satisfactory for up to 80 autoclave cycles.

Growtek was used in both static and agitated modes (at 100 rpm on a rotary shaker) for pineapple and *Chrysanthemum* shoot multiplications.

2.2 Plants and culture conditions

Extensive performance studies have been conducted using representative species of trees (sandalwood, *Santalum album* L. IITBT 08), commercial ornamentals (*Chrysanthemum*: *Dendranthema grandiflora* Tzvelev, cv. Birbal Sahni), monocotyledonous horticultural species (pineapple, *Ananas comosus* L. Merr., cv. Queen), tuber crops (potato, *Solanum tuberosum* cv. Kufri Jyoti), and a medicinal plant (Madagaskar periwinkle, *Catharanthus roseus* L. G. Don., var. pink).

The following optimised phytohormone doses were used in MS media (Murashige and Skoog, 1962) with 3 % (w/v) sucrose. Sandalwood: 4.44 µmol BAP, 1.14 µmol IAA, 0.58 µmol GA; *Chrysanthemum*: 0.88 µmol BAP, 0.57 µmol IAA; pineapple: 26.6 µmol BAP, 1.07 µmol NAA; potato: 4.44 µmol BAP, 1.14 µmol IAA; periwinkle hairy root culture: hormone-free.

The quantity of inoculum used for each vessel was as follows. Sandalwood: 1 g (fresh weight) embryogenic callus mass for embryo maturation and 400 somatic embryos for a root injury study; pineapple: 5

shoot clusters, each of about 500 mg; periwinkle hairy roots: 500 mg fresh weight; potato and *Chrysanthemum*: 5 nodal segments.

All cultures have been maintained and mass produced during several years in the optimised media. In order to retain their satisfactory performance over a reasonable period of time, a regular rotation of subculture between gelled and liquid media was followed, as in the following schedules.

Multiple shooting in pineapple, *Chrysanthemum* and potato: 2 subcultures in gelled medium followed by 4 in liquid medium for mass-production; somatic embryogenesis in sandalwood: 2 in gelled and 8 in liquid; periwinkle hairy roots: 2 in gelled and 10 in liquid. Each subculture was of 3-4 weeks duration. Performances reported here are for liquid subculture stages, in comparison to growth in gelled media as mentioned in data tables.

The other physical conditions were as follows. Media (50 ml in each vessel) were autoclaved at 104 kPa (121 ^{0} C) for 15 min. The pH of the medium was adjusted to 5.7 $\pm$ 0.1 before autoclaving. Incubation was in culture racks maintained at 25 $\pm$ 2 ^{0} C., 60-70 % relative humidity and at 16 h day length (47 µmol m^{-2} s^{-1}) provided by timer-controlled cool white fluorescent lamps. Periwinkle hairy roots were kept dark. Unless otherwise mentioned, the agar gel concentration for media for all purposes was 0.7 % (w/v) and for some somatic embryo germination it was 1.0 % (w/v).

For pineapple shoot production and sandalwood embryogenesis in liquid media, pH was always maintained in the range 4.8 – 5.8 and 5.2 – 5.8 respectively (by monitoring and control through the side tube) for consistent performance of the cultures. Change of medium between rooting and *in vitro* hardening was done through the side tube.

2.3 Growth rate (GI)

The growth rate was calculated on a dry weight basis from the following relationship-

$$GI = \frac{\text{Final biomass weight} - \text{initial biomass weight}}{\text{Initial biomass weight}}$$

The biomass was lyophilised before measuring dry weight.

2.4 Root injury index

Root injury was calculated during uprooting of plantlets/somatic seedlings from agar-gelled media (somatic embryos were 10 – 15 mm long; and rooted shoots had 4 – 6 roots). Even if the plant had only one damaged root, it was classified as 'damaged'. To determine whether or not a root was injured, observations were made using a dissecting microscope.

$$\text{Root injury \%} = \frac{\text{Number of plantlets with injured roots x 100}}{\text{Total number of plantlets}}$$

2.5 Rooting, hardening and field cultivation of pineapple

Growtek was extensively used for pineapple mass propagation that led to field trials. Rooting of pineapple shoots was achieved in 10 days (nearly 100%) in half-strength MS macro-element solution (with full strength microelement solution and 2% (w/v) sucrose) supplemented with 9.8 µmol IBA. Rooted pineapple plantlets (6-8 cm long) were hardened first *in vitro* in a Growtek for one week (in photoautotrophic mode, in quarter-strength MS macroelements and full-strength microelement solutions without sucrose and other organic supplements) and then in a greenhouse (Figure 2, C) for one month before field transfer. Plantlets were transferred to perforated polythene pots filled with sand and vermicompost (1:1). The potted plantlets were kept in sunlit greenhouse (relative humidity 75-85 %) racks, shaded partially by agronet. In the first week, 75% sunlight shading was achieved, followed by 50% shading and no shading in the 2nd and subsequent weeks, respectively. Field cultivation (Figure 2 D) was conducted in the Science & Technology Entrepreneurship Park of IIT-Kharagpur (www.stepiitkgp.com). Field survival was nearly 100%: it eventually resulted in normal fruiting.

2.6 Cost analysis for culture vessels

For Life Guard and temporary immersion systems (TIS) prices are taken from the product list of Sigma Chemical Co., USA and for Growtek from Tarsons Products, India (2002). The relative cost factor is calculated on the basis of the Growtek price.

Table 1: Efficiencies of different culture vessels for multiple shoot production of pineapple and *Chrysanthemum* in liquid media

Vessel	Number of shoots produced in 4 weeks			
	Pineapple		*Chrysanthemum*	
	Static	Agitated	Static	Agitated
Glass jar or Erlenmeyer flask (gelled medium)	14.5 ± 1.7	-	27.8 ± 1.7	-
Life Guard	39.1 ± 3.2	-	29.2 ± 2.3	-
Growtek	310.0 ± 6.2 (21.4)	338.1 ± 7.3 (23.3)	33.4 ± 2.1 (1.2)	46.3 ± 2.2 (1.7)

Data are means ± S.E. for 15 replicates for each vessel type. Figures in parentheses indicate fold of multiplication in comparison to glass jar.

Table 2: Performances of different culture vessels for biomass production during hairy root culture of *Catharanthus roseus* and multiple shoot culture of *Solanum tuberosum*

Vessel	Medium	Days to reach GI 2.0	
		Hairy root	Multiple shoot
Glass jar/ Erlenmeyer flask	Gelled	20.2 ± 1.1 (142)	28.3 ± 1.5 (137)
Life Guard	Liquid	16.5 ± 1.4 (116)	24.8 ± 1.4 (120)
Growtek	Liquid	14.2 ± 0.6	20.7 ± 0.8

Data are means ± S.E. for 15 replicates each. Figures in parentheses indicate prolonged incubation time (%; basis Growtek) at the mean values for respective vessel.

Table 3: Peripheral fungal contamination in culture for different vessel types

Vessel	Medium	Contamination % *
Phytacon	Gelled	20.1 ± 1.8 a
Life Guard	Liquid	19.7 ± 1.4 a
Magenta	Gelled	19.5 ± 1.1 a
Glass jar	Gelled	16.4 ± 0.6 b
Growtek	Liquid	3.5 ± 0.3

Data are means ± S.E. for 25 replicates for each vessel type (5 each for pineapple, potato and *Chrysanthemum* multiple shoot cultures; sandalwood somatic seedlings and *C. roseus* hairy root cultures).

* Values for other vessel types are significantly different from that of Growtek (at 0.01 level; t-test). Means followed by different letters indicate significant difference at 0.01 level (Anova and F-test) for other vessels.

3. Results

3.1 Comparison of shoot multiplication in different culture vessels

Pineapple and *Chrysanthemum* multiple shoot production was compared during 4-week periods in jars, Erlenmeyer flasks, Life Guard and Growtek devices. The data presented in table 1 clearly show the better performance in Growtek compared with other culture vessels. Pineapple responded well in liquid culture in Growtek. In static conditions a 21.4-fold increase in shoot production was observed in comparison to production in agar-gelled medium in jars, this was further enhanced when agitated (Table 1) on a rotary shaker. The health of plantlets grown in Growtek was much better than those raised in jars or Life Guard (Figure 1 B and C).

3.2 Comparison of biomass production for periwinkle hairy roots and potato multiple shoots

Figure 2 B and table 2 show the suitability of Growtek for hairy root culture. Table 2 presents data showing more biomass production in Growtek. Plants in both Life Guard and jars took longer times (116 – 120 % and 137 – 142 % respectively) to exhibit a growth rate 2.0. Faster growth was observed in Growtek, both for hairy root and for multiple shoot production. Somatic embryos grown in Growtek (Figure 2 A) also exhibited higher embryonic biomass in comparison to those from agar-gelled medium in glass jars or in Magenta vessels (Table 4).

3.3 Air-borne fungal contamination in culture

Air-borne fungal spores may contaminate cultures during incubation due to drainage of condensed water vapour from the seal of caps down the inside wall of the vessels. Such contaminants normally colonized along the periphery of the medium surface. Data presented in table 3 show that such contamination occurs to the extent of about 20%. The lowest incidence was for Growtek (3.5%), followed by glass jars (16.4%) and other vessels with push-fit types of caps (the maximum contamination; ~20%).

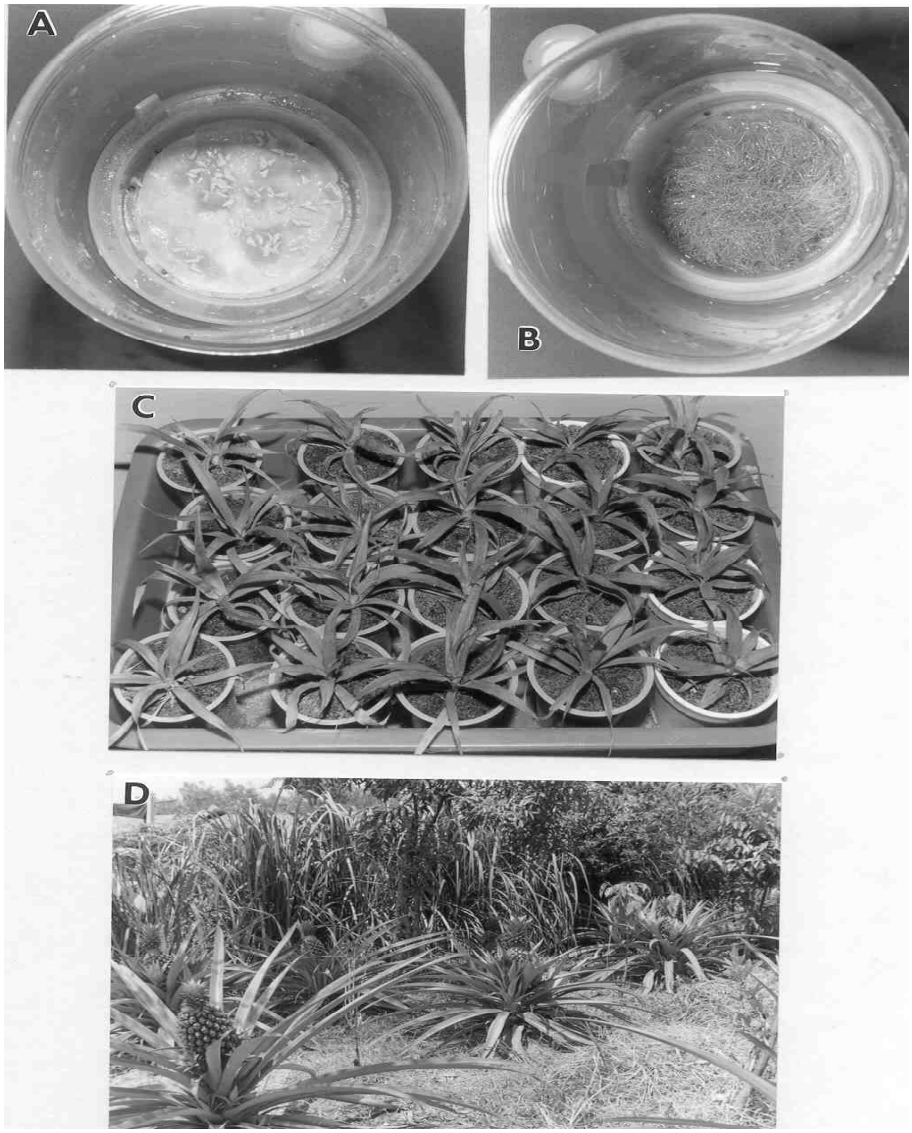

Figure 2: (A): Somatic embryogenesis of sandalwood in Growtek (top view);
(B): Hairy root culture of periwinkle in Growtek (top view);
(C): Pineapple plantlets hardened in greenhouse and ready for field transfer;
(D): Tissue-cultured pineapple at the fruiting stage in the field.

3.4 Root injury of plantlets and somatic seedlings before transfer for hardening

Both potato and *Chrysanthemum* plantlets and sandalwood somatic embryos suffered from significant root injuries (about 48%) while being uprooted from agar-gelled media. Root injury was absent in the case of plantlets and somatic seedlings raised in Growtek (Table 4).

3.5 Cost of different commercially available culture vessels

Growtek appears to be the lowest cost among the three sets of culture apparatus available on the market (Table 5). Life Guard and TIS are 1.20 were 21.84 times more expensive.

Table 4: Comparison of root injury in gelled and liquid media before transfer for hardening

Vessel	Medium	Root injury (%)		
		Potato plantlet	*Chrysanthemum* plantlet	Sandalwood somatic embryo
Glass jar/ Phytacon/ Magenta	Gelled (0.7 % agar)	44.9 ± 2.6	35.2 ± 3.2	33.6 ± 1.5 (20.1 ± 3.4)
	Gelled (1.0 % agar)	-	-	44.4 ± 3.7 (35.6 ± 3.5)
Growtek	Liquid	0.0	0.0	0.0 (248.2 ± 12.2)

Data represent means ± S.E. for 25 replicates for each plant type. Figures in parenthesis are number of normal healthy cotyledonary somatic embryos produced in each condition.

Table 5. Comparison of costs of culture vessels designed for liquid media

Vessel	Approx. price (€)	Relative cost factor (Basis Growtek)
Life Guard with membrane raft (Sigma C8062 & M7413)	7.00	1.20
Temporary Immersion System (Sigma)	126.70	21.84
Growtek	5.80	

4. Discussion

The performance of the Growtek bioreactor presented in this article is important in view of the concern expressed by many researchers about minimisation of production costs for *in vitro* mass propagation and secondary metabolite production (Dey, 2001; Sutton and Polonenko, 1999; Vasil, 1994; Goldstein, 1999; Zobayed et al., 2001; Bhattacharya et al., 1990). The response of *Chrysanthemum* (Table 1) under static condition is broadly similar in all culture vessels with gelled medium and the difference is not significant between gelled and liquid media using Life Guard. Agitated liquid medium in Growtek has however, resulted in enhanced growth (1.7 times vs. 1.2 times; in comparison to gelled medium). A production increase of 23.3-fold was obtained for pineapple grown in Growtek in the agitated mode. The explant holder, being circular and floating, offers the unique advantage of agitating cultures in liquid media, when using a rotary shaker. The number of shoots obtained per Growtek (338 ± 7.3) is probably the maximum. Escalona et al (1999) attempted automated scale-up in a bioreactor and obtained 192 'competent' plants per litre of medium in a temporary immersion system. The cultivars (Smooth Cayenne) and culture conditions were however different. Other reports concerning pineapple micropropagation (Lakshmi Sita et al., 1974; Zepeda and Sagawa, 1981) were not targeted for scale up and cost reduction. Using this protocol one million pineapple plantlets can be raised in 8 weeks using 3000 Growtek and 50 m^2 space. The 100% field survival of pineapple plantlets is better than reported earlier (Escalona et al., 1999; Soneji et al., 2002). The differences in the response of *Chrysanthemum* and pineapple may be explained by the interaction between the tissue and the support matrix in the processes of nutrient uptake. The better performance of pineapple is likely to be due to greater adherence to the explants holder, as reported for other plants (Facchini and Di Cosmo, 1991; Bhattacharya et al., 1994). We believe that pineapple shoots were able to draw a few ions more rapidly when grown in Growtek (data not presented here) because of reduced water-stress due to the specially-designed floating explants-holder. Better growth of pineapple in the Life Guard and Growtek bioreactors compared with glass jars may be attributed to the elimination of the influence of impurities (normally present in the low-cost tissue-culture grade agar used in this study), as well as reduced diffusion barriers (Debergh, 1983). The healthier shoots in Growtek (Figure 1 C) are due, possibly, to a more suitable gas /vapour phase inside the culture vessel in comparison to inside the Life Guard. The latter and the similar other vessels have a flat ceiling which accumulate (Kavanagh et al., 1991) more condensate (which also partly blocks the air passage because water droplet accumulation at the junction of

body and cap) leads to restricted gas exchange including more ethylene accumulation. About 35% light reduction through lid was also observed. The central downward slope in Growtek prevented this problem. Such accumulation in flat lids also caused higher fungal contamination as explained later in this section.

Cultures in both glass jars and Life Guard bioreactors took longer times than Growtek (116-142%; Table 2) in reaching a GI 2.0 for hairy root and multiple shoots, indicating a better physico-chemical microenvironment inside Growtek. Hairy root cultures in suspended agitated conditions in conventional bioreactors create rheological problems and more root injury (Curtis and Emery, 1993). Fragmentation of hairy roots in air-lift or stirred tank bioreactors reduce productivity, and even the metabolite profile during secondary metabolites production (Takayama, personal communication). The shear stress management for high-value product formation in hairy roots requires considerable attention (Curtis and Emery, 1993; Sharp and Doran, 2001). The possibility of using Growtek in both static and agitated modes simulates features of both gelled and liquid medium systems. This unique combination can be fruitfully utilized for laboratory-scale studies on secondary metabolite biosynthesis in hairy roots and for better somatic embryogenesis (Table 4).

The literature clearly records the problems encountered with culture contamination from bacteria (Maes et al., 1998; Cassells, 1991). Culture contamination from air-borne fungal spores is an especially serious problem in tropical and semi-tropical climate (particularly during the rainy season). This problem occurs during incubation on illuminated, but uncooled, shelves owing to drainage of along the edge of the medium along the inner wall. The effectiveness of five different vessels (Table 3) shows the order: Growtek >glass jar> Magenta/Life Guard/ Phytacon.

It is obvious that more contamination occurred in vessels with push-fit type lids (providing straight air passage between the inside and outside) having flat lids. The condensate trickles down periodically sucking in contaminated air. The better result in Growtek compared with glass jars is due to a coarser thread area in the former (Figure 1 B). Higher depth of thread rim in Growtek favours proper exchange, thereby better shoot and root health.

The perforated explant-holder (Figure 1 A, a) permits the free access of nutrient media to tissue surfaces, without sinking the latter, but perforations are small enough to prevent root entry. This surface growth of roots helps the easy and speedy transfer of rooted plantlets without injury (Table 4). This in turn offers a greater greenhouse and field survival rate for plantlets (Figure 2 C, D). It has been observed earlier that healthy and uninjured roots lead to successful field survival of plantlets (Gangopadhyay et al., 2002;

Bhattacharya et al., 1994). Root health is seriously affected by ethylene accumulation in vessels with inadequate gas exchange (Zobayed et al., 2001; de Klerk, 2001). The near 100% field survival of pineapple plantlets may be correlated with both healthy, uninjured roots.

It is clear, therefore, that Growtek is an effective bioreactor for many aspects of propagation of plant cells and tissues. It is also cheaper than other commonly used apparatus meant for the use of liquid media (Table 5). Apart from the cost, other culture vessels may require additional expenses for operation (e.g., wetting agent in Life Guard; air delivery system in TIS). The superiority of any of these available bioreactors will be dependent on eventual cost-effectiveness in mass cloning and convenience of operation without compromising the quality of the plantlets.

In conclusion, this article reports the usefulness of Growtek in terms of enhanced multiplication rates, reduced bioreactor costs, saving in incubation time, the minimisation of contamination and plantlet transfer without root injury. Experiments continue to be conducted in our institute to use Growtek for *in vitro* molecular pharming, production of secondary metabolites, bioremediation, solid-state fungal cultivation and aseptic seed germination.

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