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Morphophysiologicals Problems in Acclimatization of Micropropagated Plants in -*Ex Vitro* Conditions- A Reviews

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Abstract

Plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. Micropropagation allows rapid production of high quality, disease-free and uniform planting material. The micropropagation of high quality planting materials of ornamentals, and forest and fruit trees has created new opportunities in global trading for producers, farmers, and nursery owners, and for rural employment. A substantial number of micropropagated plants do not survive transfer from *in vitro* conditions to greenhouse or field environments. The greenhouse and field have substantially lower relative humidity, higher light level and septic environment that are stressful to micro-propagated plants compared to *in vitro* conditions. It is possible to acclimatize plantlets during *in vitro* as well as *in vivo* by various methods and thereby facilitating the successful transfer of *in vitro* cultured plantlets to soil.

Keywords: Cuticle, *Ex vitro* acclimatization, Humidity, Micropropagated plants, Stomata.

INTRODUCTION

A major limitation in large scale application of micropropagation technology is high mortality experienced by *in vitro* raised plants during laboratory to land transfer. Micropropagated plants on being transferred to *ex vitro* conditions are exposed to (altered temperature, light intensity and water stress) conditions so need acclimatization for successful establishment and survival of plantlets (Chandra *et al*., 2010). Following the survey, the candidate realized that understanding the factors which control successful acclimatization of tissue cultured plants needs to be taken up and it is important to recognize and understand the differences between an *in vitro* and greenhouse or field environment. Realizing the importance of acclimatization for micropropagated plants, the candidate has made an attempt to gather available information on morphology and anatomy of *in vitro* and *ex vitro* or field grown plants. A thorough study of the available literature on *in vitro* hardening and *ex vitro* acclimatization of tissue culturally raised plants has been undertaken to know the factors affecting acclimatization and the morphological and anatomical changes that occur in the tissue cultured raised plants during acclimatization.

Ex vitro acclimatization

Hardening is a time-consuming and labour – intensive process contributing to major portion of the production cost. The successful *ex vitro* acclimatization of micropropagated plants determines the quality of the end product and, in commercial production, the economic viability of the enterprise (Conner and Thomas, 1982).

When shoots or plantlets are transplanted from culture room to greenhouse conditions they may desiccate or wilt rapidly and can die as a result of the change in environment, unless substantial precautions are taken to accommodate them. In commercial micropropagation, this step is often the limiting factor (Poole and Conover, 1983) and at best, is challenging, labour intensive and costly (Debergh, 1988).

Traditionally, the acclimatization environment *ex vitro* is adjusted to accommodate transplants from culture, gradually weaning them towards ambient relative humidity and light levels. Transplants must undergo a period of acclimatization, more specifically, a period of transitional development in which both anatomical characteristics and physiological performance escape the influence of the *in vitro* conditions (Donnelly *et al.*, 1984).

Tissue cultured plants are susceptible to transplantation shocks leading to high mortality during the final stage of micropropagation (Dhawan and Bhojwani, 1986). This is because the growth conditions inside the culture vials induce abnormal morphology and physiology of the plants (Sutter, 1984). Understanding these abnormalities is a prerequisite to develop efficient transplantation protocols. After *ex vitro* transfer, these plantlets might easily be impaired by sudden changes in environmental conditions and so need a period of acclimatization to correct the abnormalities (Pospisilova *et al.*, 1999). Therefore, after *ex vitro* transplantation plants usually need some period of acclimatization with gradual lowering in air humidity (Bolar *et al.*, 1998). Acclimatization units have been developed with temperature, humidity, irradiance, CO_2 concentration and air flow rate controlled by computer (Table1) (Hayashi *et al*., 1988).

Morphology of plantlets raised *in vitro*

Difficulties in successfully transplanting tissue cultured shoots and plantlets to soil are well documented (Timmis and Richie, 1984) as they share certain characteristic features that are inconsistent with development under greenhouse or field conditions. The culture- induced phenotype (CIP) (Donnelly *et al.*, 1987) reflects epigenetic variation (Donnelly and Vidaver, 1984), and acclimatization to environmental conditions which exist within the closed culture containers. *In vitro* environments are characterized by a saturated atmosphere, relatively low light intensity (photosynthetic photon flux), average 12-70 μmol m^{-2} s^{-1}, relatively high and constant temperature (20-28 ^{o}C), low rates of gas exchange between the containers and the external atmosphere and high concentrations of carbohydrate and exogenous growth regulators in the medium. In the greenhouse or field, where the relative

humidity tends to be less than 100%, the ambient light levels are much higher than in culture with fluctuating temperatures and the substrate has a much higher water potential so it is necessary to convert rapidly from a mixotrophic to a fully autotrophic mode of nutrition to survive.

Ex vitro plantlets have extreme evapotranspiration rates and may guttate copiously, demonstrating impaired ability to regulate water loss. Excessive evapotranspiration is affected by reduced or nonexistent stomatal control (Wardle and Short, 1983) and large cuticular water losses (Marin and Gila, 1988) possibly due to poor epicuticular and cuticular wax formation (Sutter, 1988) or reduced trichome numbers (Sutter, 1988). No correlations have been established between *ex vitro* survival and the physical or morphological characteristics of foliar wax (Sutter, 1988). The *ex vitro* guttation rate may be affected by the large increase in substrate water potential at transplantation stage and tends to increase under conditions that augments the transpiration rate (Donnely and Skelton, 1989).

To promote *ex vitro* survival and physiological competence, especially to guard against water stress and encourage autotrophy, a transitional environment is usually supplied for an acclimatization interval, ranging in duration from one to several weeks (Grout and Millam, 1985). In this transitional environment, the relative humidity is kept in the range of 70-100% via tenting, misting or fogging and the light level should not be much greater than it was in culture.

Cuticle of *ex vitro* plants

The cuticle is a superficial non-cellular layer secreted by epidermis and composed of cutin matrix together with embedded and surface waxes that covers above ground tissues of plants. The primary function of the cuticle is to limit transpirational water loss. Water permeability through the cuticle is influenced primarily by the structure and amount of cuticular and epicuticular waxes (Martin and Juniper, 1970). Scanty deposition of protective epicuticular wax on the surface of the leaves is the most important factor responsible for excessive loss of water, leading to poor transplantation success (Hazarika *et al*., 2000).

Measurement of the amount of epicuticular wax on leaves of cultured cabbage plant revealed that the lack of crystalline structure was correlated with significantly less epicuticular wax compared to that on greenhouse grown plants (Sutter and Langhans, 1982). They also reported epicuticular wax on cauliflower and cabbage leaves *in vitro* which was only 25% that of plant grown in the greenhouse. The relationship between the amount of wax formed *in vitro* and that formed on greenhouse grown plants was not consistent or predictable in foliage plants with naturally glossy surfaces (Sutter, 1985). It was concluded that SEM alone was not a reliable method for determination of amount of wax on leaf surfaces but that gravimetric measurements were necessary as well. Consequently, conclusions about the amount of epicuticular wax were inferred from SEM micrographs without gravimetric corroboration may be suspected.

The chemical nature of wax deposited on the surface of the leaves under *in vitro* conditions is also known to differ from that formed under natural conditions allowing excessive diffusion of water from *in vitro* formed leaves (Sutter, 1984). Epicuticular wax on leaves of cultured cabbage plants differed chemically from that on greenhouse grown plants (Sutter, 1984). Wax on leaves formed *in vitro* had a higher proportion of esters and polar compounds and are less hydrophobic and afford greater water permeability than long chain hydrocarbons, it was probable that the chemical composition of the wax also contributed to water loss. Leaves of cabbage plants grown *in vitro* never became as glaucous as new leaves developed increasingly greater amount of wax and more complex crystalline structure over time (Sutter and Langhans, 1982). The reduced amount of epicuticular wax was directly correlated with substantially increased water loss in cultured shoots. Transpiration rates were significantly higher in leaves of cultured plants lacking epicuticular wax compared with those in greenhouse grown plants (Wardle *et al*., 1983a). Increased water loss directly related to the presence of epicuticular wax was shown when greenhouse grown cabbage leaves lost significantly more water after epicuticular wax was removed by chloroform (Sutter and Langhans, 1982). Sutter (1985), however, noted that the quality of epicuticular wax was not a good predictor of survival of micropropagated plantlets in the greenhouse during acclimatization.

The SEM studies by Dhawan and Bhojwani (1987) in *Leucaena leucocephala* revealed a definite increase in the amount of epicuticular wax deposited on the leaves following the transfer of plants out of culture. They reported that the micropropagated plants attained wax density comparable to that of field grown plants, within 6-7 weeks of transplantation. These observations corroborate well with data on the rate of water loss from leaves at different stages of micropropagation and hardening. The decline in the rate of water loss coincided with increase in the amount of wax deposited on the leaves. However, the efficient water economy of the naturally grown plants could not be matched by the transplanted plants even after 5 months. This may perhaps be due to the difference in the chemical nature of wax deposited, with hydrophobic wax predominantly found in the plant growing in the field. However, this hypothetical assertion needs to be confirmed by proper chemical analysis. Differences in the rate of water loss by leaves at different stages of micropropagation have been also reported in *Malus domestica* (Brainerd and Fuchigami, 1981), *Prunus insititia* (Brainerd *et al.*, 1981), *Brassica oleracea* (Sutter and Langhans, 1982) and *Solanum laciniatum* (Conner and Conner, 1984).

Stomatal functioning of *ex vitro* leaves

Stomatal structure and impaired stomatal functioning have been considered as factors contributing to excessive loss of water by cultured plants. Scanning electron microscopic (SEM) studies indicated that stomatal structure in some species of cultured plants differed markedly from that in greenhouse or field grown plants. *In vitro* raised plants showed leaves with stomata that had raised, rounded guard cells compared to normal elliptical, sunken guard cells in a variety of species including sweet gum (Wetzstein and Sommer, 1983), apple (Blanke and Belecher, 1989) and rose (Capellades *et al.,* 1990). Scanning electron microscopic (SEM) studies in citrus leaves (Hazarika *et al*., 2002) indicated that stomata with kidney shaped guard cells were observed in greenhouse leaves while crescent shaped and rounded guard cells were observed in *in vitro* leaves.

When expressed as an index per number of epidermal cells, there was no significant difference in stomatal frequency among *in vitro*, acclimatized and greenhouse grown plants (Conner and Conner, 1984). But Zaid and Hughes (1995a) reported that the stomatal frequency of greenhouse grown leaves of date palm was significantly higher than control plantlets. However, polyethylene glycol (PEG) treatment did not increase the number of stomata of *in vitro* plantlets of date palm. In *Liquidamber styraciflua, Vaccinium corymbasum* and *Nicotiana tabacam*, stomatal density decreased after transplantation (Drew *et al.*, 1992). After a short period of acclimatization, stomatal density on adaxial and abaxial leaf epidermis of *N. tabacum* plants had not changed, but later the total number of stomata per leaf was more than double in *ex vitro* plants due to enormous leaf area growth after being transferred to *ex vitro* conditions (Pospíšilovă *et al*., 1999). On the other hand, in *Prunus serotina* and *Rhododendron*, stomatal density increased and stomatal pore length decreased after transplantation (Drew *et al.*, 1992). Leaves from *in vitro* grown *Prunus cerasus, Vaccinium corymbosum* or *N. tabacum* plantlets had ring shaped stomata, but in leaves of *ex vitro* transferred plants, stomata were elliptical (Drew *et al*., 1992). Guard cells of *in vitro* grown *Rosa hybrid* plantlets contained numerous ribosomes and mitochondria, starch rich plastids and relatively large vacuoles indicating that they may exhibit metabolic activity similar to normal guard cells (Drew *et al*., 1992).

It has been reported that stomata of cultured plants show a characteristic inability to close when first removed from culture. Stomata on excised leaves of micropropagated apple plants that had been acclimatized responded as expected by closing immediately when treated with ABA (Brainered and Fuchigami, 1982). Stomata on epidermal strips of *Chrysanthemum* also similarly closed when incubated in the presence of ABA (Wardle and Short, 1983). Similarly, *in vitro* cultured sweet gum leaves did not respond to the application of ABA, whereas stomata on leaves of greenhouse grown and acclimatized plants closed in response to the treatment (Wetzstein and Sommer, 1983). Marin *et al.,* (1988) reported that up to 80% of stomata of excised leaves of *P. cerasus* closed when removed from culture and placed in 45% relative humidity. Histological study in which the cellulose, pectin, callose, cutin and birefrin-

gence patterns produced by cellulose micro fibrils in epidermal cells was studied, however, were able to revert to a functional state to some degree relatively rapidly after removal from culture, but up to 78% of stomata of cherry and sweet gum plants closed after 1 h of exposure to ambient conditions on a laboratory bench (Sutter, 1988). Sutter and Langhans (1982) stated that stomata on excised leaves of cultured cabbage plants closed after the leaves were allowed to wilt for 5 min. Shackel *et al.,* (1990) reported that stomata of intact apple shoots do have the ability to close in an atmosphere of 90% relative humidity. This steady rate, which got maintained for as long as 3 days, was indicative of a low rate of cuticular transpiration combined with transpiration from any open stomata. The size of stomatal aperture is controlled by relative volume of the guard cells depending on the turgor. Guard cell turgor in turn depends on vascular osmotic and water potential. The failure of stomata to close could be due to either abnormal cell wall properties or improper protoplast function (Zeiger, 1983). In carnation, the failure of the guard cells to contract in hypertonic solutions resulted from defects in the cell wall and correlated with abnormal orientation of cellulose microfibrils (Ziv *et al.*, 1987). However, dark treatment did not induce stomatal closure and vacuolar volumes remained unchanged. K+ content in guard cells did not vary significantly and a very low concentration of Ca^{++} was found. However, after *ex vitro* acclimatization, stomatal sensitivity to the dark was developed. Simultaneously, the light induced opening of stomata and K^+ influx into guard cells were observed and calcium amount was ten times higher than in the guard cells of *in vitro* grown plantlets (Ziv *et al.*, 1987).

Anatomy of plantlets raised *in vitro*

The poor mesophyll differentiation and weak vasculature of the leaves formed *in vitro* render the plants highly susceptible to transplantation shock. Leaves of plants grown *in vitro* were thinner and had a characteristically poorly developed palisade layer with significant amount of mesophyll air space compared to greenhouse - grown plants. Both micropropagated cauliflower (Grout and Aston, 1978) and sweet gum (Wetzstein and Sommer, 1982) plantlets failed to develop a clearly defined palisade layer *in vitro*. Leaves on micropropagated plum shoot had only one layer of palisade cells rather than usual two to three layers and greater air space in mesophyll tissue compared with leaves on greenhouse or field- grown plants (Brainerd *et al*., 1981). Such dissimilarities in leaf anatomy of *in vivo* and *in vitro* grown plants were also observed in *Liquidamber styraciflua* (Wetzstein and Sommer, 1982) and *Rubus idaeus* (Donnelly and Vidaver, 1984). Roots of *in vitro* plantlets were slender, covered with root hairs and had much less periderm than the field grown red raspberries (Donnelly *et al.*, 1985). The connection between roots and shoots was shown to be incomplete in cauliflower plants resulting in insufficient water transfer between the roots and shoots (Grout and Aston, 1977). However, xylem appeared continuous and was functional between the roots and shoot of *in vitro* rooted *Prunus* sp. (Marin *et al.*, 1988).

The changes in leaf anatomy that occurred during acclimatization were noted most in leaves that developed after the plants were removed from culture. Persistent leaves of strawberry plants became thicker due to enlargement of the palisade cells (Fabbri *et al*., 1986), but there was no change in number of layers of palisade cells or in amount of mesophyll air space. During acclimatization, leaves present at primordial stage *in vitro* assumed intermediate characteristics between leaves grown *in vitro* and greenhouse or field leaves. Only new leaves that formed completely after removal from culture resembled greenhouse grown leaves (Donnelly *et al*., 1985). Hydathodes on leaves of micropropagated blackberry (Donnelly *et al.*, 1987) and strawberry (Donnelly and Skelton, 1987) were open, whereas those on leaves of greenhouse grown plants were closed or had smaller apertures. Since the hydathodes appeared to be open and exhibited guttation, it was hypothesized that they might contribute to adaxial water loss (Donnelly *et al.*, 1985).

Photosynthetic parameters and light intensity

In vitro plantlets are generally grown under low light intensity (1,200-3,000 lux) and temperature (25±2 ^{o}C), hence direct transfer to broad spectrum sunlight (4,000-12,000 lux) and temperature (26-36 ^{o}C) might cause charring of leaves and wilting of plantlets (Chandra *et al.*, 2010).

It is therefore, necessary to accustom the plant in the natural conditions by a process of hardening or acclimatization (Lavanya *et al.*, 2009). The culture containers could be kept in the greenhouse with loose lids. Micropropagated plantlets can be left in shade for 3-6 days under diffused natural light to make them adjust to the conditions of new environment. This helps in semi-hardening of plants and leads to shoot elongation (Lavanya *et al*., 2009). Transfer of microshoots from *in vitro* to *ex vitro* conditions under direct sunlight might cause photoinhibition and chlorophyll (Chl) photobleaching. The exposure of *Calathea louisae* and *Spathiphyllum floribundum* plantlets to high irradiance immediately after transplantation caused photoinhibition and even Chl photobleaching (Van Huylenbroeck *et al.*, 1995a). No photoinhibition was found during growth in the greenhouse when *Nicotiana tabacum* plantlets were acclimatized in two phases, first in the greenhouse (low irradiance of 30-90 µmol m^{-2} s^{-1}) and then in the open air (200-1,400 µmol m^{-2} s^{-1}) (Pospíšilová *et al*., 1999). *In vitro* to *ex vitro* transfer might lead to a transient decrease in photosynthetic parameters. Net photosynthetic rate in *Solanum tuberosum* and *Spathiphyllum floribundum* plants decreased in the first week after transplantation and increased thereafter (Pospíšilová *et al.,* 1999). High sucrose and salt containing media, low light level and the carbon dioxide concentration in culture vessel are some of the important limiting factors among various physical micro environmental factors which influence photosynthesis of *in vitro* cultured plants (Hazarika, 2004). For *in vitro* growth, a continuous supply of exogenous sucrose is required (2-3%) as a carbon source (Hazarika, 2004). This is because of the exogenous supply of sucrose, which does not necessitate the normal development of photosynthetic apparatus. Therefore, *in vitro* cultured plants are either poor in chlorophyll content or the enzymes responsible for photosynthesis i.e. ribulose biphoshate carboxylase (RubPcase) are inactive or absent altogether the low RubPcase activity may be due to the presence of sucrose during the development of leaves (Donnelly and Vidaver, 1984).

Humidity

Short *et al*., (1987) reported that optimum growth of cultured cauliflower and *Chrysanthemum* occurred when plantlets were cultured with 80% relative humidity. Leaves of *Chrysanthemum* and sugarbeet which were initiated and developed at relative humidity below 100%, displayed increased epicuticular wax, improved stomatal functioning and reduced leaf dehydration (Ritchie *et al.*, 1991).

During acclimatization to *ex vitro* conditions, cuticular transpiration rates gradually decrease because stomatal regulation of water loss becomes more effective and cuticle and epicuticular wax develop (Fila *et al.*, 1998). Stomatal and cuticular transpiration similar to those in seedling were achieved between 8 and 12 weeks after transplanting and on the other hand, low stomatal conductance (g_s) observed in *Malus pumila* plantlets increased after transfer to *ex vitro* conditions (Diaz-Perez *et al.*, 1995a).

The old *in vitro* leaves

Leaves that developed in culture were retained after transplantation for a week to several months prior to senescing (Grout and Aston, 1984). Persistence depended on the plant species and the degree of environmental stress *ex vitro*. These persistent leaves increased in size slightly, mainly due to cell elongation (Grout and Aston, 1978), and accumulated dry matter under certain conditions (Donnelly and Vidaver, 1984). In some cases, wax was deposited on the leaf surface after transplantation (Ziv, 1986). Stomatal function (open-closure mechanism) was either improved (Conner and Conner, 1984) or was not established in persistent leaves (Wardle and Short, 1983). In most cases, stomatal function has been equated with closure (Wetzstein and Sommer, 1983). However, stomatal closure may only indicate the collapse of the guard cell membranes in response to exposure to low levels of relative humidity (RH) (Wardle and Short, 1983) and need not indicate the stomatal capacity to reopen. The role of the persistent leaves remains a controversial and important issue. Photosynthetic capacity appears to vary with plant species in culture and may determine the *ex vitro* contribution of persistent leaves. In the non-competent group, cultured cauliflower and strawberry leaves that developed in culture deteriorated rapidly after transplantation. Such leaves have been referred to as storage organ or pseudocotyledonary tissues (Wardle et al., 1983a).

Non- competence in strawberry has been attributed to irreversibly reduced levels of RubPcase activity in leaves developed in the presence of sucrose. Strawberry plantlets defoliated in the absence of sucrose in the medium were competent (Grout and Price, 1987). Dieffenbachia (*Dieffenbachia picta*) as well as potato (*Solanum tuberosum*) and Chrysanthemum (*Chrysanthemum morifolium*) (Grout, 1988) were photosynthetically competent *in vitro*. They achieved a positive carbon balance in culture and continued to contribute photosynthetically after transplantation. Leaves of competent species did not deteriorate rapidly after transplantation (Grout and Donkin, 1987). Persistent leaves of Asian white birch (Smith *et al*., 1986) and red raspberry (Donnely and Vidaver, 1984) seem to fall into competent group. Red raspberry plantlets photosynthesized at a low level after transplantation (Donnely and Vidaver, 1984) (Table 2).

The newly developed leaves

The phenotype of new leaves formed *ex vitro* varies with the species, the culture and transplant environments and the age of the transplant. New leaves of cauliflower (a non-competent species) that formed in the second week after transplantation, apparently exhibited greenhouse control leaves of CO_2 uptake (Grout and Aston, 1977). However, new leaves of raspberry (a competent species) were transitional in the sense that weekly flushes of new leaves became progressively larger, eventually with control type anatomy, functional stomata and improved CO_2 uptake capability (Donnelly *et al.*, 1985). The number of transitional leaves produced by a transplant may depend on the number of immature leaf buds formed in culture. The degree of transition of these leaves and how closely they resemble with control plants is probably a reflection of the stage of development of leaf primordia when the plantlets were transferred from culture and the conflicting stresses imposed on leaf developed by both the culture environment and the new ambient environment (Donnelly and Vidaver, 1984). It is likely that the retention of any culture type organ on the transplant influences the physiological status of the rest of the plant (Donnelly *et al.*, 1985).

Biological hardening of tissue cultured plants

Another major cause of high mortality of plantlets is their sudden exposure (particularly the root system) to microbial communities present in the soil as they do not possess sufficient resistance against the soil microflora (Hao *et al.*, 2010). In the last few years, trials have been done to expose the young *in vitro* raised plantlets to useful microorganisms that promote growth and encourage mutual association (Hao *et al.*, 2010). Endophyte refers to the fungi and bacteria which invade or live inside the tissues of plants without causing any disease or injury to them. They also promote growth of the host plant and the formation of secondary metabolites related to plant defense (Hao *et al.,* 2010). Biohardening is an emerging dimension of micropropagation techniques (Srivastava *et al*., 2002). *In vitro* co-culture of plant tissue explants with bacteria and vesicular arbuscular mycorrhizae (VAM) induces developmental and metabolic changes in the derived plantlets which enhance their tolerance to abiotic and biotic stresses. The induced resistance response caused by the inoculants is referred to as ‘‘biotization’’. (Srivastava *et al.*, 2002). Hao *et al.,* (2010) reported that treatment of suspension cells of *Ginkgo biloba* with fungal endophytes resulted in accumulation of flavonoids, increased ABA production and activation of phenylalanine ammonia-lyase. Bacteria associated with roots and rhizosphere of many plant species are also known as plant growth promoting *Rhizobacteria* (Ramamoorthy *et al.*, 2001). By using rhizosphere bacteria, successful biohardening of tissue cultured raised tea plants have been reported by Pandey *et al.,* (2000). The bacterial isolates *Bacillus subtilis*, *Bacillus* spp. (associates of established tea rhizosphere), *Pseudomonas corrugata* 1 and *P. corrugata* 2 (associates of established tea rhizosphere), were tested as microbial inoculates for hardening of tissue cultured tea plants raised *in vitro*. The bacterial isolates colonized the soil rapidly and influenced the survival and growth of tea plants especially shoot length and leaf number in most cases Pandey *et al.,* (2000).

Improvement of *ex vitro* acclimatization

The film–forming antitranspirants (Aquawiltless, Clear spray, DC-200, Exhalt 4-10, Folicote, Protec, Vapor Gard and Wiltpruf) were tested for amelioration of wilting of *Chrysanthemum morifolium* and *Dianthus caryophyllus* plantlets transferred *ex vitro* (Pospíšilová *et al.*, 1998) Although DC-200 had the greatest effect in reducing transpiration, it had adverse effects on plant growth. All other antitranspirants were effective in improving vigour of plants. Amaregouda *et al.*, (1994) found that stomatal resistance were more in plants treated with 1,500 ppm of Alar (B-9) phenylmercuric acetate (PMA) (20 ppm), while Alachlor (20 ppm), Sunguard (0.02%), china clay (6% w/v) and silica powder (6% w/v) maintained moderate stomatal resistance compared to control. All other antitranspirants were ineffective in improving vigour of the plants (Pospisilova *et al.,* 1998).

The exogenous ABA can serve as antitranspirant. In addition to depression in stomatal conductance, it can increase root hydraulic conductivity and accumulation of proline. Addition of ABA to the substrate immediately after transplantation alleviated "transplantation shocks" of *Nicotiana tabacum* plants (Pospisilova *et al.,* 1998). Stomatal conductance of leaves which was high during the first few days after transplantation was markedly decreased by ABA application. However in following days, stomatal conductance decreased by ABA-treated plants. After two or three weeks, stomatal conductance of transplanted plant was significantly lower than that of plantlets grown *in vitro* but similar in control and ABA treated plants. ABA treatment had slight positive effect on Chl content and other photosynthetic parameters and enhanced plant growth (Pospisilova *et al*., 1998).

Elevated CO_2 concentration can also serve as antitranspirant. Acclimatization of tobacco plantlets under elevated CO_2 concentration also decreased stomatal conductance and improve plant water status after transplantation (Pospisilova *et al.*, 1999). Elevated CO_2 concentration during *ex vitro* acclimatization promoted more effectively the growth of plants grown *in vitro* under ambient CO_2 concentration than that of plants grown during both growth phases under elevated CO_2 concentration. Elevated CO_2 concentration during acclimatization of tobacco plants markedly increased net photosynthetic rate P_N *in situ*, water use efficiency and growth, stomatal regulation of gas exchange (Pospisilova *et al.*, 1999).

CONCLUSION

Acclimatization has been defined as a process of adaption or an organism to an environmental change (Brainerd and Fuchigami, 1981). This differs from frequently used term "acclimation" which denotes the adaptation of an organism on its own to an environmental change (Conover and Poole,1984), whereas acclimatization implies the human interception in this adjustmental process. Hardening is a time-consuming and labour – intensive process contributing to major portion of the production cost. The successful *ex vitro* acclimatization of micropropagated plants determines the quality of the end product and, in commercial production, the economic viability of the enterprise (Conner and Thomas, 1984). Tissue cultured plants are susceptible to transplantation shocks leading to high mortality during the final stage of micropropagation (Dhawan *et al.*, 1986). This is because the growth conditions inside the culture vials induce abnormal morphology and physiology in the plants (Sutter, 1984). Understanding these abnormalities is a prerequisite to develop efficient transplantation protocols. After *ex vitro* transfer, these plantlets might easily be impaired by sudden changes in environmental conditions and so need a period of acclimatization to correct to abnormalities.

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Tables

Table1. Anatomical and Histological difference between roots and shoots and leaves of plants *in vitro* and after transplanting *ex vitro*

S.N.	Plant organ and Tissue	*In vitro*	*Ex vitro*	Species Studied
1.	Roots	Thin	Large root system	**Root and stem**
2.	Epidermis	Uniseriate	Uniseriate and multiseriate	*Acer rubrum L.*
3.	Periderm	Limited	Multi-layered	*Amelanchier lamarckii*
4.	Cortex	Broad, irregular enlarged, hypertrophied individual cells, numerous intercellular spaces; loose arrangement of cortical parenchyma; frequently contain plastids with chlorophyll and other pigments, starch grains	Uniform, compact cortical cell arrangement	*Betula nigra L.*
5.	Vascular bundles	Narrow, Primary, immature, development, limited secondary cambium activity	Cambium activity consistent	*Solanum melongena L.*
6.	Xylem and Phloem	Poorly developed; multiple scatterd xylem bundles with variable stele patterns, no or limited secondary xylem growth; phloem frequently contain plastids with chlorophyll and other pigments, starch grains.	Secondary xylem formation arrangement in diarch, triarch or pentarch stele patterns in different species.	*Prunus kurilensis*
7.	Root hairs	Few or no, thick, short and straight, fine delicate appearance, many fused together, usually abundant	Long, thin, slender fibrous, wiry and formed an interwoven mat	
8.	Stems	Small diameter	Large diameter	
9.	Epidermis	Limited development	Fully developed	
10.	Cortex	Limited development, little collenchymas, few sclerenchyma fibers; starch grains in old stem	Fully developed with continuous cylinder of collenchyma	
11.	Vascular bundles	Poorly developed; lower activity of cambium or not initiated large no of starch grains in the vascular bundles at the early stages	Fully developed	
12.	Xylem and Phloem	Roots frequently initiated in densly cytoplasmic phloem parenchyma; more xylem and phloem vessels in the parenchymatus cells of the xylem and phloem at early stages	Thick layers of sclerenchyma in the phloem of the stele	
13.	Pith	Limited development, thin cell walls; starch grains	Fully developed, having both thin and thick cell walls	
14.	Callus	Frequently accompanies root formation	Usually not produced	

Table 2. Anatomical and histological differences between the leaves of plants *in vitro* and after transplanting *ex vitro*

S.N.	Plant organ and Tissue	*In vitro*	*Ex vitro*	Species Studied
1.	Leaves	Small, succulent, brittle and hyperhydrated	Normal size and shape	**Leaves**
2.	Epidermis	Deformed thin cell walls irregular shaped	Normal cell wall	*Actinidia delicisa*
3.	Cuticle	thin and discontinuous	Thick and continuous	*Cucumis melo*
4.	Stomata	irregular guard cells with thin cell walls large stomata	Normal guard cells	*Cydonia oblong* *Prunus avium*
5.	Mesophyll	Irregular structure mainly spongy parenchyma	Regular structure	*Pyrus malus*
6.	Palisade	Reduced palisade tissue	Normal palisade	*Salix babylonica* *Picea sitchensis*
7.	Spongy Parenchyma	Highly vacuolated with large intercellular air spaces	Normal cells with regular air-spaces	*Gerbera jamesonii*
8.	Vascular bundles	Immature Secondary phloem	Fully developed Normally oriented sieve tube	*Olea europaea*
9.	Phloem	Limited secondary phloem	Normally oriented sieve tubes	
10.	Xylem	Reduced lignifications in the xylem vessels and sieve elements	Fully developed xylem vascular tissue	
11.	Chloroplasts	Low chlorophyll, abnormal non-functional chloroplasts, limited formation of grana, abundant stroma, starch grains	Normal chloroplasts with typical granal structure, increased chlorophyll content	