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REVIEW

Acclimatization of micropropagated plants to *ex vitro* conditions

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Abstract

The special conditions during *in vitro* culture result in the formation of plantlets of abnormal morphology, anatomy and physiology. 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. This review is focused upon contemporary information on the changes in leaf structure, water relations and photosynthesis during acclimatization of plantlets to *ex vitro* conditions. It also describes some ways of improving plant survival and for the speeding up of acclimatization.

Additional key words: antitranspirants, chlorophyll content, chlorophyll fluorescence, cuticle, epidermis, hardening, *in vitro* and *ex vitro* growth, leaf mesophyll, net photosynthetic rate, photoautotrophy, photoinhibition, photomixotrophy, stomatal conductance, stomatal density, transpiration rate, water potential.

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Abbreviations: ABA - abscisic acid; Chl - chlorophyll; E - transpiration rate; F_m - maximum fluorescence; F_v - variable fluorescence; g_s - stomatal conductance; P_N - net photosynthetic rate; RWC - relative water content; ψ_w - leaf water potential.
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Introduction

Micropropagation has been extensively used for the rapid multiplication of many plant species. However, its more widespread use is restricted by the often high percentage of plants lost or damaged when transferred to *ex vitro* conditions (greenhouse or field).

Micropropagation of many plants is achieved through the establishment of explants, their initial growth *in vitro* being followed by transplanting into greenhouse or field. During *in vitro* culture, plantlets grow under very special conditions in relatively air-tight cultivation vessels, *e.g.*, air humidity is higher and irradiance lower than in conventional culture. The use of closed vessels in order to prevent microbial contamination decreases air turbulence which increases leaf boundary layers and limits the inflow of CO_2 and outflow of gaseous plant products from the vessels. The cultivation media are often supplemented by saccharides as carbon and energy sources. This addition decreases considerably the water potential of the medium and increases the risk of bacterial and fungal contamination. Furthermore, the plantlets are usually supplied with large doses of growth regulators. These conditions result in the formation of plantlets of abnormal morphology, anatomy and physiology (for reviews see, *e.g.*, Kozai 1991, Pospíšilová *et al*. 1992, 1997, Buddendorf-Joosten and Woltering 1994, Desjardins 1995, Kozai and Smith 1995).

After transfer from the *in vitro* to the *ex vitro* conditions the plantlets have to correct the above-mentioned abnormalities. In the greenhouse, and especially in the field, irradiance is much higher and air humidity much lower than in the vessels. Even if the water potential of the substrate is higher than the water potential of media with saccharose, the plantlets may quickly wilt as water loss of their leaves is not restricted. In addition, water supply can be limiting because of low hydraulic conductivity of roots and root-stem connections (Fila *et al*. 1998). Many plantlets die during this period. Therefore, after *ex vitro* transplantation plantlets usually need some weeks of acclimatization with gradual lowering in air humidity (*e.g.*, Preece and Sutter 1991, Kadleček 1997, Bolar *et al.* 1998). Acclimatization units have been developed with temperature, humidity, irradiance, CO_2 concentration and air flow rate controlled by computer (*e.g.*, Hayashi *et al.* 1988).

Table 1. Growth parameters of 32-d-old *in vitro* grown *Nicotiana tabacum* plantlets, and of tobacco plants 17 and 45 d after transfer from *in vitro* to *ex vitro* environment (means ± SE, $n = 3$; data from Kadleček 1997).

	Plant height [cm]	Number of leaves [plant^{-1}]	Total leaf area [cm^2 plant^{-1}]	Total dry mass [g plant^{-1}]	Shoot/root ratio [g g^{-1}]	Leaf mass/ area ratio [g m^{-2}]
In vitro	2.2 ± 0.1	6.7 ± 0.3	74 ± 3	0.14 ± 0.01	2.5 ± 0.1	11.0 ± 0.6
Ex vitro, 17 d	18.0 ± 0.9	13.3 ± 0.7	365 ± 56	0.78 ± 0.12	6.5 ± 0.3	11.9 ± 1.0
Ex vitro, 45 d	42.4 ± 1.7	22.7 ± 0.3	3002 ± 252	11.00 ± 0.45	15.3 ± 3.1	22.1 ± 1.3

In many 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). However, if the *ex vitro* transplantation of plantlets is successful, the increase in their growth can be enormous; *e.g.*, total dry mass of *Nicotiana tabacum* plants was several times higher than that of plantlets grown *in vitro*: the transplanted plants were taller, had higher dry mass of leaves, stems and roots, and larger leaf area and leaf thickness (Table 1; Pospíšilová *et al.* 1989, Kadleček 1997, Kadleček *et al.* 1998). During the acclimatization of *Spathiphyllum floribundum* plantlets, two different stages were observed: an adaptation period with slow shoot growth and root formation, followed by a period of fast growth of roots and shoots (Van Huylenbroeck and De Riek 1995).

Leaf structure

After transfer of plantlets from *in vitro* cultures to the greenhouse or field, substantial changes in leaf morphology and anatomy are observed, above all in epidermal characteristics, leaf thickness, differentiation of leaf mesophyll, and chloroplast number and structure.

Leaves from *in vitro* plantlets of *Liquidambar styraciflua* had a less developed cuticle in contrary to the well developed cuticle in leaves of transplanted and field-grown plants (Wetzstein and Sommer 1982). An increase in cuticle thickness, mass and wax content from young to adult leaves was found in both *in vitro* and *ex vitro* grown *Hedera helix* plants (Gilly *et al.* 1997). The structure and quantity of epicuticular waxes on the upper surface of *Brassica oleracea* leaves, determined two weeks after transplantation, were similar to those on seedling leaves (Grout and Aston 1977). The relative wax content on an area basis of *Liquidambar styraciflua* leaves remained unchanged whereas that in *Malus domestica* leaves decreased after acclimatization (Sutter 1988). On the other hand, in *Malus pumila* plantlets, thickness of epicuticular waxes of leaves formed *in vitro* was not affected after transfer to *ex vitro* conditions, but it was higher in newly formed leaves (Díaz-Pérez *et al*. 1995b). In *Rubus idaeus,* the number of epidermal hairs was low *in vitro*, higher in leaves formed after transplantation, and the highest in greenhouse and field grown plants (Donnelly and Vidaver 1984).

In *Liquidambar styraciflua*, *Vaccinium corymbosum* and *Nicotiana tabacum* stomatal density decreased after transplantation (Fig. 1; Wetzstein and Sommer 1983, Noé and Bonini 1996, Tichá *et al.* 1999). After a short period of acclimatization stomatal density on adaxial and abaxial leaf epidermes of *Nicotiana tabacum* plants was not yet changed, but later the total numbers of stomata per leaf were more than doubled in *ex vitro* plants due to enormous leaf area growth after transfer to *ex vitro* conditions (Pospíšilová *et al.* 1998). On the other hand, in *Prunus serotina* and *Rhododendron* spp. plants stomatal density increased and stomata pore length decreased after transplantation (Waldenmaier and Schmidt 1990, Drew *et al*. 1992). Leaves from *in vitro* grown *Prunus cerasus*, *Vaccinium corymbosum* or *Nicotiana tabacum* plantlets had ring-shaped stomata, but in leaves of *ex vitro* transferred plants stomata were elliptical (Fig. 1; Marín *et al*. 1988, Noé and Bonini 1996, Tichá *et al.* 1999). Guard cells of *in vitro* grown *Rosa hybrida* 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. 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^{2+} ions 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 guard cells of *in vitro* grown plantlets (Sallanon *et al.* 1991). In *Prunus cerasifera* plantlets, the ability of stomata to close in response to abiotic factors and to re-open after treatment was much higher in young than in adult and old leaves which might be also important during transfer to *ex vitro* conditions (Zacchini and Morini 1998). In guard cells of *in vitro* grown *Solanum phureja* plantlets, consistently more chloroplasts were found than in those of *ex vitro* grown plants (Singsit and Veilleux 1991).

Fig. 1. Stomatal characteristics on adaxial and abaxial leaf sides of *Nicotiana tabacum in vitro* grown plantlets, and tobacco plants 11 and 45 d after *ex vitro* transfer (from Tichá *et al.* 1999).

In *Nicotiana tabacum* plantlets grown *in vitro*, the leaf mesophyll consisted of one layer of loosened, poorly differentiated palisade parenchyma and two to three layers of spongy parenchyma (Tichá and Kutík 1992), and in *Vaccinium corymbosum* of

only one or two layers of disorganised spongy parenchyma (Noé and Bonini 1996). Large intercellular spaces were present in the mesophyll (Brainerd *et al.* 1981, Donnelly and Vidaver 1984, Johansson *et al.* 1992, Tichá and Kutík 1992, Dami and Hughes 1995, Noé and Bonini 1996). In *Brassica oleracea*, one layer of palisade mesophyll cells was developed three weeks after transplantation into a greenhouse (Grout and Aston 1978). In acclimatized *Liquidambar styraciflua* plants, thicker leaves than in *in vitro* grown plantlets, and mesophyll tissue differentiated into palisade and spongy parenchyma were found; the spongy parenchyma had fewer and smaller air-spaces (Wetzstein and Sommer 1982). Similar results were found by Brainerd *et al.* (1981) in *Prunus insititia*, Donnelly and Vidaver (1984) in *Rubus idaeus*, Fabbri *et al.* (1986) in *Fragaria* × *ananassa*, Lee *et al.* (1988) in *Liquidambar styraciflua*, Waldenmaier and Schmidt (1990) in *Rhododendron* spp., Johansson *et al.* (1992) in *Rosa odorata* × *Rosa damascena*, and Noé and Bonini (1996) in *Vaccinium corymbosum*.

Mesophyll cells of *in vitro* grown *Liquidambar styraciflua* plantlets had large vacuoles, limited cytoplasmic content, and flattened chloroplasts with irregularly arranged internal membrane systems (Wetzstein and Sommer 1982); internal chloroplast organisation was strongly light dependent (Lee *et al*. 1985). On the contrary, the chloroplasts of acclimatized *Liquidambar styraciflua* plants had well developed grana, osmiophillic globules and frequently starch granules (Wetzstein and Sommer 1982).

Water relations

The retardation in development of cuticle, epicuticular waxes and functional stomatal apparatus during *in vitro* culture cause high stomatal and cuticular transpiration rates (E) of leaves in plantlets taken out of the cultivation vessels. 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, Santamaria *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 to close (Santamaria *et al*. 1993, Santamaria and Kerstiens 1994).

During acclimatization to *ex vitro* conditions, E typically gradually decreases because stomatal regulation of water loss becomes more effective and cuticle and epicuticular waxes develop (Grout and Aston 1977, Wardle *et al.* 1979, Short *et al.* 1984, Pospíšilová *et al.* 1987, 1988, 1997, Baroja 1993, Baroja *et al*. 1995, Fila *et al*. 1998). Stomatal E of *Nicotiana tabacum* plants leaves measured three weeks after transplantation was similar to stomatal E of tobacco seedling leaves. Cuticular E of leaves of transplanted tobacco plants was slightly higher than that of tobacco seedlings, thus the epicuticular waxes developed after transplanting more slowly than effective stomatal regulation (Pospíšilová *et al.* 1987, 1988). Similar results were found in *Brassica oleracea* (Grout and Aston 1977, Wardle *et al.* 1979), *Leucaena leucocephala* (Dhawan and Bhojwani 1987), *Prunus serotina* (Drew *et al.* 1992),

Santpaulia spp. (Short *et al.* 1984) and *Solanum laciniatum* (Conner and Conner 1984) but acclimatization was slower than in tobacco. Stomatal and cuticular E similar to those in seedlings were achieved between 8 and 12 weeks after transplanting. On the other hand, low stomatal conductance (g_s) observed in *Malus pumila* plantlets increased after transfer to *ex vitro* conditions (Díaz-Pérez *et al*. 1995b).

Immediately after transplantation, visible wilting is usually observed and corresponding low relative water content (RWC) is found. However, the water status of plants may stabilize after some days or weeks. RWC of about 88 % was, *e.g*., observed in *Malus pumila* plants three weeks after transplantation (Díaz-Pérez *et al*. 1995b). After transfer to *ex vitro* conditions, water potential (ψ_w) of *Nicotiana tabacum* plantlets grown on medium without saccharose decreased whereas ψ_w of *Solanum tuberosum* plantlets grown on that with saccharose increased (Pospíšilová *et al*. 1988).

Photosynthetic parameters

Chlorophyll (Chl) *a* and Chl *b* contents increased after transplantation (Trillas *et al*. 1995, Rival *et al.* 1997, Synková 1997, Pospíšilová *et al*. 1998). The same effect was evident in originally photoautotrophically grown *Nicotiana tabacum* plants but in originally photomixotrophically grown plants an abrupt decrease in Chl *a* and Chl *b* contents during the first week after transplantation followed by a slow increase was found (Fig. 2; Kadleček 1997, Kadleček *et al.* 1998). Chl *a* fluorescence parameters (*e.g*., variable to maximum fluorescence ratio F_v/F_m, actual quantum yield of photosystem 2) increased after transfer of *Elaeis guineensis* and *Nicotiana tabacum* plantlets to *ex vitro* conditions (Kadleček 1997, Rival *et al.* 1997, Synková 1997). However, decreased F_v/F_m in *Spathiphyllum floribundum* was observed during the first days after transplantation by Van Huylenbroeck and Debergh (1996).

Net photosynthetic rate (P_N) in *Solanum tuberosum* and *Spathiphyllum floribundum* plants decreased in the first week after transplantation and increased thereafter (Baroja 1993, Baroja *et al.* 1995, Van Huylenbroeck and Debergh 1996). After transplantation, the $^{14}CO_2$ uptake by persistent leaves of *Fragaria* and *Rubus idaeus* was similar to that in plantlets grown *in vitro* or was slightly increased, and a significantly increased $^{14}CO_2$ uptake was found only in newly formed leaves (Short *et al*. 1984, Deng and Donnelly 1993). In *Calathea louisae* the *in vitro* formed leaves were not able to photosynthesize during the first days after transfer, but in *Spathiphyllum floribundum* the *in vitro* formed leaves were photosynthetically competent and normal source-sink relations were observed. Nevertheless, in both plant species, substantial photosynthetic activities were measured when new leaves were fully developed (Van Huylenbroeck *et al.* 1998). Two weeks after *ex vitro* transplantation of *Nicotiana tabacum* plantlets, P_N, maximum photochemical efficiency, and actual quantum yield of photosystem 2 were higher than in plantlets grown *in vitro* (Pospíšilová *et al*. 1998). Similarly, higher P_N was found in *Malus pumila* plants three weeks after transplantation (Díaz-Pérez *et al*. 1995b) and more

than twice as high a maximum P_N in *Vitis vinifera* × *Vitis berlandieri* rootstocks one month after transplantation (Fila *et al.* 1998). 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). The above mentioned results suggest that photoinhibition might be the cause of the transient decrease in photosynthesis after transplantation. 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 decreased osmotic potential by addition of mannitol (Sallanon *et al*. 1998). In *Nicotiana tabacum* plantlets acclimatized to *ex vitro* conditions under slight shade in a greenhouse where irradiance varied during the day and daily maximum was usually 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. The degree of de-epoxidation of xanthophyll cycle pigments was not changed (Pospíšilová *et al*. 1999). Similar results in composition of

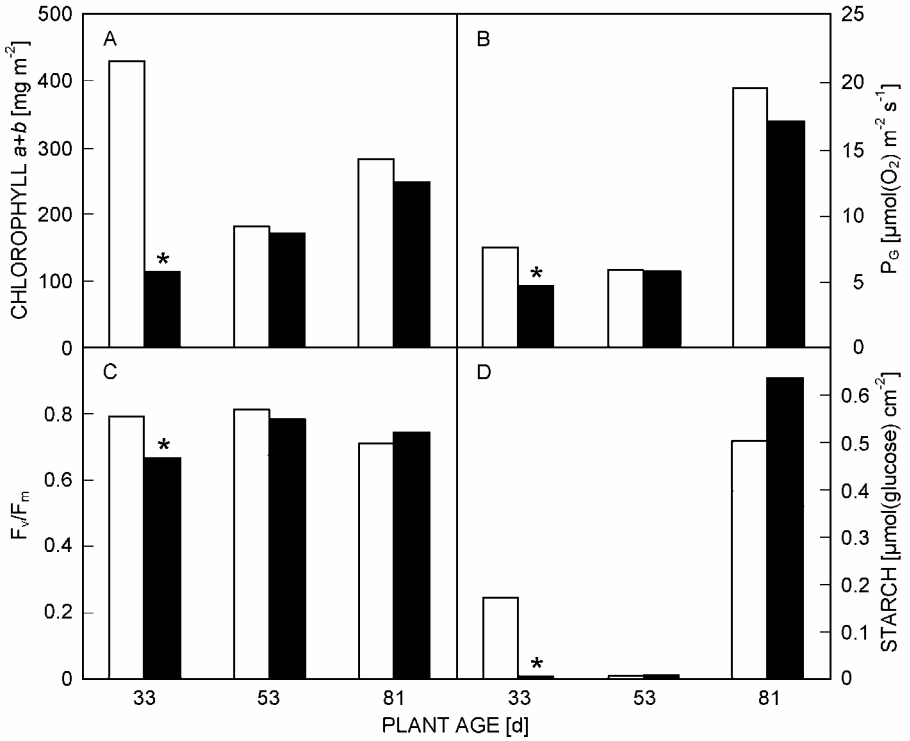

Fig. 2. Chlorophyll *a*+*b* content (*A*), gross photosynthetic rate (P_G) at saturated irradiance and CO_2 concentration (*B*), maximum photochemical efficiency F_v/F_m in dark adapted leaves (*C*), and starch content (*D*) during transfer of tobacco plants from *in vitro* to *ex vitro* conditions. Means of three plants. On the 35th day of culture the plants were transferred from *in vitro* to the greenhouse, on the 55th day from the greenhouse to open air. *Asterisks* indicate means significantly different at $P = 0.05$. *Open columns* - originally photomixotrophically grown plants, *full columns* - originally photoautotrophically grown plants (adapted after Kadleček *et al.* 1998).

xanthophyll cycle pigments and β-carotene during acclimatization of *Nicotiana tabacum* plants in an air-conditioned chamber at low irradiance (200 μmol m^{-2} s^{-1})

were found, but content of light-harvesting pigments (neoxanthin and lutein) was increased from the 3rd day after transplantation. On the contrary, during acclimatization at high irradiance (700 μmol m^{-2} s^{-1}), content and de-epoxidation state of xanthophyll cycle pigments, and content of neoxanthin were much higher. Changes in composition of other carotenoids were dependent on the presence or absence of saccharose in the medium during *in vitro* culture. In plantlets grown *in vitro* without saccharose, contents of lutein and β-carotene increased, in plantlets grown *in vitro* with saccharose they were not changed (Haisel, unpublished).

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. Furthermore, in the *in vitro* photoinhibited, photoautotrophically grown plantlets a recovery from photoinhibition appeared over several days. After transfer to the open air, F_v/F_m decreased transiently as a response to the abrupt increase in irradiance. The increase in photosynthetic capacity 46 d after transfer was accompanied by an increasing starch content in the leaves (Fig. 2; Kadleček 1997, Kadleček *et al.* 1998).

Possibilities of improvement

Hardening of plantlets *in vitro*: The hardening of plantlets *in vitro* by *1*) decreasing air humidity, *e.g.,* by using lids permeable for water vapour or by bottom cooling, *2*) increasing irradiance, or 3) increasing CO_2 concentration by forced ventilation (Wardle *et al*. 1983, Ziv 1986, Short *et al*. 1987, Capellades *et al.* 1990, Roberts *et al*. 1990, Smith *et al*. 1990, 1992, Ghashghaie *et al*. 1992, Deng and Donnelly 1993, Yue *et al*. 1993, Cassells and Walsh 1994, Díaz-Pérez *et al*. 1995a,b, Kanechi *et al*. 1998) can ameliorate wilting of plants after transplantation. However, these procedures might lead to a quick drying out of the cultivation medium and to an impairment in plantlet growth (Wardle *et al.* 1983, Ghashghaie *et al*. 1991, Sallanon and Maziere 1992, 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, Pospíšilová 1996), paclobutrazol [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pentan-3-ol], indolebutyric acid, or 6-benzyl-aminopurine (Smith *et al*. 1992, Pospíšilová *et al*. 1993, Eliasson *et al*. 1994) into the cultivation medium, or by decreased osmotic potential of the medium by polyethylene glycol (Zaid and Hughes 1995, Dami and Hughes 1997). Acclimatization can be also improved by hormonal stimulation of root development (Van Telgen *et al.* 1992, Díaz-Pérez *et al*. 1995a). Concentrations of saccharose and agar in the medium can also affect subsequent acclimatization to *ex vitro* conditions, *e.g.*, of *Brassica napus*, *Eucalyptus camaldulensis*, *Nicotiana tabacum*, *Rosa hybrida, Spathiphyllum floribundum, Triticum aestivum*, and *Vitis vinifera* (Kirdmanee *et al*. 1995, Genoud-Gourichon *et al.* 1996, Van Huylenbroeck and Debergh 1996, Kadleček 1997, Synková 1997, Fila *et al*. 1998, Voráčková *et al*. 1998).

Photoautotrophic growth of plantlets on medium without saccharides enables the development of fully functional photosynthetic apparatus. These plantlets usually need elevated CO_2 concentration and higher irradiance than conventionally used (for reviews see, *e.g.*, Kozai 1991, Pospíšilová *et al*. 1992, 1997, Buddendorf-Joosten and Woltering 1994, Jeong *et al*. 1995, Kozai and Smith 1995, Tichá 1996, Kubota *et al*. 1997). The CO_2 enrichment can be achieved either by using a gas permeable film for vessel closure and increasing CO_2 concentration around the cultivation vessels (*e.g.*, Tichá 1996, Solárová and Pospíšilová 1997), or by direct supply of CO_2 into the vessels, *e.g.,* by forced ventilation. Individual growth parameters are affected by increased CO_2 concentration differently in individual plant species and ontogenetic stages (*e.g.*, Figueira *et al*. 1991, Fournioux and Bessis 1993), and interactions with other environmental factors, especially irradiance and medium composition, have to be considered (Kozai and Iwanami 1988, Kozai *et al*. 1988a,b, Kirdmanee *et al*. 1995, Sallanon *et al*. 1995, Düring and Harst 1996, Seko and Nishimura 1996, Seko and Kozai 1997). Elevated CO_2 concentration enhanced photosynthesis of *Brassica oleracea* plantlets under photoautotrophic conditions, but inhibited it under photomixotrophic conditions (Kanechi *et al*. 1998).

In photoautotrophically cultured *Nicotiana tabacum* plantlets, growth of plants was significantly impaired during acclimatization to *ex vitro* conditions as compared to plants cultured *in vitro* on medium with saccharose, however, photosynthetic parameters were not affected (Kadleček *et al.* 1998).

Photoinhibition may restrict the use of fully photoautotrophic cultures because it was observed under rather low irradiance, *e.g.*, in *Nicotiana tabacum* (Tichá *et al*. 1995, 1998) and in *Gardenia jasminoides* (Serret *et al*. 1996). In both plant species the susceptibility to photoinhibition was dependent on saccharose concentration in the medium: 3 % saccharose decreased susceptibility to photoinhibition in *Nicotiana tabacum* plantlets whereas it increased in *Gardenia jasminoides* plantlets.

Nevertheless, the increased CO_2 concentration accompanied by increased irradiance and/or decreased relative humidity during *in vitro* culture promoted

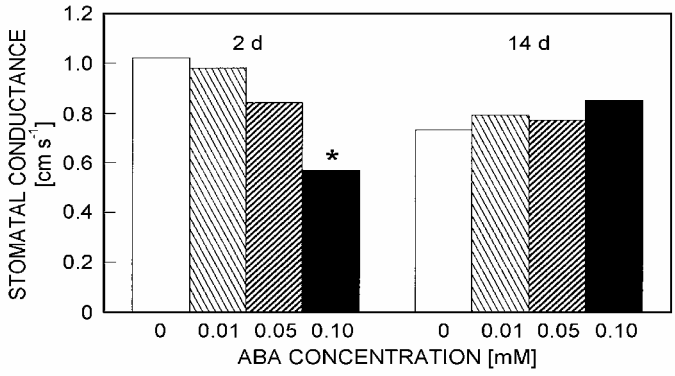

Fig. 3. Effect of the antitranspirant abscisic acid (ABA) on leaf stomatal conductance of *Nicotiana tabacum* plantlets. ABA in concentrations 0, 0.01, 0.05, or 0.10 mM was applied on the first day after transfer of plantlets from *in vitro* to *ex vitro* conditions and stomatal conductance was measured 2 and 14 d after transfer. *Asterisks* indicate means significantly different at $P = 0.05$ (data from Pospíšilová *et al*. 1998).

survival and growth of *Asparagus officinalis, Eucalyptus camaldulensis, Ficus benjamina, Fragaria* × *ananassa,* and *Rubus idaeus* plantlets during acclimatization

to *ex vitro* conditions (Laforge *et al*. 1991, Deng and Donnelly 1993, Kirdmanee *et al.* 1995, Matysiak and Nowak 1998). However, in banana plantlets elevated CO_2 concentration increased dry matter accumulation *in vitro,* but 20 d after *ex vitro* transfer the dry mass of plants was not significantly different as the growth rate of *ex vitro* plants grown under elevated CO_2 concentration *in vitro* was slower than of those cultured under ambient CO_2 concentration (Navarro *et al*. 1994).

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* (Sutter and Hutzel 1984). Although *DC-200* had the greatest effect in reducing transpiration, it had adverse

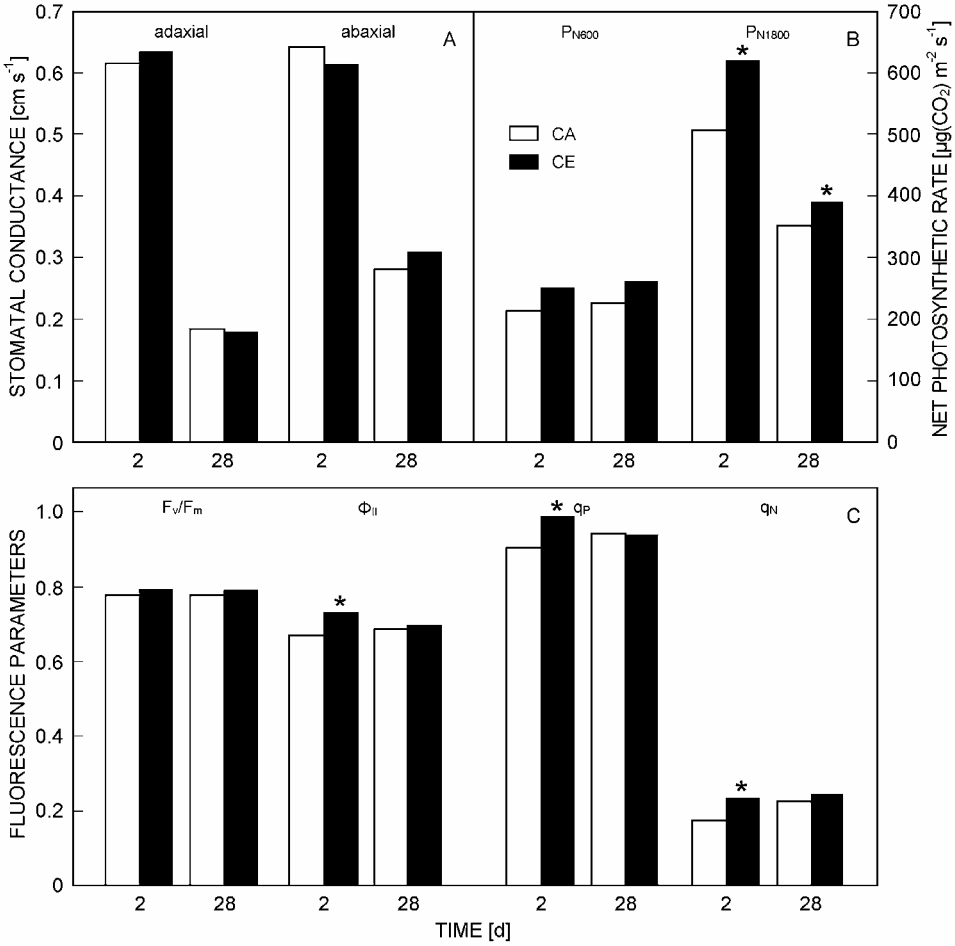

Fig. 4. Adaxial and abaxial stomatal conductances (*A*), net photosynthetic rate measured at 600 or 1800 mg(CO_2) m^{-3} (*B*), and chlorophyll *a* fluorescence kinetic parameters [variable to maximum fluorescence ratio (F_v/F_m), quantum yield of photosystem 2 (ϕ_{II}), photochemical quenching (q_P), non-photochemical quenching (q_N)] (*C*) in *Nicotiana tabacum* plantlets grown after transfer from *in vitro* to *ex vitro* conditions 2 or 28 d under ambient (CA = 600 mg m^{-3}) or elevated (CE =1800 mg m^{-3}) CO_2 concentration. *Asterisks* indicate means significantly different at $P = 0.05$ (data from Pospíšilová *et al*. 1999).

effects on plant growth. All other antitranspirants were ineffective in improving vigour of plants.

Addition of ABA to the substrate immediately after transplantation alleviated "transplantation shock" of *Nicotiana tabacum* plants (Pospíšilová *et al*. 1998). Stomatal conductance of leaves was markedly decreased. Later, g_s decreased more quickly in control than in ABA-treated plants. After two or three weeks, g_s of transplanted plants was significantly lower than that of plantlets grown *in vitro* but similar in control and ABA-treated plants (Fig. 3). ABA-treatment had slight positive or insignificant effects on photosynthetic parameters and enhanced plant growth.

Elevated CO_2 concentration can decrease g_s 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 (Fig. 4; 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).

Conclusions

1) The abnormalities in morphology, anatomy and physiology of plantlets cultivated *in vitro* can be repaired after transfer to *ex vitro* conditions. However, many plant species need gradual changes in environmental conditions to avoid desiccation losses and photoinhibition.
2) During acclimatization to *ex vitro* conditions, leaf thickness generally increases, leaf mesophyll progresses in differentiation into palisade and spongy parenchyma, stomatal density decreases and stomatal form changes from circular to elliptical one.
3) The most important changes include development of cuticle, epicuticular waxes, and effective stomatal regulation of transpiration leading to stabilization of water status.
4) For photosynthetic parameters it seems very important at which conditions *in vitro* plantlets have been grown. According to this, transfer can be accompanied with a transient decrease in photosynthetic parameters. Further, an increase in chlorophyll content, maximum photochemical efficiency, actual quantum yield of photosystem 2, and net photosynthetic rate is usually observed in dependence on the environmental conditions during acclimatization.
5) Acclimatization can be speed up by hardening of plantlets *in vitro* or after transplantation by decreasing the transpiration rate by antitranspirants including ABA, or by increasing photosynthetic rate by elevated CO_2 concentration.

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