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ADJUSTMENTS TO IN VITRO CULTURE CONDITIONS AND ASSOCIATED ANOMALIES IN PLANTS

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Plant tissue culture techniques have become an integral part of progress in plant science research due to the opportunity offered for close study of detailed plant development with applications in food production through crop improvement, secondary metabolites production and conservation of species. Because the techniques involve growing plants under controlled conditions different from their natural outdoor environment, the plants need adjustments in physiology, anatomy and metabolism for successful *in vitro* propagation. Therefore, the protocol has to be optimized for a given species or genotype due to the variability in physiological and growth requirement. Developing the protocol is hampered by several physiological and developmental aberrations in the anatomy and physiology of the plantlets, attributed to *in vitro* culture conditions of high humidity, low light levels and hetero- or mixotrophic conditions. Some of the culture-induced anomalies become genetic, and the phenotype is inherited by clonal progenies while others are temporary and can be corrected at a later stage of protocol development through changes in anatomy, physiology and metabolism. The success of protocols relies on the transfer of plantlets to field conditions which has been achieved with many species through stages of acclimatization, while with others it remains a challenging task. This review discusses various adjustments in nutrition, physiology and anatomy of micro-propagated plants and field grown ones, as well as anomalies induced by the *in vitro* culture conditions.

Keywords: Plant physiology, plant regeneration, *in vitro* culture, somaclonal variation, hyperhydricity, fasciation, acclimatization

INTRODUCTION

A plant tissue culture technique is the culture of plant cells, tissues or organs under controlled *in vitro* conditions to produce large number of true-to-type plants in short time using different starting plant material through stages of explant selection and preparation, culture establishment, regeneration and acclimatization of the plantlets to *ex vitro* conditions (George, 2008). The technology is advancing in applications for clonal propagation of medicinal, horticultural, agronomic crops and forest trees. However, success at commercial scale is constrained by formation of aberrant plantlets and low survival of the regenerate during transfer to field conditions (Gaspar et al., 1996; Sahay and Verma, 2000; Bairu and Kane, 2011). Many factors influence *in vitro* response of plants including the selected explant to be cultured, physiological state of the explant – juvenile or mature state, genotype, the health status of the explant and culture media (Roh and Wocial, 1989; Lee, 2004; Kane, 2005). The selected explant for *in vitro* studies needs physiological adjustment to the culture conditions so as to achieve enhanced clonal multiplication and for the cultivated plant to achieve physiological stability and repeated subculture to fresh media is necessary as medium nutrients get exhausted over time (Lee, 2004; Kozai and Xiao, 2006). The ability to regenerate the whole plant from cultured somatic cells, tissue or organ has been known for several decades; however, the problem of how the cultures differentiate into a whole plant and various physiological and anatomical features of the regenerated plants and during transfer to field conditions is still being studied by many research groups (Skoog and Miller, 1957; Pospisilova et al., 1999; Vogel, 2005; Kennedy and Norman, 2005; Jariteh et al., 2015). Manipulation of the *in vitro* development of plants is of paramount and applied interest as it proffers a model to characterize developmental stages at genomic and proteomic levels and also offers poten-

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tial to rejuvenate plants for increased propagation (Lee, 2004; Moyo et al., 2015). It provides opportunities for improvement of physiological adaptation to the environment with potential to improve establishment and post-establishment of micro-propagated plants (Joyce et al., 2003; Pence, 2010). Therefore, a profound understanding of the *in vitro* plant development, the morphophysiology as well as stress physiology mechanism and potential for acclimatization to *ex vitro* environment are of importance in predicting and improving the survival rate of plantlets during the *in vitro* culture conditions and acclimatization stages (Pospisilova et al., 1999; Sahay and Verma, 2000; Cassells and Curry, 2001; Moyo et al., 2015).

As *in vitro* culture of plants involves growing them in an artificial environment of culture growth conditions, physiology and growth needs are different from those of the natural environment (Lee, 2004). Hence, the protocol has to be optimized for a species as differences exist in organogenesis potential and morpho-physiological response across plant families, genera, species and even genotype of a species can show a different response to the conditions (George, 1993, 1996; Cassells and Curry, 2001; Lee, 2004). The response also varies with explant and ontogeny and recalcitrance is of common occurrence, particularly with the advance in the age of selected explants or cultures (Hagege, 1995; Brar and Jain, 1998). An important aspect of the protocol development is physiological and anatomical change which plantlets experience during the *in vitro* culture and at the stage of transition to *ex vitro* environment (Pospisilova et al., 1999; Hazarika et al., 2001, 2002; Bairu and Kane, 2011).

Success in clonal propagation is achieved when a large number of true-to-type regenerates are obtained with high survival in the field conditions and *in vitro* culture biomass accumulation results from interaction between the cultured plants, culture media composition, carbohydrate metabolism and environmental conditions in the *in vitro* and during *ex vitro* transfer (Kozai et al., 1991; Sahay and Verma, 2000). Physiological changes depend on different conditions the tissue got exposed to and may include culture medium composition, physical environment and duration of exposure to the *in vitro* culture (Gaspar et al., 1996; Lee, 2004). Several physiological and developmental problems may arise with the plants during the *in vitro* clonal propagation and can have a bearing on the performance in the *in vitro* and *ex vitro* transfer (for review see Hazarika, 2006; Bairu and Kane, 2011). These may include, among others, necrosis of the explant, fasciation, tissue proliferation, somaclonal variations, epigenetic changes and exudation of phenolic compounds during rhizogenesis (Hazarika, 2006; Bairu et al., 2009; Bairu and Kane, 2011; De Klerk et al., 2011). Physiological and anatomical deformities, common among micro-propagated plants, may include poor photosynthetic efficiency, malfunctioned stomata, hyperhydricity, marked decrease in epicuticular wax formation but regular assessment of the cultures can help overcome the problems with significant control of the abnormalities (Hazarika, 2006; Bairu et al., 2009). The epigenetic and genetic problems include loss of organogenesis potential and somaclonal variation that depends on the genotype, explant and culture environment (Cassells and Curry, 2001; Smulders and de Klerk, 2011; Uscamas et al., 2014). The above and some other conditions determine the extent of adjustments in morphological, anatomical and physiological features a cultured plantlet requires in *in vitro* and during acclimatization (Solarova et al., 1996) and developing new leaves will be of paramount importance to their establishment (Vina et al., 1999).

Many of the culture-induced variations shown by the *in vitro* propagated plants could result from oxidative stress damage to tissues during the preparation of the explant, the condition of culture factors or natural acclimatization factors during *ex vitro* transfer (Cassells and Curry, 2001). Also, stress due to imbalanced culture media and composition, poorly designed culture vessel and environment can contribute to genetic developmental and physiological variability in the *in vitro* cultured plant (van Staden et al., 2006; De Klerk, 2007; Bairu et al., 2011a) with resultant senescence without *in vitro* response or sometimes achievement of the experimental aim (Ziv, 1991; Cassells and Curry, 2001). If a plant responds, it may occur through regulation of auxin to cytokinin ratio and absolute growth regulator concentrations. In the cases of recalcitrance, it was overcome in many species through using a pulse in the sequence with auxin followed by cytokinin (Skoog and Miller, 1957; Christianson and Warnick, 1985; Cassells and Curry, 2001). Due to these and other difficult microenvironmental conditions that *in vitro* propagated plants are exposed to, normal micro-propagated plants develop physiological and anatomical features inferior to the plants grown under field conditions. These have a severe bearing on the practical use of plant tissue culture for clonal propagation and genetic manipulation of plants. A proper understanding of the physio-morphological characteristics and adjustments made by plants during the *in vitro* culture and changes they needed during acclimatization form a critical stage of propagation protocols. This review discusses the adjustments in physiology, anatomy, nutrition and metabolism by *in vitro* cultured plant cells, tissues or organs in response to culture conditions and during acclimatization. Even though the physiological response to culture conditions varies with species and genotype, a generalized discussion of the infor-

mation is attempted with emphasis on aspects necessary for the survival of plants during the *in vitro* growth and transfer to *ex vitro* conditions.

NUTRITIONAL ADJUSTMENT

Plant roots perform the role of an anchor to the substrate and uptake of nutrients while leaves regulate gaseous exchanges, carbon assimilation and allocation to different parts. The nutrient medium for plant cell, tissue, organ cultures is composed of inorganic salts, carbon source, vitamins and growth regulators and with a gelling agent when culture media need to be solidified to provide a substrate for proper growth of cultures. The mineral nutritional need of *in vitro* cultured plants is dynamic with respect to the culture media nutrients; overall nutrition involves minerals in the culture media, interaction between the supplied ions and medium substrate, movement of ions via the substrate to the plant's surface, then uptake by the plant, transport within the plant before final assimilation (Williams, 1992; Debergh et al., 1994).

An aspect of great importance to the nutrition is carbohydrate provided in the culture media as sugars or photosynthesized using CO_2 to give potential energy that is made available to plants via respiration (Welander and Pawlicki, 1994; Ticha et al., 1998). The carbon source and salts used in the culture media coupled with poor light regimes restricts photosynthetic efficiency of plantlets and the added sugars can decrease water potential of the culture medium (Mukherjee et al., 1991; Pospisilova et al., 1999; Ticha et al., 1998). Concentration of the carbon source and other nutrients, light intensity and CO_2 level in the culture vessel are limiting factors that can induce physiological and structural modifications to the cultivated plants. The plantlets can show low capacity assimilation of the inorganic carbon due to low net photosynthesis, low photochemical quenching, small but variable fluorescence ratio resulting in diminished Photo System II (PS II) primary photochemistry and higher capacity of non-photochemical quenching of radiation (van Huyelenbroeck and Debergh, 1996; Ticha et al., 1998) and also due to the added sugar in the culture media (Galzy and Compan, 1992; Ticha et al., 1998). Such plantlets could be normal but unlikely active in photosynthetic efficiency as the field grown plants, due to the carbon source uptake from the culture media that makes developing photosynthetic machinery poor or unnecessary with resultant low content of photosynthetic enzymes (Joyce et al., 2003) and overall effects on photosynthetic efficiency of the plants caused by poor chlorophyll content or photosynthetic enzymes, compared to plants grown in the *ex vitro* conditions (Wetzstein and Sommer, 1982; Donnelly and Vidaver, 1984; Yokota et al., 2007).

For *in vivo* grown plants, developing leaves and components of photosynthetic apparatus are synthesized and assembled into a functional unit to make sure adequate transduction of light energy into chemical energy occurs by photosynthesis (Chaves, 1994). The photosynthetic machinery is synthesized and assembled into a unit for leaves to transduce light energy into chemical energy as a carbohydrate that in turn are used by the plants for maintenance and building of structures (Baker, 1985; Chavez, 1994). Synthesis of the carbohydrates mainly occurs in leaves, then they are translocated to non-green cells that are often remote from leaves (Welander and Pawlicki, 1994). Certainly, differences exist in photosynthetic efficiency between field grown plants and the *in vitro* ones due to variable assemblage, genotype effects and functionality of systems in the two plants and discrepancies can sometimes be attributed to methodologies used for assessment of the photosynthetic rate (Preece and Sutter, 1991; Chaves, 1994). Therefore, *in vitro* cultured plants have to make adjustments in physiology and/or morphology to enable themselves to adapt to culture conditions of physical environment and nutrients supply provided by the culture media. Adjustments in the morphology and biochemical processes increase photosynthetic capacity under the conditions by producing thinner and larger leaves for greater light capture, but the amount of light-harvesting chlorophyll response varies with plant species and cultivars (George, 2008). Plants such as cauliflower and strawberry could not develop the ability for leaf photosynthesis in the *in vitro* conditions while others produce photosynthetically competent leaves and adapt to the autotrophic conditions (Grout, 1988).

In *in vitro* cultures, cells proliferate followed by differentiation and introducing a change in balance of growth regulators can induce organogenesis, but ontogenetic state of the explant and age of the cultures play a significant role in determining regenerability (Rout et al., 2000; Kane, 2005; van Staden et al., 2006; Smulders and de Klerk, 2011). Plant growth regulators (PGRs) supplemented in the culture media at a selected concentration result in the formation of desired plantlets but with abnormal morphology, physiology and anatomy. In most instances during transfer to *ex vitro* conditions, the abnormalities have to be corrected for successful acclimatization in the greenhouse conditions as irradiance and air humidity are much higher in greenhouse or field conditions than *in vitro* (Wardle et al., 1983; Selvapandiyan et al., 1988; Pospisilova et al., 1999; Hazarika, 2003, 2006).

Sometimes cultured cells, tissues or organs become independent of a certain substance in the

culture media for growth and development through habituation (George, 1993). Habituation often occurs in cultured plant cells, the callus can be particular about the age of tissue region with cytokinin and auxin habituation as the most common (Greenwood, 1987; Mendoza and Kaeppler, 2002). The cytokinins are added to culture media to induce morphogenesis or rejuvenation of explants while auxins are required for cellular division and differentiation, callus induction and somatic embryogenesis but cultures can become habituated over time (Greenwood, 1987; Meins, 1989; Mendoza and Kaeppler, 2002).

Explant rejuvenation, habituation and many morphological changes in the *in vitro* cultures are regulated by epigenetic changes (Smulders and de Klerk, 2011) but habituation and subculture impose a negative effect on cultured plants with resultant heritable and reversible effects (Meins and Foster, 1985; Meins, 1989; Smulders and de Klerk, 2011). Decreased cell-to-cell adhesion with the irreversible loss of regeneration capacity are fundamental features of callus habituation (Meins, 1989; Gaspar, 1998). During acclimatization, when plants are removed from culture vessel conditions, they have to overcome limitations of heterophic condition through production of more assimilates than their importation and by increasing Rubisco content to cope with increased energy necessary for *ex vitro* survival (Pospisilova et al., 1999; Hazarika, 2006). The restriction on carbon uptake due to the transient water deficits could be overcome by growing plants in an environment with increased humidity level or atmosphere with high CO_2 that will improve water-use efficiency and net carbon uptake with a resultant increase in reserve accumulation in young plants.

STRUCTURAL AND PHYSIOLOGICAL ADJUSTMENTS

In vitro propagated plants differ in physiology from the mother plant from which cultures are initiated and could cause increased propagation potential due to tissue rejuvenation over subcultures (Jones, 1994). The differences between an *in vitro* grown plant and greenhouse one are striking and could be morphologically visible, sometimes as poor cuticular wax deposition on the surface of leaves, pigmentation and various morphological features associated with the *in vitro* condition adaptations. Culture conditions of artificial support medium containing minerals, PGRs, carbon sources for heterotrophic growth, low light regimes and higher relative humidity can induce anatomical, physiological and morphological change in the plants, leading to formation of malfunctioned roots, deficient formation of vascular tissues, especially deficient connection of the root and shoot vascular system (Grout and Aston, 1978a; Kozai, 1991; Apter et al., 1993a, b).

The changes in population, the development of *in vitro* propagated plants are recognized when comparison is made with a control population and are expressed as apical dominance, number of leaves, their sizes, and more important is the flowering time and yield quality (Cassells et al., 1997; Cassells and Curry, 2001; Jiang et al., 2011). Morphological variability shown by *in vitro* cultured plants within population of clones can result from loss of specific viruses from regenerated plants, chimeral breakdown, rearrangement and/or synthesis of unstable chimeras (Kowalski and Cassells, 1999). Variabilities between the population of plantlets may arise when plants are propagated on different media or culture vessels having different characteristics (Joyce et al., 1999; Cassells and Curry, 2001). In some instances, albino shoots can be observed among the propagated plantlets but expression of the morphological variation is hard to assess due to variation between shoots, temporal differences in shoot induction and limited expansion of leaves in the *in vitro* cultures (Fig. 1c–e). Plantlets grown in a culture vessel having low light levels, medium containing sugar in various forms, other nutrients needed for growth in culture, high relative humidity in culture vessel and aseptic conditions, develop features inconsistent with plants grown under greenhouse or field conditions; the shoot of the *in vitro* grown plant may be slender with less sclerenchyma and collenchymatous tissue than the field grown one and roots of the shoots may be covered with root hairs having less periderm compared to field-grown ones (Donnelly et al., 1985). The plants may produce fewer shoots, low metabolic activity due to the low photosynthesis and carbohydrates assimilation resultant from presence of high sucrose concentration in culture media and low light level with declined ethylene production, as supported by experiments on garden geranium, cauliflower and raspberry (Grout and Aston, 1977; Donnelly et al., 1984; Desjardins et al., 1988; Hazarika et al., 2000; Wojtania et al., 2015). Low light conditions can also result in thin or irregular epidermal cells, limited palisade layer and sometimes strangely shaped palisade cells, impaired stomata and loosely organized spongy mesophyll cells (Saez et al., 2012a, b). The poorly developed mesophyll and palisade tissue encountered in leaves of *in vitro* propagated plants result from reduced cellular division, the number of stomata and chloroplast but the thickness of the mesophyll did not show variation in comparison to that of in vivo grown plants observed in *Jatropha curcas* (Hazarika, 2006; Rodrigues et al., 2014). Leaves of

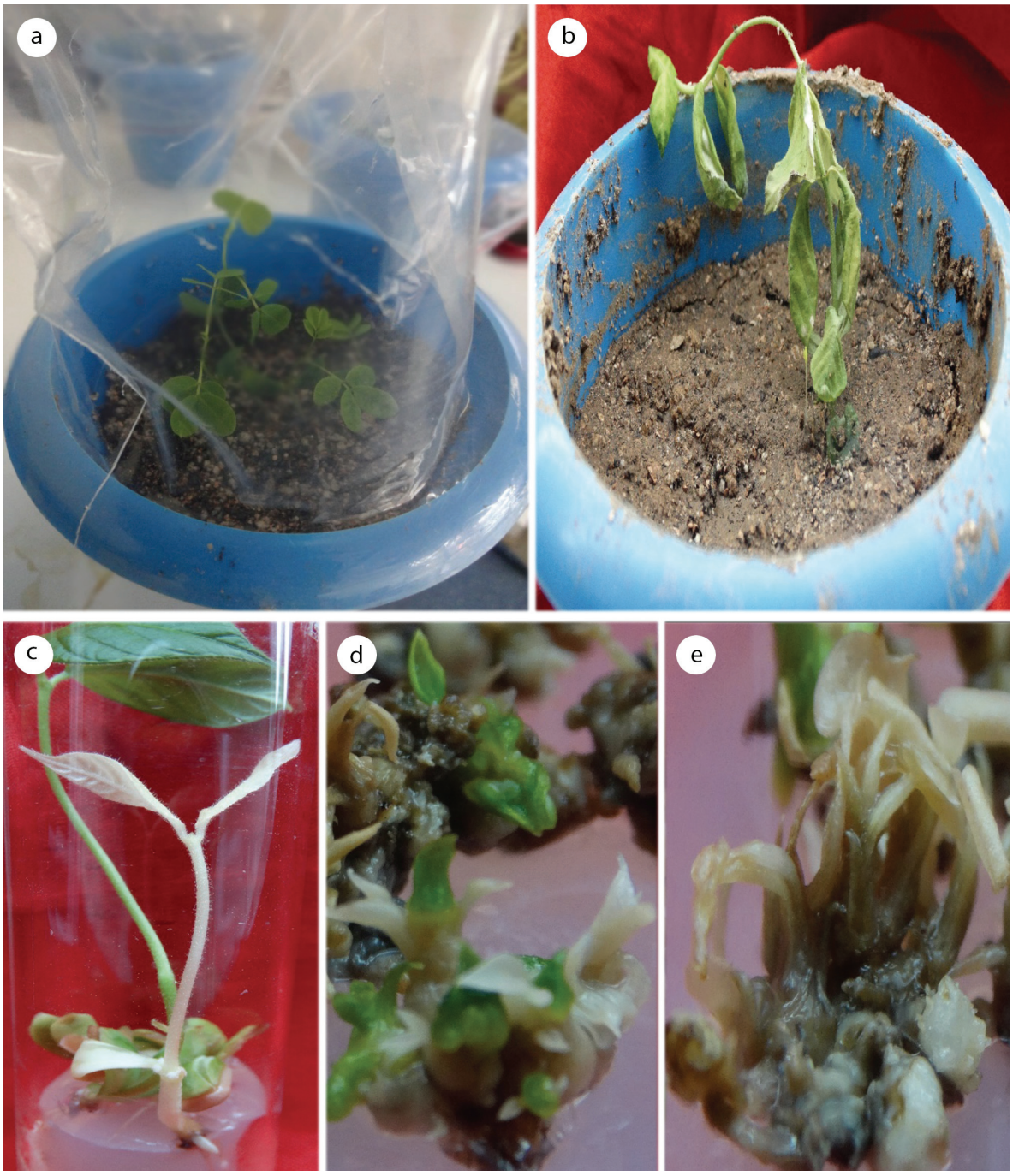

Fig. 1. (**a**) Early stage of *Senna occidentalis* acclimatization involving reduction in humidity level, (**b**) transplantation shock in Senna plantlets due to direct exposure to the outdoor environment, (**c–d**) formation of an albino shoot among the population of *in vitro* propagated plants in *Chonemorpha fragrance* (**c**) and *Caladium bicolor* cv. 'Bleeding hearts' arising due to long-term culture (d). (e) Hyperhydricity in the albino shoots of *Caladium bicolor* cv. 'Bleeding hearts'

the plants possessed a thin cell wall due to inhibited cell wall components deposition, formation of collenchyma and sclerenchymatous cells.

Culture conditions provide less physiological stress due to the carbon source supplied in media that reduces the need for photosynthesis and the aseptic condition reduces other stresses associated with pathogenic organisms (Cassels and Curry, 2001). Shoots regenerated *in vitro* are often tiny due to reduced accumulation of dry matter caused

by the *in vitro* culture. A prolonged culture of the shoots can sometimes lead to physiological aging with undermining effects on the viability of clones. The stomatal pore regulates gaseous exchange between leaves of higher plants with the atmosphere through rhythmic opening and closure to balance CO_2 acquisition for photosynthesis and prevent excessive water loss due to transpiration. Control of the gaseous exchange involves an array of responses to environmental factors and its imbalance or deformity has severe physiological and anatomical damaging effects on the *in vitro* cultivated plants. The behavior pattern can be adjusted in response to intercellular signaling that reflects the physiological state (Mansfield, 1994).

In vitro cultured plant stomatal structure and density are contributory factors to excessive water loss compared to greenhouse or field grown ones; stomata are raised with round guard cells compared to sunken elliptical normal ones in many species, e.g. *Citrus* (Hazarika et al., 2002). The most typical culture vessel conditions that induce modifications in stomata include a poor mechanism for regulating water loss due to the inefficient stomatal functionality, epicuticular wax formation and reduced development of photosynthetic tissues (Lamhanedi et al., 2003). The above stomatal features developed in leaves by *in vitro* cultured plants due to the *in vitro* conditions result in low water control mechanism that renders plantlets vulnerable to physiological disturbance when removed from the culture vessel and directly transferred to outdoor conditions (Fig. 1b). Aberrations in leaf morphology could also have severe undermining effects on the metabolic and physiological process associated with photosynthesis and transpiration of plantlets (Long et al., 1994). The water status and gaseous exchange in cultures are main factors that drive abnormal *in vitro* morphogenesis of plants and environment of cultures can affect many activities of enzymes with a resultant change in metabolic processes and responses similar to plants under stress conditions (Ziv, 1999; Cassells and Curry, 2001; Hazarika, 2006).

Although developmental and morphological variability expressed among the population of micro-propagated plants are accepted in *in vitro* phenomena, cryptic variabilities in juvenility within a population are recognized in regenerated plants by adventitious regeneration pathway with genetic variation (Leva et al., 2012). The gaseous phase and water status in the cultures are therefore determinant factors involved in aberrant *in vitro* morphogenesis of plants leading to altered leaf morphology with severe influence on metabolic and physiological processes associated with photosynthesis and transpiration in the plantlets (Ziv, 1991; Carvalho et al., 2001; Badr and Desjardins, 2007; Saez et al., 2012a). As a result, the plants develop physiological and morphological fragility when removed from the culture environment to *ex vitro* conditions (Preece and Sutter, 1991). However, change in the anatomy of leaves that includes an increase in thickness of palisade cells without a change in layers of the cells or of mesophyll air space may occur after removal of the plants from culture conditions (Fabbri et al., 1986). Therefore, the adjustment need of *in vitro* cultured plants depends on the biosynthetic capacity, developmental potentials and consideration should be given to the factors when culturing plant cells, tissues or organs during developing protocols for clonal propagation.

CULTURE-INDUCED ABNORMALITIES

In vitro propagation of plants is used for rapid clonal multiplication of many plant species and the ultimate success depends on health quality of the regenerate with large-scale and low-cost high survival when transferred to *ex vitro* conditions. Cultured plants often develop aberrations due to artificial environmental conditions of the *in vitro* culture. Many of the aberrations could be formed among plantlets remarkably and the age of cultures is associated with increased genetic instability (Kaeppler et al., 2000). The *in vitro* propagated plants are impaired by the condition of culture factors leading to metabolic, physiological and morphological anomalies that may include vitrification or hyperhydricity, translucency, succulence and glassiness (Ziv, 1991; Hazarika, 2006; Chiruvella et al., 2014). Physiological abnormalities due to hyperhydricity, genetic and epigenetic variations and poor physiological quality have a common basis in the *in vitro* propagated plants. The disorders mainly affect photosynthesis and gas exchange in leaves while anatomical anomalies manifest themselves in the stem and roots, to a lesser extent they can impede the establishment of the plantlet to the *ex vitro*. The weak formation of vascular tissues in leaves, poor differentiation of mesophyll make *in vitro* plants susceptible to transplantation shock (Fig. 1b) due to the developed thinner leaves with poorly developed palisade layers and large air space in mesophyll compared to the greenhouse-grown. In some instances, *in vitro* grown plantlets do not develop a defined palisade layer (Grout and Aston, 1978a; Wetzstein and Sommer, 1982) or even form a single layer in the place of normal two to three layers of greater mesophyll tissue in the leaves of greenhouse or field plants (Brainerd et al., 1981). The cuticles covering above ground parts also restrict water loss through transpiration and level and structure

of the cuticular, epicuticular waxes influence water permeability (Martin and Juniper, 1970). Poor formation of cuticular wax in the *in vitro* grown plants is also among factors responsible for the excessive water loss during transfer to *ex vitro* conditions (Fuchigami et al. 1981; Wetzstein and Sommer, 1982) and might be due to differences in chemical composition of the cuticular wax deposit in leaves of *in vitro* and *ex vitro* grown plants, allowing excessive water loss in the case of *in vitro* grown one.

HYPERHYDRICITY

Hyperhydricity, formerly called vitrification, is a physiological malformation in plantlets that results in excessive hydration, reduced lignification, impaired stomatal function with reduced mechanical strength of plantlets and the consequences include poor regeneration of plants without intensive acclimation to greenhouse or outdoor conditions (Kei-ichiro et al., 1998). Hyperhydric conditions may lead to leaf tip and bud necrosis and in some instances loss of apical dominance in the shoot (Cassells and Curry, 2001; Machado et al., 2014). The malformation is characterized by 'glassy appearance' of plants with thick, rigid, easily breakable stem and leaves (Fig. 1e) that have decreased chlorophyll and protein contents, low phenolics, increased water content and altered ion composition (Phan and Letouze, 1983; Kevers and Gaspar, 1986; Bottcher et al., 1988; Perry et al., 1999; Frank et al., 2004). Hyperhydric conditions may be due to excessive hydration, reduced lignification, impaired stomatal function and reduced mechanical strength of plantlets that could lead to poor regeneration. During acclimatization, because of the low chlorophyll formation and high water content in the plantlets, the condition becomes exacerbated by thin or deficient cuticular layer, reduced palisade cells layers, irregular stomatal formation, a poorly developed cell wall and large intercellular spaces in mesophyll cell layers in leaves (Kei-ichiro et al., 1998; Cassells and Curry, 2001; Franck et al., 2004; Hazarika, 2006). Hyperhydricity of *in vitro* propagated plants depends on water availability, PGRs supplemented in the culture media, mineral salts (micro and macro nutrients), the microenvironment of the culture vessel and ethylene composition (Doneso, 1987; Kataeva et al., 1991; Machado et al., 2014). Anatomical and morphological features of a leaf of vitrified plantlets differ from those of healthy and *ex vitro* grown plants and may include abnormal development of meristems that results in delicate plants with glassy hyperhydrous appearance (Ziv, 1999). Microscopic examination in most cases shows that they possess thin palisade tissue but most of unorganized mesophyll is composed of spongy parenchyma rich in intercellular spaces (Ziv, 1999). The leaves of hyperhydric plants are composed of cells with a thin cell wall, poor and large-vacoulated cytoplasm and in some species are associated with defective epidermal tissue and low deposition of epicuticular wax (Ziv, 1999; Franck et al., 2004). Hyperhydric leaves may even lack palisade tissue but possess spongy and large-vacoulated mesophyll with large intercellular space (Vieitez et al., 1985). The leaves may contain lower level of cell wall carbohydrate with resultant low guard cells turgor properties and structural defects in cells (Gaspar et al., 1987; Ziv et al., 1987; Machado et al., 2014) which can be overcome by using many approaches (Shetty et al., 1996; Perez-Tornero et al., 2001; Toth et al., 2004).

Excessive accumulation of ethylene at a later stage of *in vitro* culture could be the principal factor inducing hyperhydricity to plants and can be overcome through reducing relative humidity by improving gas exchange in the culture vessel (Buddendorf-Joosten and Woltering, 1995; Saez et al., 2012b), increasing concentration of agar in the culture media (Debergh and Harbaoui, 1981) and use of ethylene adsorbents (Zobayed et al., 2001; Sarkers et al., 2002; Mayor et al., 2003). The use of osmotic and reducing agents, enhanced aeration in the culture vessel or growth of retardants can mitigate the frequency of hyperhydric plantlets formation in cultures (Rossetto et al., 1992; Ziv, 1998; Thomas et al., 2000; Sharma and Mohan, 2006). Amending silver nitrate or low concentration of cytokinin in the culture media and daily illumination at the compensating point combined with an aerated culture vessel reduced the frequency of the formation of hyperhydric plantlets in cultures through inhibition of ethylene accumulation in the culture vessel (Sharma and Mohan, 2006; Chiruvella et al., 2014; Juturu et al., 2014)

The mechanism by which hyperhydric conditions occur in tissue cultures continues to intrigue tissue culturists and several theories have attempted to explain the mechanism of the occurrence and effect(s) of the condition to physiology, anatomy and survival of plants during the *in vitro* conditions and *ex vitro* transfer. Most of the theories center on the role of stress on protoplast and apoplast 'water logging', leading to oxidative stress and anatomical changes observed in hyperhydric plants. Recent developments in genomics and instrumentation technologies when coupled in studies of hyperhydricity hold promise in unraveling the many unknowns that predispose plants to the condition during the *in vitro* culture and *ex vitro* survival. At the moment, our knowledge is on the biochemical changes, leading to anatomical and morphological expression of the hyperhydric condition.

FASCIATION

Fasciation is an abnormal growth of plants that occurs in the growing point resulting in formation of cylindrical tissue which becomes elongated, perpendicular to the direction of growth with resultant formation of flattened, ribbon-like, crested or contorted tissue in the apical tip of stem, root, fruit or flower head (White, 1948) and could result in increased weight and volume of the plants in most cases (Albertsen et al., 1983). The *in vitro* culture fasciation is a newer culture-induced abnormality in plantlets that occurs due to flattening of the shoot, resulting from failed formation of lateral branches and separation from the main shoot in the growing cultures or fusion of organs due to deviation from normal cellular division or even transformation of the single growing point into a line (Zielinski, 1945; Vitkovskii, 1959; Clark et al., 1993; Karagiozova and Meshineva, 1977; Iliev and Kitin, 2011). The condition is associated with an increase in the size of meristem and growth of the plant shoot that arises due to abnormal enlargement of shoot apical meristem with changes in control of meristematic cells development, formation of multiple apical domes and points of growth. Although single recognized apical meristem can be found in fasciated plants, differences in meristematic layers could also be distinguished (Laufs et al., 1998a, b; Kitin et al., 2005; Fambrini et al., 2006; Iliev and Kitin, 2011). The condition may be physiological or genetic in origin; physiological fasciation could be due to culture environmental conditions or treatment with PGRs while less is known about the genetic or epigenetic source of the abnormality at the molecular level. Certainly many factors are believed to induce the condition on micro-propagated plants. Even though fasciation can appear in all or sections of the plant shoot (Iliev et al., 2003; Kitin et al., 2005) and be propagated to earlier unaffected tissues in many ways (Crespi et al., 1992; Bertaccini et al., 2005), the condition can be observed visibly in shoots. Much information is known about fasciation of the shoot or rhizome but less about leaves and roots (Iliev and Kitin, 2011). Exogenously applied cytokinins may induce fasciation in cultured plants and frequency of the induction varies within the genotype, depending on cytokinin type and concentration; it can be induced with Zeatin (Iliev et al., 2003, 2011), benzyladenine (Papafotiou et al., 2001; Kitin et al., 2005; Chiruvella et al., 2014) but not with thidiazuron (TDZ) (Mitras et al., 2009) and can be overcome by a high concentration of the TDZ or Zeatin (Fukai et al., 2000).

The development of fasciation might be followed by uneven distribution of cells in the central and peripheral zones of shoot apical meristem associated with the proliferation of meristematic cells (Kitin et al., 2005; Iliev and Kitin, 2011). The plant shows increased levels of free auxin in apical meristematic cells resulting in changes in epidermal structure, plastochron, leaves, inhibited axillary buds formation with effects on the shape of vascular cylinders and pattern of vascular tissues development (Kitin et al., 2005; Iliev and Kitin, 2011). The stem section of the fasciated shoot may be elliptical, circular or irregular in contrast to a concentric ring around pith in the proper shoot of most plants (Iliev and Kitin, 2011). The Shoot Apical Meristem (SAM) of fasciated shoots shows a unique structural change in central and peripheral zones in the shoot apex that results in an abnormal shape of the stem and lateral organs (Iliev and Kitin, 2011). *In vitro* culture fasciated shoots possess increased cortex parenchyma and pith tissues, less developed xylem and phloem fibers (Iliev and Kitin, 2011). The former is due to enhanced cellular enlargement (Nilsson et al., 1996; Kitin et al., 2005), while the latter is due to delayed differentiation of vascular tissues resulting in less cell layers without secondary wall development and callus-like cells formation in pith and cortex (Iliev et al., 2003; Kitin et al., 2005; Mitras et al., 2009). Iliev and Kitin (2011) observed prosenchymatic cambium-like cells in the longitudinal section of the shoot which explained the increased cross-sectional area of fasciated shoots to be due to the increase in cambial activity and secondary growth. However, differential anatomical features were not observed in normal Vs fasciated *in vitro*-induced shoots (Tang and Knap, 1998; Iliev et al., 2003; Kitin et al., 2005). However, epidermal cells, stomata, rhizoids and axillary buds formation in *Cymbidium kanran* showed distinct features between fasciated and non-fasciated rhizomes (Fukai et al., 2000). *In vitro* fasciated shoots showed enlarged SAM bearing a large number of cells, delayed xylem differentiation and enlarged parenchymatous cells at the later stage of organ development and atomical study of the SAM indicated the condition was triggered by changes in cellular arrangement in central and peripheral zones of apical meristem (Laufs et al., 1998a, b; Tang and Knap, 1998; Fambrini et al., 2006).

The genetic and epigenetic modulators involved in the development of fasciated plantlets are not yet understood and the evidence for their occurrence is largely based on studies on anatomical and physiological effects on *in vitro* propagated plants. The few data from these studies have provided insight into the nature of the occurrence and impact but how it occurs in cultures remains elusive, thus making the remedial measures difficult to identify.

SOMACLONAL VARIATION

Somaclonal variations are changes that occur in undifferentiated cultures, during differentiation and/or transfer to the outdoor environment which

may be due to changes in ploidy level, chromosomal aberrations and rearrangement, activation of transposable elements or even abnormalities during the cell cycle. The changes may be induced by condition culture factors with resultant effect(s) on the formation of chimeral tissues/organs, abnormal arrangement of chromosomes due to non-disjunction arising from disturbance to cell cycle and transposable elements movement within the genome.

Regenerating plants in *in vitro* conditions is associated with variability among regenerates and most common due to prolonged culture with resultant effects on genetic alteration of the cultures (Reuveni and Israel, 1990; Duncan, 1996; Us-Camas et al., 2014). Sometimes, the variations may not be due to tissue culture conditions or even occur in some plants (Bennici et al., 2004; Smykal et al., 2007; Bairu et al., 2011b). Somaclonal variation may be heritable and visible as in chimeric tissues that arise in cultures or invisible and propagated in clones but, later, over a generation of clones, manifests itself in the phenotype of clones (Fig. 1d). The variation is characterized by morphological, molecular, biochemical, genetic and epigenetic changes that can affect the plants' *in vitro* response to morphogenesis and mechanism by which it contribute to decline in vigor and regeneration capacity of cultures over time, depends on the genotype of the propagated plant (Larkin and Scowcroft, 1981; Phillips et al., 1994; Kaeppler et al., 2000; Smulders and de Klerk, 2011). The extent of somaclonal variation induction may show variation in explants and chances are higher with highly differentiated tissues such as stem, leaves and roots while axillary buds and shoot tips have fewer chances due to pre-existing meristems (Sahijram et al., 2003; Sharma et al., 2007). Highly organized explants as shoot tips were reported to show more of the variation than somatic embryos in banana (Israeli et al., 1996) while undifferentiated tissues such as pericycle, procambium and cambium reduced chances of somaclonal variation in the *in vitro* propagated plants (Sahijram et al., 2003; Bairu et al., 2011b). Chances of regenerating somaclones in the *in vitro* cultures depend on the regeneration pathway involved; in most cases they are higher when undifferentiated tissue is used (indirect regeneration), compared to differentiated ones as it involves induction of stresses of various kinds to the tissues at a higher level, leading to disturbance on cell division, genome instability with profound effects on metabolic processes and expression of variation in clones. Plant growth regulators added to culture media for induction of morphogenesis and whose mechanism of action in cells involves cell cycle disturbance can induce the variability (Peschke and Phillips, 1992). For instance, derivatives of diphenyl urea such as 2,4-D were implicated in many cultures-induced somaclonal variations (Roels et al., 2005; Siragusa et al., 2007; Radhakrishnan and Ranjitha Kumari, 2008; Bairu et al., 2011b) and their use at higher concentration in culture media to induce calli was implicated in many instances of somaclonal variation (Nehru et al., 1992; Gesteira et al., 2002; Jin et al., 2008) while imbalanced concentration along with cytokinins induced polyploidy (Swartz, 1991; Bairu et al., 2011b).

As *in vitro* morphogenesis of plants is determined by genetic, epigenetic response to culture condition, control of molecular developmental changes in gene expression leading to hormonal synthesis, signaling and regulation of transcription determine the extent of the morphogenesis and somaclonal variation. Expression of genes in the *in vitro* and *ex vitro* is regulated by molecular changes involving cross-talk between protein kinases, transcription factors, structural proteins and enzymes, resulting in molecular changes and phenotypic expression. The extent of the exposure of plant tissue to culture condition/stress(es) determines the degree of disturbance to genome and cell division leading to somaclonal variation in growing cultures. For instance, transposable elements whose molecular mechanism of transposition in genome involves selective transcription, cryptic transposition and stress-induced activation, are increasingly receiving research attention on their activation by tissue culture-induced stress that compromises genome integrity, as in maize where they constitute 85% of the genome (Schnable et al. 2009).

Somaclonal variation can lead to many changes that represent deviation from the normal plant as in abnormalities in the *in vitro* flowering through the disrupt of coordinated floral development induced by epigenetic changes due to the *in vitro* culture conditions (Jaligot et al., 2000; Sun and Zhou, 2008; Meijon et al., 2009, 2010). Sometimes, epigenetic variations arising in cultures may turn out of advantage to plantlets, as for instance, conferment to plantlets developmental maturity with possible stress cross tolerance that influence their establishment and tolerance to biotic and abiotic stresses through changes in metabolism or genetic alterations to meet metabolic demands for efficient cell division, growth and organogenesis (Gaspar et al., 1996; Joyce and Cassells, 2002; Us-camas et al., 2014). Somaclonal variation can manifest itself as morphological and physiological aberration in plantlets whereas phenotypic aberrations in the *in vitro*-raised progenies of the plants reflect the effect of uncharacterised stress imposed on the plastic genome and gene expression (Joyce et al., 2003). However, interaction between environment and *in vitro* cultured plants determines epigenetic changes that occur in the genome of cultures as well as during transfer to outdoor environment (Pospisilova et

al., 1999; Bird, 2007; Smykal et al., 2007; Chen et al., 2010; Miguel and Marum, 2011; De-la-Pena et al., 2012).

Epigenetic changes arising in *in vitro* cultures include the extensively studied histone modification and DNA methylation while miRNAs that play a significant role in regulating many biological processes as cell fate determination, gene silencing and adaptation to *in vitro* culture conditions are less studied (Us-camas et al., 2014). These changes reflect genotypic plasticity in plant cells to adapt to changing environment (Brautigam et al., 2013). Histone modifications and associated phenomena constitute epigenetic changes that play a significant role in cellular identity, differentiation and development of cultured cells and their coordinated regulation is important in regulating somaclonal variation in cultures (Ikeuchi et al., 2013). Chromatin remodeling which involves efficient modification of chromatin structure for the expression of genes, also plays a significant role in epigenetic regulation of gene expression (Jarillo et al., 2009), dedifferentiation, proliferation of cells and during organogenesis (Dean Rider et al., 2003; Grafi et al., 2007) as well as in the hormonal response of plants in the culture (Anzola et al., 2010; Furuta et al. 2011). Epigenetic changes in the *in vitro* cultures that involve DNA methylation are related to gene regulations by silencing of genes mediated by the additional methyl group to 5' position of cytosine (Allis, 2007; Fu et al., 2013; Smith and Meissner, 2013). The methylation of DNA is necessary for plant embryogenesis and its role has been elucidated in the *in vitro* plant morphogenesis, including induction and development of somatic embryos, zygotic embryos development and somaclonal variation (Lo Schiavo et al., 1989; Chakrabarty et al., 2003; Viejo et al., 2010). Methylation of DNA and somaclonal variation may occur at a low level in young explants or increase in juvenility of the cultured explant (Pierik et al., 1987; Fraga et al., 2002; Baurens, 2004; Valledor et al., 2007; Monteuuis, 2008; Wang et al., 2012) and could be related to duration of the *in vitro* culture of a plant and culture media composition (Pierik, 1987; Lo Schiavo et al., 1989), external factors (Pospisilova et al., 1999; Hao and Deng, 2003; De-la-Pena et al., 2012) or differentiation associated regeneration (Vining et al., 2013). The methylation of DNA increases with culture age and its decline is accompanied by loss of totipotency and potential for regeneration, possibly due to the accumulation of mutated cells (Lopez et al., 2010). Small RNAs that include miRNAs and trans-acting small RNAs are increasingly emerging as important regulators of epigenetics in plants through their roles in mediating post-translational gene silencing involving degradation of near-perfect complementary mRNA and cleavage or translational repression, thus assisting in silencing transcription factor genes, transposons, repetitive elements and imparting stability to the genome of plants (Almeida and Allshire, 2005). The miRNAs play an important role in plant defense and stress responses, hormonal signaling, seed germination, flowering and development and their importance during *in vitro* culture of plants has recently been unraveled (Miguel and Marum, 2011; Rodriguez-Enriquez et al., 2011 and references therein). Understanding of epigenetics of these processes and their relationship with *in vitro* morphogenesis as it relates to the occurrence of somaclonal variation are areas of research interest and cellular divisions that ensure tissue proliferation and morphogenesis through flexible coordinated epigenetic changes in the genome of cultured cells are regarded factors causing somaclonal variation in cultures (Rani and Raina, 2000; Causevic et al., 2006; Sivanesan, 2007).

Somaclonal variation can be detected using a wide range of techniques and the choice of the method depends on the task; techniques of morphological assessment of clones, cytological features of chromosomes that include their number and structure, physiological and biochemical traits, molecular tools involving the use of various molecular markers, each with advantages and drawbacks, have proved of application in detecting the variation. However, molecular techniques that use nucleic acid to detect somaclonal variation in the cultures and at the juvenile stage are regarded the best approach while morphological and physiological methods that require plant to be at the adult stage seems of less application and for a particular species a protocol for detection of the variation needs to be optimized (Bairu et al., 2011b). Molecular techniques when integrated with the modern and advanced technique of flow cytometry, fluorescence *in situ* hybridization and other live imaging techniques will prove of high success in detecting variation in cultures. For instance, in *Arabidopsis*, using flow cytometry, diploids and tetraploids were detected among regenerated plants from 2-week-old calli and chromosomal count revealed mixoploidy with high frequency aneuploidy in the calli while diploid and tetraploid were identified from 6-week-old calli. Regenerants from the 2-week-old calli were infertile with altered morphology and application of the second technique revealed some structural chromosomal translocations, deletions and duplications (Orzechowska et al., 2013).

Despite the accumulated knowledge of causes of somaclonal variation and epigenetic regulation in cultures, it remains one of the neglected aspects of designing micropropagation protocols at academic and commercial scale. While variations may pre-exist in the mother plant for which cultures are established, testing the genetic fidelity of the explant

is of paramount importance before usage to establish cultures. The variations may not be detected in the explant but express in the regenerate; therefore, it is important to identify an extremely sensitive variations testing technique in designing tissue culture protocols. One aspect of somaclonal variation that continues to frustrate tissue culturists are the harmful epigenetic variations transmitted to somaclones, their long-lasting nature and transmission during sexual propagation, even though the mother plants do not exhibit such variations. Similarly, how variations observed among *in vitro* clones arise, and the genetic and epigenetic modulators/regulators continue to pose a challenge to tissue culturists. The development in microscopy and live imaging techniques, genome sequencing of many species and its availability in databases, gene sequence amplification and spectroscopic techniques, the genomic tools as molecular markers and spectroscopic techniques open avenues for the study of somaclonal variation at anatomical, physiological and genomic levels in the *in vitro* propagated plants.

OTHER ANOMALIES

Other *in vitro* culture-induced anomalies that impose anatomical, physiological and metabolic disorders to cultured plants include, among others, tissue proliferation, shoot tip necrosis, habituation. A brief information is presented here:

Habituation is the development of autonomy on the need for growth factors by *in vitro* cultures leading to reduced productivity and vigor of the cultivated plant (Christou, 1988). Although it rarely occurs, when shown by cultures, in most cases it is due to prolonged culture duration and the most common is cytokinin habituation that results from over-expression of signaling components and receptors accompanied by degradation of signaling compounds (Pischke et al., 2006; Akin-Idowu et al., 2009). Strategies to control occurrence of habituated cultures that involve studies on physiochemical modulators, culture media and duration hold great potentials in understanding the causes and effects of habituation in cultures.

Another *in vitro* culture-induced physiological anomaly is shoot tip necrosis which may occur due to, *inter alia*, medium nutrients deficiency, type of culture media, composition and gelling agent used, imbalanced PGRs amended in the culture media, duration of subculture, pH and aeration (Barghchi and Anderson, 1996; Bairu et al., 2009). The symptoms of shoot tip necrosis may, among others, include browning of the shoot tip accompanied by basipetal necrosis, senescence with the co-commitant death of apical bud (McCown and Sellmer, 1987; Srivastava and Joshi, 2013). Medium nutrients such as calcium ions, that play many indirect physiological roles, coupled with the culture vessel conditions may determine the occurrence of the anomaly with a cultured plant; a low level of calcium ion in the culture media, high humidity and low transpiration rate in a close culture tube may cause necrotic shoots (Singha et al., 1990; Abousalim and Mantell, 1994) but increased calcium ions along with low temperature and enhanced ventilation can overcome the problem (McCown and Sellma, 1987). Similarly, increased boron concentration, to some degree, reduced the occurrence of shoot tip necrosis in cultures without symptoms of boron toxicity effects to the shoots (Abousalim and Mantell, 1994; Anirudha and Kanwar, 2008).

FROM CULTURE VESSEL TO FIELD OR GREENHOUSE CONDITIONS

Flexibility in plant metabolism enables its response to changing environment from *in vitro* to *ex vitro* through physiological change needed to survive in the conditions, mediated by changes in anatomical, physiological and molecular/metabolic processes. The events involve sensing environmental changes by plasma membranes, transduction of information from the membrane to metabolism involving secondary messengers and phytohormones, integration of carbon balance to accommodate response in plants and along the line some genes got strongly expressed while others were repressed (Pospisilova et al., 1999; Us-Camas et al., 2014).

The success of *in vitro* culture depends on physiological and anatomical change plantlets can make and the transition from *in vitro* to the *ex vitro* conditions. The procedure used to achieve higher survival, growth and establishment of plants is of paramount importance but a greater role is played by physio-anatomical features of plantlets (Sahay and Verma, 2000; Hazarika, 2006). A strategy for acclimatization should be suitable if it addresses gradual changes that include environment, culture-induced phenotype, photosynthetic competence or water relations needed during acclimatization and provides optimal survival, growth and establishment of plants over weaning stages towards ambient relative humidity and light levels (Wardle et al., 1983; Sudha et al., 2000).

Control of physical environmental conditions and culture medium are series of strategies during pre-acclimatization of plantlets that determine growth, development and proper morphological changes to cope with the acclimatization (Wardle et al., 1983; Kozai et al., 1987, 1991). Because regenerated plants are delicate due to high humidity in culture vessel, low light intensity and hetero- or mixotrophic nutrition, poor protective features of waxy cuticles, stomatal physiology and poor photo-

synthetic apparatus development, they become vulnerable to physiological disturbance when exposed to *ex vitro* environment during acclimatization (Pospisilova et al., 1999; Khan et al., 2003; Mathur et al., 2008). Certainly, understanding of the aspects will prove helpful in developing an effective protocol for transplantation with the high survival of plantlets in the field conditions. The leaves with low chlorophyll content and photosynthetic rate impede the growth of plants when exposed to lower relative humidity during the transfer to *ex vitro* conditions. Therefore, the process of acclimatization has to be gradual and accommodating to many changes in leaves, especially the shape and distribution of epidermal cells, increased thickness and differentiation of mesophyll tissues, chloroplast structure and number that may occur for a plant to survive in the *ex vitro* conditions (Wardle et al., 1983; Selvapandiyan et al., 1988; Pospisilova et al., 1999; Lavanya et al., 2009). The physiological and metabolic controlling systems in leaves can change as a function of the leaf development in the new environment through control of 'source strength' and carbon assimilation to ensure allocation to different parts of the plant (Dale, 1988; Chaves, 1994).

During the early stage of acclimatization, transpiration rate becomes higher due to poor stomatal control and cuticular water loss with resultant wilt, necrosis of leaves, senescence and possible death of leaves, plantlets or low survival (Brainerd and Fuchigami, 1982; Grout and Millan, 1985; Lee et al., 1988; Preece and Sutter, 1991; Diaz-Perez et al., 1995a, b; Machado et al., 2014). Water imbalance created by *ex vitro* conditions resulting from lack of stomatal control of transpiration can also cause a deficit in leaves with a negative consequence on carbon uptake, growth and autotrophic development of the plant (Lee et al., 1988; Preece and Sutter, 1991). However, the nature and extent of the effects depend on intensity, duration and genetic capacity for acclimatization of a plant (Chavez, 1991, 1994). The volume of guard cells regulates the size of stomata that opens/closes and their shape may be variable from rounded, kidney-shaped, crescent, normal elliptical to sunken, depending on turgor in the cells. Failure of the stomata to close could be due to the abnormal cell wall formation or abnormal function of protoplast in influx/efflux of K^+ and Ca^+ (Zeiger, 1983; Sallonon et al., 1991).

Changes in cuticular wax deposit, increased stomatal density, ion exchange in guard cells of stomata during opening and closure, increased mesophyll layers and cellular organelles can help plantlets cope with the challenging *ex vitro* conditions during acclimatization (Grout and Aston, 1978b; Wetzstein and Sommer, 1982; Sallanon et al., 1991; Gilly et al., 1997; Pospisilova et al., 1998). The features can develop through gradual acclimatization beginning while plantlets are still in a culture vessel and may involve uncapping several days before removal of plants followed by post removal treatments, then transfer to the greenhouse (Selvapandiyan et al., 1988; Preece and Sutter, 1991; Hazarika, 2006). The stomata may lack the ability to close when plantlets are first removed from the culture vessel, but with acclimatization, they gain the physiological feature (Brainerd and Fuchigami, 1982). Because plantlets are exposed to low light levels *in vitro*, they develop thin leaves resembling shade leaves and when placed under high light levels, they become chlorotic and scorched. Therefore, transfer to the higher illumination of the *ex vitro* conditions also has to be gradual through gradual reduction of sucrose concentration in the culture media, humidity and increased light levels (Fig. 1a) to enable plantlets to survive (Wardle et al., 1983; Selvapandiyan et al., 1988; Bhatt and Dhar, 2000). Techniques of shading can reduce transpiration due to excessive light that can destroy chlorophyll (Griffis et al., 1983; Preece and Sutter, 1991).

Some of the leaves developed by plantlets in the *in vitro* conditions may persist and increase in size through cellular elongation after plantlets are transferred to *ex vitro*, depending on species and stress condition (Grout and Millam, 1985; Fabbri et al., 1986; Hazarika, 2006) but their photosynthetic ability varies in cultured species (Grout and Aston, 1978b; Grout and Millam, 1985). The number and morphology of leaves formed *ex vitro* depend on the species age of the transplant and culture environment. Physiological, morphological and anatomical features may also vary within the cultural and ambient environment but retention of organs developed in the *in vitro* conditions influences physiological status of the plant (Donnelly and Vidaver, 1984; Donnelly et al., 1985; Mathur et al., 2008).

CONCLUSIONS AND PROSPECTS

Plant tissue culture techniques have gained wide applications in clonal propagation of plants and also bridged the gap in developing transgenics following gene introduction into a cell, exploiting the inherent totipotent nature of plant cells. Anomalies induced by culture conditions, fragile anatomy and physiology of the regenerate still hinder the commercial application of the technique while developing protocols for clonal propagation of many plants species. Understanding of the features of culture-induced anomalies in the *in vitro* and changes or adjustments plantlets undergo during acclimatization could help in designing culture vessels that favor fewer chances of developing anomalies to plantlets during the *in vitro* culture with enhanced features

for survival during acclimatization. Although many candidate genes and proteins playing a key role in the *in vitro* morphogenesis of plants have been elucidated, to a greater extent, based on phenotypic expression of transgenic plants, molecular processes involved that include regulators of signaling pathways with relation to developmental switch and controlling factors remain elusive to tissue culturists. However, constitutive expression of one gene may be associated with extraordinary influence on others, leading to normal or abnormal growth and development of plants under the *in vitro* culture conditions and during *ex vitro* transfer. A culture vessel with enhanced ventilation and illumination that improves *in vitro* performance with increased stomatal density and functional characteristics (Saez et al., 2012b) might be of application in the large-scale propagation of medicinal and horticultural plants that show severe anomalies when propagated in conventional culture vessels. The use of artificial light and photoperiodic duration to grow plants under tissue culture conditions possesses significant anatomical, physiological and morphogenetic effect on the *in vitro* response of the plantlets (Konow and Wang, 2001; Hsu and Chen, 2010; Moyo et al., 2014) but less attention is given to the impact in the reported protocols for clonal propagation in the modern days. The standard treatments of day/light periodic conditions are always reported, in most cases without strict adherence to the daily schedule or optimal use and assessment of the impact of the developed protocols for clonal propagation.

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