

## ***Ex vitro* phenotype stability is affected by *in vitro* cultivation**

D. HASEL\*, P. HOFMAN<sup>a</sup>\*, M. VÁGNER\*, H. LIPAVSKÁ\*\*, I. TICHÁ\*\*, C. SCHÄFER\*\*\*  
and V. ČAPKOVÁ\*

*Institute of Experimental Botany, Academy of Sciences of the Czech Republic,  
Rozvojová 135, CZ- 165 02 Praha 6, Czech Republic\**

*Department of Plant Physiology, Faculty of Science, Charles University,  
Viničná 5, CZ-128 44 Praha 2, Czech Republic\*\**

*Lehrstuhl für Pflanzenphysiologie, Universität Bayreuth,  
Universitätsstrasse 30, D-95440 Bayreuth, Germany\*\*\**

### **Abstract**

Plant phenotype stability during *ex vitro* growth, one of the main requirements of plant micropropagation, was tested on tobacco. Plants cultivated *in vitro* in the presence of 3 % sucrose under photon flux density (PFD) of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (3 % HL plants) showed the best growth and photosynthetic parameters in the course of 7-day acclimation. However, significant change in phenotype of these plants appeared under a decrease in PFD to 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  during further *ex vitro* growth (in the period of 7<sup>th</sup> - 17<sup>th</sup> day). Much higher internodia elongation was found in 3 % HL plants in comparison with plants grown *in vitro* on sucrose media under PFD of 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (3 % LL) or without sucrose either under PFD of 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  or 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (0 % LL, 0 % HL). It can be presumed that 3 % HL plants show permanent demand for high PFD. Neither ABA or chlorophyll contents nor *de novo* thylakoid membrane synthesis were related to the morphogenic effect of low PFD. Changeable contents of hexoses in leaves of 3 % HL and 3 % LL plants were in no direct correlation to the elongated growth.

*Additional key words:* ABA, chlorophyll, D1 protein synthesis, *Nicotiana tabacum*, sugars, tobacco.

### **Introduction**

Micropropagation, an important part of plant biotechnology, has still a problem to overcome: *ex vitro* transfer and acclimation of plants. Dynamics of the whole process as well as the final percentage of fully acclimated plants are related to plant species and to both *in vitro* and *ex vitro* cultivation conditions (Pospíšilová *et al.* 1999). Some plant species are unable to adapt *in vitro* formed leaves to *ex vitro* conditions, but leaves of many other plant species are fully capable of *ex vitro* acclimation and they function till new leaves are formed (Preece and Sutter 1991, Dietrich *et al.* 1992, Van Huylbroeck and Debergh 1996). Decisive role of *in vitro* cultivation

conditions introduced the question of the optimal conditions leading to maximum capability of plantlets to overcome the *ex vitro* transfer stress. Many protocols for hardening plantlets *in vitro* by decreasing air humidity or concentration of sucrose, by increasing of CO<sub>2</sub> concentration and/or irradiance were suggested but common conclusions do not exist (the mechanisms involved are still not quite clear).

To contribute to this topic, tobacco nodal cuttings of four cultivation variants (PFD 50 or 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , sucrose concentration 0 or 3 %) and the process of their 7-day acclimation after abrupt transfer into soil had been

Received 5 January 2001, accepted 15 March 2001.

*Abbreviations:* ABA - abscisic acid; Fv/Fm - maximum photochemical efficiency; HL - *in vitro* PFD 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ; HPLC - high performance liquid chromatography; LHCII - light harvesting complex II; LL - *in vitro* PFD 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ; PFD - photon flux density; SDS PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis.

*Acknowledgement:* Dr. M. Kaminek and Dr. J. Pospíšilová are thanked for critical reading of the manuscript. This work was supported by grants: VS 96 145 and CEZ: J13/98:113100003 of the Ministry of Education, Youth and Sports of the Czech Republic, and 100/1998/B BIO/Pf of the Grant Agency of the Charles University.

<sup>a</sup> - Corresponding author; fax: (+420) 2 20390461, e-mail: hofman@ueb.cas.cz

analyzed (Tichá *et al.* 1998, Hofman *et al.* 2001). Plant growth, dry matter accumulation, total leaf area, photosynthetic potential and high irradiance resistance were positively affected by cultivation on sucrose media and at higher irradiance. With regard to these *in vitro* characteristics, 3 % HL plants showed the highest readiness for *ex vitro* acclimation although the other three plant variants withstood the abrupt transfer as well. After 7-day acclimation, measured parameters like

maximum photochemical efficiency (Fv/Fm) and D1/LHCII ratios and changes in pigment stoichiometry were balanced in all the plants with exception of the 0 % HL plants, where photoinhibition had occurred during *in vitro* cultivation (Hofman *et al.* 2001).

This investigation was aimed to the effects of conditions during *in vitro* cultivation on the responses of *ex vitro* grown plants to changes in irradiance.

## Materials and methods

**Plants:** Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were derived from nodal cuttings by 38-day cultivation on Murashige-Skoog (MS) agar medium (Sigma-Aldrich, Praha, Czech Republic) either supplemented with 30 g dm<sup>-3</sup> sucrose (3 %) or without sucrose (0 %) and exposed either to PFD 50 µmol m<sup>-2</sup> s<sup>-1</sup> (LL) or to 200 µmol m<sup>-2</sup> s<sup>-1</sup> (HL). 16-h photoperiod and day/night temperature 25/18 °C were used. Tobacco plantlets of these four cultivation variants, 3 % HL, 3 % LL, 0 % LL, 0 % HL (Tichá *et al.* 1998) were transplanted into soil and exposed to 200 µmol m<sup>-2</sup> s<sup>-1</sup>. After 7 d of *ex vitro* acclimation, tobacco plants were either transferred to lower PFD (50 µmol m<sup>-2</sup> s<sup>-1</sup>) or grown under the same PFD. 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 during *in vitro* cultivation. The plants were irrigated regularly. Three *in vitro* fully developed leaves were harvested for biochemical analyses after 0, 7 and 17 d of *ex vitro* growth.

**Sugar content determination:** Sugar content determination was done by HPLC analysis with refractometric detection (refractometer Shodex RI-71, integrator ChromJet, Spectra Physics, pre-column: Hema-Bio 1000 Q + SB, Watrex, Praha, Czech Republic; column: Hi Plex Ca2+, Polymer Laboratories, Shropshire, Great Britain) (for details see Lipavská *et al.* 2000).

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

**Protein analyses:** The rate of protein synthesis was determined by *in vivo* <sup>14</sup>C pulse labelling. Leaf discs of 4 cm<sup>2</sup> total area were incubated with <sup>14</sup>C-amino acid

mixture (Amersham, Praha, Czech Republic, specific activity 1.85 GBq mmol<sup>-1</sup>), diluted with water to a concentration of 1 MBq cm<sup>-3</sup>, under shaking (40 cycles min<sup>-1</sup>) at room temperature. After that, the leaf discs were rinsed with water, gently wiped and frozen in liquid nitrogen. Isolation of thylakoid membranes from leaf discs was performed according to Leto and Arntzen (1981). Thylakoid membrane proteins were prepared for electrophoresis as described in Tichá *et al.* (1998). Proteins were separated by SDS PAGE on 15 % polyacrylamide gel containing 6 M urea. Electrophoresis ran 8 h under 12 mA per slab gel on Biometra unit (Göttingen, Germany) cooled to 9 °C. Twenty µg protein aliquots were loaded per lane. Separated proteins were silver stained. After fluorography the radioactivity of individual protein bands was assessed by means of Phosphor Imager SF (Molecular Dynamics, Shepherdstown, USA) and ImageQuant Programme. Uptake and incorporation of <sup>14</sup>C amino acids were determined using LSC Packard-TRI CARB 250 0 TR (San Francisco, USA) in standard procedure.

**Abscisic acid content:** Approximately 3 g (f.m.) of leaves were powdered in liquid nitrogen and extracted in ten times its volume of 80 % (v/v) twice distilled methanol (Fluka, Bochs, Switzerland) containing 100 mg dm<sup>-3</sup> butylated hydroxytoluene as an antioxidant. Extraction was performed in darkness at 5 °C overnight. Following steps were performed in dim light. Filtrates were collected, pooled and vacuum dried. Ten cm<sup>3</sup> of 0.5 M K<sub>2</sub>HPO<sub>4</sub> were added to each sample to reach pH of approximately 8.5. The samples were then partitioned against double distilled ether three times. Water fractions were loaded on Polyclar AT column equilibrated with 0.1 M K<sub>2</sub>HPO<sub>4</sub> and eluted with 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 (Savant, USA) and methylated with 300 mm<sup>3</sup> of ethereal diazomethane solution at room temperature for 30 min. The samples were vacuum dried again and finally dissolved in 200 mm<sup>3</sup> 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 and discussion

Physiological state and photosynthetic efficiency parameters showed that high PFD and sucrose in media (3 % HL) are optimal for both *in vitro* and *ex vitro* growth (Tichá *et al.* 1998, Hofman *et al.* 2001). Recommendation of these conditions for standard tobacco plant micropropagation required a check on phenotype stability, the main request of plant biotechnologies. High sensitivity of sucrose fed plantlets to low irradiance, resulting in prolonged internodes, appeared during *in vitro* cultivation of 3 % LL plantlets. To determine whether the morphogenic effect of low PFD was connected with the exogenous supply of

sucrose, PFD of 50 or 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  were applied on plants after 7 d of *ex vitro* acclimation.

The heights of 3 % LL, 0 % LL and 0 % HL plants increased almost independently on irradiance during 10 d of 2<sup>nd</sup> *ex vitro* acclimation phase. However, remarkable 4.4 times height increase, due to internodia elongation, was induced by PFD decrease to 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and only twice height increase under 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in 3 % HL plants (Fig. 1A). It shows that only decrease of PFD together with previous cultivation on sucrose under HL conditions leads to internodia elongation and change of plant phenotype.

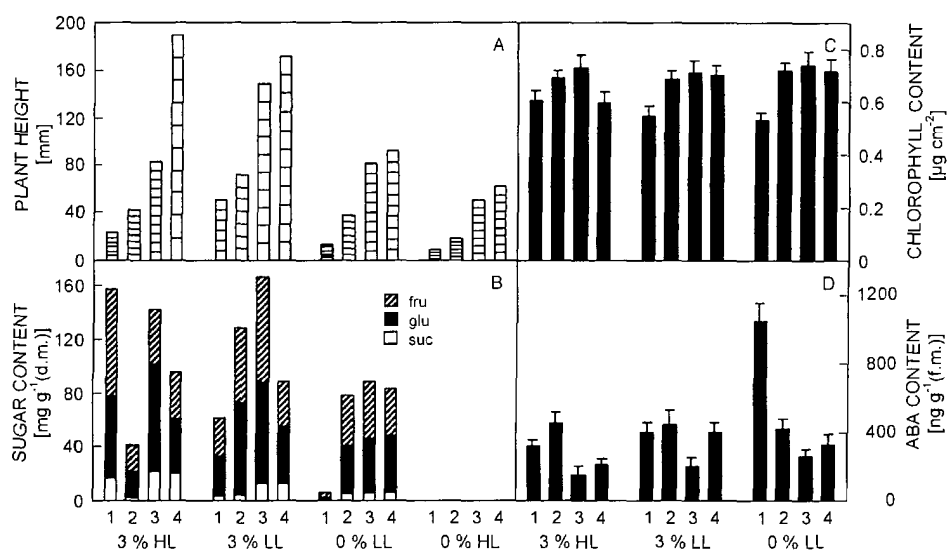


Fig. 1. Plant heights and average internodia lengths (A), and sugar (B; fru - fructose, glu - glucose, suc - sucrose), chlorophyll (C) and ABA (D) contents in leaves of individual plant cultivation variants after 38 d of *in vitro* culture (1), after 7 d in *ex vitro* under 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (2), after 17 d in *ex vitro* under 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (3) and after 17 d in *ex vitro* conditions with a PFD decreased from 200 to 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  on the 7<sup>th</sup> day of *ex vitro* acclimation (4). Vertical bars indicate SE of means of three independent measurements. SE for Fig. 1A, B are not higher than 20 %.

Lack of plant material due to continuing shedding of *in vitro* developed leaves in 0 % HL plants caused omitting this variant from next analyses. The greatest increase and further stability of total as well as relative quantities of sucrose, fructose and glucose in leaves was detected in phenotypically stable 0 % LL plants, and *vice versa*, both sucrose fed plantlets displayed broad changes in sugar contents in the course of *ex vitro* growth (Fig. 1B). But no correlation between elongation of internodia and contents of hexoses was found. Sucrose as well as hexoses play multiple roles in plants. They are involved in highly regulated mechanisms balancing

photosynthesis and sink, in hormonal regulation, they can serve as osmotic agents, energy reserves or regulators of gene expression (Paul and Stitt 1993, Genoud-Gourichon *et al.* 1996, Furbank *et al.* 1997). Almost nothing is known about the molecular basis of the series of biochemical events resulting in homeostasis of cells and survival of plants under changeable environment, but sucrose and hexoses may be involved in these processes. The obvious outlasting effect of sucrose even 17 d after transfer to soil was not reflected in other measured parameters. Decrease of chlorophyll content in 3 % HL plants transferred to low irradiance was only slight and no

changes in it occurred in other variants (Fig. 1C). ABA content, a stress marker (Hartung and Davies 1991), was low, below 400 ng g<sup>-1</sup>(f.m.) with exception of 0 % LL plants before transfer to *ex vitro* conditions (Fig. 1D).

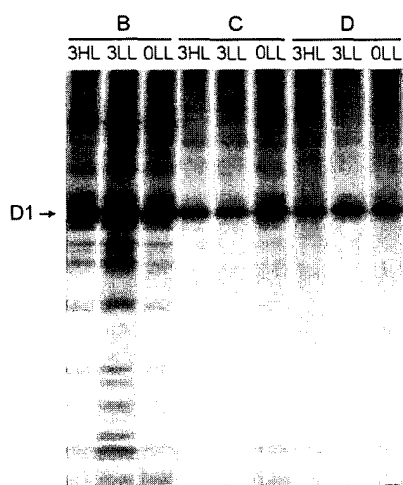


Fig. 2. *De novo* synthesis of thylakoid membrane proteins after 7 d in *ex vitro* conditions under PFD 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (B), after 17 d in *ex vitro* conditions under PFD 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (C) and after 17 d in *ex vitro* conditions with a PFD decreased from 200 to 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  on the 7<sup>th</sup> day of *ex vitro* acclimation (D).

Thylakoid membrane proteosynthesis was monitored by <sup>14</sup>C-amino acid mixture incorporation. Fluorographs

(Fig. 2) and radioactivity counts of individual protein bands (data not given) demonstrated that the decrease of PFD had only insignificant impact on *de novo* synthesis of thylakoid membrane proteins. However, an increase of preferential synthesis of D1 protein in *in vitro* developed leaves of 3 % HL, 3 % LL and 0 % LL plants was found regardless of *ex vitro* PFD. According to our pilot experiments with tobacco leaves, relative intensity of D1 protein synthesis is a good marker of stress or leaf ageing. Diluted spectra of *de novo* synthesized proteins together with increase of relative D1 protein synthesis are characteristic for ageing leaves (Droillard *et al.* 1992).

The first preliminary quantitative analyses of plant hormones showed outlasting effect of *in vitro* PFD on the concentration and distribution of auxins in leaves and stems of mixotrophically cultured plants. This differences more or less corresponded to the internodia elongation of 3 % HL plants. Therefore, hormone analyses would be the next step in the study of morphogenic effect of *ex vitro* PFD.

In summary, our data show that the effect of *in vitro* PFD and sucrose feeding outlasted to the second period of *ex vitro* acclimation (from the 7<sup>th</sup> to the 17<sup>th</sup> day *ex vitro*) and was expressed by internodia elongation in 3 % HL plants under PFD decrease. What substances are responsible for keeping information about original *in vitro* conditions remains to be elucidated.

## References

- Diettrich, B., Mertinat, H., Luckner, M.: Reduction of water loss during *ex vitro* acclimatization of micropropagated *Digitalis lanata* clone plants. - *Biochem. Physiol. Pflanz.* **188**: 23-31, 1992.
- Droillard, M.J., Bate, N.J., Rothstein, S.J., Thompson, J.E.: Active translation of the D-I protein of photosystem II in senescing leaves. - *Plant Physiol.* **99**: 589-594, 1992.
- Furbank, R.T., Pritchard, J., Jenkins, C.L.D.: Effects of exogenous sucrose feeding on photosynthesis in the C<sub>3</sub> plant tobacco and the C<sub>4</sub> plant *Flaveria bidentis*. - *Aust. J. Plant Physiol.* **24**: 291-299, 1997.
- Genoud-Gourichon, C., Sallanon, H., Coudret, A.: Effects of sucrose, agar, irradiance and CO<sub>2</sub> concentration during the rooting phase on the acclimation of *Rosa hybrida* plantlets to *ex vitro* conditions. - *Photosynthetica* **32**: 263-270, 1996.
- Hartung, W., Davies, W.J.: Drought-induced changes in physiology and ABA. - In: Davies, W.J., Jones, H.G. (ed.): *Abscissic Acid Physiology and Biochemistry*. Pp. 63-81. BIOS Scientific Publishers Limited, Oxford 1991.
- Hofman, P., Haisel, D., Komenda, J., Vágner, M., Tichá, I., Schäfer, C., Čapková, V.: Stress responses and changes in thylakoid membrane proteins and pigments of tobacco plantlets during *ex vitro* acclimation in dependence on previous *in vitro* cultivation conditions. - *Biol. Plant.*, in press, 2001.
- Leto, K.J., Arntzen, C.: Cation-mediated regulation of excitation energy distribution in chloroplasts lacking organized photosystem II complexes. - *Biochim. biophys. Acta* **637**: 107-117, 1981.
- Lipavská, H., Svobodová, H., Albrechtová, J., Kumstýřová, L., Vágner, M., Vondráková, Z.: Carbohydrate status during somatic embryo maturation in Norway spruce. - *In Vitro cell. dev. Biol. Plant* **36**: 260-267, 2000.
- Paul, M.J., Stitt, M.: Effects of nitrogen and phosphorus deficiencies on levels of carbohydrates, respiratory enzymes and metabolites in seedlings of tobacco and their response to exogenous sucrose. - *Plant Cell Environ.* **16**: 1047-1057, 1993.
- Pospíšilová, J., Tichá, I., Kadleček, P., Haisel, D., Plzáková, Š.: Acclimatization of micropropagated plants to *ex vitro* conditions. - *Biol. Plant.* **42**: 481-497, 1999.
- Preece, J.E., Sutter, E.G.: Acclimatization of micropropagated plants to greenhouse and field. - In: Debergh, P.C., Zimmerman, R.H. (ed.): *Micropropagation. Technology and Application*. Pp. 71-93. Kluwer Academic Publishers, Dordrecht - Boston - London 1991.
- Tichá, I., Čáp, F., Pacovská, D., Hofman, P., Haisel, D., Čapková, V., Schäfer, C.: Culture on sugar medium enhances high light resistance of plantlets grown *in vitro*. - *Physiol. Plant.* **102**: 155-162, 1998.
- Van Huylenbroeck, J.M., Debergh, P.C.: Impact of sugar concentration *in vitro* on photosynthesis and carbon metabolism during *ex vitro* acclimatization of *Spathiphyllum* plantlets. - *Physiol. Plant.* **96**: 298-304, 1996.