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Improvement of *ex vitro* transfer of tobacco plantlets by addition of abscisic acid to the last subculture

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Abstract

Tobacco (*Nicotiana tabacum* L.) plantlets were grown on Murashige and Skoog medium in ventilated Magenta boxes and for the last subculture 10 μM ABA was added to the medium. After three weeks plantlets were transferred into pots with *Perlite* moistened with water and grown in controlled conditions (16-h photoperiod, day/night temperature 25/20 °C, air humidity about 45 %) either under low or high irradiance of 150 (LI) and 700 (HI) μmol m^{-2} s^{-1}, respectively. Content of endogenous ABA was 271.7 pmol g^{-1}(f.m.) in ABA treated plantlets, while in control plantlets it was only 53.3 pmol g^{-1}(f.m.). After *ex vitro* transfer, stomatal conductance and transpiration rate decreased considerably in comparison with *in vitro* grown plantlets and remained lower also 7 d after *ex vitro* transfer, especially in ABA-treated plants and so wilting of plants was practically eliminated. Net photosynthetic rate also decreased 1 d after *ex vitro* transfer but after 7 d it was mostly higher than that of *in vitro* grown plantlets. Water use efficiency significantly increased in ABA-treated plants. Chlorophyll *a+b* content did not change immediately after *ex vitro* transfer, nevertheless, after 7 d chlorophyll content was higher in ABA-treated plants. Pool of xanthophyll cycle pigments (XCP) and the degree of their deepoxidation (DEPS), which are connected with harmless dissipation of light energy, increased under high irradiance. Contents of XCP and ABA precursors (neoxanthin and violaxanthin) were lower in ABA-treated plants than in control plants indicating less stress in these plants. Most chlorophyll *a* fluorescence parameters did not change considerably after *ex vitro* transfer and so the photoinhibition was not observed even under HI. Slight increase in non-photochemical quenching under HI in ABA-treated plants suggested their better photoprotection. Thus application of ABA to the last subculture can improve acclimatization of *in vitro* grown plants to *ex vitro* conditions

Additional key words: carotenoids, chlorophyll contents, chlorophyll fluorescence, net photosynthetic rate, *Nicotiana tabacum*, stomatal conductance, transpiration rate, xanthophyll cycle pigments.

Introduction

Widespread use of micropropagation is restricted by the formation of plantlets of abnormal morphology, anatomy and physiology induced by special conditions during *in vitro* culture, which might be easily impaired by sudden changes in environmental conditions after *ex vitro* transfer (for recent review see *e.g.* Pospíšilová *et al.* 2007). Sudden decrease in air humidity and increase in irradiance belong to the most harmful. The first one can cause wilting while the second one photoinhibition or formation of reactive oxygen species (ROS; Desjardins

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Abbreviations: ABA - abscisic acid; Car - β-carotene; Chl - chlorophyll; DEPS - degree of XCP deepoxidation [DEPS = (zeaxanthin + 0.5 antheraxanthin)/XCP]; F_m - maximum chlorophyll fluorescence; F_v - variable chlorophyll fluorescence; PS - photosystem; qNP - non-photochemical quenching; qP - photochemical quenching; RWC - relative water content; WUE - water use efficiency; XCP - xanthophyll cycle pigments (XCP = violaxanthin + antheraxanthin + zeaxanthin); Φ_{PS2} - quantum yield of PS 2 photochemistry.
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The article is dedicated to our colleagues Dr. Jiří Čatský and Dr. Zdeněk Šesták passing away last year.
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et al. 2009). For plant survival, the most important is regulation of transpiration and stabilization of water status but for their further growth, no less important is adequate photosynthetic rate.

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, can ameliorate wilting of plants after transplantation. However, these procedures might lead to a quick drying out of the cultivation medium (for review see Pospíšilová *et al*. 1999). Another possibility might be application of abscisic acid (ABA). The relative water loss from detached leaves of *in vitro* grown *Aronia arbutifolia*, *Tagetes erecta* or tobacco plantlets was reduced by addition of ABA into culture medium (Colón-Guasp *et al*. 1996, Pospíšilová 1996, Aguilar *et al*. 2000). Addition of ABA to the substrate immediately after transplantation of *in vitro* grown tobacco plants also alleviated their wilting (Pospíšilová *et al*. 1998, 2000, 2009).

Stomatal effects of ABA are well known even if new facts concerning their mechanisms steadily appear (*e.g*. An *et al*. 2008, Jiang and Hartung 2008, Song *et al*. 2008, Cousson 2009). Moreover, nonstomatal effects of ABA might be also important. ABA application increased chlorophyll and carotenoid contents under water stress (Agarwal *et al*. 2005, Haisel *et al*. 2006, 2008), increased resistance to photoinhibition (Ivanov *et al*. 1995, Jia and Lu 2003) and maximum photochemical efficiency under stress (Zhou and Guo 2009) or affected expression of many photosynthetic genes (*rbsS*, *rbsL*, *cab, psbA;* Giraudat *et al*. 1994, Bray 2002). In previous experiments ABA was applied immediately after *ex vitro* transfer of *in vitro* grown tobacco plantlets, and it was found that chlorophyll (*a*+*b*) and β-carotene contents were higher in ABA-treated plants, but the content of xanthophyll cycle pigments was not increased. However, the degree of xanthophyll cycle pigments deepoxidation was decreased what also suggested less stress in ABA-treated plants (Pospíšilová *et al*. 2009). A combined application of ABA and osmoticum is a routine method for stimulating somatic embryo maturation. ABA also stimulated *in vitro* flowering in *Vigna aconitifolia* (Saxena *et al*. 2008).

Comparing different data from literature Rezaei Nejad and Van Meeteren (2007) suggested that short-term effects of elevated ABA concentration on stomatal functioning are reversible while its long term effects are permanent. Daily application of ABA to *Tradescantia* during growth at high relative humidity resulted in response of stomata to dessication similar to that found in plants grown at moderate relative humidity (Rezaei Nejad and Van Meeteren 2007, 2008).

The aim of present experiments was to apply ABA to the last subculture lasting three weeks and to follow this long-term effect of ABA on plantlet response to subsequent *ex vitro* transfer and growth at low or high irradiance. The comparison with previous results (Pospíšilová *et al*. 2009) enables us to differentiate short-term and long-term effects of ABA.

Materials and methods

Experimental material was tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) seedlings grown *in vitro* for about 2 months in ventilated Magenta boxes. For the last subculture 10 μM ABA was added to the medium. Control plants were subcultured on the same medium without ABA. In preliminary experiment we found that higher ABA concentration retarded growth. After three weeks, the plantlets were transferred into pots with *Perlite* moistened with water or nutrient solution every other day. Plants were grown in controlled conditions (16-h photo-period, day/night temperature 25/20 °C, air humidity about 45 %) either under low irradiance of 150 (LI) or high irradiance of 700 (HI) μmol m^{-2} s^{-1}.

The last day before *ex vitro* transfer, the content of endogenous ABA in control and treated plantlets was measured by mass spectrometry (for detail see Pospíšilová *et al*. 2009). To check the vigour of the control and treated plantlets, "transpiration curves" were measured by weighing cut leaves in regular intervals for 3 h under controlled conditions.

Net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) were measured before *ex vitro* transfer and 1 or 7 d after *ex vitro* transfer on intact leaves using gas exchange system *LCA-4* (*ADC Bio Scientific*, Hoddesdon, UK) with leaf chamber *LC4/PLC4BT-1/E* at a temperature of 25 °C, irradiance of 750 μmol m^{-2} s^{-1}, CO_2 concentration of 350 μmol mol^{-1}, and relative humidity of about 30 %.

Contents of photosynthetic pigments were determined in acetone extracts of leaf discs by HPLC (*ECOM, s.r.o.*, Prague, Czech Republic) using a reverse phase column (*Watrex Nucleosil 120-5-C18*, 5 μm particle size, 125 × 4 mm, *ECOM, s r.o.*, Prague, Czech Republic). The solvent system was acetonitrile : methanol : water (80:12:10) followed by methanol : ethylacetate (95:5), and the gradient was run from 2 to 6 min. The flow rate was 1 cm^3 min^{-1}, the detection wavelength 445 nm. Data were captured by PC-software *Clarity* (*DataApex*, Prague, Czech Republic).

Chlorophyll (Chl) *a* fluorescence kinetic was measured on the adaxial surface of detached leaves after 25-min dark acclimation with the *PAM Chlorophyll Fluorometer* (*Walz*, Effeltrich, Germany) at room temperature and ambient CO_2 concentration. Measuring irradiance was 0.35 μmol m^{-2} s^{-1}, actinic irradiance

200 μmol m^{-2} s^{-1}, and 700-ms saturated flashes of "white light" (2 500 μmol m^{-2} s^{-1}) were applied at 300 s intervals. Data sampling, control, and calculation were served by the *DA 100 Data Acquisition System* (*Walz*, Effeltrich, Germany). The nomenclature of Van Kooten and Snel (1990) was used throughout this work.

Relative water content (RWC) was measured gravimetrically in leaf discs (0.5 cm^2) water-saturated by immersing into holes of fully moistened polyurethane foam under dark according to Čatský (1960). For "transpiration curves" RWC was calculated from gradual decrease in fresh mass of initially water saturated leaves and dry mass.

For each experiment about 180 plantlets were used and experiments were repeated twice. Means and SE were calculated using *SigmaPlot*.

Results and discussion

When tobacco plantlets were grown for 3 weeks on 10 μM ABA content of endogenous ABA increased from 53.3 (control plants) to 271.7 pmol g^{-1}(f.m.). In agreement with previous experiments (Pospíšilová 1996), ABA-treated plantlets were less prone to dehydration when taken out of the cultivation boxes as can be seen from "transpiration curves" of their leaves. Both stomatal and cuticular transpiration rate was lower in ABA-treated leaves than in control leaves (Fig. 1). Similarly, the relative water loss from detached leaves of *in vitro* grown *Aronia arbutifolia* was reduced by addition of ABA into culture medium (Colón-Guasp *et al*. 1996). In agreement with our previous experiments and data in literature, stomatal conductance (g_s) and transpiration rate (E) were high in *in vitro* grown plantlets. However, g_s and E were significantly lower in ABA-treated plantlets than in control plantlets (Fig. 2). These results proved previous suggestion (Majada *et al*. 1998, Haisel *et al*. 1999, Aguilar *et al*. 2000, Estrada-Luna *et al*. 2001, Pospíšilová *et al*. 2009) that stomata of plantlets grown in ventilated boxes and/or on medium with ABA are able to control better the water loss than plantlets grown in sealed containers.

After *ex vitro* transfer the sharp decrease in g_s (mostly due to sudden decrease in air humidity) and in consequence E was observed in many plant species (for review see Pospíšilová *et al*. 2007). In agreement with our previous experiments (Pospíšilová *et al*. 1998, 2000, 2009), the values of g_s and E were very low 1 d after transplantation and gradually increased thereafter, but 7 d after *ex vitro* transfer still remained lower than in *in vitro* grown plantlets (Fig. 2). Application of ABA further decreased g_s and E and long-term effect of ABA was similar to previous short-term effect. This rapid restriction of water loss brought about that rather high relative water content (RWC) was measured 1st day after *ex vitro* transfer in control plants (86.8 and 84.4 % under LI and HI, respectively) and namely in ABA-treated plants (93.2 and 90.3 % under LI and HI, respectively).

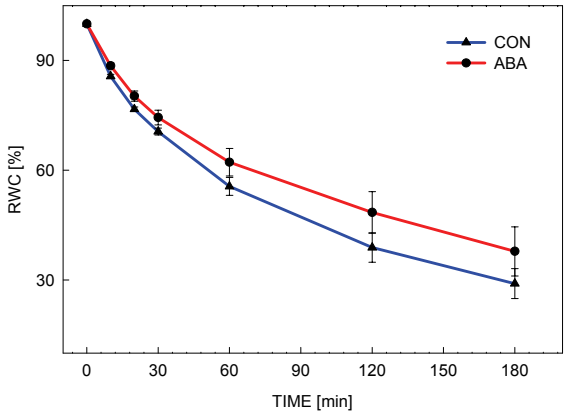

Fig. 1. Effect of 10 μM ABA added to the last subculture (3 weeks) on "transpiration curves" (changes in RWC of detached leaves with time) of ABA-treated and control plantlets taken out of cultivation vessels the last day before *ex vitro* transfer. Means ± SE, n = 15.

Net photosynthetic rate (P_N) of *in vitro* grown control plantlets was rather high due to development of functional photosynthetic apparatus in ventilated *Magenta* boxes. Due to stomatal closure, P_N decreased 1 d after transplantation but after 7 d it was higher than that of *in vitro* grown plantlets with exception of ABA-treated plants. P_N was higher in plants grown under HI than under LI. In several plant species, P_N also decreased during the first days after transplantation and increased thereafter but significant increase in P_N to values higher than during *in vitro* growth was usually found only when new leaves were fully developed (see review Pospíšilová *et al*. 2007, and recent papers Siddique and Anis 2008, Guan *et al*. 2008). No statistically significant effect of ABA was found in plantlets grown *in vitro* and also 1 d after *ex vitro* transfer while 7 d after *ex vitro* transfer P_N was lower in ABA-treated plants than in control plants (Fig. 2). When ABA was applied immediately after transplantation, P_N was decreased also 1 d after *ex vitro* transfer (Pospíšilová *et al*. 2009).

Water use efficiency (WUE) increased considerably after *ex vitro* transfer. It is not surprising because decrease in stomatal opening usually affects more E than P_N. In agreement with this fact, WUE was very high in ABA-treated plantlets 1 d after *ex vitro* transfer (Fig. 2). Similarly, ABA treatment increased WUE in previous experiments (Pospíšilová *et al*. 2009) as well as in water-stressed bean, sugar beet and maize seedlings grown in sand with nutrient solution (Pospíšilová and Baťková 2004).

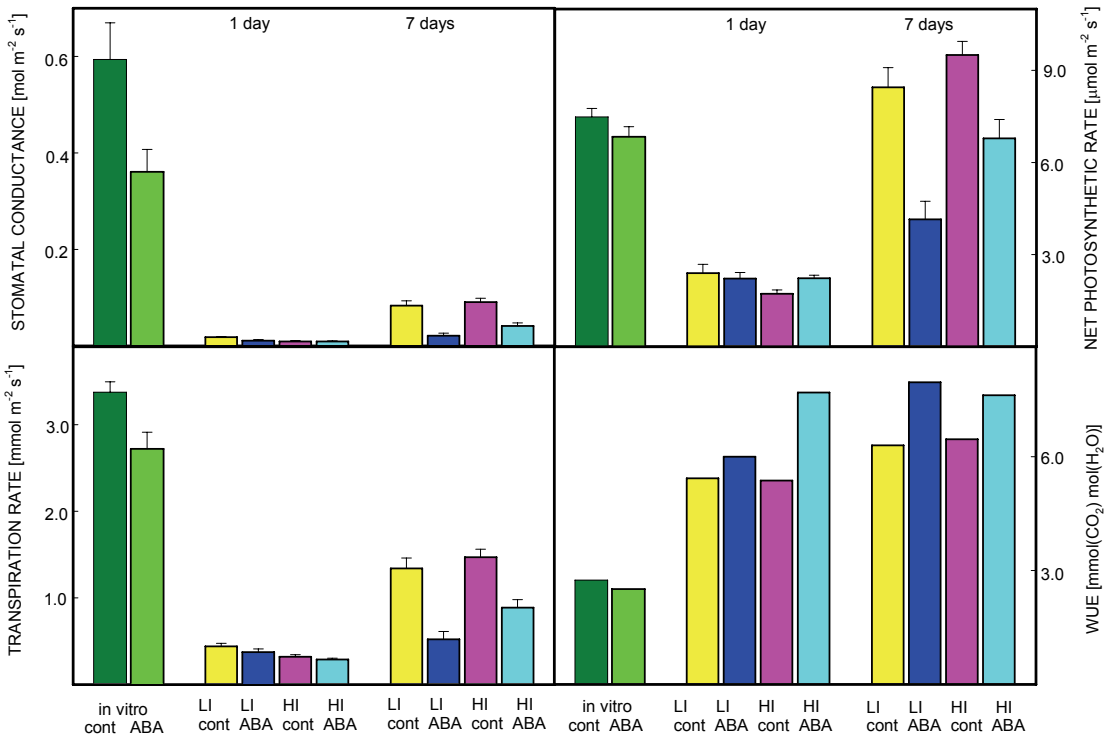

Fig. 2. Effect of 10 μM ABA added to the last subculture on stomatal conductance, transpiration rate, net photosynthetic rate and water use efficiency, in *in vitro* grown tobacco plantlets and 1 and 7 d after *ex vitro* transfer. Plants were cultivated under low or high irradiance (LI = 150, HI = 700 μmol m^{-2} s^{-1}). Means ± SE, n = 18.

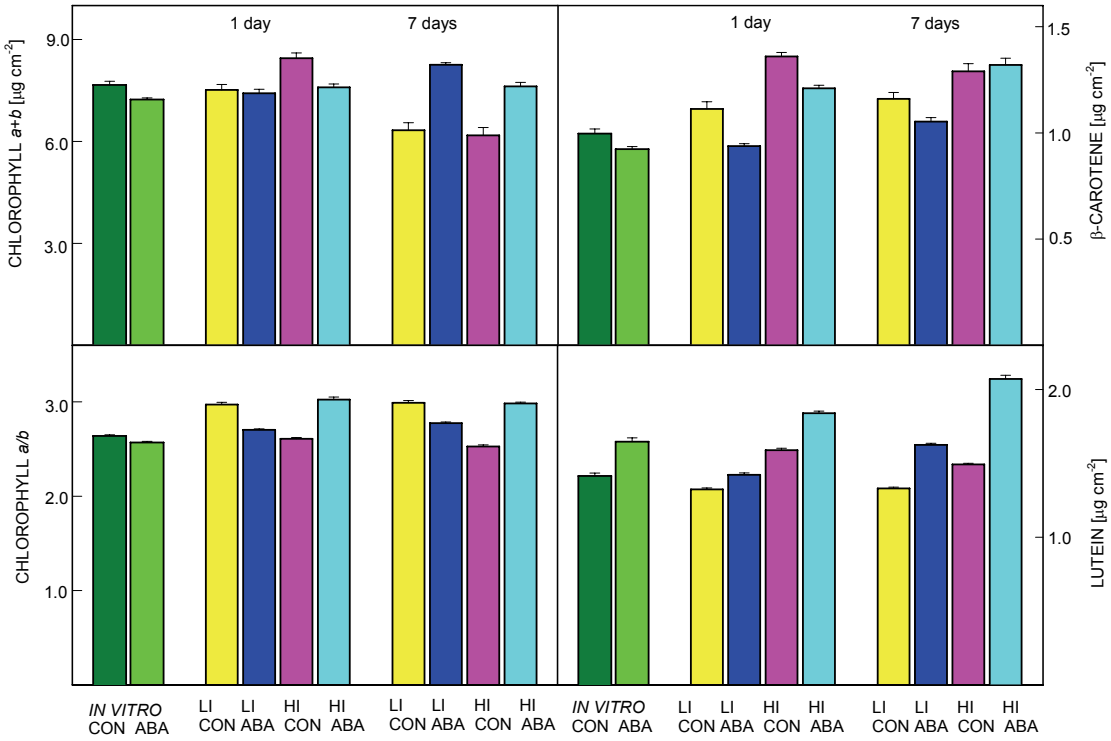

Fig. 3. Effect of 10 μM ABA added to the last subculture on chlorophyll *a*+*b* content, Chl *a*/*b* ratio, β-carotene content and lutein content in *in vitro* grown tobacco plantlets and 1 and 7 d after *ex vitro* transfer. Plants were cultivated under low or high irradiance (LI = 150, HI = 700 μmol m^{-2} s^{-1}). Means ± SE, n = 6.

In dependence on irradiance, medium composition and CO_2 concentration, chlorophyll content can be higher or lower in leaves of *in vitro* grown plants than in corresponding *ex vitro* grown plants. In our experiments, Chl *a+b* content did not change considerably after *ex vitro* transfer (Fig. 3). This is further evidence of successful development of photosynthetic apparatus in tobacco already during *in vitro* growth. Chl *a+b* content increased after *ex vitro* transfer in *Prunus* (Trillas *et al*. 1995), increased after temporary decrease in *Ocimum* (Siddique and Anis 2008) and increased in *Anoectochilus* under irradiance of 180 μmol m^{-2} s^{-1} but decreased under irradiance of 300 μmol m^{-2} s^{-1} (Pandey *et al*. 2006). Chl *a+b* content was slightly lower in ABA treated plants at the end of *in vitro* culture and in plants grown after *ex vitro* transfer for 1 d at high irradiance. However, after 7 d Chl *a+b* content was considerably higher in ABA-treated plants (Fig. 3). Higher Chl *a+b* content in ABA-treated leaves was also observed in previous experiments where ABA was applied immediately after *ex vitro* transfer. In this case, increased Chl *a+b* content was observed also 1 d after *ex vitro* transfer (Pospíšilová *et al*. 2009). Increase in Chl *a+b* content in ABA-treated plants was observed not only in persistent leaves but also 2 weeks after *ex vitro* transfer in new leaves (Pospíšilová *et al*. 1998). ABA pre-treatment also ameliorated negative effects of water stress in naturally grown barley, bean, maize, sugar beet and tobacco (Mizrahi *et al*. 1974, Agarwal *et al*. 2005, Haisel *et al*. 2006). Chl *a/b* ratio remained unchanged or slightly increased after *ex vitro* transfer. When plant were grown under LI this ratio was lower in ABA-treated than in control plants, but when the plants were grown under HI this ratio was higher in ABA-treated plants. This ABA-induced increase in Chl *a*/*b* ratio under HI might indicate decrease in light-harvesting complex associated with photosystem 2 (Špundová *et al*. 2003), which may help in their photoprotection.

In addition to its light harvesting function, β-carotene (Car) serves as precursor for biosynthesis of most other carotenoids as well as ROS scavenger. Car content increased considerably in tobacco plants grown *ex vitro* under HI, which was important for their photoprotection (Fig. 3), while Car content decreased in *Anoectochilus* plants grown under HI (Pandey *et al*. 2006). In contrast to previous experiments, where single ABA application increased Car content in all variants, after long-term ABA treatment Car content was mostly decreased (Fig. 3). On the other hand, content of another light-harvesting pigment lutein was always higher in ABA-treated plants than in control plants (Fig. 3) in agreement with previous experiments (Pospíšilová *et al*. 2009).

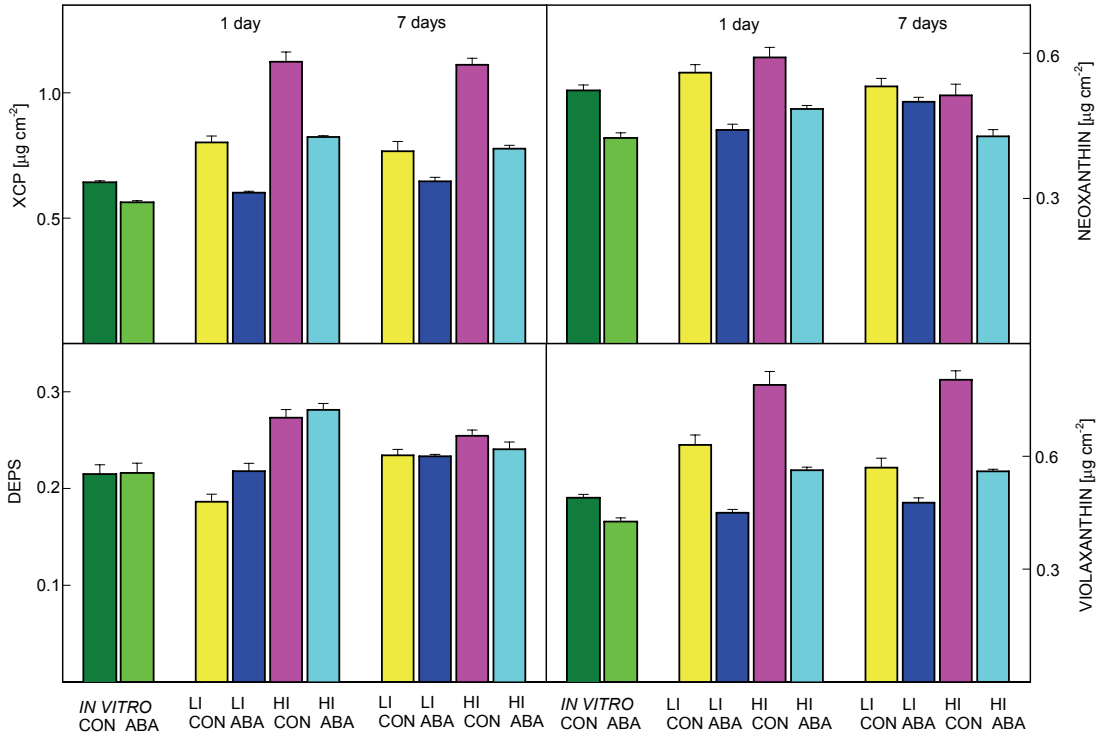

Fig. 4. Effect of 10 μM ABA added to the last subculture on content of xanthophyll cycle pigments (XCP = violaxanthin + antheraxanthin + zeaxanthin), degree of their deepoxidation [DEPS = zeaxanthin + 0.5 anteraxanthin)/XCP], and contents of ABA biosynthesis precursors neoxanthin and violaxanthin in *in vitro* grown tobacco plantlets and 1 and 7 d after *ex vitro* transfer. Plants were cultivated under low or high irradiance (LI = 150, HI = 700 μmol m^{-2} s^{-1}). Means ± SE, $n = 6$.

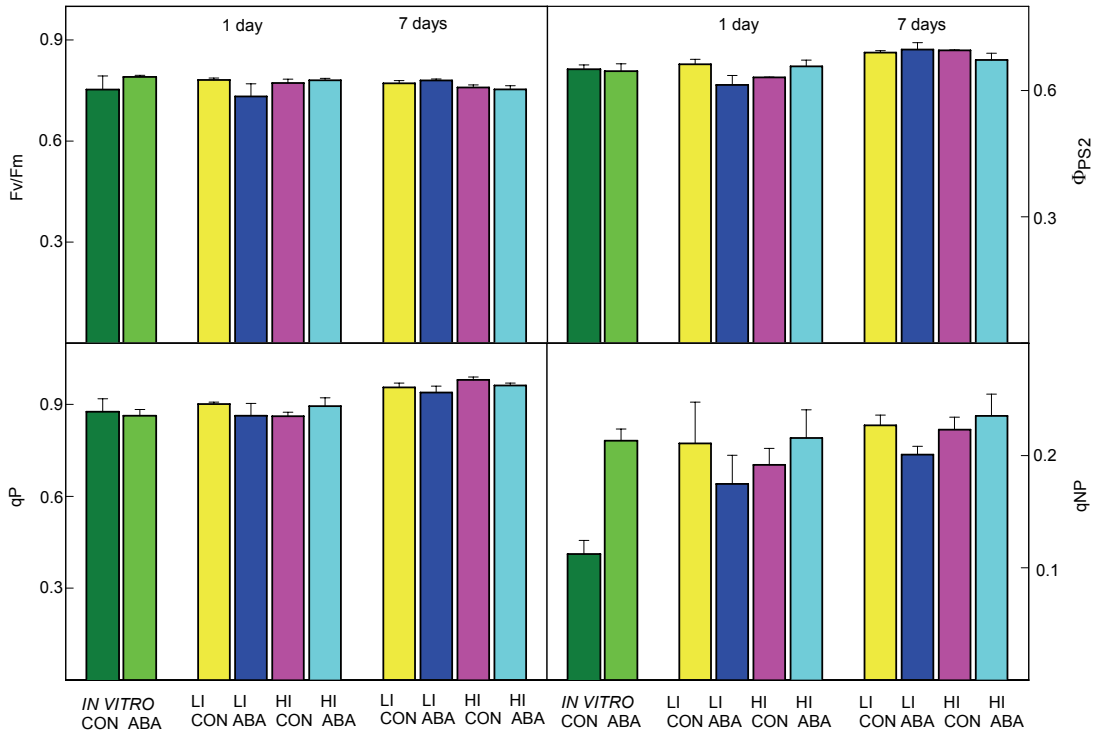

Fig. 5. Effect of 10 μM ABA added to the last subculture on parameters of chlorophyll *a* fluorescence (variable to maximum fluorescence ratio, F_v/F_m, actual efficiency of photosystem 2, Φ_{PS2}, photochemical quenching, qP and nonphotochemical quenching, qNP) in *in vitro* grown tobacco plantlets and 1 and 7 d after *ex vitro* transfer. Plants were cultivated under low or high irradiance (LI = 150, HI = 700 μmol m^{-2} s^{-1}). Means ± SE, $n = 5$.

Pool of xanthophyll cycle pigments (XCP = violaxanthin + antheraxanthin + zeaxanthin) increased after *ex vitro* transfer with exception of ABA-treated plants grown under LI. XCP content was higher under HI than under LI and always lower in ABA-treated plants than in control plants (Fig. 4), which showed less stress in ABA-treated plants. The decrease in XCP content was also observed after single ABA application with exception of plants grown under LI for 7 d (Pospíšilová *et al*. 2009). The degree of XCP deepoxidation [DEPS = (zeaxanthin + 0.5 anteraxanthin)/ XCP], which is connected with harmless dissipation of light energy, was also increased under HI (especially 1 d after transplantation). This was in agreement with our previous results (Pospíšilová *et al*. 2009) and with results of Pandey *et al*. (2006). Slight increase in DEPS in ABA-treated plants was observed in plants grown under LI for 1 d, while decrease in plants grown under HI for 7 d (Fig. 4). ABA application induced decrease in violaxanthin (Fig. 4) as well as zeaxanthin (data not shown) content, therefore DEPS was not markedly affected by ABA. The short-term ABA treatment more markedly affected DEPS than long-term treatment. The decreased DEPS due to single ABA application was observed in plants grown under both LI and HI and 1 and 7 d after *ex vitro* transfer. XCP content and DEPS were not changed markedly during acclimatization of tobacco plantlets in greenhouse under shade (Pospíšilová *et al*. 1999, 2000) but temporary increased during acclimatization at high irradiance (Semorádová *et al*. 2002). XCP content and DEPS increased in naturally grown plants during water stress and even more in plants pre-treated with ABA (Haisel *et al*. 2006). In maize, XCP content increased in long-term ABA treated plants but not in short-term ABA treated plants (Jia and Lu 2003). Increased XCP content was also observed in barley seedlings after 7-d ABA treatment (Ivanov *et al*. 1995).

Ivanov *et al*. (1995) speculated that applied ABA reduced the requirement of carotenoid precursors for its biosynthesis but it was not confirmed in their experiments with barley. In contrast, our experiments were in conformity with their hypothesis. Long-term ABA treatment led to decrease in contents of both precursors of ABA biosynthesis neoxanthin and violaxanthin (Fig. 4) in tobacco plants grown under LI and HI for 1 or 7 d. Similarly, short-term ABA application mostly caused decrease in neoxanthin and violaxanthin contents, with exception of plants grown under LI for 7 d (Pospíšilová *et al*. 2009). As far as we know, the feedback regulation of ABA synthesis was mostly focused on activities of basic enzymes of its biosynthetic pathway zeaxanthin epoxidase and 9-*cis*-epoxycarotenoid diogenase and the expression of respective genes (for review see, *e.g*., Xiong and Zhu 2003). It is not clear yet, how important

might be the above mentioned effect of ABA on contents of its precursors neoxanthin and violaxanthin, because their amount in leaves is usually much higher than their need for ABA biosynthesis.

A measurement of Chl *a* fluorescence induction kinetic is often used for the estimation of changes in photosystem (PS) 2 functioning. Generally, no statistically significant changes were found among control and ABA treated plants during our present experiment. Variable to maximum fluorescence ratio (F_v/F_m) in dark adapted leaves, which is usually interpreted as maximum photochemical efficiency, was in all variants in the range typical for non-stressed plants with only slightly lower values in *in vitro* grown control plants. ABA-treated plants grown under LI (Fig. 5) showed also lower values 1 d after transplantation. Despite a considerable reduction in g_s and CO_2 supply of chloroplasts, no signs of serious photodamage were observed either in plants grown *in vitro* or after *ex vitro* transfer. This was in agreement with our previous experiments (Pospíšilová *et al*. 1999, 2000, 2009). However, when plantlets were grown *in vitro* in tightly closed vessels, F_v/F_m temporarily decreased after transplantation (Semorádová *et al*. 2002). The fact that occurrence of photoinhibition after *ex vitro* transfer depended on conditions during previous *in vitro* cultivation was recently found by Yang and Yeh (2008). Similarly it was observed that occurrence of photoinhibition was affected by irradiance and other environmental conditions during *ex vitro* growth (Jeon *et al*. 2006, Pandey *et al*. 2006), or under natural conditions (Roy Chowdhury *et al*. 2009).

The values of actual photochemical quantum yield of PS 2 during the transition of the photosynthetic apparatus from a dark- to a light-adapted state (Φ_{PS2} ; Genty *et al*. 1989) were also very similar in all variants with a nonsignificant tendency to increase with the duration of acclimatization to *ex vitro* conditions (Fig. 5). The same holds for photochemical quenching (qP). While qP refers to the energy consumption by a charge separation in reaction centres, non-photochemical quenching (qNP) included regulatory mechanisms providing dissipation of excess of excitation energy to protect thylakoid membranes against photodamage (for review see Roháček 2002). Similarly as in previous experiments (Pospíšilová *et al*. 2009), non-photochemical quenching (qNP) seemed to be the most affected fluorescence parameter. We have no explanation for rather low qNP values in control plants grown *in vitro,* because values found for control plants in previous experiments were similar to those found here in ABA-treated plants. Effect of single ABA application was not statistically significant (Pospíšilová et al. 2009). In present experiments, qNP values were lower in ABA-treated plants than in control plants under LI while higher under HI (Fig. 5). This ABA-induced increase in qNP under HI might be useful for photoprotection in agreement with the results of Baraldi *et al*. (2008).

To sum up results obtained, application of ABA to the last subculture induced more vigorous plantlets. They were less prone to wilting after *ex vitro* transfer and better photoprotected.

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