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The role of abscisic acid in acclimation of plants cultivated *in vitro* to *ex vitro* conditions

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Abstract

The content of endogenous free abscisic acid (ABA) in the shoots of *in vitro* cultivated tobacco (*Nicotiana tabacum* L. cv. White Burley) and its changes during *ex vitro* acclimation of these plants to the greenhouse or growth chamber were estimated. The content of free ABA significantly increased at the 1st and/or 2nd day after plant transfer from *in vitro* to *ex vitro*. The ABA content of plants covered with transparent foil to maintain higher relative humidity (RH), did not significantly differ from ABA content of plants cultivated under ambient RH. Transfer to fresh medium also transiently increased the content of endogenous ABA. The ABA content in plants, which had been acclimated for 1 week to *ex vitro* conditions, decreased to the content found in the *in vitro* plants. Acclimation to *ex vitro* conditions affected the stomata on adaxial and abaxial sides differently: stomata on the adaxial side were less open than those on the abaxial one. The exogenous application of 5 μM ABA increased transiently its endogenous concentration in shoots of *in vitro* plants more than ten fold, but after 1 week the concentration in the shoots decreased.

Additional key words: Nicotiana tabacum, stomata.

Introduction

ABA is an important plant hormone involved in various processes during the life cycle of plants. An increase in ABA content has been reported in many of higher plant species which have been exposed to water stress, nitrogen deficiency, chilling, excessive radiation, heat stress, salinity, low relative humidity, air pollutants, wounding, pathogen attack, *etc.* These results have led to the idea that ABA is involved in the adaptation of plants to stress conditions. ABA plays a major role in plant-water relations and promotes stomatal closure in water stressed plants (for review see Walton 1980, Hetherington 2001, Finkelstein and Gibson 2002). It is transported via the xylem to the shoot, where it regulates transpiration, water loss and leaf growth (Hartung *et al.* 2002). During long-term water stress ABA accumulates in both the symplast and apoplast (Zhang and Outlaw 2001).

Plants cultivated *in vitro* are different from field grown plants, and represent a unique model for studying plant behaviour under stress. These differences are brought by different environmental conditions. The relative humidity of air is more than 90 % in closed vessels, and the irradiance is lower than in open-air conditions. CO_2 inside the vessels has a typical circadian fluctuation, depending upon irradiance (Šantrůček *et al.* 1991, Debergh *et al.* 1992, Pospíšilová *et al.* 1992). The resulting slow plant growth was formerly explained by

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Abbreviations: ABA - abscisic acid; GC-ECD - gas chromatography with electron capture detection; GC-MS - gas chromatography with mass spectrometry detection; LA-VPD - leaf to air vapour pressure difference; MS - Murashige and Skoog medium; PPFD - photosynthetic photon flux density; RH - relative humidity; SEM - scanning electron microscopy.
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low photosynthesis and then the cultivation media were supplemented with saccharides as carbon and energy sources. *In vitro* grown plants, especially plants supplied with high doses of growth regulators, frequently show abnormalities in morphology, anatomy and underlying processes (Phan 1991, Williams and Taji 1991, Dietrich *et al.* 1992, Ghashghaie *et al.* 1992); they can be called vitrified plants or, as it is proposed by Debergh *et al.* (1992) "hyperhydric" plants. Stomata are often large and have a changed shape and structure. Guard cells have thinner cell walls and contain more starch and chloroplasts (Marín *et al.* 1988). The lower amount of ABA in *in vitro* conditions and the absence of any changes in ABA amount during leaf development may result in changes in the wall structure of guard cells. These stomata cannot close, even under severe water deficit (Mansfield 1994). Excessive stomatal conductance and transpiration due to the loss of stomatal regulation and high irradiance during acclimation can be the cause of very high losses of acclimated plants (Pospíšilová *et al.* 1987, 1988, Diettrich *et al.* 1992). Also, environmental conditions such as concentration of sucrose and agar in the medium, and CO_2 concentration in the vessel atmosphere can affect acclimation to *ex vitro* conditions (Genoud-Gourichon *et al.* 1996).

The aim of this work was to answer following questions: *1*) Are *in vitro* plants deficient in ABA? *2*) Are plants that being acclimated able to produce enough ABA to close stomata? *3*) Are stomata sensitive to an increase of endogenous ABA, or exogenous ABA application?

Materials and methods

Plants: Seedlings of tobacco (*Nicotiana tabacum* L. cv. White Burley) were cultivated *in vitro* on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) without sucrose, solidified with 0.8 % agar. After germination on MS in Petri dishes, the plantlets were transferred into glass vessels (100 cm^3 volume) with aluminium foil caps. The plants were grown in cultivation room at 23 °C, 16-h photoperiod and PPFD of 80 μmol m^{-2} s^{-1} provided by fluorescent tubes. Relative air humidity within the vessels was approximately 90 %. The period of cultivation in glass vessels was 4 - 6 weeks.

Acclimation experiments: Experiments were undertaken either in a greenhouse or in a growth chamber with a controlled environment. The greenhouse experiments were performed during the winter (day/night temperature was 20 - 22/15 °C, RH 30 - 40 %, 16-h photoperiod (day prolonged by artificial irradiation using fluorescent tubes). Growth chamber experiments were run at a controlled day/night air temperature of 22/15 °C, a 12-h photoperiod, PPFD of 600 - 800 μmol m^{-2} s^{-1} and RH of about 40 %. *In vitro* grown plants were transferred carefully into small pots filled with sterilised soil and placed in the greenhouse or in the growth chamber. The plants were divided into 2 groups. Half of the plants were covered with microtene foil to protect against drying-out and to maintain high RH. The other half of the plants remained uncovered. A mild acclimation treatment was simulated using *in vitro* grown plants with perforated aluminium caps.

ABA analysis: Samples (leaves with stems from 15 plants) for ABA analysis were taken after 1, 2 and 7 d of acclimation, quickly cut into small pieces and plunged into liquid nitrogen and immediately freeze dried. Dry material was stored in a refrigerator in tightly capped glass vessels until analysis. *In vitro* plants were used as a control. The endogenous ABA contents in plants on MS medium with 5 μM ABA and in *in vitro* plants transferred to fresh MS medium without ABA were also measured. Standards were prepared from 2-*cis*-4-*trans*-abscisic acid (*Aldrich*, Steinheim, Germany). About 70 mg of freeze-dried plant material was powdered and then extracted in 15 cm^3 of 80 % methanol, containing 100 mg dm^{-3} butylated hydroxytoluen (BHT) (*Serva*, Heidelberg, Germany) as an antioxidant, at 4 °C in darkness overnight. An internal standard DL-*cis*, *trans*-[G-^{3}H]ABA (917 Bq) (*Amersham*, Little Chalfont, England) was added before start of shaking. A modified purification procedure described by Prinsen *et al.* (1991) was used. Extract was centrifuged at 2 260 *g* for 20 min. The supernatant was applied to a *Separcol SI C18* (*Anapron*, Bratislava, Slovakia) column, which had been conditioned with methanol, water and 80 % methanol, connected with polyvinyl pyrrolidone (*PVP - Polyclar AT*, insoluble, *Sigma*, St. Louis, MO, USA) columns. The columns used 1 × 5 cm plastic syringes with 4 cm^3 of PVP slurry in water, which had been washed with 80 % methanol. The columns were used as a filter and the eluate was kept, then columns were washed with 1 cm^3 of 80 % methanol. The combined eluates were diluted to 50 % methanol by adding water, and applied on *DEAE Sephadex A-25* (*Pharmacia LKB*, Uppsala, Sweden) columns (1 × 6 cm plastic syringe with 200 mg of sorbent, washed with 50 % methanol). *Sephadex* columns with adsorbed sample were connected with *Bakerbond SPE* C_{18} (*J.T. Baker Inc.*, Philipsburg, NJ, USA) (500 mg) cartridges conditioned with methanol, water and formic acid (pH 2.35). ABA was eluted from *Sephadex* cartridges with 2 × 5 cm^3 of formic acid (pH 2.35), and was collected on the C_{18} *Bakerbond* columns from which it was displaced with 5 cm^3 of 60 % methanol. Samples were evaporated using

a vacuum rotation pump. Dried samples were dissolved in 200 mm^3 of ethyl acetate and 200 mm^3 of phosphate buffer (pH 3.1) was added, and the upper organic layer was removed. This step was repeated. Combined ethyl acetate fractions were evaporated to dryness by a stream of nitrogen and methylated with an ethereal solution of diazomethane for 15 min under dark, with stirring. Ten mm^3 of 1 ng cm^{-3} solution of 4,4'-dibro-mobifenyl (*Supelco*, Gland, Switzerland) was added as an internal standard and 60 mm^3 of isooctane and 30 mm^3 of toluene were added. The ABA concentration was determined using gas chromatograph *HP 5880A* (*Hewlett Packard*, Palo Alto, USA) with an ^{63}Ni electron capture detector and a splitless injector and a 30 m *SPB-5* fused silica capillary column (0.25 mm I.D., 0.25 μm film thickness) (*Supelco*, Gland, Switzerland) with a temperature programme from 90 to 240 °C. The injector and detector temperatures were 230 and 280 °C, respectively. Helium served as the carrier gas at a flow rate of 25 cm^3 min^{-1}. Accuracy of the method had been confirmed by an *EI GC/MS Profile Mass Spectrometer* (*Kratos Analytical Ltd.*, Manchester, England) with a *GC HP5890* (*Hewlett Packard*, Avondale, PA, USA). The results were statistically analysed using *ANOVA*.

SEM observations: Samples were taken from fully developed non-senescent leaves on day 2 of acclimation. Leaf samples were processed as described above. Freeze-dried leaves were placed on aluminium discs, sputtered with gold and observed under scanning electron microscope *Jeol 6300* (*Jeol*, Croissy sur Seine, France). Photographs of the leaf surfaces were taken. Stomatal length and width were measured on the photographs and stomatal pore area was determined using a digitiser-graphic tablet (*HiSketch1812/D*, *Autodesk* Inc., Massachusetts, USA). Stomatal opening has been expressed in terms of pore area. Results were calculated using *Costat*. About 150 randomly chosen stomata were measured for each treatment.

Results

Shoots of *in vitro* tobacco plants had significantly less ABA than plants of the same age grown in the greenhouse (Table 1). ABA content increased during *ex vitro* acclimation in the greenhouse or growth chamber (Figs. 1 and 2). Often the ABA concentration rose during the 1st day of acclimation. The increase in ABA content was transient, and after 1 week of acclimation no differences between acclimated and *in vitro* control plants were found (Fig. 2).

Only 45 % of the acclimating plants survived the greenhouse conditions, and just 35 % of those continued to grow without a time lag. At higher humidity (plants covered with transparent foil), 85 % of plants survived and 55 % of them continued to grow without a time lag. Similar results were obtained from the growth chamber experiment.

Table 1. Content of endogenous ABA [nmol (ABA) g^{-1}(d.m.)] in shoots of tobacco grown under different conditions. Exogenous ABA was applied in concentration 5 μM into MS medium and the endogenous ABA content was measured after 2 or 7 d. Means ± SE of 3 to 5 replications. Different letters marks statistically significant differences (P = 0.05) tested by *ANOVA*.

	ABA content
Greenhouse plants	6.29 ± 0.60b
In vitro plants	2.54 ± 0.68a
In vitro + 5 μM ABA 2 d	33.75 ± 5.73d
In vitro + 5 μM ABA 7 d	13.51 ± 3.50c

Even perforation of aluminium caps on the glass vessels (Fig. 1) or transfer of *in vitro* plants to fresh medium (Fig. 2) increased the endogenous ABA concentration. Significantly higher amounts of ABA were found in plantlets one day after transition to fresh medium while during the 2nd day the ABA content reverted to pre-transfer levels (Fig. 2).

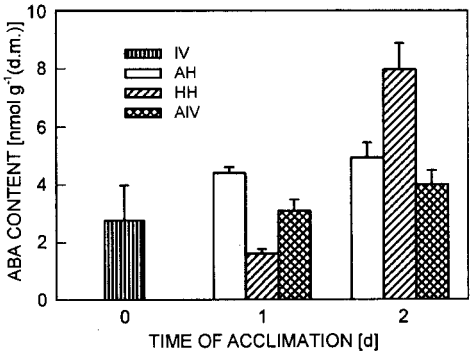

Fig. 1. Content of ABA in the shoots of tobacco on the 1st and 2nd day of acclimation in the greenhouse. Comparison of *in vitro* grown plants (IV), plants acclimated to ambient RH (30 - 40 %) (AH), acclimated to higher RH (plants covered with the transparent foil) (HH) and plants *in vitro* after perforation of the aluminium caps (AIV). Means from 3 to 5 replicates. *Vertical bars* represent SE of the mean.

ABA content in the shoots of plants given exogenous ABA increased more than ten times after exposure (Table 1). However, after 1 week the ABA content was significantly lower than on the 2nd day.

Ratios of the number of stomata (adaxial/abaxial) was

0.86 for *in vitro* grown plants and 0.39 for greenhouse grown plants. The density of the stomata were, on average, 28 and 72 per mm^2 for adaxial and abaxial side of the greenhouse grown leaves and 50 and 58 per mm^2 of the *in vitro* grown leaves, respectively. The closing of stomata on the adaxial leaf side (Fig. 3*A*) varied with acclimation and/or ABA treatment. The stomatal closing was proportional to stress during acclimation and to ABA content. *In vitro* grown plants had only 20 % of stomata closed, compared to 70 and 60 % of stomata in acclimated plants at ambient and high humidity, respectively, and almost 80 % in *in vitro* plants treated with ABA. On the contrary, more than 30 % of stomata were open or wide open in *in vitro* plants. In ABA treated plants, this category was not present. On the abaxial leaf side (Fig. 3*B*), stomata were less sensitive to ABA concentration in the root medium, and the response to air humidity was less pronounced in comparison with that on the adaxial side of the leaves. On abaxial side almost 20 % of stomata remained open and only 40 % were fully closed in ABA treated plants. Guard cells of the *in vitro* grown plants were a more circular (Figs. 4*A*, 5.), than the guard cells of greenhouse plants (Fig. 4*B*).

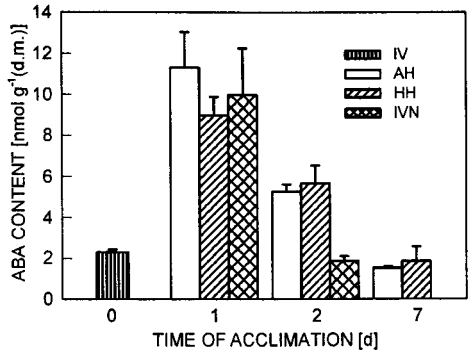

Fig. 2. Content of ABA in the shoots of tobacco on the 1st, 2nd and 7th day of acclimation in the growth chamber. Comparison of IV, AH, HH (as in Fig. 1) and *in vitro* plants after transfer to the fresh MS medium (IVN). Means from 3 to 5 replicates. *Vertical bars* represent SE of the mean.

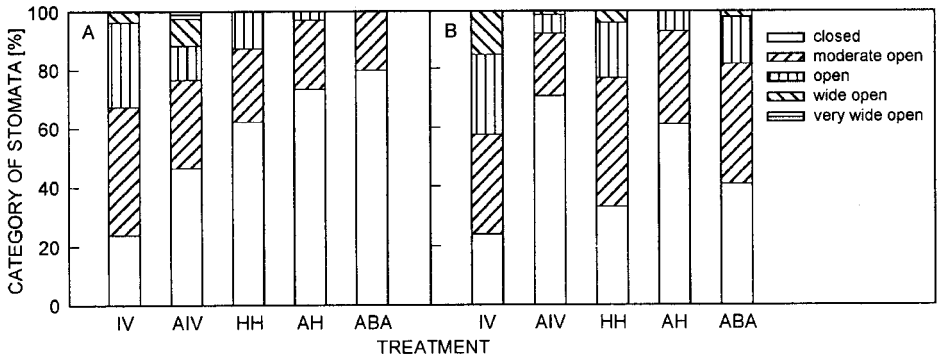

Fig. 3. Percentage of stomata in categories closed (aperture size 0 - 20 μm^2), moderate open (20 - 50 μm^2), open (50 - 100 μm^2), wide open (100 - 200 μm^2) and very wide open (200 - 300 μm^2) on the adaxial (*A*) and abaxial (*B*) sides of the tobacco leaves on the 2nd day of acclimation. Comparison of IV, AIV, HH, AH, as in Fig. 1 and *in vitro* plants with 5 μM ABA applied into the MS medium (ABA).

Discussion

We have shown that ABA was synthesized in a higher amount during *ex vitro* acclimation than during *in vitro* cultivation. The content of ABA rose in tobacco shoots, especially during the first two days of acclimation. These results correspond with the findings of the Aguilar *et al.* (2000), who reported limited ABA accumulation in *Tagetes erecta* plants cultivated *in vitro* in tightly closed containers. Ventilation stimulated an increase in endogenous ABA content. Similarly, in our experiments perforation of the caps increased the ABA content. So *in vitro* plants have a capacity to increase ABA content when exposed to decreased RH. A similar capacity to accumulate ABA in leaves of *Delphinium* grown *in vitro* was determined by Santamaria and Davies (1994). Therefore, a poor stomatal control of water loss from micropropagated *Delphinium* plants (Santamaria and Davies 1994) and high rates of transpiration of *in vitro* grown plants placed in dry air (Pospíšilová *et al.* 1987, 1988) did not result from a reduced capacity to accumulate ABA. ABA seemed to be an internal stress signal for plants. After 1 week of acclimation, the ABA concentration in shoots reverted to the previous value, and this corresponds with results presented by Haisel *et al.* (2001) and Hofman *et al.* (2002) on the *in vitro* tobacco plants acclimated to *ex vitro*. But ABA content increased only in plants cultivated *in vitro* without sucrose and with low irradiance in their experiments. These conditions are comparable with ours. When

sucrose and higher irradiance were present during *in vitro* cultivation, ABA content during *ex vitro* acclimation did not increase (Haisel *et al.* 2001, Hofman *et al.* 2002).

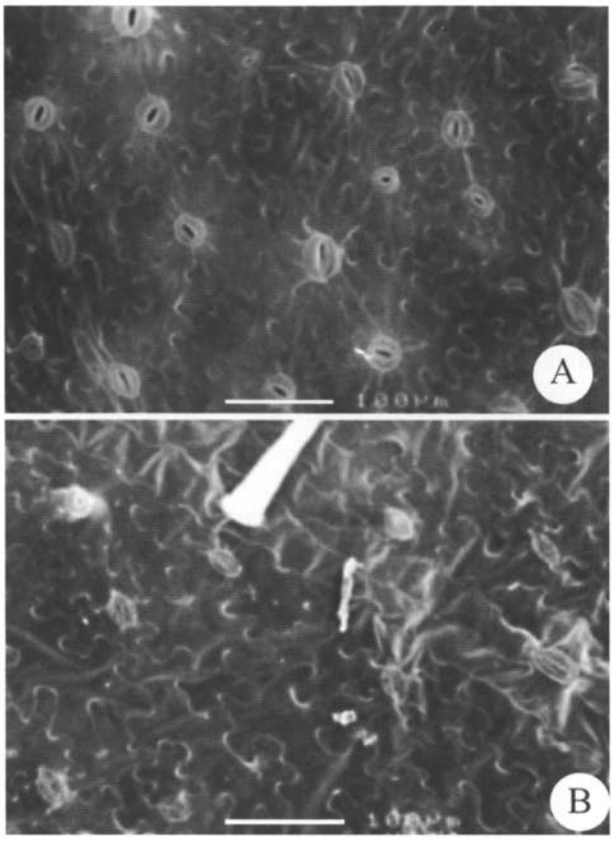

Fig. 4. SEM micrograph of the abaxial side of tobacco leaves grown in *in vitro* (*A*) and in the greenhouse (*B*).

Exogenous application of ABA in the medium increased its endogenous content in tobacco shoots more than *ex vitro* acclimation. However after 1 week, the ABA concentration in shoots markedly decreased, probably due to depletion of the sources in medium and/or its degradation. Adding ABA to the medium improved the control of water loss from the leaves and field survival in *Tagetes erecta* (Aguilar *et al.* 2000). ABA application reduces water loss from the leaves of *in vitro* grown plants (Colón-Guaspp *et al.* 1996, Hartung and Abou-Mandour 1996, Pospíšilová 1996). ABA added to the substrate on the 1st day after *ex vitro* transfer considerably decreased the stomatal conductance for a few days (Pospíšilová *et al.* 1998). ABA treatment had a positive effect, alleviating the "transplant shock" and enhancing plant growth. Photosynthetic parameters like P_N, chlorophyll and carotenoid contents and chlorophyll fluorescence characteristics were influenced insignificantly, or were slightly positive after 2 weeks of acclimation. It is evident from our experiments that the high content of ABA in shoots after exogenous application closed some stomata, even under very high air humidity in *in vitro* conditions. However, stomata were not completely closed, especially on the abaxial side of the leaf (Fig. 3*B*).

What is the reason for the uncontrolled loss of water

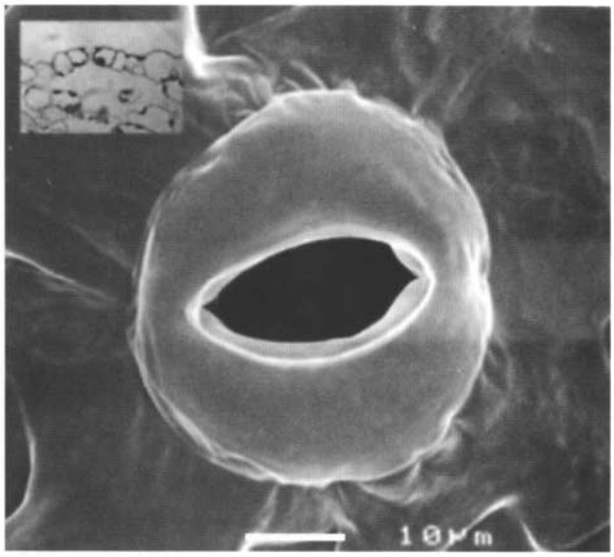

Fig. 5. Detail of guard cells from abaxial side of in *in vitro* grown tobacco. Insert: Cross-section of the leaf.

from *in vitro* plants? Two reasons were published: morphological and physiological abnormalities of the stomata and a less developed cuticle. Rapid water loss from leaves was associated only with the failure of stomatal closure, as described by Santamaria *et al.* (1993) and Santamaria and Kerstiens (1994). The cuticle permeabilities in their experiments were similar to those of *ex vitro* grown leaves. Experiments were performed on hypostomatous leaves of *Delphinium elatum*, *Doronicum* sp., *Hosta sieboldiana*, *Rodgersia pinnata* and on amphistomatous *Heuchera* sp. In contrast, retarded development of cuticle and epicuticular waxes was given as the principal cause for uncontrolled water loss (Sutter 1985, 1988) for apple, cherry, sweetgum and ornamental plants, carnation (Ziv *et al.* 1987), and rose (Ghashghaie *et al.* 1992). Zobayed *et al.* (1999) considered both reasons as a cause of extensive water loss – absence of functional stomata and reduction of leaf epicuticular and cuticular waxes in photomixotrophic cultures of potato plantlets. Several reasons were suggested for the inability of stomata to close. One reason could be insufficient flexibility of guard cell walls to allow closure of stomata even if they are subjected to extreme closing stimuli (Blanke and Belcher 1989, Wardle and Short 1983 and Ziv *et al.* 1987). But the complete mechanism for failure of guard cells to close remains unknown.

Stomata on the adaxial side of tobacco leaves were immediately more sensitive to higher ABA contents than stomata on the abaxial leaf side (Fig. 3*A*,*B*). Stomata from epidermal strips of *in vitro* growing *Delphinium* plants were not sensitive to ABA, stomata were unable to close

fully even at high concentrations of ABA (Santamaria and Davies 1994). The malfunctioning of stomata occurred in micropropagated plants irrespective of whether they were vitrified or not. After 3 weeks of plant cultivation on medium with ABA, the stomatal conductance was measured upon removal of the plants from *in vitro* culture and was lower, especially on the abaxial side, than in plants without ABA addition (Pospíšilová 1996). Possibly, the previous long-term presence of ABA was more important for stomata on the abaxial side of the leaves, because they were less sensitive to ABA content. But sensitivity of stomata to ABA was evaluated in our experiments after 2 d. We used stomatal pore area as a measure of stomatal opening. Although, the pore width (aperture) is commonly used for expression of stomatal opening instead of pore area, both are linearly related if the pore length stays constant during its movement (Kaiser and Kappen 2001). The general physical relationship between aperture and stomatal conductance g_s is still in doubt. Although it is commonly believed that g_s is often linearly related to aperture, recent measurements demonstrated a non-linear relationship, fitting a hyperbolic function (Kaiser and Kappen 2001).

Stomatal density on adaxial side of tobacco leaves were higher in *in vitro* plants compared to the greenhouse ones, which is in agreement with results obtained by Tichá *et al.* (1999). They found that acclimation of tobacco to *ex vitro* conditions decreased stomata density and also changed the size and morphology of stomata on the both sides of newly formed leaves.

Conclusions: *1*) *In vitro* plants are deficient in ABA, but they had capacity to increase its content under stress conditions. *2*) More *in vitro* plants survived the acclimation procedure when kept under increased humidity for a few days. Exogenous application of ABA into the medium increased their endogenous content in shoots more than stress conditions. *3*) Stomata on the adaxial side of the leaf were more sensitive to endogenous ABA than those on the abaxial leaf side. Higher ABA concentration close stomata heterogeneously, especially on abaxial leaf side.

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