Acclimation of Plantlets to Ex Vitro Conditions: Effects of Air Humidity, Irradiance, CO_2 Concentration and Abscisic Acid (a Review)

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Abstract

Plantlets grown in vitro might be easily impaired by sudden changes in environmental conditions after ex vitro transfer. They usually need several weeks under shade and gradually decreasing air humidity to acclimate to the new conditions and to correct all abnormalities in their anatomy and physiology induced by special conditions of in vitro culture. For plant survival, the most important changes include development of cuticle, epicuticular waxes, and effective stomatal regulation of transpiration leading to stabilization of water status. For plant growth, changes in photosynthetic parameters (chlorophyll content, chloroplast ultrastructure, efficiency of photosystem 2, net photosynthetic rate) ensuring fully autotrophic growth with the rate corresponding to naturally grown plants are the most important. Acclimation can be speeded up by hardening of plantlets in vitro or after transplantation by decreasing the transpiration rate by antitranspirants including abscisic acid, or by increasing photosynthetic rate by elevated CO_2 concentration.

INTRODUCTION

Within the last four decades, plant micropropagation has developed from a laboratory curiosity to a real industry. Its use in horticulture, agriculture and forestry is currently expanding worldwide. Micropropagation of many species can be achieved through the establishment of explants, their initial growth in vitro being followed by transplanting into the greenhouse or field. During in vitro cultivation, plantlets grow under constant temperature, very high air humidity, low irradiance, very low air turbulence, variable and often insufficient CO_2 concentration, water potential dependent on medium composition, sugars as carbon source, growth regulators in nutrient medium, ethylene and other volatiles, etc. The conditions are very dependent on the vessel and closure types (e.g., Solárová et al., 1996). Acclimation to these conditions leads to formation of plantlets with morphology, anatomy and physiology different from naturally grown plants (for review see, e.g., Pospíšilová et al., 1992, 1997; Buddendorf-Joosten and Woltering, 1994; Desjardins, 1995; Kozai and Smith, 1995; Kubota et al., 1997).

After ex vitro transfer, the plantlets need some time to correct in vitro-induced abnormalities and acclimate to autotrophic conditions, low air humidity, high irradiance, etc. Few weeks of growth under a shade and gradually lowering air humidity are usually prerequisite for successful establishment of vigorous plants. In some plant species, the leaves formed in vitro are unable to develop further under ex vitro conditions and they are replaced by newly formed leaves (Preece and Sutter, 1991; Diettrich et al., 1992).

This review is mainly focused on 1) the changes essential for stabilization of water relations of plantlets (development of cuticle, epicuticular waxes, and functional stomatal apparatus leading to effective regulation of transpiration), 2) the improvement of photosynthetic apparatus (changes in chlorophyll *a* and *b* contents, photosynthetic efficiency and net photosynthetic rate) ensuring fully autotrophic growth with the rate corresponding to naturally grown plants, 3) occurrence of photoinhibition, and 4) possibilities of improvement of ex vitro transfer by in vitro hardening or by application of abscisic acid (ABA) and/or CO_2 enrichment.

DEVELOPMENT OF CUTICLE, EPICUTICULAR WAXES, AND FUNCTIONAL STOMATAL APPARATUS

The main problem during ex vitro transfer is the high rate of water loss from shoots of plantlets taken out of the cultivation vessels. Even if the water potential of the substrate (soil or sand with nutrient solution) is higher than the water potential of media with sucrose, the plantlets may quickly wilt (e.g., Pospíšilová et al., 1988). The cause is unrestricted rate of transpiration due to the retardation in development of cuticle, epicuticular waxes and functional stomatal apparatus. Exceptions are *Malus pumila* and *Agave tequilana* with only slightly reduced capacity to control water loss (Shackel et al., 1990; Díaz-Pérez et al., 1995b; Santamaría et al., 1995) and *Delphinium elatum, Doronicum* hybrid*, Hosta sieboldiana* and *Rodgersia pinnata* with cuticle permeabilities within the same ranges as found in leaves grown ex vitro and rapid water loss associated only with failure of stomata closure (Santamaría et al., 1993; Santamaría and Kerstiens, 1994).

Stomatal density in plantlet leaves might be higher or lower than in leaves of comparable plants grown ex vitro. According to this, stomatal density in *Liquidambar styraciflua*, *Rosa odorata* × *R. damascena*, *Vaccinium corymbosum, Nicotiana tabacum* and *Cynara scolymus* decreased (Wetzstein and Sommer, 1983; Johansson et al., 1992; Noé and Bonini, 1996; Tichá et al., 1999; Brutti et al., 2002), while in *Prunus serotina* and *Rhododendron* ssp. (Waldenmaier and Schmidt, 1990; Drew et al., 1992) increased after transplantation. The changes in stomatal density were sometimes compensated by an increase in stomatal size (length, guard cell area and pore area). Leaves from in vitro grown *Prunus cerasus*, *Vaccinium corymbosum, Quercus robur*, *Nicotiana tabacum* or *Cynara scolymus* plantlets exhibited ring-shaped stomata, but in leaves of ex vitro transferred plants stomata were elliptical (Marín et al., 1988; Noé and Bonini, 1996; Sha Valli Khan et al., 1999; Tichá et al., 1999; Brutti et al., 2002). However, in *Paulownia fortunei*, ring-shaped stomata were observed only in plantlets grown under photomixotrophic conditions while elliptical stomata were found in those grown under photoautotrophic conditions (Sha Valli Khan et al., 2003).

Stomata of in vitro grown plantlets often failed to close fully in response to external stimuli. In *Rosa hybrida* and in old leaves of *Prunus cerasifera*, dark treatment did not induce stomatal closure (Sallanon et al., 1991; Zacchini and Morini, 1998). However, during ex vitro acclimation of rose, stomatal sensitivity to the dark developed. Simultaneously, the light-induced opening of stomata and K^+ influx into guard cells were observed, and calcium amount was ten times higher in subsidiary cells than in guard cells of in vitro grown plantlets (Sallanon et al., 1991). In *Vitis vinifera* plantlets, stomatal conductance was high and did not respond to changes in air humidity. One month after transplantation, stomatal conductance decreased considerably and stomata responses to air humidity, irradiance and internal CO_2 concentration resembled those of naturally grown plants (Fila et al., 1998). Similarly in *Nicotiana tabacum*, stomatal conductance and stomatal transpiration rate decreased to values found in seedlings 3 weeks after transfer to ex vitro conditions, but the cuticular transpiration rate decreased more slowly (Pospíšilová et al., 1988, 1998, 1999). Development of ability of stomata to regulate transpiration rate during acclimation period was found in many other species, e.g., *Brassica oleracea* (Grout and Aston, 1977), *Leucaena leucocephala* (Dhawan and Bhojwani, 1987), *Prunus serotina* (Drew et al., 1992), *Solanum laciniatum* (Conner and Conner, 1984) and *Solanum tuberosum* (Baroja, 1995), *Lycopersicon esculentum* (Bhatia and Asnwath, 2004) but acclimation was usually slower than in tobacco. On the other hand, low stomatal conductance observed in *Malus pumila* plantlets increased after transfer to ex vitro conditions (Díaz-Pérez et al., 1995). After ex vitro transfer of tobacco plantlets, the decrease in stomatal opening was accompanied with the increase in the content of endogenous abscisic acid (Hronková et al., 2003). On the contrary, the decrease of free and conjugated ABA contents were found after transplantation of *Dianthus caryophyllus* (Majada et al., 1998).

CHANGES IN CHLOROPHYLL *A* AND *B* CONTENTS AND NET PHOTOSYNTHETIC RATE

The development of photosynthetic apparatus is usually not retarded by conditions of in vitro cultivation as much as above mentioned parameters regulating plant water relations. The low net photosynthetic rate (P_N) and in consequence low growth rate of plantlets in situ is usually due to low CO_2 concentration in tightly closed cultivation vessels for at least half of light period. The daily dynamics of CO_2 concentration is also dependent on sucrose concentration of the medium (Morini and Melai, 2003/4). Higher aeration of cultivation vessels markedly increase P_N in many plant species (for review see e.g., Kozai et al., 1991; Pospíšilová et al., 1997). The same was proved recently in *Myrtus communis* (Lucchesini et al., 2001).

Chlorophyll (Chl) *a* and Chl *b* contents might be higher or lower in leaves of in vitro grown plantlets than in corresponding ex vitro grown plants and usually depends on irradiance and concentration of sugars in the medium (e.g., Tichá et al., 1998). More permeable closures increased chlorophyll content in potato plantlets (Chanemougasoundharam et al., 2004). Similarly chloroplast ultrastructure may be dependent on irradiance and sucrose concentration (Lee et al., 1985; Capellades et al., 1991; Serret and Trillas, 2000).

Chl *a* and Chl *b* contents usually increased after transplantation (Trillas et al., 1995, Rival et al., 1997; Synková, 1997; Pospíšilová et al., 1998). This was observed in originally photoautotrophically grown *Nicotiana tabacum* plantlets, however, in originally photomixotrophically grown plantlets an abrupt decrease in Chl *a* and Chl *b* contents during the first week after transplantation followed by a slow increase was found (Kadlecek et al., 1998). In the first two weeks after ex vitro transfer, P_N, Chl *a* and Chl *b* contents and Chl *a*/*b* ratio were higher in tobacco plantlets grown in vitro in Magenta boxes with permeable closures than in those grown in vitro in tightly closed glass vessels, but during further growth the differences almost disappeared (Pospíšilová et al., 2000). In *Calathea louisae* the chlorophyll and carotenoid contents were almost three times higher 30 d after ex vitro transfer but only in newly-formed leaves (Van Huylenbroeck et al., 2000).

Improved chloroplast ultrastructure was observes e.g., in *Liquidambar styraciflua* (Wettstein and Sommer, 1982). Also photochemical efficiency, which may be characterized by variable to maximum fluorescence ratio (F_v/F_m), increased after transfer of *Elaeis guineensis* and *Nicotiana tabacum* plantlets to ex vitro conditions (Rival et al., 1997; Synková, 1997; Pospíšilová et al., 1998).

P_N in *Solanum tuberosum*, *Spathiphyllum floribundum*, *Capsicum annuum* and *Rehmania glutinosa* plants decreased in the first days after transplantation and increased thereafter (Baroja et al., 1995; Van Huylenbroeck and Debergh, 1996; Estrada-Luna et al., 2001; Seon et al., 2000). In *Calathea louisae* and *Spathiphyllum floribundum* substantial increase in P_N was measured when new leaves were fully developed (Van Huylenbroeck et al., 1998, 2000). Three weeks after ex vitro transplantation, P_N of *Nicotiana tabacum* leaves was considerably higher that P_N of leaves of plantlets grown in vitro and responses of P_N, to irradiance and CO_2 concentration were quite similar to those of naturally grown plants (Pospíšilová et al., 1992, 1998). Similarly, higher P_N was found in *Malus pumila* plants three weeks after transplantation (Díaz-Pérez et al., 1995) and more than twice as high a maximum P_N was observed in *Vitis vinifera* × *Vitis berlandieri* rootstocks or *Vitis vinifera* plants one month after transplantation (Fila et al., 1998; Slavtcheva and Dimitrova, 2001). In *Rehmania glutinosa* plantlets grown in vitro, P_N was higher under autotrophic than under heterotrophic conditions and the difference preserved after ex vitro transfer (Seon et al., 2000).

OCCURRENCE OF PHOTOINHIBITION

It is well known that exposure of photosynthetic apparatus to excessive irradiance causes photoinhibition and that the susceptibility to photoinhibition may raise considerably under stress conditions. Low P_N in situ due to insufficient CO_2 supply might

be the cause that photoinhibiton in plantlets grown in vitro was observed under relatively low irradiance. For example in *Gardenia jasminoides* plantlets grown in media with 0.5, 1.5 and 3.0% sucrose, irradiance of 100 and 300 μmol m^{-2} s^{-1} was sufficient to induce photoinhibition: the decrease in Fv/Fm, mostly due to an increase of initial fluorescence (F_0) (Serret et al., 1996). *Gardenia* plantlets suffer less photoinhibition when cultivated in tubes with permeable caps (Serret et al., 2001b). However, irradiance of 700 μmol m^{-2} s^{-1} induced photoinhibition in *Nicotiana tabacum* plantlets grown in tightly closed glass vessel or Magenta boxes with more permeable vents and even in those plantlets where covers were completely removed (Semorádová et al., 2002).

Sudden increase in irradiance after ex vitro transfer might be dangerous. Exposure of *Calathea louisae* and *Spathiphyllum floribundum* plantlets to high irradiance immediately after transplantation caused photoinhibition and even Chl photobleaching (Van Huylenbroeck, 1994; Van Huylenbroeck et al., 1995, 2000), however, no photoinhibition was observed in plants acclimatized under low irradiance for four weeks (Van Huylenbroeck, 1994). Similarly, photoinhibition was observed in *Rosa hybrida* plantlets, but only in the first week after ex vitro transfer, and especially in those plantlets transplanted into medium with osmotic potential decreased by addition of mannitol (Sallanon et al., 1998). In this plant species, Fv/Fm decreased with the irradiance increasing from 45 to 300 μmol m^{-2} s^{-1}, but after six weeks photoinhibition was observed only in those plants grown under irradiance of 300 μmol m^{-2} s^{-1} (Genoud et al., 1999). In *Nicotiana tabacum* plantlets acclimatized to ex vitro conditions under shade (daily maximum irradiance less than that needed for saturation of photosynthesis), no photoinhibition occurred: F_v/F_m was in the range typical for non-stressed plants and did not change during acclimatization (Pospíšilová et al., 1999, 2000). On the contrary, during acclimatization of tobacco at high irradiance (700 μmol m^{-2} s^{-1}) F_v/F_m decreased after transplantation and the decrease was more marked in plantlets cultivated in vitro in tightly closed glass vessels than in those cultivated in Magenta boxes with more permeable lids (Semorádová et al., 2002). Similarly in *Gardenia jasminoides*, occurrence of photoinhibition was dependent on conditions during previous in vitro cultivation: plantlets cultured on medium with 3% sucrose and higher irradiance were less photoinhibited after ex vitro transfer than those cultured in vitro on medium without sucrose and lower irradiance (Serret et al., 2001a). 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 (200–1400 μmol m^{-2} s^{-1}), no photoinhibition was found during growth in the greenhouse, but F_v/F_m decreased transiently after transfer to the open air (Kadlecek et al., 1998).

In addition to fluorescence parameters, the increased content of xanthophyll cycle pigments [violaxanthin + antheraxanthin + zeaxanthin] and particularly the degree of their deepoxidation [DEPS = (zeaxanthin + 0.5 antheraxanthin)/(zeaxanthin + antheraxanthin + violaxanthin)] may be indicators of photoinhibition. During in vitro growth, content of xanthophyll cycle pigments was lower in tobacco plantlets grown in vessels with more permeable closures than in those grown in tightly closed vessels (Haisel et al., 1999). In the same plantlets, the xanthophyll cycle pigment contents and degree of their deepoxidation were not changed markedly during acclimation under shade (Pospíšilová et al., 1999, 2000) but temporary increased during acclimation at high irradiance (Semorádová et al., 2002).

IMPROVEMENT OF EX VITRO TRANSFER BY HARDENING OF PLANTLETS DURING THE LAST WEEKS OF IN VITRO CULTURE

The hardening of plantlets in vitro by decreasing air humidity, e.g., by using lids permeable for water vapour or by bottom cooling, or by decreased osmotic potential of the medium by addition of polyethylene glycol or sugars can ameliorate wilting of plants after transplantation (for review see Pospíšilová et al., 1999). However, these procedures might lead to a quick drying out of the cultivation medium and to impairment in plantlet growth (e.g., Solárová et al., 1996). The relative water loss from detached leaves of in

vitro grown plantlets can be reduced by application of abscisic acid (ABA) (Colón-Guasp et al., 1996; Hartung and Abou-Mandour, 1996; Pospíšilová, 1996; Aguilar et al., 2000), paclobutrazol, indolebutyric acid, or 6-benzyl-aminopurine (Smith et al., 1992; Pospíšilová et al., 1993; Eliasson et al., 1994) into the cultivation medium. The forced ventilation not only decreases air humidity but also increases turbulence and CO_2 supply (Nguyen et al., 2001). In *Dianthus caryophyllus*, forced ventilation improved stomatal function by an increasing K^+ concentration in the guard cells and free ABA content in leaves (Majada et al., 1998). Gradual opening of closures during last 9 days of in vitro growth improved ex vitro acclimation of *Picea glauca* (Lamhamedi et al., 2003).

Increased irradiance together with increased CO_2 concentration in cultivation vessels (by using a gas permeable film for vessel closure, increasing CO_2 concentration around the cultivation vessels, or direct supply of CO_2 into the vessels) improved development of photosynthetic apparatus. As was mentioned above, these treatments make easier the transfer of plantlets to full autotrophy under ex vitro conditions and decrease the risk of photoinhibition.

High concentrations of sucrose in the medium can retard development of photosynthetic apparatus but low concentrations not only stimulated plantlet growth but often also their vigour. Positive effect on further ex vitro transfer was also observed (e.g., Van Huylenbroeck and Debergh, 1996; Kadlecek, 1998; Fila et al., 1998; Serret et al., 2001; Hoffman et al., 2002; Custódio et al., 2004; Ket et al., 2004).

IMPROVEMENT OF ACCLIMATION TO EX VITRO CONDITIONS

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 shock" of *Nicotiana tabacum* plants (Pospíšilová et al., 1998). Stomatal conductance of leaves which was high during the first days after transplantation was markedly decreased by ABA application. However, in following days stomatal conductance decreased more quickly in control than in ABA-treated plants. After two or three weeks, stomatal conductance of transplanted plants 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 *a* content and other photosynthetic parameters and enhanced plant growth (Pospíšilová et al., 1998, 2000).

Elevated CO_2 concentration can also serve as antitranspirant. Acclimation of tobacco plantlets under elevated CO_2 concentration also decreased stomatal conductance and improve plant water status after transplantation (Pospíšilová et al., 1999). In addition, it can promote plant photosynthesis and ex vitro growth (for review, see, Buddendorf-Joosten and Woltering, 1994). CO_2 enrichment had no effect on *Fragaria* × *ananassa* plants growth immediately after transplantation, but from day 20 it increased P_N and in consequence biomass accumulation; this increase was more marked under higher irradiance (Desjardins et al., 1987). Elevated CO_2 concentration during acclimatization of tobacco plants markedly increased P_N in situ, water use efficiency and growth, and slightly increased Chl *a* fluorescence kinetic parameters, photochemical activities and stomatal regulation of gas exchange (Pospíšilová et al., 1999). However, 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 (Solárová and Pospíšilová, 1997).

Elevated CO_2 concentration also enhanced the effect of ABA application (Pospíšilová et al., 2000). Stomatal conductance of tobacco plantlets treated with ABA and acclimated for 2 or 7 day under increased CO_2 concentration was lower than in those plantlets acclimated under normal CO_2 concentration with or without ABA treatment and in elevated CO_2 concentration without ABA treatment. The combination of ABA and elevated CO_2 concentration also induced the highest net photosynthetic rate, content of Chl *a* and Chl *a*/*b* ratio measured 7 and 28 d after ex vitro transfer (Pospíšilová et al., 2000).

Oxidative stress also belongs among stresses occurring during ex vitro transfer. Therefore for successful ex vitro transfer sufficient content of non-enzymatic antioxidants as well as activities of antioxidative enzymes are important. These parameters are also very dependent on conditions during in vitro growth and during ex vitro acclimation (Van Huylenbroeck et al., 2000; Synková and Pospíšilová, 2002).

CONCLUSIONS

1) The abnormalities in morphology, anatomy and physiology of plantlets cultivated in vitro can be repaired during acclimation to ex vitro conditions.
2) For the development of cuticle, epicuticular waxes, and effective stomatal regulation of transpiration leading to stabilization of water status the most important factor is gradually decreasing air humidity.
3) For the improvement of photosynthetic parameters (chlorophyll content, chloroplast ultrastructure, photochemical efficiency, net photosynthetic rate) the most important factors are irradiance and CO_2 concentration during previous in vitro growth as well as during acclimation.
4) Hardening of plantlets in vitro can speed up acclimation to ex vitro conditions.
5) Ex vitro transfer can be improved by application of antitranspirant ABA and/or by elevated CO_2 concentration.

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Literature Cited

Aguilar, M.L., Espadas, F.L., Coello, J., Maust, B.E., Trejo, C., Robert, M.L. and Santamaría, J.M. 2000. The role of abscisic acid in controlling leaf water loss, survival and growth of micropropagated *Tagetes erecta* plants when transferred directly to the field. J. Exp. Bot. 51:1861–1866.

Baroja, M.E., Aguirreolea, J. and Sánchez-Díaz, M. 1995. CO_2 exchange of in vitro and acclimatizated potato plantlets. p.187–188. In: F. Carre and P. Chagvardieff (eds.), Ecophysiology and Photosynthetic in Vitro Cultures. CEA, Centre d´Études de Cadarache, Saint-Paul-lez-Durance.

Bhatia, P. and Ashwath, N. 2004. Comparative performance of micropropagated and seed-grown tomato plants. Biol. Plant. 48:625–628.

Brutti, C.B., Rubio, E.J., Llorente, B.E. and Apóstolo, N.M. 2002. Artichoke leaf morphology and surface features in different micropropagation stages. Biol. Plant. 45:197–204.

Buddendorf-Joosten, J.M.C. and Woltering, E.J. 1994. Components of the gaseous environment and their effects on plant growth and development in vitro. Plant Growth Regul. 15:1–16.

Capellades, M., Lemeur, R. and Debergh, P. 1991. Effects of sucrose on starch accumulation and rate of photosynthesis in *Rosa* cultured in vitro. Plant Cell Tissue Organ Cult. 25:21–26.

Chanemougasoundharam, A., Sarkar, D., Pandey, S.K., Al-Biski, F., Helali, O. and Minhas, J.S. 2004. Culture tube closure-type affects potato plantlets growth and chlorophyll contents. Biol. Plant. 48:7–11.

Colón-Guasp, W., Nell, T.A., Kane, M.E. and Barrett, J.E. 1996. Effects of abscisic acid on ex vitro acclimatization of *Aronia arbutifolia* (L.) Pers. J. Amer. Soc. Hort. Sci. 121:101–104.

Conner, L.N. and Conner, A.J. 1984. Comparative water loss from leaves of *Solanum laciniatum* plants cultured in vitro and in vivo. Plant Sci. Lett. 36:241–246.

Custódio, L., Martins-Loução, M.A. and Romano, A. 2004. Influence of sugars on in vitro rooting and acclimatization of carob tree. Biol. Plant. 48:469–472.

Desjardins, Y. 1995. Photosynthesis in vitro - on the factors regulating CO_2 assimilation

in micropropagation systems. Acta Hort. 393:45–61.
Desjardins, Y., Gosselin, A. and Yelle, S. 1987. Acclimatization of ex vitro strawberry plantlets in CO_2-enriched environments and supplementary lighting. J. Amer. Soc. Hort. Sci. 112:846–851.
Dhawan, V. and Bhojwani, S.S. 1987. Hardening in vitro and morpho-physiological changes in the leaves during acclimatization of micropropagated plants of *Leucaena leucocephala* (Lam.) De Wit. Plant Sci. 53:65–72.
Díaz-Pérez, J.C., Sutter, E.G. and Shackel, K.A. 1995. Acclimatization and subsequent gas exchange, water relations, survival and growth of microcultured apple plantlets after transplanting them in soil. Physiol. Plant. 95:225–232.
Diettrich, B., Mertinat, H. and Luckner, M. 1992. Reduction of water loss during ex vitro acclimatization of micropropagated *Digitalis lanata* clone plants. Biochem. Physiol. Pflanz. 188:23–31.
Drew, A.P., Kavanagh, K.L. and Maynard, C.A. 1992. Acclimatizing micropropagated black cherry by comparison with half-sib seedlings. Physiol. Plant. 86:459–464.
Eliasson, M.K., Beyl, C.A. and Barker, P.A. 1994. In vitro responses and acclimatization of *Prunus serotina* with paclobutrazol. J. Plant Growth Regul. 13:137–142.
Estrada-Luna, A.A., Davies, F.T. Jr. and Egilla, J.N. 2001. Physiological changes and growth of micropropagated chile ancho pepper plantlets during acclimatization and post-acclimatization. Plant Cell Tissue Organ Cult. 66:17–24.
Fila, G., Ghashghaie, J., Hoarau, J. and Cornic, G. 1998. Photosynthesis, leaf conductance and water relations of in vitro cultured grapevine rootstock in relation to acclimatisation. Physiol. Plant. 102:411–418.
Genoud, C., Coudret, A., Amalric, C. and Sallanon, H. 1999. Effect of micropropagation conditions of rose shootlets on chlorophyll fluorescence. Photosynthetica. 36:243–251.
Grout, B.W.W. and Aston, M.J. 1977. Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Hort. Res. 17:1–7.
Haisel, D., Pospíšilová, J., Synková, H., Catsky, J., Wilhelmová, N. and Plzáková, Š. 1999. Photosynthetic pigments and gas exchange of in vitro grown tobacco plants as affected by CO_2 supply. Biol. Plant. 42:463–468.
Hartung, W. and Abou-Mandour, A.A. 1996. A beneficial role of abscisic cid for regenerates of *Ruta graveolens* ssp. *divaricata* (Tenore) Gams suffering from transplant shock. Angew. Bot. 70:221–223.
Hofman, P., Haisel, D., Komenda, J., Vágner, M., Tichá, I., Schäfer, C. and Čapková, V. 2002. Impact of in vitro cultivation conditions on stress responses and on changes in thylakoid membrane proteins and pigments of tobacco during ex vitro acclimatization. Biol. Plant. 45:189–195.
Hronková, M., Zahradníčková, H., Šimková, M., Šimek, P. and Heydová, A. 2003. The role of abscisic acid in acclimation of plants cultivated in vitro to ex vitro conditions. Biol. Plant. 46:535–541.
Johansson, M., Kronestedt-Robards, E.C. and Robards, A.W. 1992. Rose leaf structure in relation to different stages of micropropagation. Protoplasma. 166:165–176.
Kadlecek, P., Tichá, I., Čapková, V. and Schäfer, C. 1998. Acclimatization of micropropagated tobacco plantlets. p.3853–3856. In: G. Garab (ed.), Photosynthesis: Mechanisms and Effects. Vol. V. Kluwer Academic Publishers, Dordrecht.
Ket, N.V., Hahn, E.J., Park, S.Y., Chakrabarty, D. and Paek, K.Y. 2004. Micropropagation of an endangered orchid *Anoectochilus formosanus*. Biol. Plant. 48:339–344.
Kozai, T. 1991. Micropropagation under photoautotrophic conditions. p.447–469. In: P.C. Debergh and R.H. Zimmerman (eds.), Micropropagation. Technology and Application. Kluwer Academic Publishers, Dordrecht, The Netherlands.
Kozai, T. and Smith, M.A.L. 1995. Environmental control in plant tissue culture - general introduction and overview. p.301–318. In: J. Aitken-Christie, T. Kozai and M.A.L.

Smith (eds.), Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Dordrecht, The Netherlands.
Kubota, C., Fujiwara, K., Kitaya, Y. and Kozai, T. 1997. Recent advances in environment control in micropropagation. p.153–169. In: E. Goto, K. Kurata, M. Hayashi and S. Sasa (eds.), Plant Production in Closed Ecosystems. Kluwer Academic Publishers, Dordrecht, The Netherlands.
Lamhamedi, M.S., Chamberland, H. and Tremblay, F.M. 2003. Epidermal transpiration, ultrastructural characteristics and net photosynthesis of white spruce somatic seedlings in response to in vitro acclimatization. Physiol. Plant. 118:554–561.
Lee, N., Wetzstein, H.Y. and Sommer, H.E. 1985. Effects of quantum flux density on photosynthesis and chloroplast ultrastructure in tissue-cultured plantlets and seedlings of *Liquidambar styraciflua* L. towards improved acclimatization and field survival. Plant Physiol. 78:637–641.
Lucchesini, M., Mensuali-Sodi, A., Massai, R. and Gucci, R. 2001. Development of autotrophy and tolerance to acclimatization of *Myrtus communis* transplants cultured in vitro under different aeration. Biol. Plant. 44:167–174.
Majada, J.P., Luz Centeno, M., Feito, I., Fernándéz, B. and Sanchez-Tames, R. 1998. Plant Growth Regul. 25:113–121.
Marín, J.A., Gella, R. and Herrero, M. 1988. Stomatal structure and functioning as a response to environmental changes in acclimatized micropropagated *Prunus cerasus* L. Ann. Bot. 62:663–670.
Morini, S. and Melai, M. 2003/4. CO_2 dynamics and growth in photoautotrophic and photomixotrophic apple cultures. Biol. Plant. 47:167–172.
Nguyen, Q.T., Kozai, T., Heo, J. and Thai, D.X. 2001. Photoautotrophic growth response of in vitro cultured cofee plantlets to ventilation methods and photosynthetic photon fluxes under carbon dioxide enriched conditions. Plant Cell Tissue Organ Cult. 66:217–225.
Noé, N. and Bonini, L. 1996. Leaf anatomy of highbush blueberry grown in vitro and during acclimatization to ex vitro conditions. Biol. Plant. 38:19–25.
Pospíšilová, J. 1996. Hardening by abscisic acid of tobacco plantlets grown in vitro. Biol. Plant. 38:605–609.
Pospíšilová, J., Catsky, J. and Sesták, Z. 1997. Photosynthesis in plants cultivated in vitro. p.525–540. In: M. Pessarakli (ed.), Handbook of Photosynthesis. Marcel Dekker, New York.
Pospíšilová, J., Čatský, J., Synková, H., Macháčková, I. and Solárová, J. 1993. Gas exchange and in vivo chlorophyll fluorescence in potato and tobacco plantlets in vitro as affected by various concentrations of 6-benzylaminopurine. Photosynthetica 29:1–12.
Pospíšilová, J., Haisel, D., Synková, H., Čatský, J., Wilhelmová, N., Plzáková, Š. and Procházková, D. 2000. Photosynthetic pigments and gas exchange of in vitro grown tobacco plants during ex vitro acclimation. Plant Cell Tissue Organ Cult. 61:125–133.
Pospíšilová, J., Solárová, J. and Čatský, J. 1992. Photosynthetic responses to stresses during in vitro cultivation. Photosynthetica 26:3–18.
Pospíšilová, J., Solárová, J., Čatský, J., Ondřej, M. and Opatrný, Z. 1988. The photosynthetic characteristics during the micropropagation of tobacco and potato plants. Photosynthetica 22:205–213.
Pospíšilová, J., Synková, H., Haisel, D., Čatský, J., Wilhelmová, N. and Šrámek, F. 1999. Effect of elevated CO_2 concentration on acclimation of tobacco plantlets to ex vitro conditions. J. Exp. Bot. 50:119–126, 1999.
Pospíšilová, J., Tichá, I., Kadleček, P., Haisel, D. and Plzáková, Š. 1999. Acclimatization of micropropagated plants to ex vitro conditions. Biol. Plant. 42:481–497.
Pospíšilová, J., Wilhelmová, N., Synková, H., Čatský, J., Krebs, D., Tichá, I., Hanáčková, B. and Snopek, J. 1998. Acclimation of tobacco plantlets to ex vitro conditions as affected by application of abscisic acid. J. Exp. Bot. 49:863–869.
Preece, J.E. and Sutter, E.G., 1991. Acclimatization of micropropagated plants to the

greenhouse and field. p.71–93. In: P.C. Debergh and R.H. Zimmerman (eds.), Micropropagation. Technology and Application. Kluwer Academic Publishers, Dordrecht, The Netherlands.
Rival, A., Beulé, T., Lavergne, D., Nato, A., Havaux, M. and Puard, M. 1997. Development of photosynthetic characteristics in oil palm during in vitro micropropagation. J. Plant Physiol. 150:520–527.
Sallanon, H., Berger, M., Genoud, C. and Coudret, A. 1998. Water stress and photoinhibition in acclimatization of *Rosa hybrida* plantlets. In Vitro Cell. Dev. Biol. Plant 34:169–172.
Sallanon, H., Laffray, D. and Coudret, A. 1991. Ultrastructure and functioning of guard cells of in vitro cultured rose plants. Plant Physiol. Biochem. 29:333–339.
Santamaría, J.M., Davies, W.J. and Atkinson, C.J. 1993. Stomata of micropropagated *Delphinium* plants respond to ABA, CO_2, light and water potential, but fail to close fully. J. Exp. Bot. 44:99–107.
Santamaría, J.M., Herrera, J.L. and Robert, M.L. 1995. Stomatal physiology of a micropropagated CAM plant; *Agave tequilana* (Weber). Plant Growth Regul. 16:211–214.
Santamaría, J.M. and Kerstiens, G. 1994. The lack of control of water loss in micropropagated plants is not related to poor cuticle development. Physiol. Plant. 91:191–195.
Semorádová, Š., Synková, H. and Pospíšilová, J. 2002. Responses of tobacco plantlets to change of irradiance during transfer from in vitro to ex vitro conditions. Photosynthetica. 40:605–614.
Seon, J.-H., Cui, Y.-Y., Kozai, T. and Paek, K.-Y. 2000. Influence of in vitro growth conditions on photosynthetic competence and survival rate of *Rehmania glutinosa* plantlets during acclimatization period. Plant cell Tissue Organ Cult. 61:135–142.
Serret, M.D. and Trillas, M.I. 2000. Effects of light and sucrose levels on the anatomy, ultrastructure, and photosynthesis of *Gardenia jasminoides* Ellis leaflets cultured in vitro. Int. J. Plant Sci. 161:281–289.
Serret, M.D., Trillas, M.I. and Araus, J.L. 2001a. The effect of in vitro culture conditions on the pattern of photoinhibition during acclimation of gardenia plantlets to ex vitro conditions. Photosynthetica. 39:67–73.
Serret, M.D., Trillas, M.I., Matas, J. and Araus, J.L. 1996. Development of photoautotrophy and photoinhibition of *Gardenia jasminoides* plantlets during micropropagation. Plant Cell Tissue Organ Cult. 45:1–16.
Serret, M.D., Trillas, M.I., Matas, J. and Araus, J.L. 2001b. The effect of photoautotrophy on photosynthesis and photoinhibition of gardenia plantlets during micropropagation. Photosynthetica. 39:245–255.
Shackel, K.A., Novello, V. and Sutter, E.G. 1990. Stomatal function and cuticular conductance in whole tissue-cultured apple shoots. J. Amer. Soc. Hort. Sci. 115:468–472.
Sha Valli Khan, P.S., Evers, D. and Hausman, J.F. 1999. Stomatal characteristics and water relations of in vitro grown *Quercus robur* NL 100 in relation to acclimatization. Silvae Genet. 48:83–87.
Sha Valli Khan, P.S., Kozai, T., Nguyen, Q.T., Kubota, C. and Dhawan, V. 2003. Growth and water relations of *Paulownia fortunei* under photomixotrophic and photoautotrophic conditions. Biol. Plant. 46:161–166.
Slavtcheva, T. and Dimitrova, V. 2001. Gas exchange of in vitro and ex vitro grown grapevine plants. Photosynthetica. 39:29–33.
Smith, E.F., Gribaudo, I., Roberts, A.V. and Mottley, J. 1992. Paclobutrazol and reduced humidity improve resistance to wilting of micropropagated grapevine. HortScience. 27:111–113.
Solárová, J. and Pospíšilová, J. 1997. Effect of carbon dioxide enrichment during in vitro cultivation and acclimation to ex vitro conditions. Biol. Plant. 39:23–30.
Solárová, J., Součková, D., Ullmann, J. and Pospíšilová, J. 1996. In vitro culture:

environmental conditions and plantlet growth as affected by vessel type and stopper material. Zahradnictví (Prague). 23:51–58.
Synková, H. 1997. Sucrose affects the photosynthetic apparatus and the acclimation of transgenic tobacco to ex vitro culture. Photosynthetica. 33:403–412.
Synková, H. and Pospíšilová, J. 2002. In vitro precultivation of tobacco affects the response of antioxidative enzymes to ex vitro acclimation. J. Plant Physiol. 159:781–789.
Tichá, I., Čáp, F., Pacovská, D., Hofman, P., Haisel, D., Čapková, V. and Schäfer, C. 1998. Culture on sugar medium enhances photosynthetic capacity and high light resistance of plantlets grown in vitro. Physiol. Plant. 102:155–162.
Tichá, I., Radochová, B. and Kadleček, P. 1999. Stomatal morphology during acclimatization of tobacco plantlets to ex vitro conditions. Biol. Plant. 42:469–474.
Van Huylenbroeck, J.M. 1994. Influence of light stress during the acclimation of in vitro plantlets. p.451–453. In: P.C. Struik, W.J. Vredenberg, J.A. Renkema and J.E. Parlevliet (eds.), Plant Production on the Threshold of a New Century. Kluwer Academic Publishers, Dordrecht, The Netherlands.
Van Huylenbroeck, J.M. and Debergh, P.C. 1996. Impact of sugar concentration in vitro on photosynthesis and carbon metabolism during ex vitro acclimatization of *Spathiphyllum* plantlets. Physiol. Plant. 96:298–304.
Van Huylenbroeck, J.M., Huygens, H. and Debergh, P.C. 1995. Photoinhibition during acclimatization of micropropagated *Spathiphyllum* "Petite" plantlets. In Vitro Cell. Dev. Biol. Plant. 31:160–164.
Van Huylenbroeck, J.M., Piqueras, A. and Debergh, P.C. 1998. Photosynthesis and carbon metabolism in leaves formed prior and during ex vitro acclimatization of micropropagated plants. Plant. Sci. 134:21–30.
Van Huylenbroeck, J.M., Piqueras, A. and Debergh, P.C. 2000. The evolution of photosynthetic capacity and the antioxidant enzymatic system during acclimatization of micropropagated *Calathea* plants. Plant Sci. 155:59–66.
Waldenmaier, S. and Schmidt, G. 1990. Histologische Unterschiede zwischen in-vitro- und ex-vitro-Blättern bei der Abhärtung von Rhododendron. Gartenbauwissenschaft. 55:49–54.

Wetzstein, H.Y. and Sommer, H.E. 1982. Leaf anatomy of tissue-cultured *Liquidambar styraciflua* (*Hamamelidaceae*) during acclimatization. Amer. J. Bot. 69:1579–1586.
Wetzstein, H.Y. and Sommer, H.E. 1983. Scanning electron microscopy of in vitro-cultured *Liquidambar styraciflua* plantlets during acclimatization. J. Amer. Soc. Hort. Sci. 108:475–480.
Zacchini, M. and Morini, S. 1998. Stomatal functioning in relation to leaf age in in vitro-grown plum shoots. Plant Cell Rep. 18:292–296.