Acclimation of Plantlets to Ex Vitro Conditions: Effects of Air Humidity, Irradiance, CO$_2$ Concentration and Abscisic Acid (a Review)

J. Pospíšilová, H. Synková, D. Haisel and Š. Semorádová
Institute of Experimental Botany, Academy of Sciences of the Czech Republic
Na Karlovce 1, CZ-16000 Praha 6
Czech Republic

Keywords: chlorophyll fluorescence, micropropagation, photoinhibition, photosynthesis, stomatal conductance, transpiration rate, water relations

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 Rodgeria 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; Bruttì 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; Bruttì 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 Viitis 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 CO₂ concentration resembled those of naturally grown plants (Filia 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 lacinatum (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 (Diaz-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 (Májada 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 (PN) and in consequence low growth rate of plantlets in situ is usually due to low CO₂ concentration in tightly closed cultivation vessels for at least half of light period. The daily dynamics of CO₂ concentration is also dependent on sucrose concentration of the medium (Morini and Melai, 2003/4). Higher aeration of cultivation vessels markedly increase PN 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, PN, 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 louiseae the chlorophyll and carotenoid contents were almost three times higher 30 d after ex vitro transfer but only in newly-formed leaves (Van Huylensbroeck et al., 2000).

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

PN 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 Huylensbroeck and Debergh, 1996; Estrada-Luna et al., 2001; Seon et al., 2000). In Calathea louiseae and Spathiphyllum floribundum substantial increase in PN was measured when new leaves were fully developed (Van Huylensbroeck et al., 1998, 2000). Three weeks after ex vitro transplantation, PN of Nicotiana tabacum leaves was considerably higher that PN of leaves of plantlets grown in vitro and responses of PN, to irradiance and CO₂ concentration were quite similar to those of naturally grown plants (Pospíšilová et al., 1992, 1998). Similarly, higher PN was found in Malus pumila plants three weeks after transplantation (Diaz-Pérez et al., 1995) and more than twice as high a maximum PN 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, PN was higher under autotrophic than under heterotrophic conditions and the difference preserved after ex vitro transfer (Seon et al., 2000).

OCCURRENCE OF PHOTINOHIBITION

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 PN in situ due to insufficient CO₂ supply might
be the cause that photoinhibition 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 (F₀) (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 Huylbenbroeck et al., 1995, 2000), however, no photoinhibition was observed in plants acclimatized under low irradiance for four weeks (Van Huylbenbroeck, 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: Fv/Fm 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) Fv/Fm 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 Fv/Fm 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 grown 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.

ACKNOWLEDGEMENTS
The financial support of the GA CR (grants Nos. 522/02/1099 and 522/07/0227) is acknowledged.

Literature Cited


Preece, J.E. and Sutter, E.G., 1991. Acclimatization of micropropagated plants to the


environmental conditions and plantlet growth as affected by vessel type and stopper material. Zahradnictvi (Prague). 23:51–58.