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## REVIEW ARTICLE

# STANDARDIZATION PARAMETERS FOR CRITICAL PROBLEMS ENCOUNTERED IN PLANT IN VITRO CULTURE TECHNIQUE

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**ABSTRACT**

Development of plant *in vitro* regeneration protocols has significantly contributed to research investigations and rapid propagation in commercial scale. However, due to considerable variations among plants, the technique encounters various hurdles. Major challenges are encountered during the micropropagation of woody plants, due to their range of secondary metabolites. Tissue and media browning, systemic contamination, vitrification, shoot tip necrosis (STN), tissue fasciations, somaclonal variations are some of the commonly encountered problems. Researchers in the recent years introduced various novel and unique protocols to eradicate these challenges, like nanosilver mediated sterilization, various new culture media for woody plant propagation, discovery of many new natural PGR having better properties like tapolins, diethyl aminoethyl hexanoate (DA-6), thidiazuron (TDZ) etc. have contributed immensely to the development of the subject. Although the basic concept of plant tissue culture remains same, the interpretation is subjected to development from time to time.



## INTRODUCTION

*In vitro* culture of plants has become an integral part of advance plant science research after the introduction and realization of the Haberlandts' theory of totipotency (Krikorian and Berquam, 1969; Bairu and Kane, 2011). Plant tissue culture plays a pivotal role in basic biological research, such as genetics, plant breeding, biotechnology, biochemistry and cell biology and it has also direct commercial importance (Gamborg, 2002). Due to the effectiveness in rapid multiplication to obtain uniform progeny, propagation of medicinal, aromatic and economic plants through this technique becoming popular (Sahoo *et al.*, 1997). The *in vitro* propagation technique also finds importance due to its effectiveness in propagation independent of season (Fay, 1992). Plant tissue culture is also recognized long back in *ex situ* conservation of rare and endangered plants (Vishwanath and Jayanthi, 1997) and various endangered and rare plants were already multiplied and conserved using this technique. The technique also finds new dimensions due to its involvement in secondary metabolite production. Various qualitative and quantitative modifications can be induced for the production of secondary metabolite through the alternation of the hormonal and nutrient condition (Collin, 2001). This branch of science has passed through various stages of evolution like other technologies for production efficiency in terms of quality. However, several challenges compromising the quality of the propagated plants arise, such as shoot tip necrosis, virtification, fasciations, browning of the media, habituations which needs to be adequately studied for greater effective solutions (Ruffoni and Savona, 2013).

Rigorous procedures like optimization of the chemicals, environmental and physical growth factors are to be involved during the development of the tissue culture protocols (Bairu and Kane, 2011). In recent years, various protocols were formulated and novel techniques were introduced by researchers working in this field. The main purpose of this review is to overlay a comprehensive outline of the techniques formulated and parameters standardized by various researchers in recent years to overcome the challenging problems in plant tissue culture technique.

### Contamination

Proper sterilization of the explants for the successful micropropagation is the most essential step in plant tissue

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culture. Contamination from different sources, such as bacteria and fungi, reduces the productivity and can completely prevent successful culture (Colgecen *et al.,* 2009). The growth media in which the plant tissue is inoculated is also a good source of nutrients for microbial growth. These microbes compete with plant tissue for nutrients and some of them produce phytotoxins, which result in culture mortality, tissue necrosis, and reduced shoot proliferation and rooting (Kane, 2003). The diversity and range of tolerance of various microorganisms mostly bacteria and fungi makes it quite challenging to eradicate the same through traditional approaches. The effective management of the microorganisms in tissue culture needs greater interpretation and research. Internal and external contamination of the microorganisms, mostly bacteria and fungi pose problems due to their greater growth rate than the plant cell, which drains all the nutrients and creates an unfavourable environment for the explants to grow (Cassells, 1991). Surface sterilization of explants may sometime fail to establish the aseptic condition of the culture medium due to systemic infection of various microorganisms and latent contamination can be observed which spoils the whole experimental setup. Inside the plant they have very little microbial competition and remain mostly latent and hardly show any symptoms in plants (Peñalver *et al.,* 1994; Hallman *et al.,* 1997) and therefore they cannot be sorted by visual observation. By surface sterilization, most of the epiphytic microorganisms are eradicated except the systemic or the endogenous one (Habiba *et al.,* 2002). Therefore, in various cases an interdisciplinary approach with other branches of biological science needs importance. Although varieties of techniques and protocols are formulated and optimized to minimize the microbial contamination, designing more efficient methods and techniques to eliminate the contamination so as to prevent the labour intensive and expensive experiments seems necessary (Mahna *et al*., 2013).

Sodium hypochloride (NaOCl), calcium hypochloride and mercuric chloride ($HgCl_2$), are the main surface sterilizing agent conventionally used in the tissue culture laboratory. The concentration and composition of the sterilizing agents are adjusted in such a way, that it may efficacy maximum sterilization and minimum damage to the explants. $AgNO_3$ is widely used to control contamination in woody plants, but $AgNO_3$ is very toxic and therefore high precaution is to be followed (Leifert and Woodward, 1997). Endogenous contaminants can be isolated and identified using standard microbiological methods and can be characterized by different biochemical test such as gram stain, mobility, gelatinase, oxidase, O/F, (Reed and Tanprasert, 1995), methyl red, arginine hydrolase, starch hydrolysis, casein hydrolysis, fluorescent pigment, lactose, citrate and catalase production (Collins and Lyne, 1984; Krieg and Holt, 1984; Sneath *et al.,* 1986) and microscopic observation etc. Msogoya *et al.* (2012) have isolated *Aspergillus spp., Fusarium spp*., *Penicillium spp*. and *Candica spp* as fungal contaminants and *Klebsiella spp., Proteus spp*., *Erwinia spp.* and *Staphylococcus spp* as bacterial contaminants from the banana *in vitro* cultures. Farther through the culture susceptibility test of the isolated microorganisms they have reported gentamicin and rifampicin as the effective antibiotics and ketoconazole as the effective antifungal agent. Although antibiotic and antifungal use is increasing to control the contamination they frequently reported to be phtotoxic or shows inhibitory effect in various cases (Abdi *et al.*, 2008).

Nanoparticle and nanomaterial application are emerging rapidly (Shokri *et al.,* 2014) and it also finds application in tissue culture technique. Silver nanoparticles were being widely used against several microorganisms since ancient times (Chen and Schluesener, 2008). At low concentration nanosilver exhibits antimicrobial effect and have no adverse effect on plant growth and development (Sarmast *et al.,* 2011). In recent years nanosilver finds attention as an efficient sterilizing agent for the valerian explants (Abdi *et al.,* 2008). Mahna *et al.* (2013) have reported to obtained compete decontamination and maximum survivability in the tissue culture of Potato, Arabidopsis and Tomato. Similarly, low concentration of nanosilver incorporation in culture media found to be effective as a disinfectant agent in olive *in vitro* culture (Rostami and Shahsavar, 2009). Though many workers have shown the effectiveness of nanosilver as antimicrobial agent, but its real mode action is still unclear, therefore more experimental trails is necessary to establish it as a sterilizing agent in tissue culture.

**Browning of Medium**

Browning of medium has been a frequently encountered problem associated with micropropagation of woody perennial plants along with the accumulation of inhibitory substances (Razdan, 2011). Browning of the medium occurs due to the exudation and oxidation of phenolic compound from the inoculated tissue (Alkhateeb and Ali-Dinar, 2002). Phenols are the aromatic compound bearing one or two hydroxyl constituents. They are mainly localized in the vacuole and remain associated with the sugar as glycosides (Onuoha *et al.,* 2011). From simple molecule like phenolic acid to highly polymerized tannins more than 8,000 phenolic structures are currently known to associate in the plant kingdom (Dai and Mumper, 2010). The oxidation of the phenolic compound is catalyzed by polyphenol oxidase, (PPO), a copper containing enzyme. Two different sequential reactions involving molecular oxygen is catalyzed by PPO; hydroxylation of monophenols to *o*-diphenols followed oxidation of *o*-diphenols to *o*-quinones, quinones polymerize to form undesirable toxic black or brown colour compounds (Fig. 1) which further enters the tissue and inhibits the action of other enzymes (Lee *et al.,* 2007; Gawlik-Dziki *et al.,* 2007; Feng *et al.,* 2007; Arnnok *et al.,* 2010).

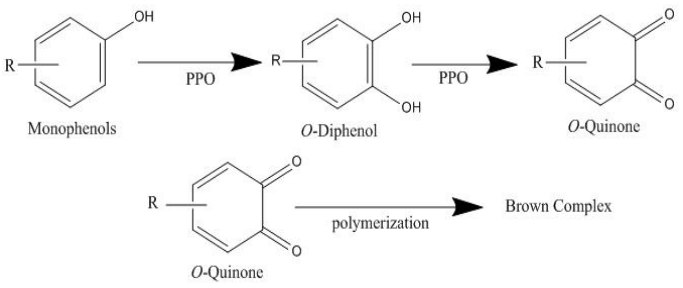


**Fig. 1. Systematic enzymatic and non enzymatic condensation of phenolic browning process**

Browning of medium leads to high mortality rate, especially in the first stage of plant tissue culture (Ko *et al.,* 2009). Pandey and Singh (2012) have successfully overcome the browning of media by frequent transfer of the explants to fresh media. However antioxidants pre-treatment is widely employed for the prevention of the browning of the medium. Antioxidants are reducing agent which checks the oxidation of the liable substrate (George and Sherrington, 1984). Phenolic browning is reduced to a great extent by leaching of the explants by agitation in antioxidant solution and by proper drying prior to inoculation (Meghwal *et al.,* 2001) For teak culture polyvinylpyrrolidone has proved to be effective amendment to the culture media (Razdan, 2011). Onuoha *et al.* (2011) have reported to obtain the best result by pre treating the plantain (*Musa paradisiaca*) explants in potassium citrate and citrate (K-C: C) antioxidant solution for 2 hours. The citrate in citric acid acts as a chelating agent on the polyphenol oxidative enzyme (PPO) responsible for the oxidation of the polyphenols (Titov *et al*., 2006). Similarly pre-treatment with 8-hydroxy-quinolinol-sulfate (8-HQS) (Machado *et al.*, 1991) and Diethyl-dithiocarbonate (DIECA ) (Kumar and Kumar, 1996) prior to inoculation also reduces the browning up to great extent. Browning of the medium can be prevented by cutting the explants under the surface of water or by incorporating ascorbic acid, citric acid (Ko *et al.,* 2009) or cysteine HCL (Mederos-Molina and Trujillo, 1999) to the culture media. Regarding the physical method, Bhat and Chandel (1991) have developed a novel technique to stop browning of tissue culture medium, by sealing the cut end of the explants with paraffin wax to stop exudation. Apart from these Alkhateeeb (2008) suggested winter culture of the plant, incubation of the plant part in darkness, addition of activated charcoal to the medium to check the browning of the medium. Some workers also suggested incubating the culture initially at low illumination, due to the known fact that the phenolic oxidation occurs at illumination.

**Vitrification of Tissue**

Vitrification or hyperhydricity is a physiological imbalance caused either due to passive diffusion of water from the media or by other strong metabolic disturbances (Paques, 1991). Presence of high concentration of ammonia, type and concentration of gelling agent, imbalanced plant growth regulators, high humidity level and gases especially ethylene inside the culture tube contribute to the vitrification of the cultured tissues (Alkhateeb, 2008; Adeyemi *et al.,* 2012). Vitrified tissues are rigid, thick and breakable; the mesophyll cells contain large vacuoles, fewer stomata and less photosynthetic activity (Leshem, 1983; Sharma and Mohan, 2006). In general the closure vessel is used to maintain the sterility of the inside environment, which in turn restricts the exchange of gases and accumulates the gases released by the tissue through metabolic processes, which may further reduce the oxygen concentration inside the vessel (Gould and Murashige, 1985). The higher plant requires an unhindered gaseous exchange for its sustainability, thus when the gaseous exchange is slowed down a critical oxygen and carbon dioxide shortage and accumulation of ethylene, volatile gases, ethanol, acetaldehydes, nitric oxide etc. is experienced (Righetti *et al.,* 1990; Jackson, 2003). An approximate figure of influx and efflux of gases within the plant parts is represented in the Fig.2.

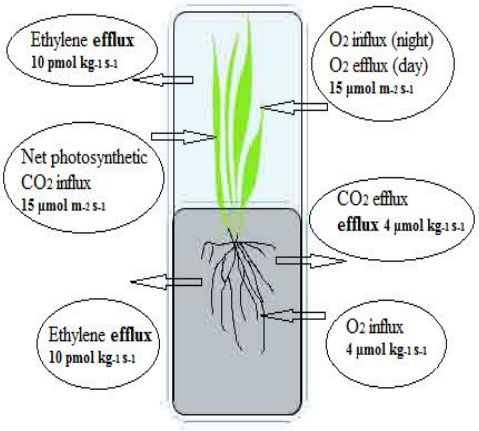


**Fig. 2. Diagrammatic representation of approximate efflux and influx of gases under normal growth. Source Jackson, M.B. (2005)**

Sharma and Mohan, (2006) succeeded in reduction of vitrified tissue in *in vitro* culture of *Chlorophytum borivilianum* by the addition of 20-50 $mgL^{-1}$ Phloroglucinol, a reducing agent and $2mgL^{-1}$ Alar B-9 a daminozide 9 (growth retardant). Ancymidol a growth retardant and inhibitor of gibberellin biosynthesis are also used in liquid cultured plants to check the shoot elongation and to induce meristematic cluster (Ziv 1991, 1992; Ziv *et al.,* 1998). The use of Rugini Olive (RO) media, replacement of agar by phytagel, incorporation of various osmotic agents like manitol and sorbitol in the medium may also help in the reduction of hyperhydricity or virtification problem (Thomas *et al.,* 2000). Tornero *et al.* (2001) developed bottom cooling system procedure in the culture vessel for a period of 3 weeks and used agargel to obtain virtificated free plantlets of apricot cultivars. A Considerable decrease in virtification and increased amount of chlorophyll content was found in the *in vitro* cultured plants of *Rubus* spp. and *Origanum vulgare* incorporated with extracellular mucoid components (EMC) of *Pseudomonas* spp. (Shetty *et al.,* 1996; Ueno *et al.,* 1997). Rossetto *et al.* (1992) while working on *in vitro* culture of various rare Australian plants have introduced a measure of virtification control through aeration where a 7 mm hole with double layer filter paper seal on the polypropylene screw lid of the culture vessels were created for proper aeration, which decreased the tissue vitrification. The culture of the plant *in vitro* close to the light compensation point, reduced BA concentration and use of the aerated capped culture bottles are some of the other measures to check the vitrification (Sharma and Mohan, 2006; Chiruvella *et al.*, 2014). $AgNO_3$ was also reported to use in the culture mediums to reduce hyperhydricity or vitrification by inhibiting ethylene gas (Juturu *et al.,* 2014). In addition, use of reducing sugars like glucose, galactose (Druart, 1988) and fructose (Rugini *et al.,* 1987) as alternate sugar source, high concentration of TDZ (1.5-2.0 $mgL^{-1}$) and repeated subculture are also reported by many workers as promising solutions for vitrification (Caboni *et al.,* 1999; Kadota and Niimi, 2003; Bosela and Michler, 2008; Noshad *et al.,* 2009; Feng *et al.,* 2010).

**Habituation to Hormones**

Habituation is the phenomenon of development of autonomous condition of various growth factors in plant tissue culture (Christou, 1988). Productivity and vigour of the plants are reduced due to habituation. Normally after prolonged period of continous sub-culture the propagated plant acquires the ability to synthesize the required hormones (Akin-Idowu *et al*., 2009) especially they acquire cytokinin independence (Melissa *et al*., 2006). Experimental evidence of habituation is very scant whereas the expression of cytokinin signalling component and overproduction of cytokinin (Catterou *et al*., 2002; Sun *et al*., 2003) may be due to increased levels of cytokinin receptor CRE1 and degradation of cytokinin signalling compound could play a role in the acquisition of habituation (Melissa *et al*., 2006). Regarding the control measure for habituation in in vitro plant culture less attention has been made in the optimization of the physiochemical parameters. Transfer of culture to hormone free media periodically, involvement of shorter periods of hormone containing media with regular intervening periods of hormone free media are some of the modifications in many protocol to control and overcome habituation (Akin-Idowu *et al*., 2009).

**Apex Necrosis**

Apex necrosis or shoot tip necrosis is a physiological disorder encountered in the *in vitro* plant culture. The early symptoms include the browning of the tip followed by the basipetal necrosis, senescence and ultimately death to the apical bud (McCown and Sellmer, 1987; Srivastava and Joshi, 2013). A complex set of factors are responsible for the shoot tip necrosis (Bairu *et al.,* 2009) which may include nutrient deficiency, unbalanced cytokinin level, medium type, aeration, $P^H$, subculture period, media and its composition and type of gelling agent (Bairu *et al.,* 2009). Of the various deficiencies the Ca and B is the most common factor for apex necrosis (Barghchi and Alderson, 1996).

Calcium alone can make up 10% of the dry weight in some plants (Hirschi, 2004). It plays an important structural and functional role in plants (Bairu *et al.,* 2009). Plant depends on the unique properties of $Ca^{+2}$ for its signalling, and enzymatic function and also strengthen the cell wall and increases the stress tolerance, apart from these, it regulates the $P^H$, acts as a regulatory ion in source and sink translocation of carbohydrate, maintaining apical dominance, takes part in cell elongation and cell division and connects various proteins and lipid at membrane surface (Marschner, 1995; Hirschi, 2004). The high humidity and low transpiration rate in the closed culture vessels may attribute to the deficiency of the Ca for the shoot tip necrosis of *in vitro* propagated plants (Sha *et al.,* 1985; Singha *et al.,* 1990; Abousalim and Mantell, 1994). Increased level of the exogenously supplied calcium level and slowing down the growth rate by low temperature to correspond the calcium supply and improving the ventilation can overcome shoot tip necrosis (McCown and Sellmar, 1987). However the calcium requirement varies from plant to plant. The form in which calcium is supplied in the culture media also affects the growth and development of the plant, for example calcium supplied as calcium chloride reduced the shoot tip necrosis and have no inhibitory effect in *Pistacia vera* cultures, whereas calcium supplied as calcium acetate found to be inhibitory (Barghchi and Alderson, 1996). On the other hand the media used to dry out when calcium chloride is supplied in excess due to increased amount of calcium ion and increased gaseous exchange and can also introduce undesirable anions (Mullins, 1987; George *et al.,* 2007). According to Roberts and Schum (2003) plant that express calcium deficiency can be overcome by the addition of calcium gluconate in the multiplication media which also have no any adverse affect. Kermani *et al.* (2010) have successfully overcomed the shoot tip necrosis problem in the *in vitro* culture of *Rosa persica* by addition of calcium gluconate (2.7 g/l) to the multiplication media. Chiruvella *et al.*, 2011 used the synergistic effect of calcium nitrate (CN) and calcium pantothenate (CP) to overcome the calcium deficiency.

Boron is mainly involved in the plasma membrane integrity and its functioning due to its influence on membrane protein and cell wall intactness (George *et al.,* 2007). The element is also used in the lignin and phenolic biosynthesis, maintenance of meristematic activity, activation of enzymes, metabolism and sugar translocation and nucleic acid synthesis (Mengel and Kirkby, 1982; Apostol and Zwiazek, 2004). It also mediates the phytochromes and responses to the gravity (Tanada, 1978). Regarding the Boron mobility two categories of plant were classified by Brown and Shelp (1997), one with restricted boron mobility and other with highly mobile boron. Sorbitol rich species like *Pyrus, Malus*, and *Prunus* boron is highly mobile in compared to sorbitol poor plants where the boron is largely immobile (Brown and Hu, 1996). Raising boron level up to considerable limit by the addition of boric acid reduced the shoot tip necrosis in many *in vitro* cultured plants without showing the symptom of acute boron toxicity (Barghchi and Alderson, 1989; Abousalim and Mantell, 1994; Anirudha and Kanwar, 2008). But boron has a narrow range of deficiency and toxicity level in comparison to other mineral nutrients (Abdulnour *et al.,* 2000). For the plant showing boron toxicity Brown and Hu (1996) suggested incorporating boron-sorbitol complex in the culture media to mediate the boron mobility so as to overcome shoot apex necrosis.

The factors other than the nutrient deficiency are also active for shoot apex necrosis in many plant species. For $P^H$ fluctuation in culture of *Populus* spp., De Block (1990) had buffered the medium with 2-(*N*-morpholino) ethanesulphonic acid and Ca-gluconate, and cultured below 25°C. Addition of $AgNO_3$ and activated charcoal in all the stages of multiplication also found to be effective in reducing shoot tip necrosis (Misra and Chakrabarty, 2009). STN under *in vitro* condition of *Syzygium cumini* was considerably reduced by the application of adenine sulphate (AdS) to the optimal media (Naaz *et al.,* 2014). Bairu *et al.* (2009) observed significant lowering of the STN in *in vitro* propagated Harpagophytum *procumbens* when supplemented with meta-Topolin riboside (*mTR*) compared to BA treatment.

**Fasciation and Tissue Proliferation**

Fasciations are morphological changes in the plant body characterized by the fusion and flattering of various organs.

Similar to the natural occurring fasciation the *in vitro* fasciations are also accompanied by increased meristematic size and enhanced growth (Iliev and Kitin, 2011). Although there were various attempts to understand the cause of *in vitro* and *in vivo* fasciation but the phenomenon is not yet clearly understood (Iliev *et al.,* 2009). It is believed to cause due to the adhesion of various sides of growth (Zielinski, 1945; Vitkovskii, 1959; Karagiozova and Meshineva, 1977) or may be due to infection, mutation, deficit of microelement or amputation of the apical meristem (Binggeli, 1990). Fasciation is a physiological disorder rather than a genetic aberration (George, 1996) but the genetic mechanism may be enacted by physiological imbalance (Boke and Ross, 1978). The hormonal imbalance mainly by the exogenously supplied cytokinin is reported to induce fasciation (Kitin *et al.,* 2005; Fatemeh and Maheran, 2010; Najmeh *et al.,* 2011; Uzelac *et al.*, 2012; Chiruvella *et al.,* 2014). However, their extent of induction varies from plant to plant. *In vitro* studies of the cultivars of *Betula pendula* showed the appearance of fasciation only in the zeatin containing media, but their formation is completely absent in media containing BA (Iliev *et al.,* 2011). Due to less data available for fasciation, remedial procedure for the same is not so much reported. Many workers optimized their hormonal condition in order to get fasciation free platelets. Antiethylene substances are also used to reduce the fasciation in *in vitro* cultures. Chaturvedi *et al.* (2003) have applied $AgNO_3$ and salicylic acid to control the embryonic fasciation in *Mangifera indica* followed by the subculture in influence of L-alpha-(2-aminoethoxyvinyl)-glycine-HCL (AVG) and in the presence of adenine sulphate (AdS).

**Somaclonal Variations**

*In vitro* raised plants are subjected to a wide range of somaclonal variations. Although the somaclonal variation is considered as agronomic and commercial important (Akin-Idowu *et al.,* 2009) many somaclonal variations however proved depressed in various essential characters. Such variations may be either due epigenetic or genetic (Evans *et al.,* 2003 and Morcillo *et al.,* 2006), through DNA methylation, point mutation, histone modification, ploidy and activation of mobile element and chromosome rearrangement (Jain, 2001; Smulders and Klerk, 2011; Sharma and Agarwal, 2012). Variations such as albinism, multiple carpel, dwarfism, dryness of apical bud, change in fruit quality etc. are mostly prevailing (Alkhateeb, 2008). The concentration beyond and above the optimum level of the growth regulator, especially the synthetic compounds (Martin *et al.,* 2006; Vidal and De Garcıà, 2007) and a prolonged callus phase are responsible for inducing genetic as well as epigenetic variation (George *et al.,* 2007). Presence of synthetic auxin especially dichlorophenoxyacetic acid (2,4-D) is reported to mainly associated with somaclonal variation by induction of polyploidy, endoreduplication of DNA due to stimulation of DNA synthesis (Neha *et al.,* 1992; Mohanty *et al.,* 2008) and abnormalities in mitotic spindle (Sogeke, 1998). DNA methylation is also increased by the application of synthetic auxins to calli or suspension culture which leads to genetic variability (Popescu *et al.,* 1997). Relative high concentration of BA also reported to cause somaclonal variations of banana and rice in *in vitro* culture (Oono, 1985; Gime´nez *et al.,* 2001). But the exact role and concentration of the cytokinin on incident of somaclonal variation needs more stringent experiments (Bairu *et al.,* 2011). Lower concentration or replacement of the 2-4-D with non chlorinated auxin like NAA (Sogeke, 1998) and reduction of hormonal concentration and culture in hormone free media in various stages of culture (Duval *et al.,* 1988) can eradicate the problem of somaclonal variation caused by hormone. The source of the explants also links to the genetic fidelity (Krikorian *et al.,* 1993). Highly differentiating tissues like roots, leaves and stems produces more variants due to their callus phase in *in vitro* culture (Kunitake *et al.,* 1995). The possibility of the somaclonal variation can be reduced by taking tissue from cambium, pericycle and procambium as starting materials for tissue culture (Sahijram *et al.,* 2003). Jaligot *et al.* (2000) and Smith *et al.* (2010) have suggested avoiding the rapid growing callus, as the multiplication rate indicates the sign of variations. Reduction of the culture period (Rohani *et al.,* 2003), support of optimum growth condition and involvement of strict quality control (Soh *et al.,* 2011) and direct organogenesis by avoiding the callus phase (Smith *et al.,* 2010) are some of the control measures adopted by various workers to control somaclonal variations in plant tissue culture.

**Conclusion**

Tissue culture is emerging technology which is catalyzing the research in various sections of advance plant sciences, improving commercial production of the various agricultural crops and conservation of rare and endangered plants. Establishment of a microenvironment and nutrient supply for the plant to grow often have various hurdles due to the complexity in understanding various peculiar phenomena which hinders the technology. However interdisciplinary approach to the technique has assured its greater effectiveness. The modern tissue culture technique is trying to understand various biological, physical and physiochemical aspects that govern the *in vitro* morphogenesis through the interpretation of various life processes rather than theoretical assumption and trial and error experiments. Sequential study of the research shows a clear development of the technology with inclusion various new techniques. Several challenging problems encountered during tissue culture are solved by the introduction of various remedial and precautionary measures. Still new approaches seem to be necessary in various aspects to understand the technology more efficiently for the future research and development.

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