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Current Approaches for Cheaper and Better Micropropagation Technologies

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ABSTRACT

There has been widespread use of plant tissue culture in micropropagation of plants of forestry, horticultural and medicinal importance. The success of mass propagation and its economic viability however, depends upon several factors which influence the scaling-up production. Poor rates of shoot multiplication, increasing cost of media ingredients, loss of cultures due to contamination and difficulties experienced during hardening and acclimatization are the major bottlenecks adversely affecting scaling-up of micropropagation technologies. A large number of *in vitro* technologies developed for a range of plant species have thus remained confined to laboratories because of such constraints. In order to extend tissue culture technology to large-scale propagation, it is necessary to develop methods that are relatively simple, have a high multiplication rate with high degree of reproducibility and give a high survival rate of microshoots or plantlets upon transfer to *ex vitro* conditions. Innovative approaches such as the use of liquid culture systems, replacing agar with other gelling agents viz. guar-gum, growing cultures under a CO_2-enriched environment, *in vitro* hardening, priming of tissue culture-raised plants using anti-transpirants or biopriming agents during the weaning period and improvement in the culture vessel environment by ventilation and other means have proved highly beneficial. Such approaches will not only allow production of micro-clones which are comparatively cheaper and better in their *post-vitro* performance in the soil environment but will also to help generate viable micropropagation technology suitable for mass production of desirable plant species.

Keywords: biopriming, CO_2 enrichment, culture vessel environment, gelling agents, *in vitro* hardening, liquid culture

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INTRODUCTION

Plant tissue culture, which relies on the concept of cell totipotency, has become an important tool-set for modern day biotechnology. It is now possible to regenerate plants from cultured cell, tissues and organs in almost every plant species (Vasil 1991). Micropropagation exploits this fundamental property of plant cells for rapid multiplication of elite genotypes on a large–scale in a comparatively short time (Bonga and Van Aderkas 1992; Rao 1993; Jain 1997). The technology is of great benefit for plants having long maturation periods, low seed viability and self–incompatibility, or those that are difficult to multiply by conventional means (Trigiano *et al.* 1992; Jain and Ochatt 2010). Production of high quality healthy planting material through rapid clonal propagation has created new opportunities in global trading for producers, farmers, and nursery owners, and for rural employment.

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Review

MICROPROPAGATION TECHNOLOGY - CURRENT STATUS

The large-scale commercial propagation of plant material based on plant tissue culture was pioneered in the USA with micropropagation of orchids in the 1950's (Morel and Martin 1952, 1955). It has seen a tremendous expansion globally between 1985 and 1990 in the number of production units as well as the number of plants produced (Govil and Gupta 1997). The worldwide commercial production of tissue culture plants increased by 50% during 1986-1993 (Anonymous 2004). An estimated production of 130 million of only ornamental plants was reported by Prakash (2006) in 1986 (**Table 1**). However, the total production was 663 million plants in 1993 and 800 million in 1997 (Anonymous 2004). Thereafter, the importance of plant production has dramatically increased through the demand for genetically engineered plants with unique characteristics for which *in vitro* techniques were/are applied as a vital intermediate production step (Kunert *et al*. 2002). Currently, the plant tissue culture represents a billion dollar industry with 500 million to 1 billion plants annually produced (Prakash 2006). In 2007, the number of countries planting biotech crops increased to 23, which included 12 developing countries and 11 industrial countries; they were, in order of hectarage: USA, Argentina, Brazil, Canada, India, China, Paraguay, South Africa, Uruguay, Phillipines, Australia, Spain, Mexico, Colombia, Chile, France, Honduras, Czech Republic, Portugal, Germany, Slovakia, Romania and Poland (James 2007).

As a result of consistent and substantial, crop productivity, economic, environmental and welfare benefits, a record 14 million small and large farmers in 25 countries planted 134 million ha (330 million acres) in 2009, an increase of 7% or 9 million ha (22 million acres) over 2008; the corresponding increase in "trait or virtual hectares" was 8% or 14 million "trait hectares" for a total of 180 million "trait hectares" compared with 166 million "trait hectares" in 2008. The 80-fold increase in biotech crop ha between 1996 and 2009, is unprecedented, and makes biotech crops the fastest adopted crop technology in the recent history of agriculture; this reflects the confidence and trust of millions of farmers worldwide who have consistently continued to plant more biotech crops every single year since 1996, because of the multiple and significant benefits they offer (James 2009). Updated global impact assessments for biotech crops indicate that for the period 1996 to 2008 economic gains of US$ 51.9 billion were generated (James 2009).

Demand for tissue culture plantlets is growing rapidly along with the rise in global production. India being a new entrant to the scene, contributed only 1% of worldwide production. In India significant development took place after the establishment of two tissue culture pilot plants at National Chemical Laboratory (NCL), Pune and The Energy and Resource Institute (TERI), New Delhi. At these micropropagation technology parks, nearly 4.0 million plantlets of eucalyptus, poplar, teak, bamboos and *Anogeissus* (desert teak) were produced and field demonstrated in an area of 3500 ha covering 17 states. These parks served as platform for technology development, training and demonstration of technology for mass multiplication of horticulture and forestry species (Govil and Gupta 1997).

However, the history of commercialization in India is a story of 'Rise and Fall, and Rise Again'. It was seen that from 1986-1989 the targets achieved were 50% of the installed capacity. In 1991, there was a decline and only 20% target was achieved. In 1996, there was a drastic reduction in the number of units, and only a mere 7% of the targeted volume was produced. The percentage increase in production decreased by 50% from 1991 to 1994 and in 1998 there was a negative percentage showing rapid decline. However, the trend reversed in 1999 and in 2001 the production level of 1996 were achieved, and since then there has been an increase in number of units produced per year. Between 1999, till date the increasing trend resulted into better capacity utilization of the existing facilities by 2002; and additional facilities are now being set up to increase the total installed capacity in the country (Prakash 2006). A market survey on tissue cultured plants by Biotech Consortium of India Ltd. (BCIL) in the year 2005 revealed that in year 2002-03 approximately 44 million plants have been produced. For the year 2003-04 the demand for TCPs had been projected at 72 million. The overall market for TCPs is expected to grow by at least 20 to 25% from 72 million in 2003-04 to 144 million over the next five years as compared to the average growth rate of 10 to 12% annually during the last two to three years (Anonymous 2005).

The potential for the domestic market is enormous and by conservative estimates it is expected to be US$ 15 billion in India with an annual growth rate of 15%. Tissue culture units in India have a capacity ranging between 1-5 million and above plants per year with an aggregate production capacity of over 200 million plantlets per year (**Table 2**). Most of these tissue culture units are located in Maharashtra, Andhra Pradesh, Karnataka and Kerala (Prakash 2006). Currently, the focus of the companies is mainly in the floriculture sector. However, micropropagation in banana and sugarcane is also gaining popularity. These units are currently producing fruit crops, forest trees, ornamental (foliage plants, flowering plants), vegetable crops and plan-

Table 1 Trend in growth of micropropagation industry at global and national level (source: Prakash 2006)

Year	No. of plants produced worldwide (in millions)	No. of plants produced in India (in millions)
1986	130	0.5
1987	180	2.5
1988	240	-
1989	300	5.0
1990	390	-
1991	513	10.0
1992	562	-
1993	663	-
1994	680	15.0
1995	722	-
1996	783	22.0
1997	800	-
1998	822	15.0
1999	865	20.0
2000	900	25.0
2001	815	22.0
2002	865	30.0
2003	922	50.0
2004	Not available	72.0
2005	Not available	Not available
2006	Not available	Not available
2007	Not available	Not available
2008	Not available	Not available
2009	Not available	144.0 (projected)

Table 2 Regional distribution of Indian commercial micropropagation units and their annual production capacity (in millions) (source: Govil and Gupta 1997).

State/City	No. of units	Annual production capacity (in million)
Maharashtra	25	45.00
Karnataka	9	30.00
Tamil Nadu	4	26.25
Kerala	4	26.00
Uttar Pradesh	3	25.00
Haryana	3	13.25
Gujarat	3	13.25
Andhra Pradesh	6	10.00
Delhi	4	06.25
West Bengal	9	05.00
Himachal Pradesh	1	05.00
Punjab	1	01.70
Total		206.70

tation crops. An analysis of plants micropropagated by Indian industry shows that ornamental plants (6.0-7.0 million) are the major items being produced, in line with the international trend, followed by tree species (1.0-2.0 million), fruit species (0.5-1.0 million) and other plants such as spices, aromatics, exotic vegetables etc. (less than 0.5 million) (Govil and Gupta 1997).

Micropropagation has been carried out in several crops at ICAR which include potato, sweet potato, yams, garlic, lemon, banana, pineapple, ginger, small cardamom, turmeric, black pepper and several medicinal and aromatic plants. Coffee, tea, pepper, cocoa, mango and citrus varieties with disease free planting material have also been produced. Banana, papaya, coconut, small cardamom, oil palm, ornamental plants have been multiplied on a commercial scale by private seed companies (Anonymous 2007).

Pathways of micropropagation

The commercial technology is primarily based on micropropagation, in which rapid proliferation is achieved from tiny stem cuttings, axillary buds, somatic embryos, cell clumps in suspension cultures and bioreactors. Micropropagation (*micro* an English word of Greek origin meaning small = small area of glass vessel; *propagation* an English word meaning to increase = to increase the number of propagules) can be defined as asexual multiplication of plants *in vitro* under controlled aseptic physico–chemical conditions. The cultured cells and tissue can take several pathways. The pathways that lead to the production of true-to-type plants in large numbers are the preferred ones for commercial multiplication. Micropropagation of plants can be achieved by four commonly used pathways namely (a) enhanced axillary branching (b) adventitious shoot bud differentiation (c) callus organogenesis and (d) somatic embryogenesis (Thorpe and Harry 1990).

1. Enhanced axillary branching

Shoot multiplication by enhanced axillary branching can be achieved by use of shoot clusters and/or microcuttings. In the first method the shoots formed by the buds, *a priori* present on the explant, develops axillary buds which grow into shoots and as a result initial explant is transformed into a cluster of microshoots. This structure is divided into small clusters of microshoots and transplanted into fresh medium for multiplication. This process can be repeated several times and continuous supply of shoots can be obtained (Hartmann *et al.* 1990). Multiplication by microcuttings is done where explant fails to form multiple shoots. In this method individual nodes are cut and axillary bud is allowed to grow into a shoot. Each shoot is cut to separate the individual nodes and the process is repeated (Lindsey and Jones 1989).

Regeneration of whole plants via enhanced axillary branching has been demonstrated in a number of plants: *Feronia limonia* L. (Purohit and Tak 1992), *Wrightia tomentosa* (Purohit *et al*. 1994; Joshi *et al.* 2009), *Chlorophytum borivillianum* (Purohit *et al*. 1994a; Dave *et al.* 2003), *Sterculia urens* (Purohit and Dave 1996), *Achras sapota* (Purohit and Singhvi 1998), *Eclipta alba*, *Eupatorium adenophorum* (Borthakur *et al.* 2000), *Celastrus paniculatus* (Bilochi 2001), *Wrightia tinctoria* (Purohit and Kukda 2004), *Terminalia bellerica* (Rathore *et al*. 2007) and *Pinus massoniana* (Zhu *et al.* 2010).

2. Adventitious shoot bud differentiation

In this pathway explants such as mature differentiated cells (e.g. microspores, ovules) and tissues (e.g. leaf discs, internodal segments) are induced to differentiate to form shoot buds *de novo* and eventually shoots, and rooted to form complete plants.

In vitro adventitious shoot bud regeneration has been achieved from a variety of explants of several plant species: *Pyrus communis* (Leblay *et al*. 1991), *Prunus* (Escalettes and Dosba 1993), *Juniperus oxycedrus* (Gomez and Segura 1994), *Pyrus* sp (Chevreau *et al*. 1997; Caboni *et al*. 2002), *Rosa hybrida* (Ibrahim and Debergh 2001), *Adenophora triphylla* (Chen *et al*. 2001), *Dalbergia sissoo* (Singh *et al.* 2002), *Platanus acerifolia* (Liu and Bao 2003), *Annona squamosa* (Nagori and Purohit 2004a), *Achras sapota* (Purohit *et al*. 2004), *Citrus* (Costa *et al*. 2004), *Feronia limonia* (Vyas *et al*. 2005), *Celastrus paniculatus* (Rao and Purohit 2006), *Actinidia deliciosa* (Prado *et al.* 2007), *Platanus occidentalis* (Sun *et al.* 2009) and *Capsicum annuum* (Song *et al.* 2010).

3. Callus organogenesis

In this pathway explants such as te apical meristem in the shoot apex, axillary buds, root tips and floral buds are cultured on relatively high amounts of auxin (e.g. 2,4-dichlorophenoxyacetic acid) to form an unorganized mass of cells, called callus. The callus can be further sub-cultured and multiplied. The callus shaken in a liquid medium produces cell suspension, which can be subcultured and multiplied into more liquid cultures. The cell suspensions form cell clumps, which eventually form calli and give rise to plants through organogenesis or somatic embryogenesis (Ammirato 1983). In organogenesis the cultured plant cells and cell clumps (callus) and mature differentiated cells (microspores, ovules) and tissues (leaf discs, inter-nodal segments) are induced to differentiate into complete plants to form shoot buds and eventually shoots, and rooted to form complete plants.

Micropropagation through callus organogenesis has been reported in several plant species: *Feronia limonia* (Purohit *et al.* 1996), *Carica papaya* (Yie and Liaw 1977), *Aristolochia indica* (Manjula *et al*. 1997), *Calamus flagellum* (Kundu and Sett 1999), *Hypericum perforatum* (Raquel and Romanto 2000), *Ocimum basilicum* (Phippen and Simon 2000), *Acacia mangium* (Xie and Hong 2001), *Pinus taeda* L. (Tang *et al*. 2001), *Areca catechu* (Wang *et al*. 2003), *Pinus elliottii* (Tang *et al*. 2006), *Echinacea pupurea* (Jones *et al*. 2007), *Abelmoschus esculentus* (Kabir *et al.* 2008), *Eleagnus angustifolia* (Karami and Piri, 2009) and *Lycopersicon esculentum* (Osman *et al.* 2010).

4. Somatic embryogenesis

Somatic embryogenesis resulting into a bipolar structure containing both root and shoot meristems represents another pathway of plantlet development (Steward *et al.* 1970; Ammirato 1985). Somatic embryos may be produced directly on the explant or may involve an intervening callus phase. Although diploid, the somatic embryos are not the product of sexual fusion, and their position on the explant is not predetermined (Dodeman *et al.* 1997; Gray 2000).

Detailed morphological observations have revealed that four phases can be recognized in the development of somatic embryos. In phase 'O', a somatic cell (stage 1) becomes a competent cell (stage 2) which develops into an embryogenic cell (stage 3). Subsequently, the phase I is induced in which the cell clusters proliferate slowly and apparently in undifferentiated manner. Thereafter, the cell cluster develops into globular embryos (phase II) followed by their development into heart and torpedo shaped embryos (phase III) (Komamine and Kawahara 1992; Feher *et al.* 2003). Auxins are most important growth regulators that allow the formation of cell cluster in phase 'O'. However, auxins are inhibitory for embryogenesis in phase I and subsequent development. Feher *et al.* (2003) reviewed the molecular mechanisms that determine the transition of somatic plant cell to an embryogenic state.

Plantlet formation via somatic embryogenesis has been achieved in *Mangifera indica* (Litz *et al.* 1982; PateNa *et al.* 2002), *Carica papaya* (Litz and Conover 1982; Chen *et al.* 1987; Fitch 1993), carrot (Roustan *et al*. 1990; Kamada *et al*. 1993), *Prunus avium* (Bhansali *et al.* 1990; Garin *et al.*

1997), *Punica granatum* (Bhansali 1990), *Vitis rotundifolia* (Gray 1992), cucumber (Lou and Kako 1994), *Chlorophytum borivilianum* (Purohit *et al*. 1994b), *Aegle marmelos* (Islam *et al.* 1996), *Pimpinella anisum* (Chand *et al.* 1997), *Tamarindus indica* (Jaiswal *et al.* 1998), *Malus domestica* (Hofer *et al.* 1999), *Commiphora wightii* (Ramawat *et al*. 2003), *Leucopogon verticillatus* (Anthony *et al*. 2004), *Annona squamosa* (Nagori and Purohit 2004b), mature oak trees (Valladares *et al*. 2006), grapevine (Yang *et al.* 2008) and *Withania somnifera* (Sharma *et al.* 2010). Besides these, this pathway has been chosen for regeneration of a number of recalcitrant monocot species such as *Apluda mutica* (Purohit *et al.* 1992), *Themeda quadrivalvis* (Purohit and Kukda 1993; Habibi *et al.* 2009), *Sehima nervosum* (Purohit and Kukda 1995), *Heteropogon contortus* (Purohit and Kukda 1996), maize (Conger *et al.* 1987) and rice (Bajaj and Rajam 1995).

Stages of micropropagation

The whole objective of micropropagation is to produce large number of plants which are able to survive under natural environmental conditions. In any chosen pathway of micropropagation, a series of activities are involved to achieve success. Micropropagation, in contrast to conventional propagation methods, is a multi–stage process and every stage is important to realize the goal of producing plants in culture.

Notwithstanding the advantage or disadvantage of various methods of micropropagation, each method involves 5 different stages (with exception in somatic embryogenesis) to produce transplantable propagules: Stage 0: Management of donor plant/s (source of explant); Stage I: Aseptic establishment and initiation of cultures; Stage II: Shoot multiplication and/or elongation; Stage III: Rooting of shoots; Stage IV: Hardening, acclimatization and transplantation in soil.

These stages are universally applicable in large-scale multiplication of plants. The individual plant species, varieties and clones require specific modification of the growth media, weaning and hardening conditions. A rule of the thumb is to propagate plants under conditions as natural or similar to those in which the plants will be ultimately grown *ex-vitro*. For example, if a chrysanthemum variety is to be grown under long day-length for flower production, it is better to multiply the material under long-day length at stages III and IV. There is a wide option to undertake production of plant material up to a limited number of stages. For example, many commercial tissue culture companies undertake production up to Stage III, and leave the remaining stages to others.

1. Stage 0: Management of donor plant

The pre-propagation stage (also called stage 0) requires proper maintenance of the mother plants in the greenhouse or in open under disease- and insect-free conditions with minimal dust. Clean enclosed areas, glasshouses, plastic tunnels, and net-covered tunnels, provide high quality explant source plants with minimal contamination. Collection of plant material for clonal propagation is done after appropriate pretreatment of the mother plants with fungicides and pesticides to minimize contamination in the *in vitro* cultures. The explants are then brought to the production facility, surface sterilized and introduced into culture. They may at this stage be treated with antibiotics and fungicides (Kitzinger *et al*. 1997) as well as anti-microbial formulations (Guri and Patel 1998).

2. Stage I: Culture establishment

This stage refers to the inoculation of the explants on sterile medium to initiate aseptic culture. Initiation of explants is the very first step in micropropagation. A good clean explant, once established in an aseptic condition, can be multiplied several times; hence, explant initiation in an aseptic condition is regarded as a critical step in micropropagation. The explants are transferred to *in vitro* environment, free from microbial contaminants. The process requires excision of tiny plant pieces and their surface sterilization with chemicals such as sodium hypochlorite, ethyl alcohol and repeated washing with autoclaved double distilled water.

3. Stage II: Multiplication and/or elongation

Stage II is the propagation phase in which the explants are cultured on the appropriate media for multiplication of shoots. The primary goal is to achieve propagation without losing the genetic stability. Repeated culture of axillary and adventitious shoots, cutting with nodes, somatic embryos and other organs from Stage I leads to multiplication of propagules in large numbers. The propagules produced at this stage can be further used for multiplication by their repeated culture. Sometimes it is necessary to subculture the *in vitro* derived shoots onto different media for elongation.

4. Stage III: Rooting

The *in vitro* shoots obtained at Stage II are rooted to produce complete plants. If the proliferated material consists of bud-like structures (e.g. orchids) or clumps of shoots (banana, pineapple), they are separated after rooting and not before. Many plants (e.g. banana, pineapple, roses, potato, chrysanthemum, strawberry, mint, several grasses and many more) can be rooted on half-strength-MS (Murashige and Skoog 1962) medium without any plant growth regulators (PGRs) (Ahloowalia *et al.* 2004).

5. Stage IV: Hardening, acclimatization and soil establishment

At this stage, the *in vitro* micropropagated plants are weaned and hardened. This is the final stage of the tissue culture operation after which the micropropagated plantlets are ready for transfer to the greenhouse. The hardening of the tissue-cultured plantlets is done gradually from high to low humidity regimes (using evaporative cooling systems) and from low light intensity to high intensity conditions. If grown on solid medium, most of the agar is removed gently by rinsing with water. Plants are left in shade for 3 to 6 days where diffused natural light conditions them to the new environment. The plants are then transferred to an appropriate substrate (sand, peat, compost, etc.), and gradually hardened.

Factors influencing micropropagation

The stimuli that evoke *in vitro* regeneration response may comprise of biological, physical and chemical factors synergistically influencing the process of organogenesis and differentiation (Hussey 1986).

Cells and tissues when isolated from a plant and grown *in vitro* need suitable chemical milieu provided in the form of a balanced nutrient media (Williams 1992). Most of the plant tissue culture media contain inorganic macro and micronutrients, vitamins, reduced nitrogen, PGRs and energy source (Earle and Torrey 1965). C, P, N, K, S and Mg are macronutrients and required in millimolar quantities while Fe, Cu, Mn, Co, Ni, B, I, Zn and Cl are required in micromolar quantities and called as micronutrients. All these elements are provided to the medium in the form of their salts. The requirement of various ingredients in the medium may vary for different stages of *in vitro* regeneration and also for genotypes, type of explant and regeneration pathway. This led to the development of various media formulations like MS (Murashige and Skoog 1962), B_5 (Gamborg *et al*. 1968), SH (Schenk and Hildebrandt 1972), BTM (Chalupa 1981) and WPM (Llyod and McCown 1981) and a few others. These media formulations mainly differ in the number and amount of mineral nutrients. Although MS medium is the most widely used medium for

micropropagation (Pruski *et al.* 1990; Zimmerman and Swartz 1994; Jain and Babbar 2003; Bhattacharya *et al.* 2003/04) still WPM (Snir 1984; Murai *et al.* 1997), B_5 (Mathew and Hariharan 1990) and SH media (Purohit and Singhvi 1998) have also been used for many plant species.

The importance of PGRs in initiating and regulating organized development is well established (Thorpe *et al.* 1991). The type, concentration and a suitable combination of PGRs are very essential for any regeneration system. Differentiation in plants is controlled by an interplay between auxin and cytokinin and the exogenous requirement of hormones in the medium depends on their endogenous level in the cultured plant (Skoog and Miller 1957). A high auxin to cytokinin ratio generally produces callus while a low ratio leads to organogenesis and shoot induction. 6-Benzylaminopurine (BAP, equivalent to 6-benzyladenine or BA), Kinetin (Kn), thidiazuron (TDZ) and zeatin are the main cytokinins used for shoot induction and multiplication. Of these, BAP is the the most commonly used cytokinin and has proved to be the best for adventitious shoot bud differentiation in a number of cases using a variety of explants *Litsea cubeba* (Mao *et al.* 2000), *Cuminum cymium* (Tawfik and Noga 2001), *Leucaena leucocephala* (Saafi and Borthakur 2002), *Annona squamosa* (Nagori and Purohit 2004a) *Achras sapota* (Purohit *et al.* 2004), *Feronia limonia* (Vyas *et al.* 2005) and *Wrightia tomentosa* (Joshi *et al.* 2009). Besides other cytokinins, TDZ, a chemical with cytokinin-like activity, has also been reported to induce adventitious shoot buds in *Malus* (Fasolo *et al.* 1989; Korban *et al.* 1992) and blackberry (Swartz *et al.* 1990). Cytokinins are generally incorporated in the medium in the concentration range of 0.5–5.0 mg L^{-1}. Higher levels of cytokinins tend to induce callusing (Zimmerman and Broome 1981), cause hyperhydricity and bring morphological abnormalities in cultures. Further, at higher concentrations of cytokinins, a large number of shoots are formed but they remain stunted. Thus, an additional *in vitro* step of shoot elongation is required. Transfer of shoots to the medium containing lower concentration of cytokinin has been found to promote shoot elongation (Schoofs 1992; Satheeshkumar and Seeni 2000; Dave *et al.* 2003). Haissig (1965) has explained the promotory role of cytokinins in combination with auxins on account of induction of DNA doubling, mitosis and cytokinesis leading to development of meristematic areas. The role of auxins incorporated in the medium individually or in combinations with cytokinins in shoot bud differentiation has been reported in a number of cases *Campanula carpatica* (Sriskandrajah *et al.* 2001), *Pyrus* sp. (Caboni *et al.* 2002), *Epipremnum aureum* (Qu *et al.* 2002), *Juniperus phoenicea* (Loureiro *et al.* 2007). Thus, auxins, cytokinins, and auxin–cytokinin interactions are usually considered to be the most important for regulating growth and organized development in plant tissue and organ culture (Evans 1981; Vasil and Thorpe 1994). However, abscisic acid, ethylene, gibberellins and other hormone–like compounds have regulatory roles in culture. Abscisic acid (ABA) in combination with BAP has been shown to promote adventitious bud regeneration in epicotyl and hypocotyls explants of sweet orange (*Citrus sinensis*) (Maggon and Singh 1995).

A solidified medium or a support matrix is almost indispensable for good *in vitro* growth and thus, medium is generally solidified with agar or other gelling agents such as agarose, gellan gum, carageenan and alginate (Cameron 2008). The gelling agents are usually added to the culture medium to increase its viscosity as a result of which plant tissues and organs remain above the surface of the nutrient medium. Agar is the most commonly used gelling agent because of its convenient gelling properties, stability and resistance to metabolism during use (Henderson and Kinnersley 1988). Agar quality has always been correlated with high gel strength (Scholten and Pierik 1998). It contributes to the matrix potential, the humidity and affects the availability of water and dissolved substances in the culture containers. It has been assumed that the gel-plant interaction is a dynamic process (Williams 1992) and that changes in the gel may affect the regeneration of plants or tissues. The type of agar influencing physical and chemical properties of the medium (Debergh 1983) and shoot proliferation (Singha 1982) has been reported. Owens and Wozniak (1991) have found that water availability from the gels to the growing explants contributed greatly to the growth of sugarbeet callus on different agars. Various brands and grades of agar are available commercially, which differ in the amounts of impurities, and gelling capacity (Debergh 1983). Agar is usually used at 6-8 g L^{-1} (w/v) for preparation of solid and semi-solid media. A semi-solid medium ensures adequate contact between the plant tissue and the medium. It is beneficial to growth as it allows better diffusion of medium constituents, and is easily removed from plantlets before their transfer to *in vivo* conditions. For these reasons, a semi-solid medium is often preferred over solid medium (Prakash *et al*. 2000).

The type and quantity of carbon source (sugars) in the medium have been reported to influence morphogenesis (Friends *et al.* 1994). Absolute requirement for an exogenous supply of carbohydrate as a carbon and energy source in cultured plant tissues has been very well recognised. The effect of carbohydrates on shoot formation and organogenesis from callus has been studied and reviewed extensively (Thorpe 1980; Thorpe and Biondi 1981). The effect of carbohydrate sources and concentration on somatic embryogenesis in cucumber (Lou and Kako 1994, 1995) and in carrot (Kamada *et al.* 1993) has been studied. Though sucrose is most commonly used as a carbon source in tissue culture, other sources like glucosein *Potentilla fruticosa* cultivar 'Tangerine' and *Ficus lyrata* (Wainwright and Scarce 1989), cork oak (Romano *et al.* 1995), *Wrightia* spp. (Sharma 2002) and the carob tree, *Ceratonia siliqua* (Custódio *et al.* 2004); fructose in *Carica papaya* (Drew *et al*. 1993) and *Morus alba* (Oka and Ohyama 1982); commercial sugar in banana (Ganapathi *et al.* 1995); sugar cubes in *Leucaena leucocephala* (Dhawan and Bhojwani 1984); jaggery in *Terminalia bellerica* (Bilochi 2001; Rathore 2004); lactose in creeping bentgrass and *japonica* rice (Asano *et al.* 1994) and maltose in wheat (Last and Brettel 1990) and rice (Ghosh Biswas and Zapata 1993) have also been used.

Besides chemical factors, physical factors like the type of vessel, vessel ventilation, relative humidity inside culture vessel, light, temperature, etc. also play a vital role in maximizing the shoot growth and multiplication (Joung *et al.* 1993). The number of air exchanges have been reported to influence the *in vitro* and *ex vitro* growth of grapevine rootstock (Shim *et al.* 2003). By increasing the vessel ventilation the *in vitro* propagation of *Phillyrea latifolia* was highly enhanced (Lucchesini and Mensuali-Sodi 2004). Culture tube closure-type affected the overall growth and pigment accumulation in potato Chanemougasoundharam *et al.* 2004). The photosynthetic efficiency was affected by the temperature during the night and daytime in grapevine cultures (Bertamini *et al.* 2007). Various vessel characteristics that play significant role during *in vitro* culture include the type of vessels, their internal volume, neck to bottom area, narrow or wide opening, their closure type and transparency (Kozai *et al.* 1992). Vessel type and closure affect the number of air exchanges permitted by the vessel which in turn affect the internal gaseous composition, relative humidity and temperature inside the vessel (Pospíšilová *et al.* 1996; Kim 2002; Shim *et al.* 2003). Different types of containers are used for culture initiation, maintenance of mother cultures and sub-culture for multiplication. Irrespective of the type, the containers used for maintaining *in vitro* plants should be transparent to facilitate illumination and easy inspection. Most often, the culture containers are sealed using tight caps in order to prevent the bacterial or fungal contamination. The *in vitro* cultures are usually incubated under artificial lighting at controlled light and temperature regimes (Kodym *et al.* 2001). Many sets of successive horizontal shelves are arranged in the culture room, and many vessels are placed on the shelves.

In order to make the *in vitro* plantlets survive upon transplantation, they are subjected to hardening and acclimatization. At this stage, the *in vitro* micropropagated plants are weaned from the *in vitro* environment and are transferred to polybags containing soilless media like Soilrite™ or vermiculite. Hardened plants are then exposed to low humidity regimes under high light intensity. For acclimatization, humidity is gradually reduced to the ambient level over a period of 2–4 weeks. This helps plants to restore their photosynthetic machinery and enables them to withstand the *ex vitro* conditions of low relative humidity and high light intensity (Bhojwani and Razdan 1996).

CONSTRAINTS IN COMMERCIAL MICROPROPAGATION

Micropropagation protocols developed in a laboratory as a part of R&D programmes sometimes fail to generate technology suitable for large-scale production of desired clones. In most of the cases, the cost of micropropagule production precludes the adoption of the technology for large-scale commercial propagation (George and Sherrington 1984). Besides the cost, the reproducibility and efficiency of protocol are also of paramount importance in commercial micropropagation.

Cost considerations

In many developed countries, conventional tissue culture-based plant propagation is carried out in highly sophisticated facilities that may incorporate stainless steel surfaces, sterile airflow rooms, expensive autoclaves for sterilization of media and instruments, and equally expensive glasshouses with automated control of humidity, temperature and day-length to harden and grow plants. Many such facilities established at a high cost are high-energy users, and are run like a super-clean hospital. The requirements to establish and operate such tissue culture facilities are expensive, and often are not available in the developing countries. In the developing countries, the establishment cost of facilities and unit production cost of micropropagated plants is high, and often the return on investment is not in proportion to the potential economic advantages of the technology. For example, the cost of electricity in the developed countries is much lower, and its supply far better assured than in the developing countries. The same can be said of the supply of culture containers, media, chemicals, equipment and instruments used in micropropagation. Hence, alternatives to expensive inputs and infrastructure have to be sought and developed to reduce the costs of plant micropropagation (Savangikar 2004).

The composition of culture media used for shoot proliferation and rooting has a tremendous influence on production costs. Of the medium components, the gelling agents such as agar, contribute to 70% of the budget. Sucrose costing US$ 40.0/kg also adds significantly to the media cost (Prakash *et al.* 2004). Incorporation of agar (Puchooa *et al*. 1999) and sucrose (Kozai *et al*. 1992) in the medium are also the major cause of contamination causing further economic losses.

The majority of costs associated with micropropagation are also related to manual labour (Prakash and Giles 1998). The high cost of labour of micropropagation is a major bottleneck in the European Union (EU) to fully exploit *in vitro* culture technology. In the EU, labour currently accounts for 60-70% of the costs of the *in vitro* produced plants (Savangikar 2004). Labour required in conventional micropropagation systems includes (1) medium preparation, dispensing and discarding, (2) vessel washing handling and transportation, (3) autoclaving, (4) excising and transplanting plants and explants, (5) capping and uncapping, (6) removal and discarding of dead and contaminated plants, (7) acclimatization, equipments and room sterilization, (8) recording jobs and labeling on culture vessels and (9) supervision (Kozai 2005). A much higher rate of multiplication for pineapple plantlets had recently been achieved under commercial conditions; *i.e*., up to 500-fold over a 6-month period (Firoozabady and Gutterson 2003). However, even a 500-fold multiplication in a 6-month period, using labor-intensive *in vitro* methods did not result in a product whose cost could compete with the cost of field-propagated material (the cost of *in vitro* materials was 35% more). Given the large numbers of pineapple plants needed annually for both established and new farms (planting density of 60,000 per ha or more), the commercial utility of the standard micropropagation approach seemed to be limited (Firoozabady and Gutterson 2003).

Protocol efficiency

Another most significant factor during large-scale production is multiplication rate; less than three-fold being unacceptable (Vasil 1991). High multiplication rate is an important factor governing success of large-scale production. It reduces the number of subcultures required in mass cloning and thus cuts labour cost. High rate of shoot multiplication can partly compensate for the losses on account of contamination and also during the process of rooting, hardening and acclimatization. Theoretically, shoot multiplication can be carried out indefinitely. In practice, however, it has been observed that the shoot cultures lose their vigour after repeated subculturing and thus become prone to infection and also multiplication rates decline (Vasil 1985; Varshney and Dhawan 1998; Naik *et al.* 2003). Shoot cultures of *Rhododendron chapmani* produced 5.8 shoots per explant during the establishment phase. This increased to 7.8 shoots during the first three cycles of multiplication, but declined to 5-fold multiplication in fifth subculture (Barnes 1985). Loss of morphogenetic potential has also been reported in callus cells of *Rhododendron* × *exbury* (Harbage and Stimart 1987) and *Tagetes erecta* (Kothari and Chandra 1986). Mendes *et al.* (1999) reported that the multiplication rate in one of the banana cultivars decreased with time: after the seventh subculture, new shoots formed at a very low rate. According to Gaspar *et al.* (2000) the loss of regenerative ability leads to genetic variation in the cell population, epigenetic changes and disappearance of an organogenesis-promoting substance. Delay in subculture has also been reported to result in reduced multiplication rate in *Pelargonium* (Cassells and Minas 1983). The current progress of the micropropagation industry is encouraging but the expected rapid growth has not taken place due to decreased multiplication rates. A total of 40 million plants annually are produced against the installed capacity of 110 million plants (Rout *et al*. 2006).

In addition to declining rates of multiplication with passage of time, several other problems are encountered during the multiplication phase of micropropagation. One of the serious problems is loss of cultures due to microbial contamination (Hussain *et al*. 1994; Prakash and Giles 1998). Microbial contamination of cultures is known to wipe out work of months, and can turn into a nightmare. Bacteria and fungi are the frequently encountered contaminants in cultures of many plant species, particularly in large-scale commercial operations (Young *et al*. 1984; Skirvin *et al*. 1999). Such microorganisms are either present in the explant or arise as laboratory contaminants, both natural and man-made (Sheilds *et al*. 1984). Avoiding contamination in small R&D laboratories is not a difficult task where only a low number of cultures are handled. However, commercial production involves handling of thousands of cultures each day. It is also essential to maintain such cultures in large numbers under contamination-free conditions, until they are used for either further subculture or hardening and growing further.

A large number of *in vitro* technologies developed for a range of plant species have remained confined to laboratories because of several difficulties experienced during their field transfer. The abnormal plant development due to maintenance of cultures under special conditions of high

humidity and low temperature contribute significantly to the high rate of mortality during greenhouse and field transfer (Kozai 1991b). The main cause of plant mortality is the water loss by plant upon *ex vitro* transfer (Preece and Sutter 1991).

Variability in cultures

Micropropagation technology can be rewarding only if complete genetic fidelity of micropropagules is maintained (Karp and Bright 1985; Debergh and Read 1990; Jain *et al.* 1998). Assessment of genetic fidelity at various culture stages has revealed that specific culture conditions induce somaclonal variations among the tissue culture raised plantlets. Such variation is a serious problem confronting researchers or propagators who require uniformity. Several convincing examples of the incidence of occurrence of somaclonal variation has been reported in a number micropropagated plants (for review see Rani and Raina 2000). Several morphological, cytological, biochemical and molecular markers have been used recently to detect variability in a number of plant species such as *Populus tremuloides* (Rahman and Rajora 2001), *Syzygium travancoricum* (Anand 2003), *Curcuma amada* (Prakash *et al*. 2004), *Gypsophila paniculata* (Rady 2005) and *Actinidia deliciosa* (Prado *et al*. 2007). A more detailed description about these markers and their utility in detecting the somaclonal variations in *in vitro* raised cultures of several plants have been presented in an another section of this review.

Microbial infection

The tissue culture propagation, also does not exclude the infection of viruses, viroids, phytoplasmas and bacteria unless the parental material used for tissue culture production are tested and maintained free from above mentioned pathogens as well as stock-cultures maintained free from above pathogens (Harper *et al*. 1999; Ndowora *et al.* 1999; Liefert and Cassels 2001). The need for a certification programme for the tissue culture plants is imperative since inadvertent micropropagation of virus infected plants will not only result in its poor performance, but also in undesirable spread of viruses wherever such plants are grown. Also, failure to use prescribed standard protocols will result in variations in the plants produced. The most deleterious variants in tissue culture raised plants are those that effect yield, genetic fidelity and carry infection of viruses, and other fastidious pathogens, which are difficult to diagnose. It is therefore essential that proper testing of material is done for the purpose of certification (Liefert and Cassels 2001; Savangikar and Savangikar 2004).

Hyperhydricity

During multiplication phase of tissue culture, morphological deformities in shoots or leaves like water-soaked, translucent and 'glassy' appearance have often been observed (Llorente and Apostolo 1998). Debergh *et al.* (1981) proposed the term 'vitrification' to describe these morphological abnormalities. The term was widely adopted by other researchers and became more and more loosely applied to explants in culture that had abnormal morphology of various types. As the phenomenon became more widely studied, it became clear that changes in the physiology of the explant preceded the appearance of the visual symptoms. Thus, some of the characteristics of the leaf anatomy and morphology of *in vitro* cultured shoots that differed markedly from that of *ex vitro*–produced leaves might be considered to be precursors of the vitrified condition. Debergh *et al.* (1992) recommended that the term 'vitrification' should be substituted by the term 'hyperhydricity' to indicate plant material with an abnormal morphological appearance and physiological function.

Shoots grown *in vitro* are exposed to unique micro–environment i.e. ample of sugar and nutrient level, low level of light, aseptic conditions and high humidity to get rapid growth and multiplication. But these cultural conditions induce many morphological, anatomical and physiological abnormalities in plants (Capellades *et al.* 1990; Chaturvedi 1992). Hyperhydricity is a serious problem encountered during *in vitro* culture of plants which directly affects the production at commercial level. While hyperhydric shoots may continue to grow and multiply at acceptable rates however, they cannot usually be rooted. When they can be rooted, they do not establish successfully in soil (Constantine 1986; Kevers *et al.* 2004). A number of factors have been listed to be responsible for hyperhydricity viz. the consistency of the medium as related to the concentration and type of gelling agent (Debergh *et al.* 1981; Debergh 1983; Ziv *et al.* 1983 and Pasqualetto *et al.* 1988), the level and type of cytokinin (Leshem *et al.* 1988), container closure type (Dillen and Buysens 1989), carbon dioxide and ethylene concentration in the head space of container (De Proft *et al.* 1985) and other environmental factors (Debergh *et al.* 1992). Hyperhydricity commonly encountered in certain families of herbaceous plants such as Caryophyllaceae has been reported (Evans *et al.* 1986; Stimart 1986 and Ault 1992).

INNOVATIVE APPROACHES

Poor rate of shoot multiplication, increasing cost of media ingredients, problems of contamination and hyperhydricity and difficulties experienced during hardening and acclimatization are the major bottlenecks adversely affecting scaling-up of micropropagation technologies. Recently, innovative approaches such as use of liquid culture system, replacing agar with other other low-cost gelling agents, growing cultures under CO_2 enriched environment, *in vitro* hardening, priming of tissue culture raised plants using antitranspirants or biopriming agents during weaning period and improvement in culture vessel environment by ventilation and other means have proved highly beneficial. Such approaches allow production of micro-clones which are comparatively cheaper and better in their *post-vitro* performance in the soil environment.

Liquid and immersion culture systems

In vitro regeneration of plants is influenced to a great extent by the composition of culture medium (Meins 1986). Plant tissue culture is mostly carried out on agar-gelled semi-solid media. *In vitro* cultivation of plants on agar–gelled semi–solid media requires labour intensive steps including repeated subculturing. Moreover, gelling agents account for 10–20% cost of the culture medium (Nagori *et al.* 2009). To overcome these drawbacks of agar-gelled semi-solid media, it has become necessary to search for alternatives. One such approach is to completely eliminate the gelling agents and culture the plants on liquid medium. Liquid media are ideal in micropropagation for reducing plantlet production costs, increasing rate of multiplication and for automation (Debergh 1988; Aitken–Christie 1991). Indeed, liquid culture systems can provide much more uniform culturing conditions, the media can easily be renewed without changing the container, sterilization is possible by microfiltration and container cleaning after a culture period is much easier. In comparison with culturing on semi-solid media, much larger containers can be used, and transfer times can be reduced.

The increased *in vitro* growth and shoot multiplication on liquid medium can be explained by the fact that shoots are bathed in nutrients, presenting a larger surface area for the rapid uptake of nutrients by cells and speedy nutrients replacement at the cell surface by diffusion and movement form outlying liquid. It is always associated with the rapid uptake of the growth regulators like cytokinins present in the formation of new buds and shoot elongation. In a gel, this is a slow process, generating gradients of concentrations for each nutrient in the zone of gel next to the cells

Table 3 Type of mechanical supports used during different stages of micropropagation of several plant species.

Type of support	Stage of micropropagation	Plant	Reference
Unglazed earthenware, used plaster of Paris	Culture establishment	Barley	Brown and Morris 1890
Filter paper bridges	Multiplication	*Chrysanthemum* and potato	Bhattacharya *et al*. 1994
	Multiplication and rooting	*Terminalia* and *Boswellia*	Suthar 2009
Cotton fibre	Callus organogenesis	*Artemisia annu*a	Moraes-Cerdeira *et al*. 1995
Luffa sponge	Multiplication and rooting	*Philodendron* spp.	Gangopadhyay *et al.* 2004
Paddy straw, jute, coir	Rooting	*Nicotiana*, *Beta*, *Chenopodium*, *Tectona*, *Musa*, etc.	Gangopadhyay *et al*. 2002
Coir	Microcorm production	*Gladiolus*	Roy *et al.* 2006
Sugarcane baggase	Rooting	Apple	Soccol *et al*. 2004
Peat pellets	Rooting	Sunflower	Pearson *et al.* 2007
Glass beads	Culture establishment	*Trifolium repens* (white clover)	Mc Leod and Nowak 1990
	Rooting	Rhododendron microcuttings	Mc Culloch *et al*. 1994
	Multiplication	*Terminalia*, *Celastrus*, *Feronia*, *Boswellia*, *Chlorophytum*	Vyas *et al*. 2008; Nagori *et al*. 2009
Glass wool	Multiplication	*Chrysanthemum*	Bhattacharya *et al*. 1994
	Culture establishment	Barley	Brown and Morris 1890
Wood pulp	Adventitious shoot bud multiplication	*Lycopersicon* (tomato)	Ichimura *et al.* 1995
Rock wool	Shoot development	*Eucalyptus citriodora*	Nagae *et al.* 1996; Nhut *et al.* 2002
		Spathiphyllum	Tanaka *et al.* 1992
Nylon cloth	Multiplication	*Chrysanthemum*	Bhattacharya *et al*. 1994
Foam supports			
Polyurethane foams	Multiplication	*Nicotiana* and *Vitis*	Conner and Meredith 1984
		Gladiolus	Prasad and Dutta-Gupta 2006
Foam plastics	Adventitious root development	*Rhododendron*	Pierik 1989
Synthetic matrices			
Polyester squares	Multiplication	*Musa*	Bonga and von Aderkas 1992
Polyester rafts	Multiplication	*Anthurium*	Teng 1997
Florailite and vermiculite	Multiplication	*Ipomoea batatas* (sweet potato)	Xiao and Kozai 2006
Polypropylene Membrane Rafts	Multiplication	*Gladiolus*	Prasad and Dutta-Gupta 2006

and hence, slowing the growth. For these reasons, lower concentrations of nutrients are usually optimal compared to those used in gel media formulation. Due to faster diffusion rate in liquid systems, exuded growth inhibitors such as phenolics are rapidly diluted to innocuous levels. Negative effects on growth are thereby minimized. Moreover, liquid medium can be filter sterilized, facilitating aseptic transfer into large closed vessels such as bioreactors. Handling of plant tissue for harvest and/or transfer is more amenable to mechanization, which can save labor and time. Scale-up of liquid cultures also requires less space than for their solid counterparts (Gupta and Timmis 2005). Plant tissues from numerous species have performed better in liquid medium rather than on semi-solid medium. For instance, a larger number of shoots was produced in peach (*Prunus persica* L.) (Hammerschlag 1982) and more somatic embryos were produced in wheat (*Triticum aestivum*) (Jones and Petolino 1988) and cotton (*Gossypium hirsutum*) (Gawel and Robacker 1990). The promotory effect of liquid medium on shoot elongation in *Pinus carribaea* (Skidmore *et al.* 1998) and biomass production in tobacco (Puchooa *et al.* 1999) has been reported.

However, in many cases liquid culture imposes stress signals that are expressed in developmental aberrations. Explants cultured on fully immersed liquid medium have a tendency for unusual apoplastic accumulation of water resulting in physiological, anatomical and gross morphological abnormalities termed as hyperhydricity. Hyperhydration renders the cultured tissues recalcitrant and the affected plantlets unfit for field transfer. Submerged tissues exhibit oxidative stress, with elevated concentration 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 (Ziv 2005). Puchooa *et al*. (1995) reported that explants of *Nicotiana* produced no shoots in static submerged liquid culture. This was most likely due to asphyxiation of the explants as a result of their complete immersion in the liquid medium.

Various approaches to overcome hyperhydricity include the use of mechanical matrices in order to keep plants above the liquid surface in apple (Pasqualetto *et al.* 1986), ornamental plants Debergh and Maene 1981), *Nicotiana* and *Vitis* (Conner and Meredith 1984), rose (Barve *et al.* 1986), *Chrysanthemum* (Roberts and Smith 1990), *Gladiolus* (Prasad and Dutta Gupta 2006; Roy *et al.* 2006; Dutta-Gupta and Prasad 2010) and *Melaleuca alternifolia* (de Oliveira *et al.* 2010) and *Rhodophyllum* species (Munoz *et al.* 2009), the use of temporary immersion culture system in banana (Alvard *et al*. 1992; Etienne and Bethouly 2002; Roels *et al*. 2005; Aragon *et al.* 2010), woody trees of arid and semi-arid regions (Nandwani *et al*. 2004) and *Charybdis* sp. (Wawrosch *et al*. 2005), and the use of an agitated liquid culture system in banana (Bhagyalakshmi and Singh 1995), *Nicotiana* (Puchooa *et al*. 1999), *Morus alba* (Tewary and Oka 1999) and *Chlorophytum borivilianum* (Rizvi *et al*. 2007) in shake flask cultures.

Recently, a number of techniques to support plants over stationary liquid to reduce hyperhydricity have been explored. Support matrix allows shoot growth at very high levels of aeration while facilitating uninterrupted and easy nutrient uptake. It allows harmful phenolic exudates to be dispersed in the medium. The static nature of supports also nullifies the shear stress and mechanical injury resulting from aeration and agitation associated with shake flask cultures (Prasad and Dutta Gupta 2006). Some sort of solid matrix is also essentially required for most of the plant systems for multiplication, proper rooting and better anchorage in different types of culture vessels. The use of support matrices is also economizing as it saves the cost of adding costly gelling agents, reduces the cost of washing and cleaning. Chances of contamination can be reduced during maintenance of such type of cultures as subculturing can take place only in the form of addition of sterile liquid media (Gangopadhyay *et al*. 2002, 2009). However, a mechanical support when used should be autoclavable, inert, non-toxic, resistant to plant digesting enzymes and porous. A number of mechanical supports are now available and their use has been effectively demonstrated in different plant systems by different workers (**Table 3**). In majority of the cases the overall growth was favoured with a substantial

reduction in the the production cost. For instance, the cost of cotton fiber is about $2/kg, and of agar approx. $100-200/kg. Similarly, sugarcane bagasse was used as support matrix for rooting of apple rootstock (Soccol *et al*. 2004). The cost of a good quality plant grown on the sugarcane bagasse was much (13.4%) cheaper than the plant grown on agar-gelled medium. When the number of good-quality rooted plants incremented above 1000, about 35% reduction in the total cost was achieved. Glass beads were effectively used as low cost agar alternatives by Prakash (1993) for culturing ginger and turmeric. The media cost was reduced by 94% in his trials. He also demonstrated that when glass beads were used as support matrices, the amount of medium required was only 15-18 ml per culture container (Erlenmeyer flask of 100 ml capacity). In this way, one liter medium gave 50 culture containers (only 30 containers are filled in case of agar-gelled semi-solid medium) resulting in a substantial saving in medium cost. Ginger and turmeric plants multiplied on glass bead liquid-medium performed as good as on agar-based medium. A similar type of response was observed for vanilla. *Ficus* cv. 'Mini lucii' showed higher multiplication rate although with a slight vitrification. *Saintpaulia*, *Syngonium*, *Philodendron* and *Spathiphyllum* also showed higher multiplication rates and better growth on glass bead liquid-medium (Prakash 1993). McLeod and Nowak (1990) used glass beads for the propagation of raspberry and white clove and reported a 60% saving in media cost (McLeod and Nowak 1990). The authors further demonstrated that the subculture could be successfully done without moving the plants from their place. Glass beads proved to be satisfactory for maintenance of callus and shoot organogenesis (McCulloch *et al*. 1994) in *Rhododendron*. The beads can be reused after washing with acid.

Nagori *et al*. (2009) and Vyas *et al*. (2008) effectively employed glass bead-supported liquid medium as a rapid and cost effective method for the *in vitro* multiplication of some economically important plant species e.g., *Celastrus paniculatus*, *Chlorophytum borivilianum*, *Terminalia bellerica* and *Boswellia serrata*. Liquid medium promoted shoot multiplication, shoot elongation and accumulation of total fresh and dry weight in all these plants. The shoots grown on this medium had more number of leaves with larger leaf area and expanded leaf lamina. Increment in chlorophyll *a*, *b* and total chlorophyll content was observed for *C. paniculatus* and *B. serrata* shoot cultures. Use of glass beads proved highly beneficial and did not cause any deterioration on account of hyperhydricity in liquid culture. The glass beads allowed easy removal of plantlets from the medium.

To avoid the problems associated with submerged culture, temporary immersion systems have also gained popularity. In a temporary immersion system/reactor (TIR), the entire culture or plant tissue is temporarily wetted with nutrient solution followed by the draining away of the excess nutrient solution under gravity. This allows the plant tissue a better access to air. Usually a periodic wetting and dry cycle (3-6 hrs) is set depending upon the type of plant and stage of micropropagation. The principle components of a TIR are similar to an airlift or bubble column type of bioreactor. A fixed or floating raft support system is present inside the culture vessel to support the explants. Liquid medium is pumped into the culture vessel from a storage tank usually located underneath the vessel or from a separate bottle in case of a twin bottle system. The medium remains in the vessel for few minutes, after which it drains back to the storage tank for reuse (Afreen *et al*. 2002). Major advantages of a temporary immersion bioreactor are reduction of hyperhydricity as plants are immersed in the liquid medium only for 5-10 min in every 3 or 6 hours thus minimizing the physiological disorders. Plantlet growth is improved because during every immersion the plant is in direct contact with the medium and thereafter a thin film of liquid covers the plant throughout the interval period. The mechanical stress on plant tissues is generally low compared to other agitated systems. Manipulative and automated control is easily possible (Afreen 2008).

An efficient and cost effective method based on temporary immersion system was developed by Firoozabady and Gutterson (2003) for commercial micropropagation of Smooth Cayenne pineapple. Micropropagation of pineapple plants has many advantages over conventional methods of vegetative propagation. For example, this technique could allow for an efficient and rapid increase of selected varieties. In last few years considerable progress was achieved by *in vitro* methods whereby an increase of 500-fold over a period of 6 months was reported (Firoozabady and Gutterson 2003). However, even a 500-fold multiplication in a 6-month period, did not result in a product whose cost could compete with the cost of field-propagated material (the cost of *in vitro* materials was 35% more). The major costs were associated with labour intensive steps involving sequential culturing on solid medium for axillary shoot-bud multiplication. The cultures were initiated form apical crown meristems by initially placing them in liquid shake flasks (500-ml flask on a gyratory shaker at 80 rpm) for four weeks. Using this method approximately 30 shoots were obtained per initial crown meristem within 10 weeks). Multiplication was then carried out inside a 10-l Nalgene vessel with shoots immersed in liquid medium for 5–10 min/h (periodic immersion bioreactor, PIB). The relative lengths of the immersion and non-immersion periods could be varied using a 4-h timer. In the PIB, liquid medium was placed inside a reservoir vessel separate from the bioreactor growth vessel. The medium was pumped into the bioreactor growth vessel every 60 min to immerse the shoots for about 6–10 min. Using the above micropropagation method and the PIB, approximately 6,000–8,000 shoots from two initial shoots were produced in less than 6 months. The shoots were then induced to form roots and transferred to soil. In total a multiplication rate of 3000-4000 fold was obtained in a period of six month. The PIB method reduced the cost of production by 35% in comparison with the conventional method, as labour intensive steps of cutting and subculturing was omitted, loss of plantlets due to contamination was negligible and the number of containers and need for shelves in culture room was minimized. The PIB method is successfully being employed for commercial cultivation of pineapple and banana in Costa Rica and for ginger, Pineapple and banana in Indonesia (Firozabady and Gutterson 2003).

A brief review of micropropagation of a number of plant species carried out under different types of liquid culture systems has been discussed in **Table 4**.

1. Automated micropropagation in bioreactors

Large-scale plant production through cell tissue and embryo cultures using bioreactors is now being viewed as a rapid technology for industrial plant propagation. Bioreactors devoted to mass propagation include systems for cultivating cells, tissues, somatic embryos or organogenic propagules in liquid medium. A large number and variety of reactor systems are now available for the above purpose (for reviews refer Saje *et al*. 2000; Honda *et al*. 2001; Paek and Chakrabarty 2003; Paek *et al*. 2005; Kämäräinen-Karppinen *et al.* 2010). The reactors used for plant tissue and organ cultures differ considerably in design and operation from those used for microbiological purposes. Paek *et al.* (2005) has classified the bioreactors used for plant cell cultures into two major types on the basis of agitation methods and vessel construction. These are (1) Mechanically agitated and (2) Pneumatically agitated or non agitated. Among the first category fall the Stirred tank bioreactor, Rotating drum bioreactor and Spin filter bioreactor. Pneumatically agitated bioreactors include Simple aeration bioreactor, Bubble column bioreactor, Air lift bioreactor and Balloon type bubble bioreactor (BTBB). The design and operation of a bioreactor are mainly determined by biological needs and engineering requirements, which often include a number of factors such as efficient oxygen transfer and mixing, low shear

Table 4 List of plants micropropagated on liquid medium.

Plant	Type of liquid culture system	Result	Reference
Banana, plantain	Temporary immersion bioreactors	The number of competent plants was increased from 80.0% to 91.0% at the end of the *in vitro* phase and the survival percentage from 95.71% to 99.80% during *ex vitro* hardening.	Aragon *et al.* 2010
Solanum tuberosum	Thin layer liquid culture	About 75 microtubers of sufficient size and mean weight (200 mg) per culture vessel were produced.	Kämäräinen-Karpinnen *et al.* 2010
Chlorophytum borivilianum, *Boswellia serrata*, *Feronia limonia*, *Terminalia bellerica*, *Celastrus paniculatus*	Static liquid medium	More than 2-fold increase in rate of shoot multiplication with no sign of hyperhydricity.	Vyas *et al*. 2008; Nagori *et al*. 2009
Watsonia spp.	Liquid shake culture	Significant increase in the growth index and corm formation.	Ascough *et al*. 2007
Echinacea purpurea	Shake culture method	Regeneration time of plantlets from somatic embryos was reduced.	Jones *et al*. 2007
Gladiolus	Static suspension	MR system resulted in 33.46 % increase in shoot regeneration than semi-solid system.	Prasad and Dutta-Gupta 2006
Wasabia japonica	Agitated liquid medium	A two-fold increase in shoot length and weight was observed with enhanced rate of multiplication and minimum amount of hyperhydricity.	Hung *et al.* 2006
Hosta	Thin-film liquid system	Increased multiplication, space utilization, sugar availability and worker efficiency was demonstrated to be greater in thin-film liquid than more conventional agar-based system.	Adelberg 2005
Carrot	Static suspension culture	Enhanced ratio (1.8 times) of torpedo-shaped embryo.	Li and Kurata 2005
Chrysanthemum	Shoot multiplication in bioreactor	Shoot length, leaf area and fresh weight was nearly doubled.	Hahn and Paek 2005
Philodendron	Multiplication and rooting in liquid medium supplemented with luffa sponge	Increased rate of multiplication with 100% rooting response and 100% transplantation survival was observed.	Gangopadhyaya *et al.* 2004
Cucumis sativus	Liquid medium in air-lift bioreactors and shake flasks	Considerable increase in biomass production.	Konstas and Kintzois 2003
Solanum tuberosum	Temporary immersion system	Plant growth and tuberization of *Solanum tuberosum* L. (potato) were stimulated by temporary immersion *i.e.* approximately 500–960 tubers for a 10 weeks culture. Total tuber weight and homogeneity also increased.	Etienne and Bethouly 2002
Chenopodium, *Beta* spp. Banana	Rooting in coir supplemented liquid medium	100% root induction was attained, the roots were normal, positively gravitropic, stout and most importantly, with adequate root hair zones.	Gangopadhyaya *et al.* 2002
Ananas comosus	Temporary immersion system	400 % increase in rate of shoot multiplication without any symptoms of hyperhydricity.	Escalona *et al.* 1999
Dierama luteoalbidum	Shake flask culture	Increased number of meristemoid formation with doubled growth index, without any signs of hyperhydricity.	Madubanya *et al*. 2006
Rose	Static-liquid culture	Growth of miniature roses in liquid (20 ml) medium was greater as compared to those cultured on solid medium.	Pati-Pratap *et al.* 2006
Apple	Static-liquid culture	Rooting was 100% as compared to 94%in agar-gelled medium.	Soccol *et al.* 2004
Terminalia bellerica, *Boswellia serrata*	Rooting on filter paper rafts	Increased rate of rooting with sturdier roots.	Suthar 2009
Sunflower	Peat pellets in liquid media for rooting	Transplanting plants rooted in liquid medium with peat pellets to soil is was destructive and resulted in healthy plants than plants grown in agar.	Pearson *et al.* 2007
Pineapple	Partial immersion bioreactor	Provided an excellent multiplication of over 3,000-fold in a 6-month period, also reduced the cost of production by 35%.	Firoozabady and Gutterson 2003
Asiatic lily hybrids	Liquid stationary culture	Proposed the formation of 9.68×10^5 bulblets from a single scale segment in 1 year.	Varshney *et al.* 2000

and hydrodynamic forces, effective control of the physico-chemical environment and ease of scale-up.

Paek and co workers (2005) have defined the configurations of the different types of bioreactors used for plant cell culture as explained hereafter. The stirred tank bioreactor is a typical bioreactor designed with numerous modifications and used for most of the plant cell cultures in the earlier times. It however, presented several limitations such as high power consumption, high shear forces and problems with sealing and stability of rotating shafts in tall bioreactors. With the advent of newer concepts more designs came in to being. Air-lift bioreactors combining high loading of solid particles and good mass transfer for three-phase fluidized beds were more commonly used. The air-bubbles generated efficient mixing of liquid phase using internal or external recirculation loops. They have a relatively simple design with low energy requirements and offer less shear forces. Rotary drum reactors have significantly higher surface area to volume ratios than other reactor types. As a result, mass transfer is achieved with comparably less power consumption. In a bubble column bioreactor the bubbles create less shear forces, so that it is useful for plant organ cultures especially for culture of shoots, bulbs, corms tubers etc. The disadvantage of air-lift and bubble column bioreactors is excessive foaming induced by large volumes of air in the headspace of vessel. To overcome this, a balloon type bubble bioreactor was designed that has a larger top-section. A concentric tube was used for cell lifting at the riser part of the vessel base which reduced foaming considerably. A novel type of ebb-and-flow bioreactor system (a periodic

immersion system) has been used for the mass propagation of several plant species. The principal equipment in an ebb and flood bioreactor is the same as that in the BTBB. The only difference is that to avoid complete submersion of explants in the liquid medium, a supporting net is used to hold the plant material. The medium is pumped from a storage tank into the culture vessel. A series of channels help to supply nutrient solution evenly to the plant material, resulting in uniform growth. The medium remains in the vessel for a said period of time, after which it drains back to the storage tank for re-use. The drainage process is controlled by a solenoid valve at intervals of between 4 and 8 hrs, depending on plant species and explant type.

Automation of micropropagation via organogenesis or somatic organogenesis in a bioreactor has been advanced as a possible way of reducing costs (Takayama and Akita 1994; Leathers *et al*. 1995; Chakrabarty and Paek 2001; Paek *et al.* 2001). Organogenic plant propagules are cultivated intensively in bioreactors for the end result of producing transplants for mass production. Intensive cultivation of such structures as potato microtubers and bulblets of lily is another strategy for producing propagules, which can be handled for direct planting in the field. Micropropagation by axillary shoot proliferation is typically a labour-intensive means of producing elite clones, but recently the adaptation of air-lift, bubble column, BTBB, ebb and flood and temporary immersion bioreactors for propagation of shoots and bud-clusters has provided a workable means for scale-up. Some of the most advanced plant tissue culture work that has been progressed to research-scale bioreactors is based on production of crop species such as *Stevia rebaudiana*, *Begonia*, *Chrysanthemum*, apple, grape, pineapple, garlic and *Phalaenopsis* (Paek *et al.* 2001).

Somatic embryogenesis also offers a potential system for large-scale plant propagation in automated bioreactors. Conventional micropropagation requires intensive labour which often limits its commercial viability and application. Somatic embryos could be easier to handle since they are relatively small and uniform in size, and they do not require cutting into segments and individual implanting onto media during proliferation. In addition, somatic embryos have the potential for long-term storage through cryopreservation or desiccation, which facilitates flexibility in scheduling production and transportation and therefore, fits large-scale production. The production of somatic embryos in bioreactors has been reported for a number of species (reviewed by Denchev *et al.*1992; Moorhouse *et al.* 1996; Ibaraki and Kurata 2001; Paek and Chakrabarty 2003), but many improvements are needed for the practical automatic somatic embryo production systems that can cope with synchronization of the somatic embryo development, identifying the occasional embryo abnormality during culture, and overcoming difficulties in embling acclimatization.

Alternative to agar and other gelling agents

The growth of cultures and production of shoots or roots is strongly influenced by the physical consistency of the culture medium. Agar is most widely used gelling agent for plant tissue culture media, since its first use by White (1939). It is however, the costliest ingredient and often contains impurities that may affect the growth of cultured plant cell and organs (Debergh 1983). Moreover, local accumulation of heat, hinderance to the access of dissolved oxygen (Tulecke and Nickell 1959; Anonymous 1988), to the cultured plant part in media and contamination of media

Table 5 Commonly used gelling agents in plant tissue culture.

Gelling agent	Make	Approx. cost (US $/500 g)	Concentration used (%)	Cost per litre (in US $)
Agar	Difco-bacto	109.03	0.9	1.96
	Qualigens	19.00	0.9	0.342
	Hi Media	28.16	0.8	0.506
	Sigma	152.7	6-12	3.66 at 12%
	CDH	28.00	0.9	0.504
	SRL	79.00	0.9	1.42
Agarose	Sigma	772.0	0.9	13.90
	Hi Media	567.0	0.9	10.92
	SRL	420.0	0.9	7.52
Gellan gum (Phytagel)	Sigma	189.0/ 250 g	0.2	1.51
Gelrite (Gellan gum)	Sigma	80.7/ 250 g	0.2	0.65
Carageenan	Sigma	131.1	1.0	2.622
Alginate (sodium salt)	Sigma	247.5	2.0	9.9
Alternative gels and mixtures				
Starches	All Local Made	≤ 1$	1-3	≤ 1$
Wheat starch				
Laundary starch				
Semolina				
Potato powder				
Rice powder				
Sago				
Cassava starch				
Barley starch				
Tapioca starch				
Corn starch				
Isubgol	Telephone Brand	2.0	3.0	0.12
Galactomannan gums				
Guar gum	Sigma	62.12	3.0-4.0	0.192
	HiMedia	2.4	1.0-3.0	0.024
	Local Made	0.4	1.0-3.0	< 1 $
Locust bean gum	Sigma	74.44	-	-
Cassia gums				
Gum katira	Local Made	3	3.0	0.18
Xanthan gum	Sigma	187.9	10.0	37.58
	Hi Media	19.5	10.0	3.9
Ficoll	Sigma	2020	10.0-40.0	1616 at 40%

through agar sticking on neck of culture vessels, *etc*. are reported to be other disadvantages of agar. During the last two decades, there have been increased efforts to look for suitable substitutes for agar (Jain and Babbar 2005). A comprehensive list of gelling agents and their cost analysis is presented in **Table 5**. Other gelling agents such as carrageenan (Bromke and Fugira 1991) and alginates (Johansson 1988) gel only in the presence of specific ions and therefore are not suitable substitutes for agar. Agarose (Kao 1981) and Ficoll are cost-prohibitive (Tremblay and Tremblay 1991). According to Boxus (1978) it is usually unnecessary to use high purity agar or gelling agents for large-scale micropropagation; several low cost options instead of agar have been successfully used for industrial scale micropropagation.

Cheaper alternatives to agar include various types of starches and plant gums (Pierik 1989; Nagamori and Kobayashi 2001). The National Research Development Corporation, India (NRDC 2002) has listed low cost agar alternatives, which are worth evaluating for routine use in commercial micropropagation. Prakash *et al*. (2004) suggested the use of (all units in g L^{-1}) wheat flours (80 and 100), laundry starch (60), semolina (50), potato powder (70), rice powder (110), sago (70) and a laundry starch + potato powder + semolina (2: 1: 1) combination for micropropagation of *Zingiber* (ginger) and *Curcuma* (turmeric). Although the media prepared by using these sources were poorly gelled and sloppy, but however favored normal growth in some cases. The combination of laundry starch, potato starch and semolina reduced the cost of gelling agent by 70-82% (Prakash 1993). Kodym and Zapata-Arias (2001) reported the replacement of Gelrite with starch-Gelrite mixture during banana micropropagation. A 90% resource cost reduction was achieved by using this protocol. Sorvari (1986) observed significantly accelerated potato disc shoot differentiation on barley starch media. Similar observations were recorded for barley anther culture on starch media (Sorvari and Scheider 1987). A combination of barley starch (20 g L^{-1}) along with agar (7 g L^{-1}) and agarose (5 g L^{-1}) was tried out for anther culture of *Hordeum vulgare* by Tiwari and Rahimbaev (1992). Also anthers incubated on potato starch produced more embryos in case of *Solanum* anther culture (Calleberg *et al.* 1989). Corn-starch (CS) as a gelling agent was used along with low concentration of 'Gelrite' (0.5 g 'Gelrite' + 50.0 g CS L^{-1}) for the propagation of fruit trees, such as apple, pear and raspberry, banana, and sugarcane, ginger and turmeric (Stanley 1995; Zimmerman 1995). The shoot proliferation was better on corn starch-medium than on agar. The cost of CS was US$ 1.8/kg as compared with US$ 200/kg of agar. Cassava flour (90% starch) was used for bud culture of *Ananas* by Costa *et al.* (2007). Addition of 80 g L^{-1} tapioca starch to the MS medium was found to be a good substitute for 'Bacto-agar' for potato shoot-culture (Getrudis and Wattimena 1994). Tapioca produced from cassava flour can be moulded into beads or pearls by heating moist flour in shallow pans. Sago an another low-cost gelatinized starch material was used as the sole gelling agent at 0.8% (8 g L^{-1}) for micropropagation of 10 potato varieties (Naik and Sarkar 2001) and *Chrysanthemum* (Bhattacharya *et al.* 1994). Sago was obtained originally from the stem piths of sago palm, *Metroxylon sagu* Rottb., and more recently it is obtained from industrially processed cassava potato and sweet potato starch. The cost of sago is US$ 0.5/kg that is approximately $1/18^{th}$ of the price of agar which makes it the choice of gelling agent for commercial micropropagation (Naik and Sarkar 2001). 'Isubgol', a colloidal mucilaginous husk (chiefly composed of pentosans) derived from the seeds of *Plantago ovata*, has a good gelling activity, and has reasonable clarity in gelled form. 'Isubgol' at 30 g L^{-1} in MS medium has been used for the propagation of *Chrysanthemum* (Bhattacharya *et al*. 1994), *Syzygium cuminii* and *Datura innoxia* (Babbar and Jain 1998) and *Dendrobium* (Jain and Babbar 2005).

In the quest for cheaper gelling agents a number of medium gelling galactomannans have been investigated in last few years. Sharma and Purohit (2001) for the first time reported guar-gum (10-30 g L^{-1}) as a better and cheaper alternative to agar in plant tissue culture medium. A number of cases of stimulatory effects of guar-gum on rate of shoot multiplication have been reported in *Wrightia tomentosa* (Sharma and Purohit 2001), and *Dendrobium chrysotoxcom* (Jain and Babbar 2005). Better growth of shoots with broad and greener leaves was obtained on guar-gum solidified media in both of these plants. Lower concentration (10 and 20 g L^{-1}) of guar-gum favoured better shoot elongation (average 5.0 cm) as compared to control in *Chlorophytum borivilianum* (Joshi 2005). It was also reported to suitably replace agar in *Celastrus paniculatus* (Bilochi 2001), *Wrightia spp*. (Sharma 2002), *Achras sapota* (Dave 2004) and *Terminalia bellerica* (Rathore 2004). Guar-gum extracted from a legume *Cyamopsis tetragonoloba* is considered as nutritionally rich (Jain *et al.* 2005). When used in tissue culture, the impurities present in it may act as a source of nutrients to the growing cultures. The batch to batch variability – a problem commonly associated with gelling agents can be minimized by using guar-gum obtained from single source. Also, guar-gum has poor gelling ability, which allows higher absorption and hence, enhanced growth of culture. Being of plant origin, it is biodegradable and does not pose any threat to the environment on being disposed-of after use. Guar-gum production in India is abundant and is much cheaper (US$ 0.8/kg) as compared to other tissue culture grade gelling agents (Rathore 2004). Other medium gelling galactomanans in wide industrial use are locust bean gum (alternatively carob bean gum) extracted from the seeds of the carob tree, *Ceratonia siliqua* and cassia gum, extracted from a Brazillian tree, *Cassia fastuosa* and other *Cassia* sps (Cameron 2008). A mixture of locust bean gum and agar was effectively used during the single node culture of *Ceratonia siliqua* and *Rhododendron* (Goncalves and Romano 2005). Use of cassia gum resulted in 17% increased shoot regeneration during the micropropagation of *Pyrus* (Lucyszyn *et al*. 2006). Similarly, cassia gum favoured shoot production and rooting in *Nicotiana* (Lucyszyn *et al*. 2007) and *Fragaria* (Lucyszyn *et al*. 2006).

Gums like gum katira or karaya gum obtained from *Sterculia* spp. do not dissolve in water but swell into a jelly like transparent mass (Nair 2003) and has been tested for shoot formation and rooting from *Syzygium* epicotyl segments (Jain and Babbar 2002). It also proved to be useful for SE production from hypocotyls segments of *Albizzia* (Jain and Babbar 2002).

Recently, xanthan gum, a polysaccharide produced by the bacterium *Xanthomonas campestris*, has also been reported in various tissue culture applications. Jain and Babbar (2006) made comparisons between 100 g L^{-1} of xanthan gum-gelled and 9 g L^{-1} of agar-gelled media for *in vitro* seed germination, shoot proliferation and rooting of *Albizzia*, androgenesis from floral buds of *Datura* and somatic embryogenesis in callus cultures of *Calliandra.* In most of the cases xanthan gum and agar performed equally well. The number of somatic embryos produced was however doubled per culture on the xanthan gum media.

Several other gels such as xyloglucan in Marubakaido and Jonagored apples (Lima-Nishimura *et al.* 2003), parenchymatic medium solidifier – PMS *in Betula pendula*, *Gerbera jamesonii*, *Floribunda rose*, *Daucus carota* and *Chenopodium album* (Titel *et al.* 1987), microcrystalline cellulose – MCC in *Nicotiana tabacum* (Gorinova *et al.* 1993) and hydrogels for shoot cultures of lettuce (Teng and Liu 1993) have also been tried out as innovative approaches against the use of agar.

CO_2 as a carbon source

During plant tissue culture the culture container is small and sealed, the concentration of CO_2 in the microenvironment is relatively low. The plantlet growth is restrained for the shortage of CO_2 in the culture vessel. Further the depletion of CO_2 concentration from 5-10 mM in the dark period to

Table 6 List of plants cultivated photoautotrophically.

Plant	Concentration of CO_2 used	Reference
Paspalum dilatatum	700 µl L^{-1}	Soares *et al*. 2008
Spinach (*Spinacia oleracea* L.) and fenugreek (*Trigonella foenum-graecum* L.)	600 µmol mol^{-1}	Jain *et al*. 2007
Alfalfa	700 µmol mol^{-1}	Erice *et al*. 2007
Sea oats	1500 µmol mol^{-1}	Valero-Aracama *et al*. 2007
Sweet potato plantlets	*v/v* 1.8×10^{-3}	Xiao and Kozai 2006
Paulownia and coffee	1600 µmol mol^{-1}	Nguyen and Kozai 2005
Samanea saman	1000 µmol mol^{-1}	Mosaleeyanon *et al*. 2004
Lettuce	10,000 µmol mol^{-1}	Tisserat and Silman 2000
Eucalyptus camaldulensis	800 and 900 mmol mol^{-1}	Zobayed *et al*. 2001
	1200 µmol mol^{-1}	Kirdmanee *et al*. 1995
Tobacco	Data not available	Pospíšilová *et al*. 2000
Rice	100 Pa	Makino *et al*. 2000
Phaseolus sps	700 µmol mol^{-1}	Salsman *et al*. 1999
Liquidambar styraciflua	ambient + 200 µl l^{-1}	Herrick and Thomas 1999
Ryegrass	60 Pa	Rogers *et al*. 1998
Carnation	Data not available	Solárová and Pospíšilová 1997
Wheat	550 µmol mol^{-1}	Nie *et al*. 1995
Red raspberry	1500 ppm	Deng and Donnelly 1993
Vitis rupestris	Data not available	Kozai *et al*. 1992
Coffea arbusta	1000 µmol mol^{-1}	Afreen *et al.* 2002
Pinus radiata	850 µmol mol^{-1}	Aitken-Christie *et al.* 1992
Azadirachta indica	450 µmol mol^{-1}	Kozai and Nguyen 2003
Garcinia mangosteen	1300 µmol mol^{-1}	Ermayanti *et al.* 1999
Gmelina arborea	400 µmol mol^{-1}	Nguyen and Kozai 2001
Petunia, *chrysanthemum*, tomato	650 µmol mol^{-1}	Qu *et al*. 2009
Phalaenopsis	1000 µmol mol^{-1}	Yoon *et al.* 2009
Cymbidium hybrids	300 µmol mol^{-1}	Teixeira da Silva *et al*. 2007
	10,000 µmol mol^{-1}	Norikane *et al.* 2010
Actinidia deliciosa	300, 600 and 2,000 µl l^{-1}	Arigita *et al.*2010
Banana plantain	1200 µmol mol^{-1}	Aragon *et al.* 2010

100 µM during light periods and low irradiance adversely affect the photosynthetic activity of plants grown in culture (Nguyen and Kozai 2005). To compensate for such deficit the tissue culture media are supplemented with sugars as carbon and energy sources (Thompson and Thorpe 1987). However, addition of sucrose and/or other carbohydrates adds significantly to the media cost, considerably decreases the water potential of the medium and increases the risk of microbial contamination (Pospíšilová *et al.* 1997). Also, it forces the shoot cultures to develop hetero- or mixo–heterotrophy (Kozai *et al.* 1992). Sucrose in the medium also suppresses the Rubisco activity (Hdider and Desjardins 1995) This and other *in vitro* conditions result in the formation of shoots/plantlets with small thin leaves, decreased epicuticular and cuticular waxes, less developed support tissues (collenchyma and sclerenchyma) and a higher water content percentage (Donelly and Tisdall 1993). All these factors together create difficulties in hardening and acclimatization of tissue culture plantlets.

One of the ways to avoid problems associated with sugar nutrition is photoautotrophic cultivation of plantlets by reducing or eliminating saccharides in the medium (Kozai *et al*. 1992), and increasing carbon dioxide concentration. Photoautotrophic cultivation refers to micropropagation with no sugar added to the medium. Photoautotrophic micropropagation has many advantages with respect to improvement of plant physiology and operation/management in the production process. Net photosynthetic rate, and thus the growth rate of *in vitro* plantlets, is often enhanced when the plantlets are cultured photoautotrophically under a properly controlled environment, compared with those cultured conventionally probably due to enhanced RuBisCo activities (Desjardins *et al*. 1995). High photosynthetic rate, normal anatomical structure and functional stomata (Zobayed *et al.* 1999) thus reduce hyperhydricity and contribute to enhanced survival percentages upon transfer to the *ex vitro* environment. Plantlets produced under such conditions are less susceptible to microbial contamination (Jeong *et al.* 1995), more vigorous with larger root systems, able to photosynthesize and do not require acclimatization for post *vitro* establishment (Van Huylenbroeck and Debergh 1996). Further, the requirements of plant growth regulating substances, vitamins and other organic substances can be minimized, automation is easy and better. Environmental control can be achieved using a larger culture vessel. The efficacy of CO_2 enrichment is well demonstrated to produce photosynthetically active plants during the micropropagation (**Table 6**).

Purohit and co-workers attempted photo-autotrophic micropropagation of some woody tree species. *In vitro* propagation of *Achras zapota* (Purohit *et al*. 2004), *Feronia limonia* (Vyas *et al*. 2005), *Chlorophytum borivilianum* (Dave *et al.* 2003), *Celastrus paniculatus* (Rao and Purohit 2006), *Terminalia bellerica* (Rathore *et al*. 2008), *Vitex negundo* (Rathore 2004) and *Wrightia tomentosa* (Purohit *et al*. 2004) was achieved under traditional photomixotrophic conditions. However, in all these cases the media contained sucrose which limited the shoot multiplication rate. Also, the plantlets derived from sucrose supplemented media showed poor survival during hardening, acclimatization and greenhouse growth. Experiments were designed to grow the plants photoautotrophically under controlled CO_2 environment in small chambers. Acrylic boxes having an airtight lid with a volume of 7500 cm^3 (25 × 20 × 15 cm; L × B × H) were designed to achieve control of CO_2 concentration (Solárová *et al*. 1996). The CO_2 concentration of 0.6 g^{-3} (0.03%) in the chambers was created and controlled 0.1 M solutions of $NaHCO_3$ and Na_2CO_3 mixed in a ratio of 77/23 (v/v). Higher concentrations (10.0 and 40.0 g (CO_2) m^{-3}; 0.5 and 2.0%, respectively) were created by using 3.0 M solutions of $KHCO_3$ and K_2CO_3 mixed in a ratio of 50/50 and 73/27 (v/v), respectively. A saturated solution of potassium permanganate was kept inside the boxes to absorb ethylene produced by growing cultures (Lemos and Blake 1996). The plantlets during different stages of growth were placed in these chambers and various growth parameters studied. Cultures kept under CO_2 enriched environment without sucrose grew photoautotrophically. The rate of multiplication under CO_2 enriched condition was almost twice of that recorded under conventional micropropagation sys-

tem. The best concentration of CO_2 was 10.0 g m^{-3} for *W. tomentosa* (Vyas and Purohit 2003), *C. paniculatus* (Rao and Purohit 2008) and *F. limonia* (Vyas and Purohit 2005) and 40.0 g m^{-3} for *A. zapota* (Dave and Purohit 2004), *V. negundo* (Purohit *et al.* 2007), *C. borivilianum* (Joshi 2005) and *T. bellerica* (Suthar and Purohit 2009). In all the cases highest leaf area and fresh and dry weight per shoot cluster promoting shoot growth and multiplication was recorded. Effective quantum yield for shoot and cultures of *F. limonia* growing at 10.0 gm^{-3} CO_2 on sucrose free medium was (0.084) for which highest ETR_{max} (22.78) was also recorded. The PAR_{max} for such cultures was 651.11 indicating that cultures growing under this concentration of CO_2 had a very healthy photosynthetic pigment and electron transport system resulting in efficient photoautotrophic growth of cultures (Vyas 2006).

A significant promotion in rooting response and shoot growth of *C. paniculatus* was observed on an enriched environment having 0.6 g (CO_2) m^{-3}. On this concentration an average of 10.83 (increased by 1.1 times) roots were produced per shoot. The average root length of was 2.98 cm as compared to 2.70 cm under control. The shoots attained a maximum height of 10.5 cm (8.5 cm in case of control) producing *ca*. 10.33 (8.5 on control) leaves per shoot. Fresh and dry weight contents were 1.5 and 1.3 times higher (Rao and Purohit 2008).

A CO_2-enriched environment also improved the acclimatization of *Achras zapota*, *Terminalia bellerica* and *Feronia limonia* plantlets under *ex vitro* conditions. Best response was achieved with 0.6 g m^{-3} and 40.0 g m^{-3} for *A. sapota* (Dave 2004), *T. bellerica* (Suthar 2009) and *F. limonia* (Vyas 2006) plants. In all cases, an increase in the number of roots/shoot, root length, fresh and dry weight was recorded (Purohit and Habibi 2010).

The cultures grown under additional CO_2 supply appeared to have luxury consumption and fertilizing effect (Kohlmaier *et al*. 1989) in the cultures grown *in vitro*. Best response on higher concentration of CO_2 can be explained by the fact that the concentration of CO_2 increases the rate of photosynthesis and improves the plantlet growth (Griffin and Seemann 1996). The present system did not employ any complex equipment and hence saved additional cost.

Xiao and Kozai (2002) and Zobayed *et al.* (2004) designed an automated photo-autotrophic micropropagation (PAM) system for the commercial production of calla lily (*Zantedeschia elliottiana*), a herbaceous flowering plant for which there is currently a large demand, is conventionally propagated by tubers, resulting in a limited multiplication rate. Although conventional micropropagation system enhanced the rate of multiplication, but its wide application is still limited by the high cost of production, poor plantlet growth, higher rate of contamination and labour intensive work (Lorenzo *et al.* 1998). The culture vessel unit consisted of a plexiglass box (115 cm wide × 52 cm deep × 20 cm high; 120-L volume capacity and approx. 0.6 m^2 area) with two air inlets and six air outlets for forced supply of CO_2. Three trays (48 × 36 × 7 cm) were placed in each vessel. Agar and sucrose were completely eliminated form the media and vermiculite was used as mechanical support. A forced ventilation unit for supplying CO_2-enriched air consisted of a CO_2 container with gas tubes, pressure gauges, airflow meters, an air pump and valves, an air disinfection and humidification tank, and a CO_2 concentration controller. A conventional photomixotrophic micropropagation unit (PMM) was also set aside for comparison. The PMM system comprised of a large number of culture vessels (*ca*. 500) of smaller volume (370 ml) filled with MS medium supplemented with 6 g L^{-1} agar and 30 g L^{-1} sucrose.

The growth in PAM was similar to or greater than the growth on day 30 under the PMM system. Shoot length, leaf area, fresh and dry weight per plantlet on day 15 were 2.0 times greater in the PAM than in PMM. The morphology and quality of plantlets also seemed suitable for *ex vitro* acclimatization. The loss due to contamination was 0% on day 15 and 5% on day 30 in the PMM. The monthly production capacity of calla lilly plantlets in PAM was about 3 times higher than that in the PMM. The PAM also shortened the rooting period by half when compared with that of PMM. Per cent rooting *in vitro* was 98% in PAM. The number of plantlets produced per year were 1,52,000 and 52,000 under the PAM and PMM, respectively. The labour cost in the PAM was less than half of that in the PMM. The reduced labour cost in the PAM significantly reduced the cost for *in vitro* multiplication and rooting. The unit price of plantlets produced under PAM and PMM was 56.18 and 101.56 US cents, respectively.

A similar method was also employed for China fir (*Cunnighamia lanceolata* (Lamb.) Hook) a rapid-growing woody plant valued for its timber in ornamental industries. The major problem faced by the conventional micropropagation method is excessive callus formation at the base in turn hindering *in vitro* (65%) and *ex vitro* (16%) root formation. The PAM system thus employed resulted in an overall increase in the rooting percentage *i.e.* 93% in case of in *in vitro* rooting and 97% during *ex vitro* conditions (Xiao and Kozai 2002; Zobayed *et al.* 2004).

Pospíšilová *et al.* (2000) demonstrated that tobacco plantlets produced under a CO_2-enriched environment and transplanted into pots saturated with ABA were able to conserve water and quickly developed fully functional photosynthetic capability. Plant biomass was doubled as a result of growth at high CO_2 and the shoot: root ratio was decreased. Stomatal density was increased in the leaves of the high CO_2 grown plants, which had greater numbers of smaller stomata and more epidermal cells on the abaxial surface (Soares *et al*. 2008).

Improvement in culture vessel environment

Tissue culture plantlets *in vitro* are planted in a culture vessel. The growth conditions of plantlets are affected by the internal microclimate of vessels such as light quantity and quality, light distribution, and air temperature. The internal microclimate of culture vessels is affected by the outside conditions and physical properties of culture vessels. The caps or closures and walls of tissue culture vessels isolated the inside environment from the containment of outside environment. Hence, by altering the interface between inside and outside environments, the effects of the conditions of the culture room microenvironment can be regulated. Huang and Chen (2005) have identified four selective criteria for a tissue culture vessel *viz.*, 1) light transmittance; 2) isolation from water loss and contamination; 3) allowance for some gas exchange; and 4) provison of an adequate growing area.

Culture tubes or vessels tightly sealed with closures have higher relative humidity (Bottcher *et al*. 1988) and water retention strength in semi-solid culture medium (Debergh *et al*. 1981). Besides, hermetic closures inhibit the gaseous exchange (McClelland and Smith 1990; Zobayed *et al*. 2001b) thereby altering the concentrations of ethylene and carbon dioxide in culture tubes/vessels, which results in abnormalities in plantlets morphology (Lentini *et al*. 1988; Perl *et al*. 1988; Genoud-Gourichon *et al*. 1993). The irradiance and spectra of light that reach the top of plantlets are also affected by the transmittance of the caps (Smith and Spomer 1995).

Recent reports suggest that loose vessel closures or vent on vessel lid facilitate better gaseous exchange, thus reducing hyperhydricity and avoiding contaminants. The effect of vessel has been studied and advantages of vented closures have been reported in a number of cases. By adjusting the air exchange rates between outside air and inside air through proper ventilation the accumulation of ethylene can be minimized considerably (Huang and Chan 2005). Majada *et al*. (2000) reported that anatomical variability of *in vitro*-grown plants of *Dianthus caryophyllus* cv. 'Nelken' to be a consequence of ventilation. The plants were cultured *in vitro* under different ventilation rates and it was revealed that ventilation modified the anatomical characteristics of shoots and leaves described for plants grown in

non-ventilated vessels: the cuticle became thicker, there was a decreased cell size and intracellular space size. Also, there were more photosynthetic and supportive tissues, including thicker cell walls. Increased ventilation promoted *in vitro* hardening of micropropagated carnation shoots, and pushed the culture-induced phenotype closer to that of *ex vitro* acclimatized plants. Also stomata of *in vitro* leaves grown in ventilated culture vessels were more functional than plants grown in non-ventilated types (Majada *et al*. 2001). The use of culture tubes closed with cotton plugs and steristoppers while culturing *Solanum tuberosum* exhibited higher fresh mass and shoot length, low senescence index with higher chlorophyll contents and without any morphological abnormalities that favoured acclimation to *ex vitro* conditions (Chanemougasoundharam *et al*. 2004). Plantlet growth of *Scrophularia yoshimurae* in vessels using dispense paper (DP) for ventilation as closure of culture vessels produced maximum number of shoots and were healthier and all plantlets survived after being transplanted to soil (Tsay *et al*. 2006) as compared to control AF (aluminium foil) as closures. The increase in number of air exchanges during *in vitro* cultivation of sweet potato exhibited higher photosynthetic rates and better survival percentage during field transfer (Xiao and Kozai 2006). During rooting of *Phillyrea*, the use of ventilated vessels in comparison with the closed ones enhanced development of roots, and doubled the dry weight of plantlets. The vessel ventilation was also beneficial for *in vitro* acclimatization of rooted *Phillyrea* plantlets (Lucchessini and Mensuali-Sodi 2004). Growth was substantially enhanced and vitrification (stunting and epinasty of leaves and hooking of stem apices) was reduced by increasing the efficiency of ventilation, the effects being greatest with forced ventilation in case of micropropagation of potato (Zobayed *et al.* 2001). Replacement of unvented caps of culture vessel (glass bottle) with indigenously designed vented caps proved highly useful in promoting the rate of shoot multiplication and increasing the fresh weight content of *Chlorophytum borivillianum* (Joshi 2005), *Feronia limonia* (Vyas 2006), *Celastrus paniculatus* (Rao 2007) and *Terminalia bellerica* (Rathore 2004; Suthar 2009). Such caps were made of autoclavable polypropylene having a central opening (ϕ 5.5 cm) with a small stopper between which a pad of cotton was inserted. The shoots grown under ventilation were dark green, lustrous, had broad lamina and did not show any symptoms of hyperhydricity. The wet weight of shoots was significantly higher per shoot cluster. On the contrary, shoots grown in bottles with unvented caps were thick, fleshy and hyperhydric. Better air exchange in the culture vessel provided congenial environment and showed cultures free from contamination.

Fal *et al.* (2002) in an experiment conducted on *Dianthus caryophyllus* demonstrated that PPFD inside the vessel was a linear function of the light transmittance of the vessel material and type of cap. This transmittance, and consequently PPFD, was higher in vessels closed with polypropylene than with metal caps.

Besides, ventilation physical methods like providing bottom cooling to the culture vessel 2–3°C below air temperature of the culture room (Ziv 1991), providing a higher light intensity (Rice *et al.* 1992) and gradual opening of vessels over a period of a few days prior to transplanting (Ziv 1986) have been suggested as effective ways of lowering relative humidity in the vessel which allowed better transplant survival of *in vitro* derived plantlets.

In addition to the culture vessel environment the type of culture vessel influences the efficiency of transfer during subculture and production of propagules per unit area. Proper choice of containers is an essential requirement during the different stages of production. Prakash *et al*. (2004) recommended the use of different types of containers depending upon the stage of propagation. He suggested the use of smaller vessels such as glass test tubes during the culture establishment stage and larger vessels conical flasks of 150-250 ml capacity for shoot multiplication and suspension cultures. The size of the flask may be increased depending upon the scale of production. However, smaller vessels with

Table 7 Types of vessels used during different stages of micropropagation.

Type of vessel	Maker	Specifications	Cost (in US$)
Culture tubes			
Borosilicate glass	Borosil	25 × 150 mm	0.42
Glass (autoclavable)	Sigma	25 × 150 mm	1.60
Conical flasks (borosilicate)			
Narrow mouth	Borosil	100 ml	1.4
Wide mouth	Borosil	100 ml	1.5
Petri dishes			
Glass (autoclavable)	Borosil		
Polycarbonate (pre-sterilized)	Hi Media	90, 100, 110 × 15 mm	0.12-0.15
Round vessels			
Culture bottles (glass)	Local	400 ml	0.1
Baby food jars (glass)	Sigma	72 mm	0.023
		95 mm	0.025
Phytajars (polypropylene)	Hi Media	250 ml	1.12
Phytacon™ round vessels (polypropylene)	Sigma	116 × 78 mm	0.44
		116 × 110 mm	0.48
		116 × 135 mm	0.58
Square vessels			
Magenta™ vessels (polypropylene)	Sigma	77 mm	1.35
		110 mm	1.44
Phytajars			
polystyrene	Hi Media	370 ml, 500 ml	0.40-0.90
polypropylene	Hi Media	370 ml, 500 ml	0.50-1.10
polycarbonate	Hi Media	370 ml, 500 ml	1.08-2.39
Trays			
Phytatray™ (square polystyrene)	Sigma	114 × 86 × 65 mm	0.06
		114 × 86 × 102 mm	0.35
Cellulose plug containing polyethyleneterepthalate tray	Sigma	145 × 190 × 81 mm (with 60-103 plugs)	9.0
Culture box			
polypropylene	Sigma	107 × 107 × 94 mm	2.2
polycarbonate	Sigma	109 × 109 × 96 mm	2.6

narrow mouth are more expensive and make manipulations of cultures difficult. In addition, irrespective of the type, the containers used for maintaining *in vitro* plants should be transparent to facilitate illumination and easy inspection. Munoz *et al.* (2009) reported that by using larger (350 mL) flasks with higher (50 mL) media volume, 100% more fresh weight of microbulb was obtained that treatment with smaller flasks (45 mL) and media volume (10 mL) in *Rhodophiala* species.

Recently, glass bottles, baby-food jars with polypropylene caps, transparent plastic containers, such as Magenta™, containers made of polypropylene and polycarbonate and polystyrene, disposable, non-autoclavable food containers and sandwich boxes made from polystyrene are more commonly used while performing tissue culture operations. The wide mouth makes culture manipulation easy and approximately 15-20 explants can be inoculated in each bottle. Such containers are widely available and cost *ca*. US$ 0.09 (**Table 7**). A study conducted on physical properties of culture vessels revealed that round vessels had more uniform transmittance than that of conical square or rectangular type of vessels. In addition to this the irradiance that reached the plantlet level was affected by the incident light and vessel materials. Therefore, polypropylene vessels could pass the wavelength ranged between 380 and 760 nm whereas polycarbonate filtered out the transmittance for the wavelength below 400 nm (Huang and Chen 2005).

To test the suitability of vessel for best shoot multiplication, shoot cultures of *Feronia limonia* (Vyas 2006), *Terminalia bellerica* (Suthar 2009), *Celastrus paniculatus* (Rao 2007) and *Chlorophytum borivilianum* (Joshi 2005) were grown in a variety of culture vessels *viz.*, narrow mouth conical flasks of 100 ml capacity (height 10.5 cm, bottom ϕ 6.0 cm, neck ϕ 2.5 cm), 150 ml capacity (height 12.4 cm, bottom ϕ 6.0 cm, neck ϕ 2.5 cm), wide mouth conical flasks of 100 ml capacity (height 11.0 cm, bottom ϕ 7.0 cm, neck ϕ 3.4 cm) and neutral glass bottle of 400 ml capacity (height 12.5 cm, mouth ϕ 5.5 cm). In all the cases best growth was achieved in 400 ml bottles. This was possibly due to better air exchange, larger growing area and high transmittance due to round surface of culture bottles.

Differences were shown in the environmental conditions inside different types of culture vessels leading to varied growth responses of plantlets that were incubated in the same external growth room conditions by Fal *et al.* (2002). The culture vessels included glass jam jars, 370 ml in volume, glass baby-food jars, 200 ml in volume, the same type of glass jars capped with polypropylene and Magenta GA7 polycarbonate vessels, 275 ml in volume. The best shoot production from these cultivars was in Baby food jars due to an intermediate value of air exchange rate and desiccation. The apical explants of four *Mentha* (mint) spp. were cultured in four different culture vessels viz., industrial glass jar (IG), Magenta vessel (MV), Erlenmeyer flask (EF) and culture tube with (CT). Magenta vessel GA 7 showed the best *in vitro* performance (Islam *et al.* 2005).

Tanaka and co-workers while experimenting with ornamental plants designed a number of culture vessels which were able to improve the *in vitro* growth and multiplication of these plants. Tanaka *et al*. (1988) first developed a film culture vessel, the *Culture Pack* (CP), which is made of fluorocarbon polymer films (*Neoflon*® PFA films) and supported by a stainless frame. Tanaka *et al.* (1996) later developed the *Miracle Pack* (MP), the practical model of the CP. The MP-PFA system made of PFA film and supported by a clear polycarbonate frame with RW as supporting material, had enhanced *in vitro* growth of many plant species such as *Anthurium*, *Syngonium*, *Spathiphyllum*, *Agapanthus*, and hascup (Tanaka *et al*. 1996), *Cymbidium* (Tanaka *et al*. 1999), and *Eucalyptus* (Nhut *et al*. 2002). In their recent experiments with *Spathiphyllum* they employed a novel disposable gas-permeable *Vitron* culture vessel, which is of similar size and shape as the MP-PFA. The *Vitron* is made from a novel *OTP*® film, a multi-layer film consisting of TPX (4-methyl-1-pentane polymer) and CPP (a polypropylene), which has similar physical characteristics as PFA film. The frame of the *Vitron* apparatus is made of polypropylene, which also greatly reduces the cost. Vitron vessel also proved to be beneficial for micropropagation of *Epidendrum* orchid and sweet potato (Teixeira da Silva *et al.* 2006)

In vitro hardening

Ex vitro acclimatization of tissue culture raised plants is a labour intensive and expensive process (Anderson and Meagher 1978). Mortality of tissue culture derived plants on account of excessive loss of water due to high transpiration rates and sluggish movement of stomata is a common phenomenon during *ex vitro* transfer. Plantlets grown *in vitro* are usually smaller than their field counterparts. The diminutive size, modified morphology, anatomical structure and physiological functions are the results of growth conditions *in vitro*, i.e. low irradiance, high relative humidity, heterotrophic or mixotrophic nutrition and restricted volume of the stoppered vessels (Pospíšilová *et al*. 1992). Leaves formed *in vitro* often have poor cuticular wax, high stomatal frequency, impaired stomatal structure and movement. These characteristics are responsible for excessive water loss upon transplantation to low humidity conditions and result into wilting and death of the plantlets (Diettrich *et al*. 1992; Santamaria and Kerstiens 1994).

In order to make the micropropagated plantlets survive upon transplantation, *in vitro* hardening as an intervening exercise during hardening and acclimatization of tissue culture plants has been applied in large number of plants (Purohit *et al*. 1998; Raste and Bhojwani 1998). Transfer of tissue culture plants directly to polybags for hardening and acclimatization may not be successful due to transplantation shock leading to heavy mortality during weaning period (Chaturvedi 1992). Alternatively, tissue culture plants can be hardened *in vitro* where ex-agar plants are transferred to soilless media (e.g. Soilrite™) moistened with nutrient salt solution without sucrose in culture bottles stoppered with polypropylene caps under aseptic conditions. *In vitro* hardening has been employed for the hardening and acclimatization of several plants: *Wrightia tomentosa* (Tak 1993; Purohit and Kukda 2004), *Sterculia urens* (Samar 1999), *Annona squamosa* (Nagori and Purohit 2004a), *Achras sapota* (Dave 2004; Nagori *et al.* 2009), *Chlorophytum borivilianum* (Dave 1994; Joshi 2005), *Celastrus paniculatus* (Bilochi 2001; Rao and Purohit 2006), *Feronia limonia* (Vyas *et al.* 2005) and *Terminalia bellerica* (Rathore *et al.* 2007). Shoots rooted *in vitro* were taken out of the culture vessels and washed gently in distilled water to remove adhering agar. These plantlets were transferred to culture bottles filled with Soilrite™ moistened with quarter strength of salts solution and were allowed to grow for 10 days in culture room conditions. For acclimatization, *in vitro* hardened plants were initially kept under high humidity conditions and then gradually exposed to low humidity regime (under high light intensity) and external environment. Hence, bottles were shifted to greenhouse which uses fan–pad evaporative cooling system. Two methods were adopted to expose the plants to low relative humidity. In one method, plantlets were exposed to *ex vitro* conditions by gradual opening of caps and then transferring the plantlets to plastic pots containing Soilrite™ moistened with ¼th salts solution. Such plants were allowed to grow under greenhouse conditions. In the other method, the caps were opened and plantlets were directly transferred to the plastic pots and kept in glass trough covered with transparent polythene maintaining high humidity. Plants under such conditions were allowed to grow for 25 days and subsequently exposed to environmental conditions by pricking holes in the polythene. Plastic covering was removed completely after 15 days. Hardened plantlets were transferred to the pots (15.0 cm height and 13.0 cm diameter) filled with potting mixture (sand, soil and leaf manure in 1: 1: 1 ratio). Growth of the plants was analyzed in terms of shoot length, root length, number of roots/shoot, fresh and dry weight per plantlet, total number

of leaves and leaf area after 40 days. It was well demonstrated in all the above cases that rooted plants transferred directly to pots without prior hardening showed yellowing of leaves followed by defoliation within 1-2 days. With *in vitro* hardening of plantlets in culture bottles containing Soilrite™, prior to their transfer to greenhouse for acclimatization, higher rates of plantlet survival and their soil establishment had been observed.

Thus *in vitro* hardening helped plants to withstand the *ex vitro* conditions of low humidity and high light intensity (Bhojwani and Razdan 1996). *In vitro* hardening of plants by decreasing humidity using gas permeable lids, increasing irradiance and increasing CO_2 concentration minimized the wilting of plants after transplantation (Deng and Donnelly 1993; Cassells and Walsh 1994; Kanechi *et al*. 1998).

Priming of tissue culture plantlets

The ability of the *in vitro* derived propagules to withstand transplanting stress very often determines the success or failure of tissue culture operations (Nowak and Pruski 2004). Therefore, the manipulation of growing environment prior to and upon transplanting forms an integral part of tissue culture and is called as priming (Nowak and Shulaev 2003). 'Priming' has been defined as a phenomenon associated with the ability of tissue culture propagules to induce molecular mechanisms of resistance to abiotic and biotic stresses encountered during transplanting and during early growth of transplanting (Nowak and Shulaev 2003). Priming is significant as shoots grown *in vitro* are exposed to unique microenvironment which induce many morphological, anatomical and physiological abnormalities in plant (Donnelly *et al.* 1985; Capellades *et al.* 1990; Chaturvedi 1992) that contribute to high mortality during greenhouse and field transfer (Kozai 1991b). Mortality of tissue culture derived plants on account of excessive loss of water due to high transpiration rates and sluggish movement of stomata is a common phenomenon during *ex vitro* transfer. High stomatal densities and impaired stomatal mechanism have been attributed for such responses. Non–closure of stomata could be due to guard cell wall (Ziv *et al.* 1987) and/or deformation of stomata (Blanke and Balcher 1989; Sallanon *et al.* 1991) which cannot be repaired by manipulation of external factors. Reduced epicuticular wax has also been found responsible for excessive transpiration (Pierik 1989; Ziv 1991).

Through micropropagation, about 50% of flori-horticultural plants are produced. However, at the weaning stage, about 10-40% (<70%) of plantlets either die or do not attain market standard, thereby causing significant losses at the commercial level (Varma and Schűepp 1995; Cassells *et al.* 1996).

Incorporation of various chemicals like jasmonates (Ravnikar *et al.* 1992; Dolcet-Sanjuan and Claveria 1995; Gaspar *et al.* 1996), salicylic acid (Kunkel and Brooks 2002; Ton *et al.* 2002), phloroglucinol (Hoque and Arima 2002) and growth retardants (Uosukainen *et al.* 2000; Hazarika 2003) like paclobutrazol (Smith *et al.* 1990) in the rooting medium has been known to induce resistance in plants. For priming of *Eclipta alba* microshoots, 6.3 µM of chlorocholine chloride was found most effective (Ray and Bhattacharya 2008). The major changes observed in 30-days-old treated shoots were, production of increased number of roots, elevation of chlorophyll level in leaves and increase in plant biomass. Furthermore, arrested undesirable shoot elongation made the plants sturdier and more suitable for acclimatization. The primed micropropagated *E. alba* plants were healthy and survived by higher frequency (100%) in soil in comparison to the non-treated plants (84% survival). Several chemicals also influence the development of the root system. Abscisic acid (Hooker and Thorpe 1998) and jasmonic acid (JA) is known to affect initiation of new lateral roots and overall changes to the root architecture. In addition, low JA concentrations (0.5-1.0 µM) stimulate both root and shoot growth (Conrath *et al*. 2002). In potato, nodal explants from JA primed stock plants tuberized earlier and more uniformly, and produced more microtubers than the non-primed controls (Pruski *et al*. 2002). Salicylic acid (SA) is another major signalling compound capable of inducing abiotic and biotic stress tolerance in plants (Conrath *et al*. 2002). It also induces adventitious roots in cuttings and stomata closure, and inhibits ethylene biosynthesis in detached leaves (Huang *et al*. 1993). Use of anitranspirants like polyvinyl resin (Wardle *et al.* 1979), ABA (Pospíšilová *et al.* 1998), phenyl mercuric acetate (Rao 1985), silicone rubber (Fuchigami *et al.* 1981) and acrylic latex polymer (Voyiatzis and McGranaham 1994) during *ex vitro* transplantation have been reported to improve transplant survival of tissue culture plants. Addition of low concentration of the growth retardant daminozide to hormone-free medium enhanced post-transplanting acclimatization of potato plants (Tadesse *et al*. 2000). Other supplements like activated charcoal (Gantait *et al.* 2009), seaweed concentrate during *in vitro* rooting of potato (Kowalski *et al.* 1999), pyroligneus acid in case of *in vitro* rooting of Japanese pear (Kadota *et al*. 2002), and dilution of salts in the basic MS medium during culture of two clones of a cold-tolerant *Eucalyptus grandis* X *E. nitens* hybrid (Mokotedi *et al*. 2000) can also aid root initiation and growth (Nowak and Shulaev 2003). A protocol was developed for *in vitro* rooting of *Dendrobium chrysotoxum* Lindl. cv. 'Golden Boy' where different auxin sources (indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA)) were evaluated in the presence of activated charcoal. A combination of 0.2 mg l^{-1} IBA plus 2 g l^{-1} AC in MS proved excellent in terms of earliness in root induction, root number, and length as well as response to rooting of *in vitro* generated microshoots (Gantait *et al.* 2009). Fe-EDTA (1 mg l^{-1}) and activated charcoal (1 g l^{-1}) were reported to stimulate total fresh weight and PLB formation in the presence of PGRs in *Cymbidium* (Teixeira da Silva *et al.* 2006). More detailed reviews on priming of tissue culture raised plantlets are presented by Nowak and Shulaev (2003) and Beckers and Conrath (2007).

More recently it is being increasingly realized that "biopriming" using microbes and their association plays an important role in establishment and subsequent growth of tissue culture propagules. Microbial inoculants have been used to induce stress resistance in plant propagules produced *in vitro* prior to their transplantation (Herman 1996; Balla *et al.* 1997). Enhanced biotic and abiotic resistance in response to some microbial inoculant(s) leading to developmental and physiological changes in the *in vitro* culture derived plantlets has been referred to as 'biotization' or 'biopriming' (Herman 1996; Nowak 1998).

In vitro (as co cultures with plant, tissue or organ) and *ex vitro* biopriming of micropropagated plants with such organisms improve plant performance under stress environments and consequently enhance yields (Nowak 1998; Rai 2001). Many plant beneficial, naturally associated algae, bacteria and fungi (especially, vesicular–arbuscular mycorrhizae – VAM) have been shown to enhance stress resistance in micropropagated plants (Hooker *et al*. 1994; Elmeskaoui *et al*. 1995; Lazarovits and Nowak 1997; Wilhelm *et al*. 1997; Duffy *et al*. 1999; Nowak *et al*. 1999). Varma *et al*. (1999; 2001) have reported promotory role of an endophytic root fungus in growth and overall biomass production in a number of tissue culture raised plants. Sahay and Verma (2000) reported 90% post–transplantation survival of micropropagated tobacco and brinjal treated with *Piriformospora indica*. Biopriming has also been achieved with microbial derivatives like culture filtrates, acetone precipitated fractions of bacteria etc. (Hussain *et al*. 1994; Chang *et al*. 1997). **Table 8** lists some important plants which performed better upon inoculation with microbial filtrates prior to transplantation (for review see Nowak and Shulaev 2003; Kapoor *et al.* 2008). Besides this, biopriming has also been reported to increase resistance against pathogenic attacks (Harish *et al.* 2008).

The roots of tissue culture raised plantlets of *Boswellia*

Table 8 Biopriming in micropropagation.

Biopriming agents	Plant	Reference
***In vitro* bacterization**		
Pseudomonas fluorescence	*Arabidopsis thaliana*	Pieterse *et al*. 1996; Van Wees *et al*. 2000
Paenibacillus polymyxa	*Arabidopsis thaliana*	Timmusk and Wagner 1999; Cheong *et al.* 2002
Burkholderia sp.	Potato	Nowak 1998
Burkholderia phytofirmans	Grapevine	Barka *et al.* 2006
	Potato, tomato, sweet pepper, cucumber and grape	Nowak *et al.* 2007
Azospirillum brasiliense	Wheat plantets	Creus *et al.* 1998
Rhizobium isolates	Rooted black locust (*Robina pseudoacacia* L.)	Balla *et al.* 1997
Agrobacterium rhizogenes	*Pinus* sp.	Villalobos-Amador *et al*. 2002
Pseudomonas putida	*Brassica campestris*	Lifshitz *et al*. 1987; Hall *et al*. 1996
Pseudomonas spp.	Oregano	Shetty *et al*. 1995
Bacillus subtilis	Chestnut	Wilhelm *et al*. 1997
	Pigeon pea	Podile and Laxmi 2008
Acetobacter diazotrophicus	Sugarcane	Reis *et al*. 1999
Azotobacter zettuovii	*Daucus carota*	Varga *et al.* 1994
Pseudomonas fluorescens, *Azospirillum brasilense*, *Trichoderma harzianum*	*Camellia sinensis*	Thomas *et al.* 2010
Mycorrhization		
Piriformospora indica	Rica and carrot	Sudha *et al.* 1998
	Nicotiana tabacum and *Bacopa monnieri*	Varma *et al.* 1999
	Terrestrial orchids	Blechert *et al.* 1999
	Tobacco and brinjal	Sahay and Verma 2000
	Withania somnifera, *Spilanthes calva*	Rai *et al*. 2001
	Barley	Rai *et al.* 2004
	Pelargonium, *Poinsettia* and *Petunia*	Waller *et al.* 2005; Deshmukh *et al.* 2006; Druege *et al.* 2007
	Arabidopsis thaliana	Peškan-Berghöfer *et al.* 2004; Shahollari *et al.* 2007; Oelmüller *et al.* 2009
Glomus intraradices	Tobacco	Maier *et al*. 1999
	Potato	Gallou *et al.* 2010
Glomus fistulosum	Potato, strawberry, azalea	Vosatka *et al.* 2000
Gigaspora species	*Babtista tinctoria*	Grotkass *et al*. 2000
Tuber melanosporum	*Cistus incanus*	Wenkart *et al*. 2001
Scutellospora species	*Psidium guajava*	Estrada-Luna *et al.* 2000
	Garlic	Lubraco *et al*. 2000
Crlomus spp.	*Withania somnifera*	Rai and Varma 2002
Pisolithus tinctorius	*Pinus massoniana*	Zhu *et al.* 2010

serrata (Suthar 2009), *Celastrus paniculatus* (Rao 2009), *Feronia limonia* (Vyas *et al.* 2008) and *Termianlia bellerica* (Suthar and Purohit 2008) were colonized with *Piriformospora indica* in order to improve growth and *ex vitro* survival of plantlets and to define the role of this fungus as biopriming agent. *P. indica*, an endophytic, cultivable fungus was maintained on Kaefer's medium (Kaefer 1977). Uniformly rooted plantlets immediately after their removal from agar-gelled medium were subjected to *in vitro* hardening in culture bottles containing autoclaved Soilrite™ and covered with polypropylene caps. The Soilrite™ was moistened with ¼ strength of MS salts solution and 50 mg (fresh weight per plant) of fungal mycelium was mixed with it. Bottles were kept in standard greenhouse conditions and their caps were opened gradually.

About 70% of *B. serrata* plantlets showed successful root colonization when inoculated with *P. indica* during *in vitro* hardening. Root colonization was observed intracellulary in cortical cells where it formed round bodies *P. indica* did not invade the stellar tissues or traversed upward into shoot. As compared to treated (inoculated with *P. indica*) plantlets, roots of control plants (without inoculated) contained no fungal structures. Plantlets inoculated with *P. indica* attained maximum height of 6.5 cm along with increased number of leaves (5.8) per plantlet as observed after one month of their growth in the greenhouse as compared to control plantlets where an average of 5.6 cm height with 4.6 leaves per plantlet was recorded. Inoculated plantlets also showed significant increase in number of roots (2.1) and root length (2.9 cm) per plantlet, which accounted for their improved growth. *P. indica* treated plantlets also showed increase in total fresh (1.230 g) and dry weight (0.980 g) were recorded in comparison to control. When transplanted to the potting mixture in the polybags, the inoculated plantlets showed better growth and higher survival percentage (75%) as observed after their growth under greenhouse conditions. Root colonized plants on their transplantation in the polybags and grown under greenhouse conditions showed more than 90% survival as compared to 75% survival of untreated control plantlets (Suthar 2009).

Inoculation of *T. bellerica* plantlets with *P. indica* was beneficial both in terms of overall growth and *ex vitro* survival in comparison to untreated control plantlets. Root colonization was observed after 10 days of inoculation where fungal mycelium grew intra-cellularly. Consequently, a pronounced increment in overall growth and height of treated plantlets was recorded. The average total number of leaves (16.5) produced per plantlet was also higher as compared to control (9.5 leaves per plantlet). Such leaves showed an average area of 562.33 mm^2 per leaf as against 313.33 mm^2 recorded for untreated control. Significant increase in root length (10.10 cm) and number of laterals in treated plantlets was observed which accounted for their improved growth. However, no significant difference in total number of roots produced in treated and untreated plantlets was observed. Highest total fresh (1.55 g) and dry weight (0.150 g) of plantlets was also recorded with treated plantlets. In control plantlets total 0.940 g fresh and 0.105 g dry weight was recorded per plantlet (Suthar and Purohit 2008).

In comparison to the untreated controls, the plantlets of *C. paniculatus* inoculated with *P. indica* attained maximum height of 13 cm along with increased leaf area (86 mm^2) and number of leaves (12.5) per plantlet as observed after

one month of their growth in the greenhouse. When transplanted to the potting mix in polybags, the treated plants showed better growth and higher survival percentage (*ca*. 80%) as observed after 3 months of their growth under nurseryshed conditions (Rao 2007).

A positive influence of root colonization with *P. indica* on vegetative growth and development in micropropagated plants of *F. limonia* was observed during the present study. The treated plants showed pronounced growth relative to the uninoculated controls. Similar reports of increased growth on inoculation with *P. indica* have been published in case of *Spilanthes calva* and *Withania somnifera* (Rai *et al.* 2001). Varma *et al.* (1999) reported an increase in plant height, fresh and dry biomass and larger leaf area in micropropagated *Artemisia annua*, *Bacopa monnieri* and tobacco. The differences in growth observed between treated and control plants have been suggested to be caused by greater absorption of water and nutrients due to extensive colonization of roots by *P. indica* (Rai *et al.* 2001). The plants have been reported to show enhanced phosphorus uptake also. The increase in fresh and dry weight has been shown to be related to increased phosphorus uptake (Sudha *et al.* 1998). The more intense root proliferation in treated plants here may be due to the synthesis of yet unidentified extracellular phytohormones by *P. indica* (Singh *et al.* 2000; Varma *et al*. 2001). The biochemical parameters studied revealed an increase in peroxidase, SOD and NR activity in biotized plantlets. This was indicative of a better defense mechanism in the tissue and high oxidation rates which supported plantlet development. These observations are also supported by increase in protein contents among biotized plants as compared to normal ones (Vyas *et al.* 2008).

Microscopic examination of stained root samples recorded a high colonization in more than 80% of plantlets. Colonization was observed intracellularly in epidermal and cortical cells where it formed coils and branches or spores but typical arbuscules as generally developed by mycorrhizal association was not observed.

Thirty-day-old, *in vitro* rooted plants of grape cv. 'Pusa Urvashi' and 'Pusa Navrang' were subjected to root colonization with five arbuscular mycorrhizal fungal (AMF) strains viz. *Glomus mossae*, *G. manihotis*, *G. deserticola*, *Gigaspora gigantia* and *Acaulospora laevis* along with an un-inoculated control. Mycorrhizal plantlets showed improved vegetative growth, high shoot and root fresh and dry weight, leaf area, chlorophyll, sugars and phenol contents. Photosynthetic rates were enhanced upto two times in AMF treated plants. The foliar tissues in treated plants showed improved nutrient contents especially for the P, Mg, Zn and Mn. High plantlet survival was noted for the mycorrhizal plants after glasshouse and field transfer (Singh *et al*. 2004).

In addition, micropropagated banana plantlets were tested for the presence of BBTV by ELISA, DIBA and PCR and the uninfected plants were biohardened with two rhizobacterial (*Pseudomonas fluorescens*, Pf1, CHA0) and endophytic bacterial (EPB5, EPB22) strains. Plants treated with mixtures of rhizobacterial and endophytic bacterial formulations viz., EPB5 + EPB22 + Pf1 + CHA0 was significantly effective in reducing BBTV under field conditions recording 33.33% infection with 60% reduction over control (Harish *et al.* 2008). Inoculation experiments were conducted to study the effects of *P. indica* on adventitious rooting in poinsettia and pelargonium.. Inoculation with *P. indica* dramatically enhanced the number and length of the adventitious roots in both the plants (Druege *et al.* 2007).

For the first time both priming and biopriming approaches were integrated into the sugarcane micropropagation technology by temporary immersion bioreactors (TIBs). When combined with the inoculation of the endophytic *Gluconacetobacter diazotrophicus* during transplanting, a significant improvement of the percentage of survival has been attached through this critical step (Bernal *et al.* 2008).

The micro-cloned plantlets of *Chlorophytum borivilianum* registered more than 95% establishment in soil following treatment with various bio-inoculants namely; *Glomus aggregatum*, *Trichoderma harazianum* and *Piriformospora indica* whereas *Azospirullum* sp. (CIM-azo) and Actinomycetes sp. (CIM-actin) showed only up to 85% plantlet establishment. The un-rooted shoots were also treated with these bio-inoculants, for *in vivo* root induction and increased survival rate/establishment frequency when transferred to soil. The un-rooted shoots also showed *in vivo* rooting (50%) when treated with mycorrhiza *Glomus aggregatum* (VAM) and *Trichoderma harazianum* (Mathur *et al.* 2008).

Micropropagated sea oats from were inoculated with AM fungal communities and were grown in the greenhouse for 8 weeks and outplanted in the field. After 1 year, root colonization was evaluated, and after 2 years, root colonization, shoot and root dry masses, and shoot- and root-P contents were determined. Overall, sea oats planted in field had greater percent root colonization, shoot dry mass, and shoot-P content than those that were non-inoculated (Agely and Sylvia 2008).

Quality assurance

Quality checks are essential to ensure the production of high quality plants and to have endusers' confidence. Quality standards require the establishment of suitable tests to maintain quality control. The choice of explant source, freedom of the donor plant from viruses, disease causing fungi, bacteria, viroids, phytoplasmas, vigour and conformity of the variety, and elimination of somaclonal variants are critical for maintaining plant quality. Variety identification by proper labeling at all stages is essential to ensure varietal identity.

1. Virus indexing

One of the main advantages of tissue culture-raised plants is the production of disease free true-to-type planting material. This is however possible only if a proper protocol has been adopted. It is therefore, very necessary that the planting material produced through tissue culture is certified before being distributed for plantation. Recently, the testing of explants before being used as mother plants, to produce virus-free stocks is more common in use. The technique is more commonly referred to as virus indexing. Many viruses have a delayed resurgence period in cultured plants. This necessitates the indexing of plants several times and only those plants are labeled as virus free which give consistent negative results. Procedures to detect the presence of viruses include visual examination for viral symptoms, infection tests on indicator plants, serological tests, electron microscopy, and direct detection of RNA using molecular techniques. Internationally approved and recognized detection systems include serological and molecular laboratory assays, and indicator hosts in the greenhouse. Virus Indexing is an essential requirement for the commercial production of plants. To facilitate this, the Department has set up National facility for Virus Diagnosis and Quality Control of Tissue Culture raised plants in India (Prakash 2006). The main facility is at IARI, New Delhi and has 5 satellite centers at NCL, Pune; TERI, New Delhi; IHBT, Palampur; IIHR, Bangalore; and SPIC, Chennai. As per the project objective, the centers are concentrating on virus diagnosis of horticultural and plantation crops especially spices. The facility functions in a network manner and each center has its defined role and responsibilities. The virus indexing is being done for known and unknown pathogens. Diagnostic kits are being developed. The planting material is being indexed and certified for the industry. Antibodies for threee viruses, CMV (*Cucumber mosaic cucumovirus*), LmoV (*Lily mottl potyvirus*) and LSV (*Lily symptomless carlavirus*) have been produced and primers for 4 viruses, Carlavirus and Carmovirus (group specific), Chrysanthemum aspermy and peanut stunt cucumoviruses have been designed/synthesized andRT-PCR based diagnostic protocols have been standardized. ELISA and RT-PC-based detection

has been standardized for a large number of viruses including some new ones (Prakash 2007).

This facility is aimed at providing necessary support to the industry and other research institutes/organization involved in large scale production of tissue culture plants. The 3 virus testing virus centers have been recently Notified under Gazette of India Notification has Inspection Authorities for Post-Entry Quarantine testing for Imported Tissue Culture plants DBT along with Ministry of Agriculture has also criteria/guidelines for Assessment of Tissue Culture Units/Laboratories. Standard guidelines for certification of different tissue culture raised plants are being prepared. Guidelines for potato minituber have been finalized and the same for Sugarcane and Banana are under preparation (Prakash 2006).

Internationally approved and recognized detection systems including serological and molecular laboratory assays and indicator hosts in greenhouse and field indexing were updated regularly by the ISHS International Working Group on Fruit Tree Viruses in occasion of their meetings 1991 in Vienna (Aburkhes 1992), 1997 in Bethesda (Roy 1998) and 2000 in Canterbury (Candresse 2001). In last few years increasing numbers of programs have developed their own capabilities for producing pathogen free material in North America and Europe. Currently, 15 out of 16 programs in Europe report being self-sufficient in this respect, whereas, in North America, 16 out of 21 programs rely on other agencies or commercial companies in addition to their own. Heat treatment for virus eradication is being used by 75% of the programs in North America and 67% in Europe (Jones 1988). The EPPO panel on Certification of Fruit Crops was created in 1985 with an aim to produce certification schemes for fruit crops which are of importance to the Euro-Mediterranean region. So far 11 certification schemes have been officially approved by the organization. They cover pome and stone fruits, small fruits, citrus and also grape vine (Roy 1998). Microbiological quality assurance systems (e.g. Hazard Analysis Critical Control Point; HACCP procedures) have been formulated and adapted to the needs of commercial plant tissue culture laboratories. These are aimed at, preventing the introduction of pathogens, into tissue cultures at establishment and in the laboratory (Leifert and Cassels 2001).

Quality of the plants can be guaranteed by certification according to internationally recognized standards for production, such as ISO or HACCP. The health and identity can be guaranteed by the certification of the plants according to the NAKTUINBOUW-Elite® regulations. These include testing for all known pathogens of the crop and for homogeneous and true-to-type flowering. As with certification of the production process, certification of plants using this system has to be renewed every year. The system was adapted for tissue culture after the devastating outbreak of *Tobacco mosaic virus* in *Petunia*. It proved to be so successful that it was extended to all vegetatively propagated bedding plants. This increased the demand for certified plants from tissue culture tremendously (van der Linde 2000).

The North American Plant Protection Organization (NAPPO) is the regional plant protection organization representing Mexico, the USA, and Canada. NAPPO produces guidelines for trade in plant material among the member countries and for importation into the NAPPO region with the goal of preventing the introduction and/or spread of serious pathogens. The NAPPO ad hoc Fruit Tree and Grapevine Nursery Stock Certification Standards Panel is currently developing: a) certification guidelines for fruit crops, b) lists of the "virus" pathogens in each crop, c) acceptable testing methods for "viruses", and d) distribution of "viruses" in the NAPPO region and worldwide. Once these standards are accepted by the NAPPO countries, they will provide a framework for harmonization of existing and new certification programs (Thompson 1998).

2. Test of clonal fidelity

The technique of micropropagation is advocated theoretically for providing true-to-type clones, but this is not the case. In several *in vitro* regeneration systems, it has been observed that progenies of plants derived from the same initial source material and multiplied following a strictly identical culture protocol, including the same length of time in culture, exhibit variable percentages of off-types (Fukui 1983; Sree Ramulu *et al*. 1984; Karp and Maddock 1984; Benzion and Phillips 1988; Wang *et al*. 1992). The term 'somaclonal variation' has been proposed to describe the variability produced by *in vitro* multiplication (Larkin and Scowcroft 1981). The occurrence of somaclonal variation is associated with point mutations and chromosomal rearrangements and recombination, DNA methylation, altered sequence copy number, transposable elements, and seems to be influenced by the genotype, explant type, culture medium, age of the donor plants (Veilleux and Johnson 1998; Jain *et al*. 1998; Jain 1997b). Depending on the plant type, the number of subcultures is another important aspect that can lead to more variation. Tissue culture system itself acts as a mutagenic system because cells experience traumatic experiences from isolation, and may reprogramme during plant regeneration which are different than under natural conditions. Reprogramming or restructuring of events can create a wide range of epigenetic variation in newly regenerated plants (Jain 2000).

In view of extensive reports on the occurrence of tissue culture induced variations (Olmos *et al.* 2002; Kawiak and Lojkowska 2004; Rady 2006; Prado *et al.* 2007) there is a strong need to verify clonal fidelity of regenerated plant and assess the reliability of micropropagation protocols. In the light of various factors that lead to genetic instability at cellular and molecular levels, assessment of clonal fidelity using a multidisciplinary approach initially and during different culture passages is essential. Genetic analysis of stability/variability at various culture stages may help in identifying specific culture age and conditions that can induce variations and suitable measures can be suggested to guarantee fidelity of tissue culture raised plantlets (Rani and Raina 2000). A wide range of markers based on morphological cytological, biochemical and molecular traits have been recommended to evaluate tissue culture plantlets for clonal fidelity. Evaluation and documentation employing a combination of parameters can generate useful data for a particular plant species (Fourre *et al.* 1997). Furthermore, they provide broad spectrum of characteristics to derive conclusions regarding suitability of protocols and culture conditions for long-term culture of plants.

a) Morphological markers: Visual detection of off-types in micropropagated plantlets were used to detect variants prior to the advent of techniques of molecular biology. Morphological markers correspond to qualitative and observable traits that can be scored visually. Being simple and omnipresent, these are irreplaceable and have been used to evaluate genetic stability in tissue culture derived plants of several genera such as *Pelargonium* (Cassells *et al.* 1997), *Ananas* (Das and Bhowmik 1997), *Phalaenopsis* (Chen *et al.* 1998), *Arachis* (Eapen *et al.* 1998), *Musa* (Grajal-Martin *et al.* 1998) and *Saintpaulia* (Paek and Hahn 1999). Rani and Raina (2000) and Hazarika (2006) have extensively reviewed the use of morphological markers in the identification of somaclonal variants. In spite of their wide use, morphological descriptors have several limitations. They are often limited in number, developmentally regulated and environmentally influenced (Lu *et al.* 1996; Galderesi *et al.* 1998) leading to errors in scoring. Morphological markers require extensive observations of the plants until maturity. Furthermore, some changes induced by *in vitro* culture cannot be observed because the structural difference in the gene product does not always alter its biological activity to such an extent that it modifies a phenotype (Palombi and Damiano 2002). **Table 9** presents a consolidated list of

Table 9 Morphological off-types during micropropagation of plants.

Plant	Phenotypic variability	Reference
Strawberry	Hyperflowering, fruit malformation, small plants, lower yields, change in bloom date, and runnerless female sterile plants.	Swartz *et al.* 1981
Banana	Morphologically aberrant.	Hwang 1986
Grapevine	Jagged and pubescent leaves, lower side of the limb having short, erect hairs, branches with more accentuated anthocyanic pigmentation, lower yields.	Grenan 1992
Pineapple	Plants either totally or partially spiny, fruits, showing albino stripes and spininess.	Wakasa 1979; Moore *et al.* 1992
Sugarbeet	Variations observed in the leaves and reproductive structures in micropropagated plants.	Saunders *et al.* 1990
Lotus corniculatus	Plants were short, flowered at a lower node, and had a reduced leaflet length: width ratio.	Orshinsky and Tomes 1984
Hermaphrodite papaya	Entirely pistillate plants.	Litz 1987
Thornless blackberries	Plants with increased vigour, smaller fruit size with differences in leaf size and pattern.	Swartz *et al*. 1983
Wheat	Variation was detected in plant height, grain number per spike, kernel weight, seed yield, total dry weight, and harvest index.	Ryan *et al*. 1987; Mohmand and Nabors 1990
Oil palm	The stamens develped into carpel like structures, no pollen formation in male inflorescences.	Corley *et al*. 1986; Paranjothy *et al.* 1990
Dendrobium	Variations in the shape and color of flowers.	Vajrabhaya 1977
sugarcane	Extremely narrow leaves, profuse tillering, twisting of the leaf lamina giving a crinkled appearance, and enclosure of the spindle leaf within the elongated leaf sheath, albino, pale green, dark green and purple leaf sheath, and purple leaf off-types.	Ahloowalia and Maretzki 1983; Taylor *et al*. 1995
Celery	Abnormal leaf and petiole morphology, stunting, premature flowering and multiple growth centers.	Fujii 1982
Maize	Dwarfs, necrotic forms, faulty pigmentation, striping in abnormal seedlings and plants, and defective kernels.	Earle and Kuehnle 1990
Coleus	Leaf color variants in shoot cultures.	Marcotrigiono 1990
Sorghum bicolor	Plants with several types of chlorophyll deficiency, short culms, seed sterility, narrow and ragged leaves, chimeric patterns, and multibranched heads.	Cai *et al*. 1990
Nicotiana tabacum, Liquidamber styraciflua	Difference in stomatal frequency.	Ticha *et al.* 1999
Strawberry	More than twice as many runners were formed by *ex vitro*, than by *in vitro*-rooted plants.	Borkowska 2001
Citrus	Stomata with kidney-shaped guard cells were observed in greenhouse leaves while crescent shaped and rounded guard cells were observed in *in vitro* leaves.	Hazarika *et al.* 2002
Carnation	Hyperhydric carnation leaves showed high peroxidase activity, low lignification and high malondialdehyde content, suggesting oxidative damage.	Piqueras *et al.* 2002
Sunflower	Hyperhydricity in shoot cultures	Mayor *et al.* 2003
Prunus avium	Hyperhydric shoots	Frank *et al.* 2004
Dianthus caryophyllus	Hyperhydric leaves had large vacuolated mesophyll cells, showing hypertrophy of cells.	Saher *et al.* 2005
Rheum rhaponticum (rhubarb)	Alterations in leaf trichomes, stomatal characteristics and epidermal cellular features	Zhao *et al.* 2006
Musa	All of them were characterized as plants with modified or deformed leaf shape, with very low chlorophyll content.	Adel El-Sawy 2007
Carnation	Higher water content, lower chlorophyll content, and defective deposition of epicuticular waxes.	Cassanova *et al.* 2008
Rhubarb	Prior to transplanting to the field the acclimated plants showed a high degree of uniformity with respect to foliage size, development and vigour but had substantial variation in root development.	Zhao *et al.* 2009
Handroanthus impetiginosus	Hyperhydric shoots presented numerous anatomical abnormalities at the proliferation stage. Disorganized cortex, epidermal holes, epidermal discontinuity, collapsed cells, and other structural characteristics were observed.	Jausoro *et al.* 2010

examples of morphological variations detected during micropropagation of some important plant species.

b) Cytological markers: Cytogenetical analysis is one of the most informative and reliable techniques to ascertain whether any changes occurred in the nuclear material during the process of regeneration and organogenesis (Rao *et al.* 1992). Among cytogenetic aspects, karyological studies are of paramount importance as they often provide authentic information pertaining to chromosome number, structure and in general, their gross morphology (Darlington and La Cour 1976). The description of chromosomal feature is based upon their size, centromere position, mitotic configuration and occurrence of satellite, which are observable following staining (Dyer 1979). Cytological evaluation in terms of karyotype, pairing behaviour of chromosomes and their segregational pattern have been reviewed in detail by Rani and Raina (2000) for the assessment of genetic stability in a number of micro-propagated plants (**Table 10**). Although, chromosome analysis has been very common parameter for evaluation of fidelity but its application in a number of cases has proved limiting on account of small size and high number of chromosomes and difficulty in obtaining metaphase cells required for such analysis (Varshney *et al.* 2001; Thiem and Sliwinska 2003). In addition, karyological analysis cannot reveal alternation in specific genes or small chromosomal rearrangements (Isabel *et al.* 1993). The morphological similarity, relatively small size of the chromosomes and low metaphasic indexes obtained in root meristems have hindered karyotypic characterization and the application of banding techniques in *Coffea*. A method based on the use of cell suspension aggregates treated with amiprophos-methyl (APM) and macerated in enzymatic solution was developed. This method generated cytogenetic preparations in which the chromosomes showed well-defined primary and secondary constrictions, facilitating the pairing of homologues and assembly of the karyogram, as well as the identification of active NOR and heterochromatin associated with the secondary constriction. This alternative technique could help on the analysis of other species with similar karyotypic characterization problems (Ronildo and Carvalho 2006).

Flow cytometry is another parameter to study cytogenetic aspects. It is a fast and accurate method for estimation of nuclear DNA content of different plant species (Thiem and Sliwinska 2003; Sliwinska and Theim 2007). This method

Table 10 Techniques employed in several plant species to detect variability and stability of tissue culture-raised clones.

Plant	Technique	Reference
Acacia mangium	RAPD	Nanda *et al*. 2004
Actinida deliciosa	AFLP	Prado *et al*. 2005, 2007
	RAPD	Huang *et al*. 2002
	SSR	Palombi and Damiano 2002
Aegle marmelos	RAPD, SSR, MiniSt.	Mishra *et al*. 2008
Allium sativum	Karyotyping, RAPD karyotyping	Al-Zahim *et al*. 1999
A. tuberosum		Rao *et al.* 1992
Ananas comosus	RAPD	Feuser *et al.* 2003; Santos *et al*. 2008
	Isozymes	Feuser *et al.* 2003
Anigozanthos viridis	AFLP	Turner *et al.* 2001
Arachis retusa	RAPD, AFLP	Gagliardi *et al*. 2007
Asparagus	Karyotyping, RAPD	Raimondi *et al.* 2001
Azadirachta indica	AFLP	Singh *et al.* 2002
Betula pendula	RAPD karyotyping	Rynanen and Arone 2005
Brassica oleracea	ISSR	Leroy *et al*. 2001
Camellia sinensis	ISSR, RAPD, RFLP	Devarumath *et al*. 2002
Carya illinoinensis	AFLP	Vendrame *et al*. 1999
Castanea sativa x *C. crenata*	RAPD	Carvalho *et al*. 2004
Celastrus paniculatus	RAPD	Mathur *et al*. 2008
Centaurea ragusina	karyotyping	Radic *et al*. 2005
Chlorophytum arundinaceum	RAPD, karyotyping RAPD	Lattoo *et al*. 2006
C. borivilianum		Mathur *et al*. 2008
Citrus limon	RAPD, FCM	Orbovic *et al*. 2008
Citrus sinensis	RAPD karyotyping	Hao and Deng 2002
Coffea arabica	RAPD, ISSR, RAPD	Rani *et al*. 2000
Cordyline terminalis	Isozymes	Ray *et al.* 2006
Curcuma longa	RAPD	Tyagi *et al.* 2007
Dictyospermum ovalifolium	ISSR	Chandrika *et al.* 2008
Digitalis obscura	RAPD	Gavidia *et al*. 1996
Dioscorea bulbifera	RAPD	Dixit *et al*. 2003; Narula *et al*. 2007
	ISSR	Leroy *et al*. 2007
Drocera angelica	RAPD	Kawiak and Lojkowska 2004
D. binata		
Eucalyptus camaldulensis	RAPD	Rani *et al.* 2001
Festuca pratensis	RFLP, karyotyping	Valles *et al*. 1993
Foeniculum vulgare	RAPD, karyotyping	Bennici *et al*. 2004
Gossypium hirsutum	RAPD, SSR, FCM, karyotyping	Jin *et al.* 2008
Gypsophila paniculata	RAPD	Rady 2006
Hagenia abyssinica	RAPD	Feyissa *et al.* 2007
Hypericum perforatum	karyotyping	Urbanova *et al*. 2002
Humulus lupulus	AFLP	Peredo *et al*. 2008
Inula verbascifolia	FCM	Sliwinska and Thiem 2007
Lilium	RAPD	Yamagishi 1995; Varshney *et al*. 2001
Malus pumila	RAPD	Modgil *et al*. 2005
Melia azedarach	Karyotyping, RAPD, isozymes	Olmos *et al*. 2002
Mucuna pruriens	RAPD	Sathyanarayana *et al.* 2008
Musa	RAPD, ISSR isozymes	Ray *et al*. 2006a; Vankatchelam *et al*. 2008
	karyotyping	Dutta *et al.* 2003; Sandoval *et al*. 1996
Oenothera paradoxa	FCM	Sliwinska and Thiem 2007
Oryza staiva	RFLP	Muller *et al*. 1990
	karyotyping	Kharabian and Darabi 2005
	Isozymes	Medina *et al*. 2004
Panax regenerants	RAPD	Carvalho *et al*. 2004
Pennisetum purpureum	Isozymes	Shenoy and Vasil 1992
Philodendron	RAPD	Gangopadhyay *et al.* 2004
Phoenix dactylifera	RAPD; AFLP	Saker *et al.* 2006
Pinus thunbergii	RAPD	Goto *et al*. 1998
Plantago asiatica	FCM	Makowczynska *et al.* 2008
Platanus occidentalis	RAPD	Sun *et al.* 2009
Plumbago zylanica	RAPD	Rout and Das 2002
Polownia tomentosa	RAPD	Rout *et al*. 2001
Populus tremuloides	SSR	Rahman and Rajora 2001
Prunus spp.	Karyotyping	Helliot *et al*. 2002
Prunus dulcis	RAPD, ISSR	Martins *et al*. 2004
Pueraria lobata	FCM	Sliwinska and Thiem 2007
Quercus robur	RAPD	Valladares *et al.* 2006
Quercus spp.	AFLP, RAPD, FCM	Wilhelm 2000
Rheum rhaponticum	karyotyping	Zhao *et al*. 2005
Rubus	RAPD, FCM	Gajdosova *et al*. 2006
	SSR, AFLP	Castillo 2007
Rubus chamaemorus	FCM	Thiem and Sliwinska 2003; Sliwinska and Thiem 2007
Saccharum sp.	Isozymes, RAPD	Srivastava *et al.* 2005

Table 10 (Cont.)

Plant	Technique	Reference
Secale cereale	AFLP	Puente *et al.* 2008
Solanum tuberosum	ISSR	Aversano *et al.* 2009
	RFLP	Potter *et al.* 1991
Solidago graminifolia, *S. virgaurea*	FCM	Sliwinska and Thiem 2007
Sugar beet	RAPD	Jazdzewska *et al*. 2000
Sugarcane	RAPD	Zucchi *et al*. 2002
Sweet potato	RAPD	Sharma *et al*. 2004
Swertia chirata	Karyotyping, ISSR	Chaudhuri *et al.* 2007; Joshi and Dhawan 2007
Tectona grandis	RAPD	Gangopadhyay *et al*. 2003
Vaccinium	FCM, RAPD	Gajdosova *et al.* 2006
Vanilla planifolia	RAPD, ISSR	Sreedhar *et al.* 2007
Vitis vinifera	RAPD	Yang *et al.* 2008
Zingiber officinales	RAPD	Rout *et al.* 1998

RAPD, Randomly Amplified Polymorphic DNA; AFLP, Amplified Fragment Length Polymorphism; SSR, Simple Sequence Repeat; ISSR, Inter Simple Sequence Repeat; FCM, Flow Cytometry; RFLP, Restriction Fragment Length Polymorphism

allows control of, genetic fidelity of micropropagated plants, which can be disturbed due to somaclonal variation. Indeed, some variation in DNA content has been observed in tissue cultures (Sree Ramulu and Dijkhuis 1986; Kubalakova *et al.* 1996; Rival *et al.* 1997; Kevers *et al.* 1999). Flow cytometry can also provide information on the distribution of cells containing different DNA amount in plant organs, calli or cell suspensions which can be useful for the *in vitro* culturing process (Dolezel *et al.* 1989; Winkelmann *et al.* 1998; Ochatt *et al.* 2000; Sliwinska and Lukaszewska 2005). Although, flow cytometry is a rapid and efficient method for routine studies of ploidy level, it only reveals large scale difference in genome size (over 2%).

Karyotyping through an Image Analyzing System in two species of *Nigella* (Ghosh and Dutta 2006) was used to evaluate interspecific genetic distance. Fluorescent *in situ* hybridization (FISH) has been reported as a powerful tool for comparative karyotype analysis in species having very similar karyotypes (Shibata and Hizume 2008). Ribosomal RNA (5S and 45S) genes were determined simultaneously by double fluorescence *in situ* hybridization (FISH) in the crucifer *Orychophragmus violaceus* (Zaiyun *et al.* 2005). The genetic variability among 1 African and 5 Asian varieties of *Jatropha curcas* were analyzed by multicolor fluorescence *in situ* hybridization (McFISH) using 5S and 45S ribosomal RNA genes (rDNAs). One locus of 5S rDNA and 2 loci for 45S rDNA were detected at specific regions of the chromosomes in all the materials. Also, telomeric repeats (TTTAGGG) were localized on the terminal regions of all chromosomes. The results confirmed the stability of these major repeated sequences among *Jatropha* lines (Witkowska *et al.* 2009).

c) Protein/isoenzyme profiles: Protein polymorphism may also serve as a genetic marker as they are quite polymorphic and generally highly heritable (Gepts 1990) which can be analysed through HPLC (Smith and Smith 1986) and SDS-PAGE (Fergusan and Grabe 1986; Raymond *et al*. 1991). Among protein-based markers, isozyme electrophoresis has been recognized as a promising technique to determine the genetic variation, if any, among *in vitro*-derived plants. Bouman and De Klerk (1996) suggested the use of developmentally and physiological stable enzymes including alcohol dehydrogenease, malate dehydrogenase, phosphoglucomutase and phosphoisomerase for analytical studies. The variation characterized has been summarized into three categories: (i) altered electrophoretic mobility; (ii) loss/gain of protein bands; and (iii) altered level of specific protein. The added advantages of inexpensive isozyme analysis over phenotypic and cytogenetical markers include codominant expression, and ease of performance. However, the limitations associated with these markers which preclude their wider application are that they may be biased since only small portion of the genome is represented by them, sensitivity to environmental and developmental conditions and also they exhibit very little polymorphism (Mowrey *et al.* 1990; Agarwal and Nath 2001; Singh and Srivastava 2004). Protein polymorphisms based on isozyme-based polymorphic patterns as reviewed by Rani and Raina (2000) and a few others have been cited in **Table 10**.

d) DNA-based molecular markers: Nowadays DNA based markers are being preferred over others to test the genetic stability in tissue culture derived plants. This is mainly due to the inertness of DNA to developmental, physiological or environmental changes for screening the variation induced under *in vitro* conditions (Anand 2003). Presently, besides RFLP, RAPD, SSR, AFLP and ISSRs, many other DNA based markers like IRAP (Inter–Retrotransposon Amplified Polymorphism), SCAR (Sequence Characterized Amplified Regions), ASAP (Allele Specific Associated Primers), STS (Sequence Tagged Sites), EST (Expressed Sequence Tags), SSCPs (Single Strand Conformation Polymorphism), asymmetric–PCR–SSCPs, RAMPO (Random Amplified Microsatellite Polymorphism), TRAP (Target Region Amplification Polymorphism) and SNP (Simple Nucleotide Polymorphism) are in vogue and their uses have been extensively reviewed by Teixeira da Silva *et al.* (2007) and Agarwal (2008). Their suitability depends upon their properties and the purpose of study (Nesbitt *et al*. 1995; Weising *et al*. 2005). Since different markers can produce different results, they can significantly influence the scope of variation under examination (Hodgkin *et al.* 2001). A brief review of a large number of plants screened for clonal fidelity using cytological, biochemical and molecular markers is presented in **Table 10**.

Modern approaches to detect undesired plant off-types in the *in vitro* propagation process could include the application of the "DNA-microchip" technology using DNA microarrays carrying hybridization targets isolated from undesirable plant variants. At the commercial level genetically unstable plants of low quality may cause severe production losses. This 'chip-based' approach involves the hybridization of fluorescence-tagged DNA from test plant DNA to microarrays carrying chemically homogenous plant off-type-derived hybridization targets (Lemieux *et al*. 1998). Moreover, these are very expensive techniques and information about their usefulness is still in its infancy.

The National Facility for Virus Diagnosis and Quality Control of Tissue Culture Raised Plants, IARI, New Delhi along with its five satellite centres is also engaged in testing the genetic fidelity of the TC plants vis-à-vis the mother plants. The quality control especially of the forest trees and other crops which may exhibit somaclonal variation is being certified using molecular markers and DNA fingerprinting techniques (Prakash 2006). Similar efforts are also being carried out by EPPO and NAPPO as mentioned above (Roy 1993; Thompson 1998).

CONCLUSIONS

High production cost is the major problem associated with commercial tissue culture technology (George and Sherrington 1984). Micropropagation protocols developed in a laboratory as a part of R&D programmes should generate viable technology suitable for large-scale production of desired clones. The success of commercialization largely depends upon the defined protocol available for a species and the benefits/risks associated with it. *In vitro* propagation of several economic plant species is often hampered by the lack of modern methods to overcome intensive labour manipulation (Levin and Vasil 1989). Scaling-up using innovative and cheaper alternatives discussed above can reduce the unit cost of micropropagules and plant production. Such protocols must be tested at pilot-scale to ascertain their viability.

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