

In vitro Plant Propagation: A Review

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ABSTRACT : Micropropagation is an alternative mean of propagation that can be employed in mass multiplication of plants in relatively shorter time. Recent modern techniques of propagation have been developed which could facilitate large scale production of true-to-type plants and for the improvement of the species using genetic engineering techniques in the next century. An overview on the *in vitro* propagation via meristem culture, regeneration via organogenesis and somatic embryogenesis is presented. The usefulness of the plants in commercial industry as well as propagation techniques, screening for various useful characteristics and the influence of different cultural conditions in the multiplication, rooting and acclimatization phases on the growth of tissue cultured plant discussed.

Keywords : Meristem culture, Micropropagation, Regeneration, Somatic embryogenesis

INTRODUCTION

Micropropagation is a plant tissue culture technique used for producing plantlets and implies the culture of aseptic small sections of tissues and organs in vessels with defined culture medium and under controlled environmental conditions and has become an increasingly important tool for both science and commercial applications in recent years. It is the foundation on which all biotechnological research rests, because almost all uses of plant biotechnology ultimately require the successful culture of plants cells, tissues or organs. This technique has many advantages over conventional vegetative propagation, as e.g. the propagation of a great number of pathogen-free plants in a short time with high uniformity. The success of micropropagation involves several factors, as the composition of the culture medium, culture environment, and genotype. The development of procedures for rapid *in vitro* clonal micropropagation of any plants may be a great commercial value to the industry. This review disc-

usses the different micropropagation techniques and various factors affecting the micropropagation of plants.

MICROPROPAGATION

Principles of tissue culture

Plant micropropagation is an integrated process in which cells, tissues or organs of selected plants are isolated, surface sterilized, and incubated in a growth-promoting aseptic environment to produce many clone plantlets (Altman, 2000). The technique of cloning isolated single cells *in vitro* demonstrated the fact that somatic cells, under appropriated conditions, can differentiate to a whole plant. This potential of a cell to grow and develop a multicellular organism is termed cellular totipotency. This potential of cells or tissues to form all cell types and regenerate a plant is the basic principle of tissue culture. *In vitro* culture is one of the key tools of plant biotechnology that exploits the totipotency nature of plant cells, a concept

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proposed by Haberlandt (1902) and unequivocally demonstrated, for the first time, by Steward *et al.* (1958). Tissue culture is alternatively called cell, tissue and organ culture through *in vitro* condition (Debergh and Read, 1991). It can be employed for large-scale propagation of disease free clones and gene pool conservation. Now a day's industry has applied immensely *in vitro* propagation approach for large-scale plant multiplication of elite superior varieties. As a result, hundreds of plant tissue culture laboratories have come up worldwide, especially in the developing countries due to cheap labour costs. However, micropropagation technology is more costly than conventional propagation methods, and unit cost per plant becomes unaffordable compelling to adopt strategies to cut down the production cost for lowering the cost per plant. The micropropagation process can be divided in five different stages:

Phase 0: growing mother plants under hygienic conditions. It involves the production of stock plants in greenhouse.

Phase I: initiation of culture. The purpose of this stage is to initiate axenic cultures. It involves the selection of explants, disinfestations and the cultivation under aseptic conditions.

Phase II: rapid regeneration and multiplication of numerous propagules (multiplication phase). Masses of tissues are repeatedly subcultured under aseptic conditions onto new culturing media that encourage propagule proliferation. The culture can supply shoots for the subsequent propagation phases as well as material that is required to maintain the stock.

Phase III: elongation and root induction or development (rooting phase). This phase is designed to induce the establishment of fully developed plantlets. It is the last period *in vitro* before transferring the plantlets to *ex vitro* conditions.

Phase IV: transfer to *ex vitro* condition (acclimatization). Acclimatization is defined as the climatic or environmental adaptation of an organism, especially a plant that has been moved to a new environment (Kozai and Zobayed, 2000).

Micropropagation via meristem culture or axillary bud/shoot tip culture and regeneration

In vitro propagation through meristem culture is the best possible means of virus elimination and produces a large numbers of plants in a short span of time. It is a powerful tool for large-scale propagation of plants. The term 'meristem culture' specifically means that a meristem with no leaf primordia or at most 1-2 leaf primordia which are excised and cultured. The pathway of regeneration undergoes several steps. Starting with isolated explants, with de-differentiation followed by re-differentiation and organization into meristematic centres. Upon further induction the cells can form unipolar structures i.e. organogenesis, or bipolar structures called somatic embryogenesis. The organization into morphogenetic patterns can take place directly on the isolated explant or can be expressed only after callus formation, which is called indirect morphogenesis. When shoots are developed directly from leaf or stem explants it refers to direct morphogenesis. Micropropagation is an alternative method of vegetative propagation, which is well suited for the multiplication of elite clones. It is accomplished by several means, i.e., multiplication of shoots from different explants such as shoot tips or axillary buds or direct formation of adventitious shoots or somatic embryos from tissues, organs or zygotic embryos. Many commercial plants are being propagated by *in vitro* culture on the culture medium containing auxins and cytokinins (Preil, 2003; Rout and Jain, 2004). Several different explants have been used for direct shoot formation. Mayer (1956) succeeded first time regeneration of Cyclamen shoots from tuber segments on MS medium supplemented with 10.7 μM NAA. Furthermore, plants have been regenerated from leaf tissues and petiole segments of *Jatropha curcas* (Kumar, 2009; Kumar and Reddy, 2010; Kumar *et al.*, 2010a; Kumar *et al.*, 2010b; Kumar *et al.*, 2010c; Kumar *et al.*, 2011a; Kumar *et al.*, 2011b). Preil (2003) noted that the regeneration potential of isolated cells, tissue or organs and the callus cultures is highly variable. Furthermore, petiole cross sections cultivated on auxin and cytokinin containing medium give

rise to adventitious shoots from epidermal cells and subepidermal cortex cells, never from pith cells of the central regions of the petiole. The direct shoot bud formation without any callus phase from appropriate explants is of great success for large-scale clonal multiplication of desired plants/clone.

Micropropagation via somatic embryogenesis

Somatic embryos, which are bipolar structures, arise from individual cells and have no vascular connection with the maternal tissue of the explants (Haccius, 1978). Embryos may develop directly from somatic cells (direct embryogenesis) or development of recognizable embryogenic structures is preceded by numerous, organized, non-embryogenic mitotic cycles (indirect embryogenesis). Somatic embryogenesis has a great potential for clonal multiplication. Under controlled environmental conditions, somatic embryos germinate readily, similar to their seedling counterpart. The commercial application of somatic embryogenesis will be accomplished only when the germination rate of somatic embryos is high up to 80-85%. Considerable success has been achieved in inducing somatic embryogenesis in many plants like *Dendrothema grandiflorum* (May and Trigiano, 1991; Tanaka *et al.*, 2000). Castillo and Smith (1997) induced direct somatic embryogenesis from petiole and leaf blade explants of *B. gracilis* on MS medium supplemented with 0.5 mg/l kinetin and 2% (v/v) coconut water. Somatic embryos were obtained with greater frequency from petiole explants than from leaf blade sections. Ostermack *et al.* (1999) succeeded in achieving somatic embryos from hypocotyl tissues of *E. pulcherrima* on MS medium supplemented with 2.0 mg/l IAA. About 1400 embryos were developed from 320 calli derived from outer regions of the hypocotyls. However, only 8% developed normal plantlets. Pueschel *et al.* (2003) succeeded in plant regeneration via somatic embryogenesis of *C. persicum* and maintained the regeneration ability for prolonged period. There are advantages and disadvantages of somatic embryogenesis in large-scale plant multiplication (Jain, 2001). The major advantages are large-scale

somatic embryo production in bioreactors, encapsulation, cryopreservation, genetic transformation and clonal propagation. The major limitations are genotypic dependence of somatic embryo production and poor germination rate.

Factors affecting *in vitro* propagation

Media

Significant effect of media has been observed on plant regeneration from different parts of plant (Sharswat and Chand, 2004). Various basal media like White medium, Nitsch and Nitsch medium, B5 medium and Gamborg medium for micropropagation (Khan *et al.*, 1988; Prakash and Gurumurthi, 2005; Diallo *et al.*, 2008), have been employed, but most widely used culture medium is Murashige and Skoog (1962) (MS medium), because most of the plants respond favorably to MS medium, since it contains all the nutrients essential for plant growth *in vitro*. Selection, strength and combination of media are also one of important parameter for optimizing the regeneration protocol (Khan *et al.*, 1988; Zukar *et al.*, 1997; Prakash and Gurumurthi, 2005; Diallo *et al.*, 2008).

Type of explant

Type of explant is also one of the important factors in optimizing the tissue culture protocol. Type of explants like leaf, petiole, cotyledonary leaf, hypocotyle, epicotyle, embryo, internode and root explant significantly effect on tissue culture process of plants (Khan *et al.*, 1988; Sujatha and Mukta, 1996; Tyagi *et al.*, 2001; Gubis *et al.*, 2003; Alagumanian *et al.*, 2004; Ali and Mirza, 2006; Kumar *et al.*, 2011b). This may be due to the different level of endogenous plant hormones present in the plants parts. Leaf is the most commonly used explant for regeneration due to more surface area available (Sujatha and Muktha, 1996; Tyagi *et al.*, 2001). Tyagi *et al.* (2001) used root, shoot, and leaf explant and maximum regeneration efficiency was observed from leaf explants in *Cajanus cajan*. Sujatha and Mukta (1996) used different explant like leaf, petiole, hypocotyle and maximum regeneration frequency was observed from leaf explant of *Jatropha curcas*. Alagumanian *et al.* (2004) used leaf and stem

explant and maximum regeneration efficiency observed from stem explant in *Solanum trilobotam*. Gubis *et al.* (2003) used hypocotyle, epicotyle, cotyledons, leaf, petiole, internode and maximum response were obtained from hypocotyle in Tomato. Ali and Mirza (2006) used root, stem, leaf and petiole but maximum responses were observed from stem explant in *Citrus jambhiri* Lush. An example of the effect of the type of explants on regeneration in *Jatropha curcas* is shown (Fig. 1).

Genotype

Genotype is also one of the most important factors affecting regeneration (Tyagi *et al.*, 2001; Gubis *et al.*, 2003; Gandonou *et al.*, 2005; Feyissa *et al.*, 2005; Chitra and Padmaja, 2005; Landi and Mezzeti, 2006; Reddy *et al.*, 2008; Kumar and Reddy, 2010). Genotypic effect on shoot regeneration and elongation has been described in many species, and could be due, in part, to differences in the levels of endogenous hormones, particularly cytokinins



Fig. 1. Plant regeneration from leaf explants of non-toxic *Jatropha curcas*. Plant regeneration from (A) in vitro mature leaf explant (bar 5 mm), (B) Field-grown mature leaf explant (bar 5 mm), (C) in vitro cotyledonary leaf explant (bar 5 mm) and (D) Glasshouse-grown cotyledonary leaf explants (bar 5 mm) on MS medium with 2.27 μM thidiazuron (TDZ) after 6 weeks. (E) Shoot proliferation of regenerated shoot buds on MS medium with 10 μM kinetin (Kn) + 4.50 μM 6-benzyl aminopurine (BAP) + 5.50 μM α -naphthaleneacetic acid (NAA) after 4 weeks (bar 75 mm). (F) Elongation of proliferated shoot on MS medium with 2.25 μM BAP + 8.5 μM indole-3-acetic acid (IAA) after 6 weeks (bar 5 mm). (G) Elongation of proliferated shoot on MS medium with 2.25 μM BAP + 2.5 μM indole-3-butyric acid (IBA) after 6 weeks (bar 5 mm; arrow indicate proliferation of axillary buds). (H) Elongation of proliferated shoot on MS medium with 2.25 μM BAP + 8.25 μM NAA after 6 weeks (bar 5 mm; arrow indicate formation of callus at the base). (I) Elongated shoot cultured on half strength basal MS liquid medium supplemented with 15 μM IBA + 5.7 μM IAA + 5.5 μM NAA for root induction (bar 1 mm). (J) Development of roots at the base of auxins treated elongated shoot on half strength basal MS medium with 0.25 mg/l activated charcoal after 4 weeks (bar 1 mm). (K) Regenerated plant in polythene bags after 2 weeks (bar 100 mm) (Source; Kumar *et al.*, 2011b)

levels during the induction period although the precise mechanism remains unclear (Pellegrineschi, 1997; Schween and Schwenkel, 2003). Henry *et al.* (1994) reported that genotypic differences with respect to embryogenesis and regeneration result from quantitative or qualitative genetic differences.

Source of explant

Source of explant i.e. *in vitro* and *in vivo* is also important for regeneration (Reddy *et al.*, 2008; Kumar *et al.*, 2010a). *In vitro* explant is considered to be the most suitable for organogenesis (Reddy *et al.*, 2008). The fact that source of explant has different capacity of regeneration are well documented (Feyissa *et al.*, 2005). *In vitro* explant in general has better potential to organogenesis as compared to *in vivo* explant (Reddy *et al.*, 2008). The difference may be due to the level of endogenous hormones present in the plant explant. Seedling explant is more

responsive or meristematic than mature plants (Teng, 1999; Feyissa *et al.*, 2005) due to different level of plant hormones present in the plants. An example of the effect of the source of explants on regeneration in *Jatropha curcas* is shown (Fig. 1).

Orientation of explant

Orientation of explant in the culture medium also affects the regeneration efficiency (Sharma and wakhlu, 2001; Arockiasamy *et al.*, 2002; Kumar and Reddy, 2010). In general regeneration efficiency is higher in horizontal position as compared to vertical condition of explant due to little contact of explant to medium in vertical position as compared to horizontal position. The initiation site, polarity, and efficiency of bud regeneration were altered by explant orientation is well documented in *Dionaea muscipula* (Teng, 1999). Cotyledons placed in abaxial (lower surface facing down) orientation consistently produced better

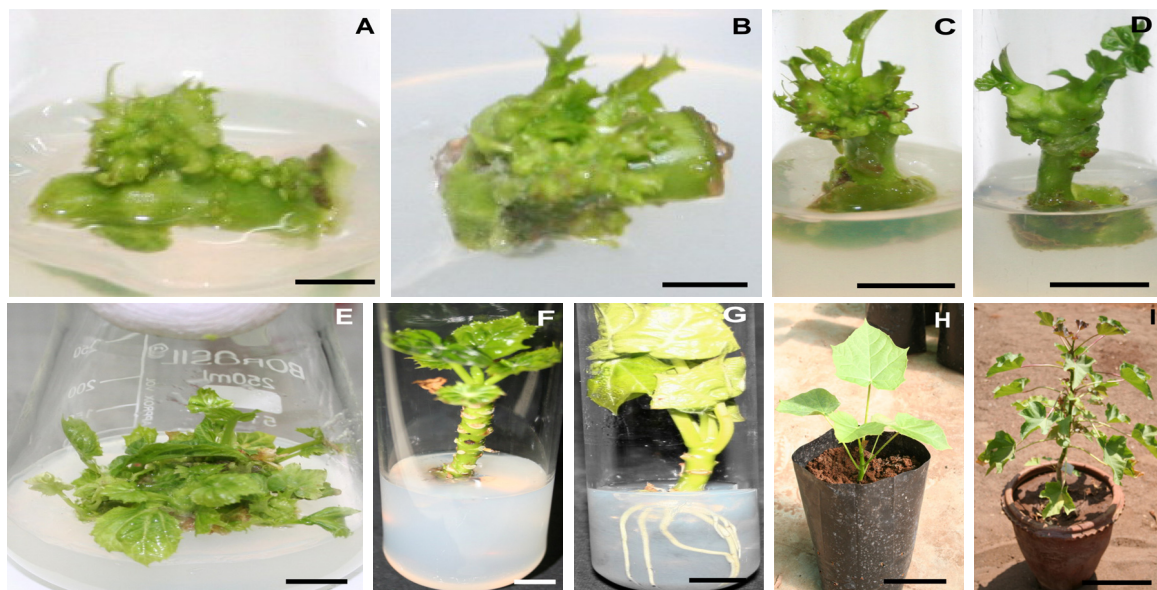


Fig. 2. Direct induction of shoot buds from petiole explants of *Jatropha curcas*. Direct induction of shoot buds from (A) *in vitro* petiole in horizontal position (bar 5 mm), (B) *in vivo* petiole in horizontal position (5 mm), (C) *in vitro* petiole in vertical position (bar 5 mm) and (D) *in vivo* petiole in vertical position on MS medium with 2.27 μM TDZ after 6 weeks (bar 5 mm). (E) Shoot proliferation of induced shoot buds on MS medium with 10 μM Kinetin + 4.5 μM BAP + 5.4 μM NAA after 4 weeks (bar 100 mm). (F) Elongation of proliferated shoot on MS medium with 2.25 μM BAP and 8.5 μM IAA after 6 weeks (bar 1 mm). (G) Development of roots at the base of elongated shoot on half strength of MS medium with 2% sucrose + 15 μM IBA + 5.7 μM IAA + 5.5 μM NAA + 0.25 mg/L activated charcoal after 4 weeks (bar 1 mm). (H) Regenerated plants in polythene bags after 4 weeks (bar 100 mm). (I) A six month old regenerated plant in pot under natural condition (bar 100 mm) (Source; Kumar and Reddy, 2010)

shoot regenerative response and produced greater numbers and taller shoots compared to those inoculated in adaxial (upper surface facing down) orientation (Bhatia *et al.*, 2004, 2005). An example of the effect of the orientation of explants on regeneration in *Jatropha curcas* is shown (Fig. 2).

Mineral nutrition

Minerals are important components of the culture medium. There is a large choice of combinations of macro- and micro-salt mixtures. The most widely used culture medium is described in Murashige and Skoog (1962) (MS medium), because most plants react to it favorably. It contains all the elements that have been shown to be essential for plant growth *in vitro*. It is classified as a high salt medium in comparison to many other formulations, with high levels of nitrogen, potassium and some of the micronutrients, particularly boron and manganese (Cohen, 1995). Due to the high salt content, however, this nutrient solution is not necessarily always optimal for growth and development of plants *in vitro* (Pierik, 1997). For that reason, the use of dilute media formulations has generally promoted better formation of roots, since high concentration of salts may inhibit root growth, even in presence of auxins in the culture medium (Grattapaglia and Machado, 1998). The ability of rose explants to produce shoots and initiate roots was studied by Kim *et al.* (2003). They concluded that optimum shoot proliferation was obtained in full-strength MS salts, while rooting improved with 1/4 strength. Sauer *et al.* (1985) reported that 1/3 strength MS salts proved to be suitable for rooting of rose. For globe artichoke, 1/2 strength MS salts have been used in the rooting medium (Ancora, 1986; Lauzer and Vieth, 1990).

Carbon source

Sucrose is by far the most used carbon source, for several reasons. It is cheap, readily available, relatively stable to autoclaving, and readily assimilated by plants. Other carbohydrates can be also used, such as glucose, maltose and galactose as well as the sugar-alcohols glycerol and sorbitol (Fowler, 2000). The carbohydrates

added to the culture medium supply energy for the metabolism (Caldas *et al.*, 1998). The addition of a carbon source in any nutrient medium is essential for *in vitro* growth and development of many species, because photosynthesis is insufficient, due to the growth taking place in conditions unsuitable for photosynthesis or without photosynthesis (in darkness). Normally, green tissues are not sufficiently autotrophic under *in vitro* conditions (Pierik, 1997) and depend on the availability of carbohydrates in the growing medium.

Growth regulators

Growth regulators are organic compounds naturally synthesized in higher plants, which influence growth and development. Apart from the natural compounds, synthetic chemicals with similar physiological activities have been developed which correspond to the natural ones (Pierik, 1997). There are several classes of plant growth regulators, as e.g. cytokinins, auxins, gibberellins, ethylene and abscisic acid. Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously (George, 1993). A balance between auxin and cytokinin is most often required for the formation of adventitious shoots and roots. In tobacco cultured *in vitro*, it was found that the formation of roots and shoots depended on the ratio of auxin to cytokinin in the culture medium. High levels of auxin relative to cytokinin stimulated the formation of roots, whereas high levels of cytokinin relative to auxin led to the formation of shoots (Taiz and Zeiger, 1991). The balance of growth regulators depends on the objective of the cultivation *in vitro* (as e.g. shoot, root, callus or suspension culture) and on the micropropagation phase considered (initiation, multiplication or rooting). In the multiplication phase, the level of cytokinins should be normally higher than of auxins. In the rooting phase, in turn, the use of cytokinin is, in some cases, not necessary and higher levels of auxins can be supplemented to the culture medium (Torres *et al.*, 2001). The cytokinins are derived from adenine (aminopurine) and play an important role in the *in vitro* manipulation of

plant cells and tissues (Torres *et al.*, 2001). Cytokinins stimulate plant cells to divide, and they were shown to affect many other physiological and developmental processes. These effects include the delay of senescence in detached organs, the mobilization of nutrients, chloroplast maturation, and the control of morphogenesis (Taiz and Zeiger, 1991). Added to the culture medium, these compounds overcome apical dominance and release lateral buds from dormancy (George, 1993). The most common cytokinins used are kinetin, BA and 2iP (Pierik, 1997). Also auxins (IAA, IBA, NAA or 2,4-D) are often added to the culture medium to promote the growth of callus, cell suspensions or organs, and to regulate morphogenesis, especially in combination with cytokinin (George, 1993). Auxins are involved in the regulation of several physiological processes, as e.g. apical dominance and formation of lateral and adventitious roots. This growth regulator generally causes cell elongation and swelling of tissues, cell division (callus formation) and the formation of adventitious roots as well as the inhibition of adventitious and axillary shoot formation (Pierik, 1997). Normally, the concentration of auxin used in the culture medium varies between 0.01 and 10 mg L⁻¹ (Torres *et al.*, 2001). The IAA is a natural auxin, whereas 2,4-D and NAA are synthetically produced and have similar effect in comparison to natural-occurring auxins. According to most of the studies that have been published concerning the effect of auxin type and concentration in rose, low concentrations of this growth regulator should be used in the culture medium. The rooting of rose shoots was improved with IAA (considered a weak auxin) supplementation at 1.0 mg L⁻¹ (Kim *et al.*, 2003), 0.1 mg L⁻¹ NAA (Rahman *et al.*, 1992; Leyhe and Horn, 1994) or even in absence of auxin (Ibrahim and Debergh, 2001). The combination of two types of auxin can be also used to increase root formation in rose. Kosh-Khui and Sink (1982) found that the best combination for the production of rooted plants was 0.1 mg L⁻¹ NAA with 0.05 mg L⁻¹ of either IAA or IBA. The combination of two auxins was more effective for root formation than either auxin alone. In globe artichoke, the most effective auxin for rooting was NAA (0.1-2.0 mg L⁻¹) also combined with

IAA (2.0 mg L⁻¹) Gibberellins are a group of compounds that is not necessarily used in the *in vitro* culture of higher plants. In some species, these growth regulators are required to enhance and in others to inhibit growth (Razdan, 1993). Gibberellic acid (GA₃) is the most common gibberellin used. It induces the elongation of internodes and the growth of meristems or buds *in vitro* (Pierik, 1997). Furthermore, the use of gibberellins in the rooting medium may reduce or prevent the formation of adventitious roots and shoots, although it can stimulate root formation when present in low concentrations. Morzadec and Hourmant (1997) showed the beneficial effect for globe artichoke of using gibberellin at a concentration of 1.0 or 5.0 mg L⁻¹ GA₃ in the rooting medium, resulting in a rapid root expression and in the formation of high quality explants. An example of the effect of the plant growth regulators on regeneration in *Jatropha curcas* is shown (Fig. 1).

Gelling agents

Culture media can be classified as liquid or solid. The liquid media have the advantage of faster (and cheaper) preparation than the solid ones. Furthermore, liquid media are more homogeneous, since gradients of nutrients may appear during tissue growing in solid media. This phenomenon is not observed in liquid media (Caldas *et al.*, 1998). Furthermore, it has been shown that the propagation ratio of some species is higher in liquid than in solid media (Debergh *et al.*, 1981; Pateli *et al.*, 2003). One serious disadvantage of using liquid media for shoot growth and multiplication is that shoots, which are perpetually submerged in liquid cultures, may become hyperhydric and will then be useless for micropropagation (George, 1993; Debergh, 2000). Ebrahim and Ibrahim (2000) reported that the solid medium should be used to overcome the production of vitrified shoots of *Maranta leuconeura* and to insure obtaining vigorous plants with higher chlorophyll content. Agar has traditionally been used as the preferred gelling agent for tissue culture, and is very widely employed for the preparation of semi-solid culture media (Torres *et al.*, 2001). It is a polysaccharide extracted from

species of red algae which are collected from the sea (Torres, 1999). Concerning the optimal agar concentration in the culture medium, large differences between two rose cultivars were observed by Acker and Scholten (1995). The cv. 'Motrea' preferred higher concentrations of agar (7 g L^{-1}). At this concentration, completely developed shoots were formed. The cv. 'Sweet Promise', in turn, showed the best results with extremely low concentrations (4 g L^{-1}). Paques (1991) pointed out that there is a strong connection between culture medium hardness, proliferation ratio and hyperhydration. Normally, an increase in the agar concentration promotes a reduction in the occurrence of hyperhydration symptoms in plants. However, the propagation rate can be drastically reduced and, consequently, the efficiency of micropropagation (Debergh, 2000). The concentration of agar in the medium may also affect the formation of roots. Rahman *et al.* (1992) reported that rooting performance of rose decreased with increasing agar concentration (from 6 to 15 g L^{-1}). At 6 g L^{-1} , optimal rooting induction was achieved. An alternative to agar is the use of a gelling agent named gelrite. Gelrite is a gellan gum - a hetero-polysaccharide produced by the bacterium *Pseudomonas elodea* (Kang *et al.*, 1982). Gelrite is an attractive alternative to agar for plant tissue culture because its cost per liter of medium is lower, and it produces a clear gel which facilitates the proper observation of cultures and their possible contamination (George, 1993). Williams and Taji (1987) found that several Australian woody plants survived best on a medium gelled with gelrite rather than agar.

PHYSICAL ENVIRONMENT

Gas exchange and relative air humidity inside the vessel

The response of plant tissue culture *in vitro* can be significantly affected by the gaseous constituents in and adjacent to the culture vessel. Carbon dioxide, oxygen and ethylene are the most frequently studied constituents of the culture atmosphere (Read and Preece, 2003). The culture vessel is usually a closed system, but some gas exchange may occur depending upon the type of vessel, the closure and how tightly they are sealed together. The

sealing of the vessels must allow sufficient ventilation to prevent significant accumulation of ethylene and depletion of CO_2 (Buddendorf-Joosten and Woltering, 1994). Carbon dioxide concentrations inside the vessels alter due to respiration and photosynthesis of the plant. In the dark, CO_2 concentrations increase due to respiration, whereas during the light period the concentration decreases (Buddendorf-Joosten and Woltering, 1994). The utilization of tightly closed vessels that reduce the gas exchange may affect negatively the normal growth and development of plants during cultivation *in vitro*. Several studies have shown the advantages of using closures with filters or vented vessels, which allow gas exchange, increasing the photosynthetic capacity, the multiplication rate, and the survival of plants after transfer to *ex vitro* conditions (Chuo-Chun *et al.*, 1998; Murphy *et al.*, 1998; Zobayed *et al.*, 2000; Benzioni *et al.*, 2003). The increased availability of CO_2 by using vessels with filter may also influence the amount of photosynthetic pigments. *Nicotiana tabacum* plants grown in vessels with closures with microporous vents (better supplied with CO_2) had higher contents of chlorophyll a, b and β -carotene, higher photochemical activity of photosystem II and electron transport chain. Furthermore, plants grown under this condition had higher net photosynthetic rate, lower transpiration rate and stomatal conductance under *ex vitro* conditions than plants grown in glass vessels tightly closed (Haisel *et al.*, 1999). Water status of cultures is influenced by the growing medium used, the culture vessel itself, and the physical environment. The medium affects water status in various ways, including the gelling agent used (or its absence), the osmotic pressure (influenced by e.g. salt concentration, amount and type of carbohydrate and quantity and type of other constituents), and changes in the medium with time (Zimmerman, 1995). It is generally accepted that the relative humidity in the vessel is approximately 98-100% (Altman, 2000). The plants that develop under higher relative humidity *in vitro* have more transpiration and more anatomical abnormalities under *ex vitro* conditions, which may result in high mortality rate during acclimatization. Therefore, different methods to reduce the relative

air humidity inside the vessel have been tested, including the opening of culture containers for some days before acclimatization (Brainerd and Fuchigami, 1981; Kirdmanee *et al.*, 1996), the use of special closures that facilitates water loss (Gribaudo *et al.*, 2003) or the cooling of container bottoms, which increases the condensation of water vapour on the gel surface (Ghashghaie *et al.*, 1992). However, methods to improve gas exchange and reduce relative humidity inside the vessel should be carefully used, in order to prevent excessive water loss during cultivation. A study carried out with grapevine shoots testing different hole diameters in the vessel closure showed that shoots cultivated in vented vessels were taller than shoots grown in unvented ones, and had higher chlorophyll content. On the other hand, the largest holes (40 mm) caused an excessive water stress. Shoots became more resistant to wilting, but their growth was seriously retarded (Gribaudo *et al.*, 2003).

Light

Light is an important environmental factor that controls plant growth and development, since it is related to photosynthesis, phototropism and morphogenesis (Read and Preece, 2003). The three features of light, which influence *in vitro* growth, are wavelength, flux density and the duration of light exposure or photoperiod (George, 1993). Several studies showed that light enhanced root formation and shoot growth (Kumar *et al.*, 2003), whereas in others darkness favored root formation (Hammerschlag, 1982). The reduced rooting in presence of light is due to the degradation of the endogenous IAA (George, 1993). Some species may react positively to an increase in the photosynthetic photon flux, especially under photoautotrophic/mixotrophic growing conditions (low sugar levels and CO₂ enrichment). *Limonium* grown under photoautotrophic conditions *in vitro* associated with a higher light intensity (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) had more leaves, higher chlorophyll and sugar contents, higher net photosynthetic rate and percent survival of plants *ex vitro* than plants grown at lower light intensities (50 and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) (Lian *et al.*, 2002). Under heterotrophic growing conditions,

no significant changes were observed with increasing light intensity. Matysiak and Nowak (1998) investigated the influence of CO₂ concentrations (350 and 1200 $\mu\text{mol mol}^{-1}$) on the growth of *Ficus benjamina* microcuttings at two light levels (50 and 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) under *ex vitro* conditions.

Temperature

Temperature influence on various physiological processes, such as respiration and photosynthesis, is well known and it is not surprising that it profoundly influences plant tissue culture and micropropagation. The most common culture temperature range has been between 20°C and 27°C, but optimal temperatures vary widely, depending on genotype (Altman, 2000; Read and Preece, 2003). Horn *et al.* (1988) studied the effect of different temperatures in the multiplication phase of 14 rose cultivars. The best overall results were obtained at 18°C, but certain ‘thermonegative’ cultivars gave best results at 12°C, while other ‘thermopositive’ cultivars had their optimum at 18°C or 24°C. Alderson *et al.* (1988) observed that the temperature during the rooting phase affected the timing of root emergency, the final rooting percentage and shoot health. For the cv. ‘Dicjana’, roots emerged first at 25°C and approximately 2 and 4 days later at 20°C and 15°C, respectively, but at 25°C the final rooting percentage was lower. The shoots remained green and looked healthiest at 20°C.

CONCLUSION

Successful *in vitro* propagation of plants is now being used for commercialization. Many commercial laboratories and national institutes worldwide use *in vitro* culture system for rapid plant multiplication, germplasm conservation, elimination of pathogens, genetic manipulations, and for secondary metabolite production. Annually, millions of plants are routinely produced *in vitro*. The great potential of micropropagation for large-scale plant multiplication can be tapped by cutting down the cost of prod-

uction per plant by applying low-cost tissue culture, which is to adopt practices and proper use of equipment and resources to reduce the unit cost of micropropagule and plant production without compromising the quality. Somatic embryogenesis facilitates cryopreservation, synseed development, mutations, and genetic transformation. Recent progress in genetic manipulation of plant cells has opened new possibilities for improvement of plants which is totally depends on tissue culture.

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(Received April 21, 2011; Accepted May 17, 2011)