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Impact of *in vitro* cultivation conditions on stress responses and on changes in thylakoid membrane proteins and pigments of tobacco during *ex vitro* acclimation

P. HOFMAN[a]*, D. HAISEL*, J. KOMENDA**, M. VÁGNER*, I. TICHÁ***, C. SCHÄFER**** and V. ČAPKOVÁ*

*Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 135, CZ-16502 Praha 6, Czech Republic**
*Institute of Microbiology, Academy of Sciences of the Czech Republic, CZ-37981 Třeboň, Czech Republic***
*Charles University, Faculty of Science, Department of Plant Physiology, Viničná 5, CZ-12844 Praha 2, Czech Republic****
*Lehrstuhl für Pflanzenphysiologie, Universität Bayreuth, Universitätsstrasse 30, D-95440 Bayreuth, Germany*****

Abstract

Four physiologically and phenotypically diversified tobacco (*Nicotiana tabacum* L. cv. Samsun) plantlet variants had been generated by cultivation on media either lacking or containing sucrose (0 and 3 %, m/v) under two different photon flux densities (PFD), 50 μmol m^{-2} s^{-1} (LL) and 200 μmol m^{-2} s^{-1} (HL). Plantlets were transferred into soil without any pre-acclimation and grown either under PFD of 200 μmol m^{-2} s^{-1} or 700 μmol m^{-2} s^{-1}. Sucrose feeding *in vitro* resulted in reduced degree and duration of wilting after transfer. The highest readiness for *ex vitro* acclimation was found in 3 % HL plants, in which changes of photosynthetic apparatus and stress responses were the smallest. On the contrary, the steepest decline of Fv/Fm ratio on the first day after transplantation, doubled chlorophyll content and almost tripled D1/LHC 2 ratio after 7 d of *ex vitro* growth under 700 μmol m^{-2} s^{-1} characterized 0 % HL plants, which had suffered chronic photoinhibition *in vitro*. Remarkably high abscisic acid content at the end of *in vitro* cultivation and during acclimation as well as increased synthesis of both D1 and LHC 2 proteins even at the end of analyzed acclimation period were found only in 0 % LL plants. Increase of D1/LHC 2 ratio and chlorophyll contents demonstrate that *in vitro* developed leaves of all plant variants are able to acclimate to new environment. The most surprising result in the whole study is the drop of D1 protein synthesis in all plants on the 3rd day. Five times decline of photoprotection level of xanthophylls in plants after *ex vitro* transfer into the same PFD showed stress character of *in vitro* cultures.

Additional key words: abscisic acid, chlorophyll, D1 protein, fluorescence, *Nicotiana tabacum*, photoautotrophy, photoinhibition, photomixotrophy, stress, xanthophyll cycle.

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Abbreviations: A - antheraxanthin; ABA - abscisic acid; Chl *a* - chlorophyll *a;* Chl *b* - chlorophyll *b*; F_m - maximum fluorescence; F_0 - initial fluorescence; F_v - variable fluorescence; HPFD - high photon flux density *ex vitro* (700 μmol m^{-2} s^{-1}); HL - high PFD *in vitro* (200 μmol m^{-2} s^{-1}); HPLC - high performance liquid chromatography; LHC 2 - light harvesting complex 2; LL - low PFD *in vitro* (50 μmol m^{-2} s^{-1}); LPFD - low photon flux density *ex vitro* (200 μmol m^{-2} s^{-1}); PFD - photon flux density; PS 2 - photosystem 2; RC 2 - reaction centre 2; SDS PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMP - thylakoid membrane proteins; V - violaxanthin; Z - zeaxanthin.

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[a] Corresponding author: fax: (+420) 2 20390461, e-mail: hofman@ueb.cas.cz

Introduction

Broad application of plant biotechnologies for crop improvements stresses the necessity to study and overcome the critical point of plant micropropagation, the *ex vitro* transfer and subsequent acclimation of plantlets.

Most of *in vitro* cultures have relied on sugar feeding, however, photoautotrophic cultivation on sugarless medium appears to have some advantages (*e.g.*, Kozai 1991). Species-specific sink demands and sugar concentration in which downregulation of photosynthesis is induced, are the reasons for existence of both positive and negative effects of exogenous sugars on *in vitro* as well as on *ex vitro* growth (Kovtun and Daie 1995, Genoud-Gourichon *et al.* 1996, Koch 1996, Furbank *et al.* 1997). Moreover, sugars serve as osmotic agents, nutrient and energy reserves and specific regulators of gene expression (Galzy and Compan 1992, Paul and Stitt 1993, Furbank *et al.* 1997). All these rather inconsistent data justify further investigation of the impact of sugars in *in vitro* cultivation media on *ex vitro* acclimation.

In vitro cultivation of tobacco nodal cuttings at two different photon flux densities (PFD) either in absence or in presence of sucrose had resulted in four morphologically and physiologically distinct plantlet variants. Positive effect of exogenous sucrose on the development of photosynthetic apparatus and on the prevention of harmful effects of high light (HL) had been demonstrated while cultivation without sucrose at HL led to photoinhibition (Tichá *et al.* 1998). Leaves of sucrose fed variants contained up to forty times higher contents of hexoses (Tichá *et al.* 1998). The plantlet variants provided an interesting model to study their ability to overcome the stress caused by transfer from agar medium into soil and by exposure to high PFD. To distinguish between the transplantation shock and high irradiance stress during acclimation, plantlets were *ex vitro* exposed either to high PFD or to the same PFD as they were cultivated *in vitro*.

Materials and methods

Plants: Nodal cuttings were derived from tobacco plants (*Nicotiana tabacum* L. cv. Samsun) and cultivated on Murashige and Skoog's (MS) agar medium (*Sigma-Aldrich*, Praha, Czech Republic) either supplemented with 30 g dm^{-3} of sucrose (3 %) or without sucrose (0 %). Each nutrition variant was combined with two light regimes, *i.e.* low PFD (LL, 50 μmol m^{-2} s^{-1}) or high PFD (HL, 200 μmol m^{-2} s^{-1}) under 16-h photoperiod and 25/18 °C day/night temperature. The effect of these four combinations of culture conditions (3 % HL, 3 % LL, 0 % LL, 0 % HL) on plantlet *in vitro* growth had been described in detail in Tichá *et al.* (1998) and Haisel *et al.* (2001). After 39 d tobacco plantlets were transplanted into soil and exposed to PFD of 200 μmol m^{-2} s^{-1} (LPFD) or PFD of 700 μmol m^{-2} s^{-1} (HPFD). Incident PFD was measured with *LI-189* quantum meter equipped with *LI-190SA* quantum sensor (*Li-COR*, Lincoln, USA). The photoperiod and temperature were the same as *in vitro*. The plants were irrigated regularly. There were no precautions taken to reduce transpiration. During the first seven days of *ex vitro* culture, the last three *in vitro* fully developed leaves were harvested for physiological and biochemical analyses.

Chlorophyll fluorescence measurements: Chlorophyll fluorescence of the upper leaf side was measured with pulse amplitude modulation fluorometer (*PAM, Walz*, Effeltrich, Germany). Minimum (F_0) and maximum (F_m) fluorescence were determined after 30 min of dark. Maximum photochemical efficiency of PS 2 [$(F_m - F_0)/F_m = F_v/F_m$] was calculated according to Schreiber *et al.* (1994). Two leaves per plant were analyzed for each acclimation period and all measurements were repeated three times.

Pigment analyses: Leaf discs (6 cm^2) were extracted with acetone. The pigments were separated by HPLC (*Spectra-Physics*, San José, USA), using a reversed phase column (150 mm × 3 mm *Sepharon SGX C18*, 5 μm particle size, *Tessek*, Praha, Czech Republic) and acetonitrile/methanol/water (80:12:6, v/v/v) solvent system followed by 100 % methanol. The gradient was run from 8 to 12 min at a flow rate of 1 cm^3 min^{-1} and the detection wavelength was 445 nm.

Protein analyses: For the isolation of thylakoid membranes three leaf discs of total area of 4 cm^2 were grinded in liquid nitrogen and extracted with buffer of Leto and Arntzen (1981). Thylakoid membrane proteins (TMP) were prepared for electrophoresis as described by Tichá *et al.* (1998). Proteins were quantified according to Schäffner and Weissmann (1973).

Proteins were separated by SDS PAGE on 15 % polyacrylamide gel containing 6 M urea. Electrophoresis was ran for 8 h under 12 mA per slab gel on *Biometra* unit (Göttingen, Germany) cooled to 9 °C. Separated proteins were silver stained or Western-blotted on 0.2 μm pore size nitrocellulose membrane (*BioRad*, Hercules, USA). The blots were developed with polyclonal antibodies to D1 protein (generous gift from R. Barbato,

Padua, Italy) and to LHC 2 proteins (kind gift from K. Apel, Zürich, Switzerland) using goat-anti-rabbit alkaline phosphatase conjugate as a secondary antibody. Twenty μg or 3-6 μg protein aliquots were loaded per lane for silver staining and for immunodetection, respectively. The rate of protein synthesis was determined by *in vivo* ^{14}C pulse labelling. Leaf discs were incubated with ^{14}C-amino acid mixture (*Amersham*, Praha, Czech Republic, specific activity 1.85 GBq milliatom^{-1}(carbon), diluted with water to 1 MBq(^{14}C) cm^{-3}, under shaking (40 cycles min^{-1}) at room temperature. Then the leaf discs were rinsed with water, gently wiped and frozen in liquid nitrogen. Membrane proteins were extracted and separated as described above. Uptake and incorporation of ^{14}C amino acids were determined using scintillation counter *LSC Packard-TRI CARB 250 0 TR*, (San Francisco, USA) in standard procedure (Čapková *et al.* 1983). The radioactivity of individual protein bands was assessed by means of Phosphor Imager SF (*Molecular Dynamics*, Shepherdstown, USA) and *ImageQuant Programme*.

Abscisic acid content: Approximately 3 g (fresh mass) of leaves were powdered in liquid nitrogen and then extracted in 30 cm^3 of 80 % (v/v) twice distilled methanol (*Fluka,* Bochs, Switzerland) containing 100 mg dm^{-3} butylated hydroxytoluene as an antioxidant. Extraction was performed in darkness at 5 °C overnight. Following steps were performed in dim light. Extract was filtrated through *Whatman* filter paper grade 1 (*Whatman International Ltd.*, Maidstone, UK) and then vacuum dried. Ten cm^3 of 0.5 M K_2HPO_4 were added to the sample to reach pH of approximately 8.5. The sample was then partitioned three times against double distilled ether. Water fractions were loaded on *Polyclar AT* column (20 cm^3 *Polyclar* in glass tube with inner diameter 8 mm), equilibrated with 0.1 M K_2HPO_4 and eluted with 20 cm^3 of the same buffer. Eluates were acidified with 2.8 M phosphoric acid to pH 2.7 and partitioned against ether. Ether fractions were collected, dried in a speed-vac concentrator (*Thermo Savant*, New York, USA) and methylated with 300 mm^3 of ethereal diazomethane solution at room temperature for 30 min. The samples were again vacuum dried and finally dissolved in 200 mm^3 of ethyl acetate (U.V. grade, *Fluka,* Bochs, Switzerland). ABA concentration was determined using gas chromatography (*GC HP 5890,* Avondale, USA) equipped with *OV-1* column and electron capture detector. Accuracy of the method had been confirmed by mass selective detector (*HP 5970,* Avondale, USA).

Results

The *ex vitro* transfer from agar media to soil is associated with a decrease of air humidity and an increase in PFD. These changes disturb plant water regime. Despite this, all experimental plants survived (Fig. 1). Previous sugar feeding *in vitro* resulted in less severe wilting. Shedding one or two oldest leaves was observed only in photoautotrophic variants under HPFD. In all experiments, HPFD induced stronger plant responses than LPFD, and therefore, mainly results concerning *ex vitro* acclimation under HPFD are presented.

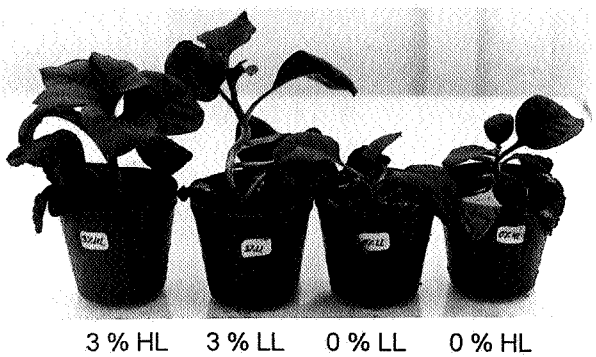

Fig. 1. Four experimental variants, 3 % HL, 3 % LL, 0 % LL and 0 % HL, of tobacco plants after 3 d of *ex vitro* growth under HPFD (700 μmol m^{-2} s^{-1}). Stem cuttings were cultivated *in vitro* for 39 d on media containing (3 %) or lacking (0 %) sucrose under irradiance of 200 μmol m^{-2} s^{-1} (HL) or 50 μmol m^{-2} s^{-1} (LL).

Maximum photochemical efficiency of PS 2, expressed as variable to maximum fluorescence (F_v/F_m), decreased significantly in plants of all variants on the first day after transfer (Fig. 2*A*). The highest decline down to value 0.6 was found in 0 % HL plants at HPFD. Since the 2nd day F_v/F_m ratios increased to about 0.8 in all plant variants and they remained stable for the rest of acclimation period. The only exception was a decline of F_v/F_m in 3 % LL plantlets on the 4th day of acclimation at HPFD.

A decline in D1/LHC 2 proportion was found in all variants on the 1st acclimation day, however, it was more pronounced in LL plantlets than in HL ones (Fig. 2*B*). Since the 1st day D1/LHC 2 ratio was increasing in all plant variants and after 7 d its values were higher than prior the transfer. During 7-d acclimation the photoinhibited 0 % HL plants increased their D1/LHC 2 ratio 3 times so they caught up with plants that had been cultivated on sucrose media.

The role of thylakoid membrane proteosynthesis in overcoming transfer stress and in the acclimation was assessed by ^{14}C-pulse labelling leaf discs. Transient stress response on the 1st day after transfer resulted in about 50 % decline of ^{14}C amino acids uptake (data not shown) and in corresponding lowering their incorporation into proteins (Fig. 2*C*). D1 protein was intensively synthesized in all variants. An increase of D1/TMP synthesis ratio was found in all plants (Fig. 2*C*), however,

D1/LHC 2 synthesis ratio increased only in plants which had been cultivated on sugarless media (Fig. 2*D*). An unexpected temporary change in the synthesis patterns of TMP occured in all variants on the 3rd acclimation day. The synthesis of D1 protein distinctly dropped and a set of new unidentified proteins appeared. Subsequently, the original protein synthesis pattern was restored (Figs. 2*C*, 3). Preferential synthesis of D1 (Fig. 2*C*) as well as of LHC 2 proteins (Figs 2*C*, *D*) was found in 0 % LL plants on the 7th day of acclimation. All these changes in TMP synthesis were highly reproducible. Modulation effect of PFD on the synthesis of thylakoid membrane proteins during the acclimation period was apparent. Distinctly higher synthesis of LHC 2 under LPFD was noticeable in all variants (Fig. 3).

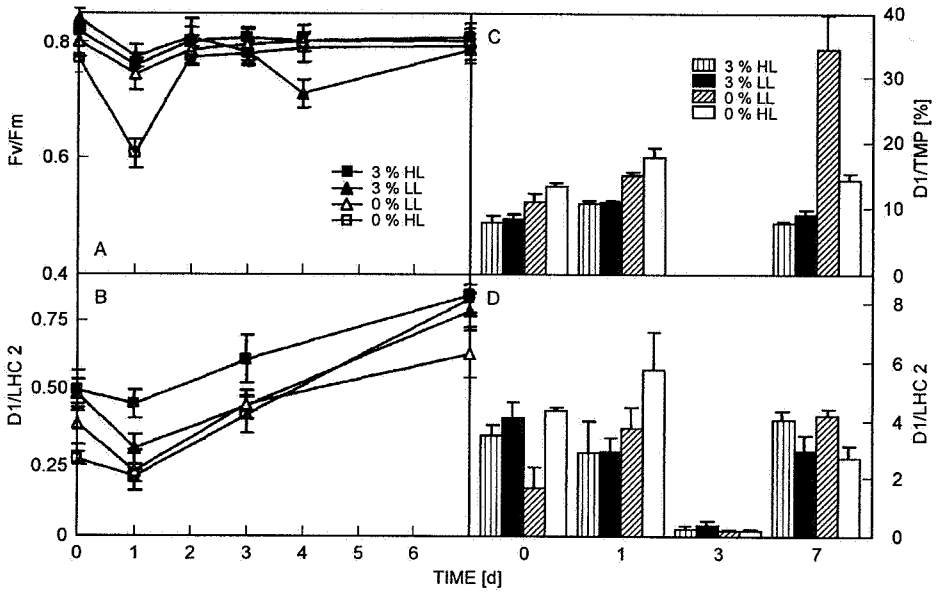

Fig. 2. Photochemical efficiency of PS 2 (Fv/Fm) of tobacco leaves (*A*), proportions of D1 to LHC 2 in thylakoid membrane proteins of tobacco leaves estimated from immunoblot data (*B*), ratios of *de novo* synthetized D1 to TMP (*C*) and *de novo* synthetized D1 to LHC 2 (*D*) calculated by means of *Image Quant Programme* at the end of *in vitro* cultivation (0) and during *ex vitro* acclimation under HPFD (700 μmol m^{-2} s^{-1}). The data are means ± SE of three independent experiments.

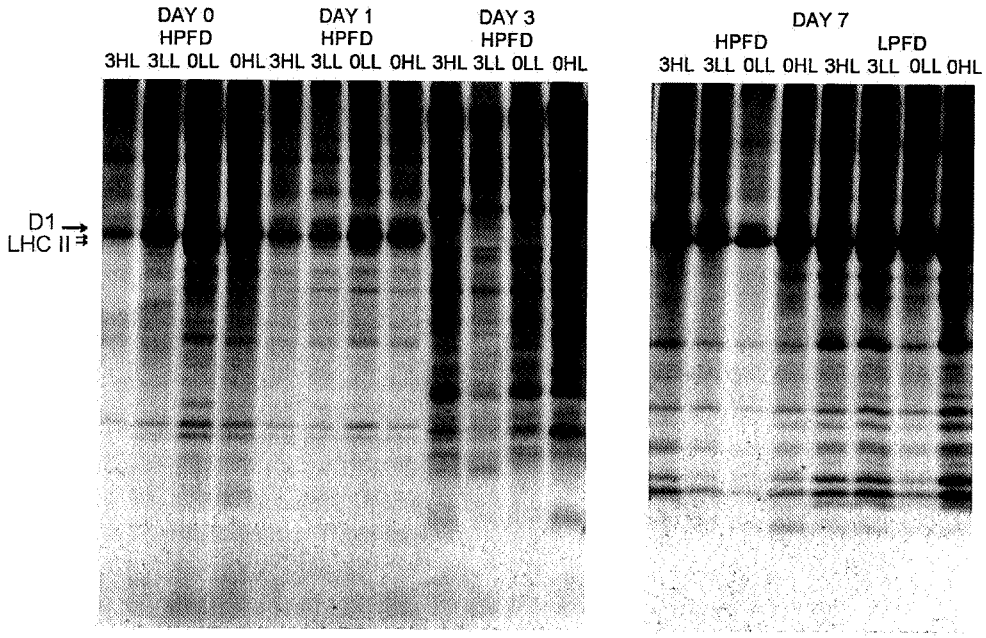

Fig. 3. *De novo* synthesis of thylakoid membrane proteins (TMP) in tobacco leaves during *ex vitro* acclimation. SDS PAGE patterns of ^{14}C amino acids pulse labelled TMP fraction. Plants were exposed to HPFD (700 μmol m^{-2} s^{-1}) or to LPFD (200 μmol m^{-2} s^{-1}).

During acclimation period the content of Chl *a+b* increased in all variants (Fig. 4*A*). As high as twice increase was found in 0 % HL plants. HPFD stimulated Chl accumulation in 3 % HL plants but suppressed it in 0 % HL ones. Changes of pigment stoichiometry were the most remarkable in 0 % HL plants where Chl *a*/Chl *b* ratio increased from 2.5 before the transfer to 2.8 under LPFD and to 3.0 under HPFD at the end of acclimation. At the end of *in vitro* growth, four times higher PFD induced four to six times higher content of deepoxidated forms of xanthophylls per chlorophyll [1000 (Z + 1/2 A)/(Chl *a+b*), Fig. 4*B*]. However, instant high decrease of the ratio appeared in HL plants after their transfer to identical PFD of 200 μmol m^{-2} s^{-1}. Similarly, contents of

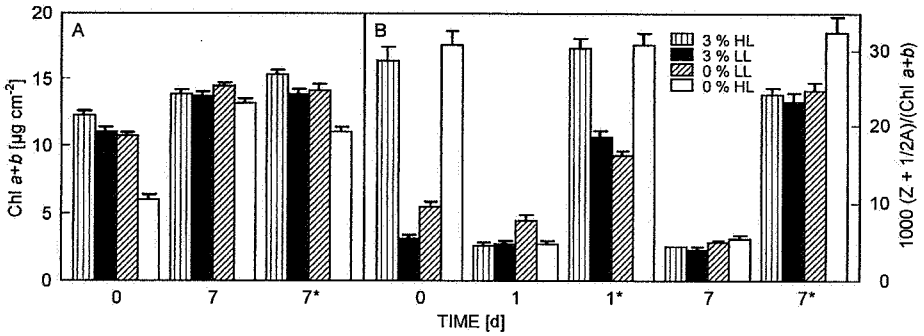

Fig. 4. Content of Chl *a+b* (*A*) in tobacco leaves at the end of *in vitro* cultivation (0), after 7 d of *ex vitro* acclimation at LPFD (200 μmol m^{-2} s^{-1}) (7) or at HPFD (700 μmol m^{-2} s^{-1}) (7*). Ratio of deepoxidated xanthophyll cycle pigments to chlorophylls [1000 (Z + 1/2 A)/(chl *a* + chl *b*)] (*B*) in tobacco leaves at the end of *in vitro* cultivation (0), after 1 and 7 d of *ex vitro* acclimation under LPFD (1, 7) or under HPFD (1*, 7*). Pigments were determined by HPLC. The data are means ± SE of three independent experiments.

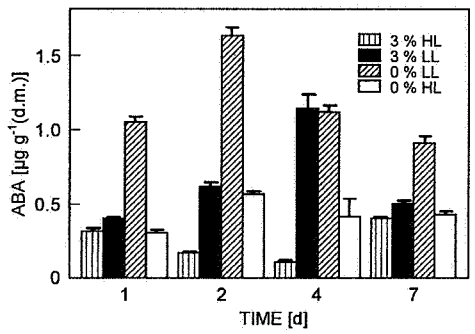

Fig. 5. Abscisic acid content in tobacco leaves at the end of *in vitro* cultivation and during *ex vitro* acclimation at HPFD (700 μmol m^{-2} s^{-1}). ABA contents were determined by gas chromatography. The data are means ± SE of three independent experiments.

deepoxidated forms of xanthophylls per chlorophyll did not increase under four times higher *ex vitro* PFD. An expected increase of this ratio was detected only in LL plants exposed to HPFD. After 7-d acclimation under LPFD, ratio of deepoxidated xanthophylls to chlorophylls varied between 4.0 - 5.4 in all variants. Under HPFD, this ratio reached 23.1 - 24.6 in 3 % HL, 3 % LL and 0 % LL, but it was higher (32.5) in 0 % HL plants (Fig. 4*B*).

Higher ABA content in 0 % LL plants was found already before transfer and further short-time increase was detected on the 2nd day of acclimation. Presence of sucrose suppressed ABA demand in *in vitro* culture, however, transiently higher content of ABA was detected in 3 % LL plants on the 2nd and 4th days (Fig. 5).

Discussion

Positive effect of sucrose on cultivation of plantlets *in vitro*, on their growth, dry matter accumulation, total leaf area, leaf thickness, Chl content and light-saturated rate of photosynthetic oxygen evolution had been already described (Tichá *et al.* 1998, Vinterhalter *et al.* 2001). This higher readiness of photomixotrophic plantlets was evident during the *ex vitro* acclimation. High accumulation of hexoses in leaves may represent another advantage of sucrose fed plantlets. Sugars are considered as source of metabolites and energy for the development of new leaves (Capellades *et al.* 1991, Van Huylenbroeck *et al.* 1996). Indeed, more new leaves were formed in both photomixotrophic variants during 7-d acclimation (data not shown).

Not only formation of new leaves but also adaptation of *in vitro* developed leaves plays an important role in *ex vitro* acclimation. Increased D1/LHC 2 ratio and chlorophyll contents of *in vitro* developed leaves were detected during *ex vitro* acclimation in all variants (Figs. 2*B*, 4*A*). However, the most evident changes appeared in 0 % HL plants. Chl contents were almost doubled and D1/LHC 2 ratio was tripled after 7 d of *ex vitro* growth

under HPFD. In *Spathiphyllum*, the plant prone to high irradiance, similar leaf acclimation appeared later (Van Huylenbroeck *et al.* 1995).

Stress was evident immediately after the transplantation, especially under higher *ex vitro* PFD, and it disappeared after 7-d acclimation. The first visible reaction was wilting. Less severe wilting in plants cultivated *in vitro* on sugar media could be connected with higher thickness of their leaves (Tichá *et al.* 1998) and/or with decrease of transpiration (Solárová *et al.* 1989). Decrease of F_v/F_m and D1/LHC 2 ratios, indicating photoinhibition (Baker 1991, Lütz *et al.* 1992, Serret *et al.* 1996), was found on the 1st day after transplantation (Figs. 2*A,B*). The steepest decline of F_v/F_m ratio was revealed in 0 % HL plants, while the decrease of D1/LHC 2 ratio was most extreme in LL plants. It can be supposed that substantial decline of F_v/F_m ratio in 0 % HL plants may be caused by activation of photoprotective mechanisms, while lower photoprotective potential in LL plants could not prevent an extensive destruction of RC 2 and loss of D1 protein.

Elevated ABA content, an indicator of stress (Hartung and Davies 1991, Norman *et al.* 1990), was detected during acclimation period only in LL variants (Fig. 5). Presence of sucrose suppressed and postponed ABA accumulation. It could be explained by higher root development in 3 % than in 0 % variants (Tichá *et al.* 1998).

Transient stress responses also appeared on the level of thylakoid membrane protein synthesis. One day after transplantation, when plants were wilted, *de novo* synthesis rate of D1 protein increased in all experimental variants (Fig. 2*C*). Different stresses usually accelerate inactivation and degradation of D1 protein, therefore acceleration of D1 synthesis seems to be an important photoprotective mechanism (Rintamäki *et al.* 1994). Dannehl *et al.* (1995) noted an increase of preferential synthesis of D1 protein together with a loss of maximum photochemical efficiency of PS 2 (F_v/F_m) under nutrient deficiency in spinach plants.

Although *in vitro* photoautotrophically cultured plants enhanced preferential D1 synthesis in comparison with LHC 2 after 1 d of acclimation, no increase but a slight decrease of D1/LHC 2 ratio on immunoblot was detected (Fig. 2*B*). This indicates higher degradation than synthetic rates of D1 protein. The increase of relative synthesis of D1/LHC 2 and a decrease of D1/LHC 2 ratio was most considerable in the 0 % LL plants, thus we can assume the highest photodegradation of D1 protein in these plants. Low capacity of D1 protein repair cycle in plants adapted to low PFD was documented by Tyystjärvi *et al.* (1992).

An unexpected temporary drop in D1 protein synthesis and an expression of a number of new proteins appeared in all variants on the 3rd d of *ex vitro* acclimation under both PFDs. This seems to be connected to the transplantation of plants from media to soil. When plants were exposed to *ex vitro* conditions on their agar media, neither decrease of D1 protein synthesis nor such evident change of spectra of *de novo* synthetised proteins were detected (data not shown). Decline of D1 synthesis on the background of continuous increase of D1 proportion (Fig. 2*C*) can be explained by its phosphorylation, which is induced by different stresses. Phosphorylation of D1 protein blocks its degradation and consequently D1 synthesis is slowed down (Giardi *et al.* 1994, Rintamäki *et al.* 1995, Ebbert and Godde 1996, Anderson *et al.* 1997). Decrease of F_v/F_m, usually accompanying this important photoprotective mechanism (Kruse *et al.* 1997), was found only in the 3 % LL plants on the 4th day of the acclimation (Fig. 2*A*).

After 7 d of the acclimation, when transient stress was overcome, an increase of preferential D1 and LHC 2 proteins synthesis on the background of decrease of total proteosynthetic activity was found in the 0 % LL plants grown on HPFD (Fig. 2*C, D*). Similarly, highly preferential synthesis of D1 protein was described by Droillard *et al.* (1992) in ageing bean leaves. Substantial decline of proteosynthetic activity detected in the 0 % LL plants could also be a signal of ageing.

Interesting effects of *ex vitro* PFD on the synthesis of LHC 2 and on deepoxidation of xanthophyll cycle pigments were revealed. Higher *ex vitro* PFD caused lower synthesis of LHC 2, and *vice versa* (Fig. 3). These results are in agreement with the data of *e.g.* Anderson (1986). Almost unchanged content of antheraxanthin and zeaxanthin per chlorophyll unit after *ex vitro* transfer to 3.5 - 4 times higher PFD and their immediate decline after transfer into the same PFD (Fig. 4*B*) show higher sensitivity of *in vitro* cultures to irradiance and therefore their stress character in general. Stress-induced increase of content of antheraxanthin and zeaxanthin with supposed photoprotection role has been described, *e.g.*, by Adams and Adams (1996).

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