Acclimatization of tissue-cultured plants

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A substantial number of micropropagated plants do not survive transfer from *in vitro* conditions to greenhouse or field environment. The greenhouse and field have substantially lower relative humidity, higher light level and septic environment that are stressful to micropropagated plants compared to *in vitro* conditions. The benefit of any micropropagation system can, however, only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro*. Most species grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil. This article reviews current and developing methods for the acclimatization of micropropagated plantlets.

CONSIDERABLE efforts have been directed to optimize the conditions for *in vitro* stages of micropropagation, but the process of acclimatization of micropropagated plants to the soil environment has not fully been studied. Consequently, the transplantation stage continues to be a major bottleneck in the micropropagation of many plants. Plantlets or shoots that have grown *in vitro* have been continuously exposed to a unique microenvironment that has been selected to provide minimal stress and optimum conditions for plant multiplication. Plantlets were developed within the culture vessels under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity. These contribute a culture-induced phenotype that cannot survive the environmental conditions when directly placed in a greenhouse or field. The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field. Although specific details of acclimatizations may differ, certain generalizations can be noted.

Sugar in the medium

Sucrose is the most common carbon source used in plant cell, tissue and organ culture media. Studies have shown that sucrose may also influence secondary metabolism in cell and organ culture[1,2]. This strategy assumes that larger persistent leaves packed with greater amounts of storage compounds would contribute more after transplantation. Increasing the concentration of sugar in the medium might maximize the nutrient function of persistent leaves[3]. To some extent, this strategy has been discounted as apt to heighten evapotranspiration losses in transplants. However, it seems to hold promise for some plants[4,5]. Wainwright and Scrace[6] found that maximum values for shoot height, fresh and dry weight of *Potentilla fruticosa* and *Ficus lyrata* were obtained *in vivo* when previously conditioned with 2 or 4% sucrose. Plantlet establishment declined when sucrose was not used. Sucrose concentration of 40 g l^{-1} prior to transferring watercress microcutting to *in vivo* conditions was shown to maximize the dry weight of established plantlets[7]. Preconditioning by addition of high concentration of sucrose was reported to influence the *in vivo* rooting and establishment of cuttings[6], but lowering the sucrose concentration in the medium increases the photosynthetic ability, thereby improving plantlet survival in rose plant[8]. Koroch *et al.*[9] reported that preconditioning at different sucrose concentration prior to acclimatization had no effect on plant establishment, but influenced plant quality. Sucrose is essential in the medium for many species. In some cases, independent growth could not be achieved on a medium without sucrose during rooting[10]. Singh and Shymal[11] evaluated different requirements for *in vitro* rooting of hybrid tea rose cv. Sonia and Super-star and found that both the cultivars differed in their demand for sucrose during rhizogenesis. The microshoots of Sonia gave maximum rooting (92.6%) with 40 g l^{-1} sucrose, while for Super-star the best rooting (84.5%) was recorded with sucrose at 25 g l^{-1}. Alleviated sucrose levels favoured rooting and root quality, but a slight declining trend was noted with the highest level (40 g l^{-1}) studied. Like any other morphogenetic process, rooting is an energy-consuming process and hence level of carbon source is desired. The difference in their specific requirement for media might be due to genotypic effect. Root initiation in apple was decreased proportionately with decreasing sucrose level[12] and shoots without sucrose did not survive after transferring to greenhouse[13]. Capellades *et al.*[14] reported that size and number of starch granules increased with level of sucrose in the culture medium. George and Sherrington[15] had drawn the conclusion that for optimal growth and multiplication, 2–4% sucrose was found to be optimum. Hazarika *et al.*[16] reported that *in vitro* preconditioning of citrus microshoots with sucrose concentration of 3% was found optimum for subsequent *ex vitro*

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survival and growth. There was a linear increase of biochemical constituents, viz. reducing sugar, starch, total chlorophyll on addition of sucrose to the medium[17]. High frequency *in vitro* shoot multiplication of *Plumbagu indica* was possible in a medium containing 3% sucrose[18]. Kumar *et al.*[19] obtained 68.8% *ex vitro* survival in kinnow mandarin by *in vitro* regeneration from epicotyl segment of *in vitro*-grown plant using 3% sucrose in the medium. Induction of multiple shoots using shoot tips of gerbera was accomplished on MS medium supplemented with 3% sucrose and other phytohormones and almost 100% survival rate was obtained after transfer[20]. Mehta *et al.*[21] reported that increase in sucrose concentration from 2 to 4% in the medium increases caulogenic response in tamarind plantlets from 34 to 48% in explants. Further increase of sucrose to 6% induced browning of media which was detrimental for growth of the shoots. Misra and Datta[22] reported acclimatization of Asiatic hybrid lilies under stress conditions after propagation through tissue culture. They reported that liquid medium having 9% sucrose and other phytohormones was found suitable for growth of bulblets in the isolated unrooted shoots. Due to high concentration of sucrose, the size of the bulblets increased from less than 0.5 cm in diameter to approximately 1–1.5 cm within 2 months of inoculations. Ticha *et al.*[23] reported that plant growth, dry matter accumulation and total leaf area were higher under photomixotrophic than photoautotrophic conditions. Not only biomass formation, but photosynthesis was also positively affected by exogenous sucrose. Photomixotropic growth of plantlets prevented the occurrence of photoinhibitory symptoms. Moreover, they concluded that the plant response will be species-dependent because reports on gardenia[24] showed that sucrose-feeding increases the susceptibility to photoinhibition. Takayama and Misawa[25] suggested that the effect of sucrose on the bulb growth of *Lilium auratum* could be mediated through changes in osmotic potential. Apparently, high sucrose levels were more stressful for the shoots, which exhibited reduced green leaves and poor development. Sugars were reported to have an osmotic role and also act as a source of energy and carbon in inducing shoot regeneration in tobacco callus[26] and from leaf pieces of *Solanum melongena*[27]. Photoautotrophic cells have well-developed and physiologically active chloroplasts, in contrast to heterotrophic cells.

Photoautotrophic micropropagation

The concept of photoautotrophic micropropagation has recently been proposed as a means of reducing production cost and automation/robotization of the micropropagation process[28]. Labour costs for multiplication, rooting and acclimatization of plantlets are said to account for approximately 60% of the total production cost in conventional micropropagation. Automation or robotization or both at the culture stages are therefore essential for drastic reduction of production cost in the micropropagation industry[29,30]. This strategy assumes that autotropic cultures will have persistent leaves that live longer and would be more photosynthetically productive *ex vitro*[3]. The objective is to modify culture-induced phenotype towards autotrophy in culture. To do this the oxygen concentration can be reduced in the culture environment, which depresses the photorespiration rate[31]. Alternatively, the sugar is reduced or completely eliminated from the medium[28], while the photosynthetic photon flux[28,32] and the carbon dioxide concentration[33] are increased[34]. Increasing the light intensity alone cannot raise the net photosynthetic rate for cultures at their CO_2 compensation point. Such a photoautotropic tissue-culture system has the added advantage that microbial contamination is less of a problem when sugar is eliminated from the medium[35]. Kozai[36] reported that the growth of plantlets *in vitro* is often greater under photoautotropic conditions than under heterotrophic conditions, provided that the *in vitro* environment is properly controlled for promoting photosynthesis. He also reported that the use of plant growth-regulating substances, vitamins and other organic substances can be minimized because some of these will be produced endogeneously in sufficient quantities by plantlets growing photoautotropically. Short *et al.*[37] found that growth by photoautotrophy could be stimulated by culturing meristems of chrysanthemum on sucrose-free medium. Plantlet culture under this regime exhibited comparable rates of photosynthesis to those found in seedlings. These procedures, therefore, can be used to facilitate the successful transfer of tissue culture-derived plants to soil condition and thereby obviate the need for any hardening regime. Dang and Donnelly[38] reported that sucrose in the medium promoted plantlet growth but depressed photosynthesis and reduced *in vitro* hardening. Root hairs were more abundant and longer on rooting of photoautotropic plantlets than mixotropic plantlets. Photoautotropically grown C-3 plants lose up to 50% of photosynthetically fixed carbon due to photorespiration in a normal atmospheric concentration of O_2 (21%) and CO_2 (345 μmol mol^{-1}). However, photorespiration is repressed with decreasing O_2 concentration and is almost completely repressed at 2% O_2 (ref. 36). The photoautotrophic tissue-culture method makes it possible to use a larger culture vessel without risk of increasing the loss of plantlets due to contamination. Use of a large vessel facilitates the automation–robotization and automatic environmental control[36].

Growth retardants

Plant growth retardants generally induce a shortening of the internode of higher plants *in vivo* and have some additional effects such as a reduction in leaf size, intensifica-

Table 1. Classification, chemical derivation and growth retardants

Substances affecting GA biosynthesis
Compounds with heterocyclic ring containing N
Triazole deriatives
Paclobutrazol*:* (2RS, 3RS)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pentan-3-ol); MW 293.79
Triapenthenol*:* ((E)-(RS)-1-cyclohexyl-4,4-dimethyl-2-(III-1,2,4 triazol-1-yl) pent-1-en-3-ol); MW 263.38
Uniconazol*:* (E)-1-(*p*-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl) penten-3-ol); MW 291.78
Pyrimidine derivatives
Ancymidol: (*α*-cyclopropyl-*α*-(4-methylphenyl)-5-pyrimidine methanol); MW 256.30
Flurprimidol: (*α*-(1-methylethyl)-*α*-[4-(trifluoromethoxy) phenyl]-5-pyrimidine methanol); MW 312.29
Norbornenodiazetine derivative
Tetcyclacis: (5-(4-chlorophenyl)-3,4,5-9,10-pentaazatetracyclo (5,4,1,0^{26},$0^{8.11}$) dodeca-3-9-dien); MW 273.72
Onium compounds (with positively charged ammonium, phosphonium or sulphonium or moiety)
Sulphonium carbamate derivative
BTS 44584*: (S-2, 5-dimethyl-4-pentamethylene carbamoyloxyphenyl-*SS*-dimethylsulphonium *p*-toluenesulphonate); MW 465.59
Substituted choline derivative
Chlormequat chloride: (2-chloroethyl) trimethylammonium chloride); MW 158.07
Piperidium derivative
Mepiquat chloride: (1,1-dimethyl piperidinium chloride); MW 149.66
Phosphonium salt derivative
Chlorphonium chloride: (2,4-dichlorobenzyltributyl phosphonium chloride); MW 397.79
Substance not affecting GA biosynthesis
Succinamic acid derivative
Diaminozide (butanedioic acid mono (2,2-dimethylhydrazide)); MW 160.17

MW, Molecular weight; *Manufacturer's code name.

tion of green colouration of leaves and thickening of roots[39]. Smith *et al.*[40] suggested that several growth retardants can be used in micropropagation to reduce damage due to wilting without deleterious side effects. The chemical derivation and known effects of these growth retardants on the isoprenoid pathway leading to gibberellins and sterol are summarized in Table 1 and Figure 1. Paclobutrazol reduces leaf water potential in *Malus domestica*[41,42]. Paclobutrazol inhibits kaurene oxidase and thus blocks the oxidative reactions from ent-kaurene to ent-kaurenoic acid in the pathway leading to the gibberellic acids[39]. Paclobutrazol is active as a growth retardant in a broad spectrum of species[43]. Smith *et al.*[44] reported that paclobutrazol (0.5–4 mg l^{-1}) in the rooting medium resulted in reduced stomatal apertures, increased epicuticular wax, shortened stems and thickened roots, reduction in wilting after transfer to compost, and also increased chlorophyll concentration per unit area of leaf. Paclobutrazol was effective in inhibiting shoot growth[45,46] and regulating various metabolic processes on apple trees. Treatments with paclobutrazol resulted in a shift in the partitioning of assimilates from the leaves to the roots, increased carbohydrates in all parts of apple seedlings, increased chlorophyll, soluble protein and mineral element concentration in leaf tissue, increased root respiration, reduced cell-wall polysaccharide and water loss, and accumulation of water stress-induced abscisic acid (ABA)[47–51]. Mckinless and Alderson[52] reported that successful clonal propagation of *Lapageria rosea* cv Nashcourt could be achieved by a rhizome-bud proliferation stage(s) in the presence of paclobutrazol followed by adventitious-root-emergence stages in the presence of paclobutrazol. It is possible to induce the formation of rhizome buds from aerial shoot auxillary buds in *L. rosea* by including the gibberellin biosynthesis inhibitor paclobutrazol in the culture medium[53]. Smith *et al.*[40] found that plantlets treated with paclobutrazol, flurprimidol, triapenthenol, chlorophonium chloride, uniconazol and ancymidol showed dose-related reduction in wilting up to a concentration of 3 mg l^{-1}. Triadimephon increases both stomatal resistance and shoot water potential in *Phaseolus vulgaris*[54] and increases stomatal resistance in *Lycopersicon esculentum*[55]. Hazarika *et al.*[56,57] reported that preconditioning citrus microshoots with paclobutrazol influence higher *ex vitro* survival by intensi-

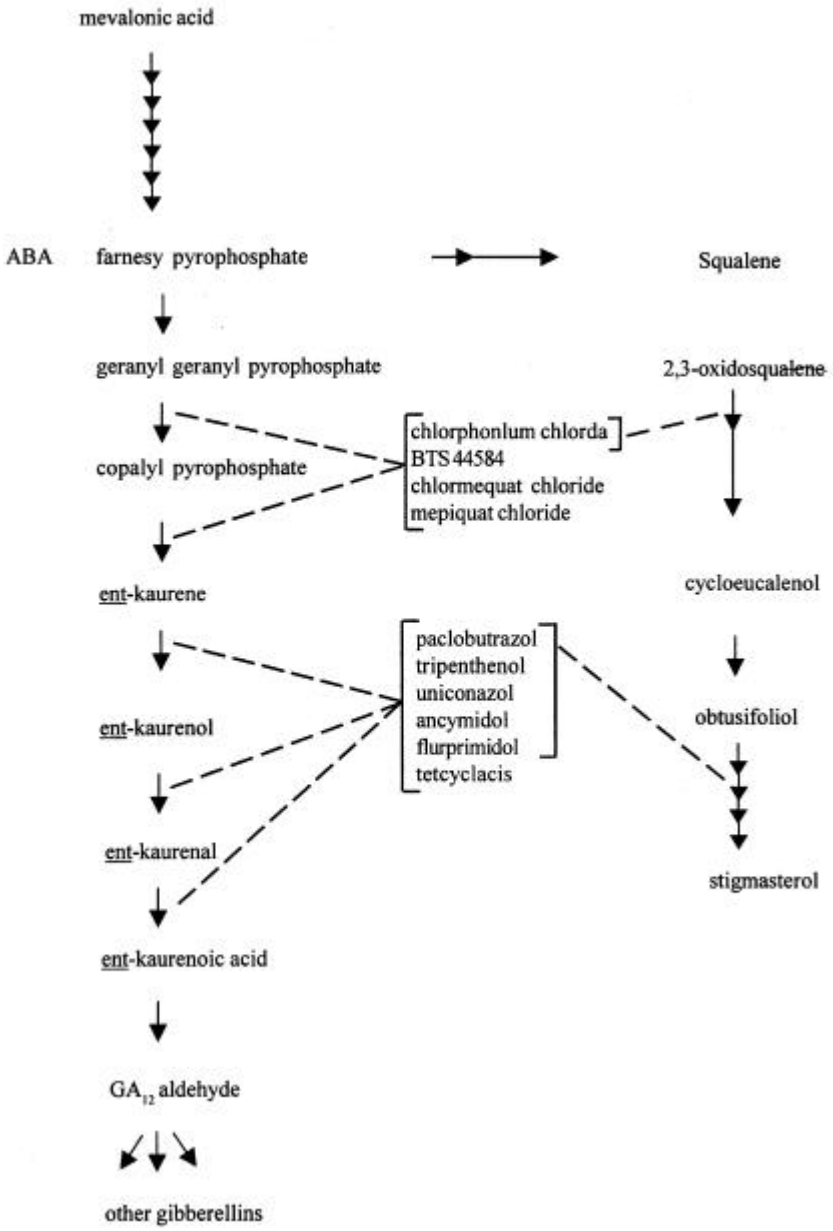

Figure 1. Known points of inhibition on the isoprenoid pathway leading to gibberellins and sterols.

fying internode length, thickening of root and reducing leaf dehydration by regulating the stomatal function and increasing epicuticular wax per unit area of leaf, besides more chlorophyll synthesis (Figure 2 *a*). Inclusion of paclobutrazol in growth medium produced smaller stomata with minimum apertures (Figure 2 *c*) unlike normal stomata (Figure 2 *d*) possibly due to a general reduction in cell expansion caused by anti-gibberellin like activity[58]. However, micropropagated plants seem less responsive to paclobutrazol than the seedlings. This may be due to increase in secondary roots of seedlings at early stages than micropropagated plantlets, which helped the plants in absorption of paclobutrazol[59].

Reduced humidity

The plants that develop under lower relative humidity have fewer transpiration and translocation problems *ex vitro*, and persistent leaves that look like normal ones. The low deposition of surface wax, stomatal abnormalities and a non-continuous cuticle are typical anatomical features of herbaceous plants growing under conditions of abundant moisture. This typical *in vitro* anatomy can be prevented by increasing the vapour-pressure gradient between the leaf and the atmosphere. Lowering the relative humidity *in vitro* has been done experimentally with varying results. A range of methods have been used in-

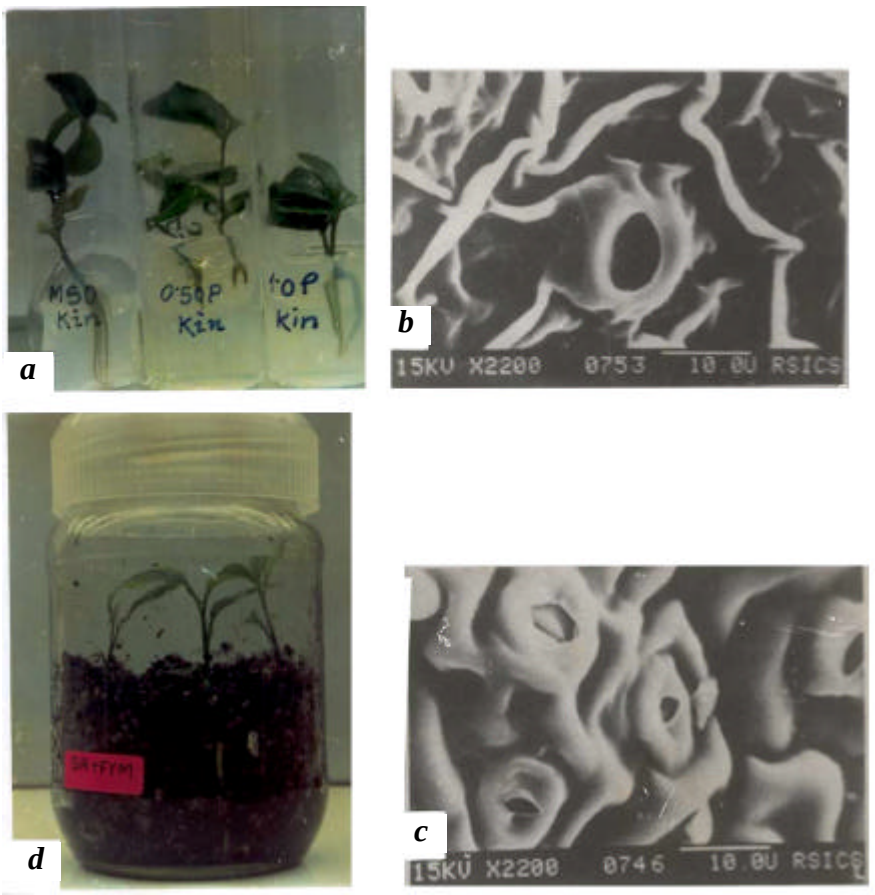

Figure 2. ***a***, Thickening of roots in rooting medium containing paclobutrazol. ***b*** and ***c***, Scanning electron micrograph of stomata from leaves without and with paclobutrazol treatment respectively. ***d***, Direct rooting of citrus microshoots in soilrite.

cluding the use of desiccants, by coating the medium with oily materials or both[60,61] by opening culture containers[62], adjusting culture closures or using special closures that facilitate water loss[63] or by cooling container bottoms[64]. A relative humidity of 85% decreased the multiplication rate of carnation but increased the number of glaucus levels. Increasing the sugar or agar concentration or adding osmotic agents such as polyetheleneglycol to the medium will also lower the relative humidity and in some cases, serve the same purpose as desiccants[65]. Wardle *et al.*[66], in their studies using silica gel and lanolin oil to reduce humidity in chrysanthemum, recorded high mortality and less roots. Improved plant survival rates after transplantation have been promoted by the reduction of relative humidity[67]. Humidity inside the culture vessel has been reduced to improve the internal structure of plantlets and give a more successful establishment in the glasshouse[61]. Short *et al.*[68] reported that optimum growth and *in vitro* hardening of cultured cauliflower and chrysanthemum occurred when plantlets were cultured at 80% relative humidity. Leaves of chrysanthemum and sugar beet, which were initiated and developed at relative humidity below 100%, displayed increased epicuticular wax, improved stomatal functioning and reduced leaf dehydration[69].

Antitranspirants

The use of antitranspirants to reduce water loss during acclimatization has had mixed results. Antitranspirants have not proven useful in promoting *ex vitro* survival or performance; phytotoxicity and interference with photosynthesis were both cited as possible reasons[70]. Other leaf-surface covering agents such as glycerol, paraffin and grease promoted *ex vitro* survival of several herbaceous species, but have not been evaluated over a long term or examined on woody species[71]. Wardle *et al.*[72] were able to substantially decrease stomatal transpiration of micropropagated cauliflower plantlets with a leaf spray of 10 mM ABA on persistent leaves. ABA, however did cause a large increase in stomatal resistance on leaves of seedling plants and new leaves that formed on acclimatized plants after removal from culture. An average of 56% of the stomata on leaves of *in vitro*-grown plants was closed after 4 h of mannitol-induced water stress and af-

ter 1 h of folicote to micropropagated apples, resulted in equal or better plant survival in a potting medium compared to acclimatization under mist. Phenylmercuric acetate (PMA) and CCC induced stomatal closure and delayed wilting in tomato plants[73,74]. Santakumari *et al.*[75] observed stomatal closure with alachlor and nearly complete closure with PMA application. Amaregouda *et al.*[76] found that stomatal resistance was more in plants treated with B-9 (1500 ppm) and 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. The relative water content followed reverse trend in these treatments. Hazarika *et al.*[77] reported that 8 HQ 2 ml/l was effective in controlling water loss from *in vitro*-grown citrus plantlets and subsequently helps in *ex vitro* survival. Voyiatzis and McGranahan[78] used latex polymer in acclimatization of tissue cultured walnut plantlets and found that the survival rate of plants dipped in latex was higher than that of the control. Plantlets treated with latex accumulated significantly more dry matter, apparently because their newly formed leaves were able to photosynthesize under favourable conditions of the open environment, longer than the control. They reported that the latex dipping procedure is a simple method that can be used for acclimatizing micropropagated walnut plantlets. Exogenous application of ABA to cell cultures can induce rapid hardening of cells to a significant level[79]. Application of ABA to whole plants has consistently shown less dramatic hardening responses compared to cells[80,81]. Inadequate uptake, rapid metabolism and microbial degradation are suggested as possible reasons for the minor hardiness in whole plants following ABA application[79]. ABA treatments alone were not able to harden plantlets to the extent attained under low temperature acclimation conditions, suggesting that factors other than or additional to ABA are involved in hardening. Further, ABA could not maintain the hardiness levels of cold acclimating treatments and plantlets de-acclimated to –9°C in BAP + ABA media. However, it is not known whether a pre-exposure to ABA before cold acclimation could maintain hardiness level.

Simultaneous rooting and acclimatization

Many commercial laboratories do not root microcutting *in vitro*, because it is labour-intensive and expensive. The process of rooting *in vitro* has been estimated to account for approximately 35 to 75% of the total cost of micropropagation[82]. When cauliflower shoots were rooted *in vitro*, the transition zone between root and shoots was abnormal. The vascular connection was poorly formed and narrow when observed at the time of plantlets removed from culture. This restricted water uptake from the root into the shoot. After acclimatization, the vascular connections were more substantial, but water uptake remained less than the seedlings[83]. An approach combining advantages of *in vitro* and *ex vitro* rooting had been successful for apples[84]. Sharma *et al.*[85] reported that acclimatization and hardening in tea micropropagation could be accomplished as a one-step procedure within a short period of time before transplanting. Optimization of time of harvesting of microshoots, shoot size, soil pH (4.0–6.4), plant growth regulator treatment (IBA 500 mg l^{-1}, 30 min), CO_2 enrichment and light (15 µmol m^{-2} s^{-1}) conditions in specially designed hardening chambers made a significant impact on the per cent of success for hardening in tea micropropagation. Das *et al.*[86] reported that direct rooting of tea shoots was achieved by dipping the cut ends in IBA (50 mg l^{-1}, 30 min) and subsequently planting these in a soil : peat moss :: 1 : 1 mixture. Shoots which were directly rooted in soil showed higher per cent survival in the field than those rooted under *in vitro* conditions. Labour costs can be dropped considerably if rooting happens *ex vitro*. Rooting *ex vitro* can be done on a stonewool substrate moistened with auxin solution during the induction phase[87]. However the *in vivo* conditions during root formation are not so important provided optimal plant material is produced at the end of the *in vitro* cycle[87]. Driver and Suttle[88] reported simultaneous rooting and acclimatization of walnut and peach microshoots directly in the field. Sahay and Varma[89] used *Priformospora indica* as a potential agent for use in the acclimatization of micropropagated tobacco and brinjal. Under *in vitro* conditions, *P. indica* was grown in a culture bottle containing minimal medium for 7 days in the dark and the regenerated shoots and the regenerated plantlets (having root and shoot) were co-cultured for 10–15 days. The morphology of the inoculated plants showed better revival and regeneration than untreated controls. During the acclimatization process, *P. indica*-inoculated plants showed more than 90% survival rate. In contrast, the untreated control plantlets had a comparatively low survival of about 62%. This is due to the fact that after root colonization, they become empowered with extra molecular weapons to tackle the situation. Since *P. indica* promotes growth like arbuscular mycorrhizal fungi (AMF), it seems that during the process of root colonization, it initiates the plant–microbe interaction AMF and the synthesis and expression of defence-related proteins and enzymes get stimulated in a controlled manner. But when a pathogen comes in contact with these plants, it is capable of boosting the production of defence mechanism-related secondary metabolites. These chemicals (isoflavonoid phytoalexins, isoflavonoids glyceollin coumestrol, coumestin isosojagol) provide protection, which results in higher plant survival. Rooting and acclimatization *in vitro* or *ex vitro* or both, can be achieved more easily in photoautotropic–micropropagation[36]. Plant regeneration was achieved from chlorophyllous root segments derived from *in vitro*-rooted plants of *Holostemma annulare* and showed 80% survival after a hardening period of four weeks by adjust-

ing the humidity conditions inside the mist chamber by removing the polythene covering for 1 h during the first week and increasing the exposure time in subsequent weeks[90]. In citrus[91] and curry-leaf micropropagation[92], simultaneous *ex vitro* rooting and acclimatization can be achieved using soilrite and soilrite with farmyard manure as a carrier (Figure 2 *d*). Better rooting (80–91%) and very high *ex vitro* survival (90–97%) was achieved in citrus using these carriers by Parthasarathy *et al.*[93], while Singh *et al.*[94] recorded 60% survival in citrus. Hazarika *et al.*[95] reported *ex vitro* acclimatization of *Aegle marmelos* using 2-cm long microshoots from proliferating cultures rooted in soilrite, after pulsing them with IBA or NAA at 10 ppm for 2 min. They found up to 79.46% rooting, which was an improvement over the conventional rooting in ager-based medium, as reported by Hossain *et al.*[96].

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