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BRIEF COMMUNICATION

Stomatal morphology during acclimatization of tobacco plantlets to *ex vitro* conditions

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Abstract

Image analysis was used in studying stomatal morphology during acclimatization of tobacco plantlets to *ex vitro* conditions. 45 d after transfer leaf area was 15 times, and total number of stomata per leaf four times increased. During acclimatization stomatal density was decreased considerably on both leaf sides, and was compensated by an increase in stomatal sizes, *e.g.*, in stomatal length and in stomatal area (both guard cells and pore). Elongation of stomata was increased indicating that the originally circular stomata of *in vitro* plantlets were changed into elliptical ones in *ex vitro* acclimatized plants.

Additional key words: epidermis, *Nicotiana tabacum* L., stomatal density and sizes

Acclimatization of *in vitro* grown plantlets after transfer to *ex vitro* environment is often a critical but necessary period in micropropagation. The high air humidity, usually low irradiance, CO_2 shortage, presence of sugars and phytohormones in the medium, *etc.* during *in vitro* culture result in plantlets with specific structure and functioning (*e.g.*, Kozai 1991, Pospíšilová *et al.* 1997). After transfer to open air and soil, plants have to overcome the sudden change in environmental conditions (decrease in air humidity, increase in irradiance and CO_2 concentration, *etc.*) and have to acclimatize to the new environment. During acclimatization important changes in plant structure as well as in their physiology have been reported (for reviews see, *e.g.*, Preece and Sutter 1991, Pospíšilová *et al.* 1997). Among important

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structural ones a decrease/increase in stomatal density and a pronounced change in stomatal shape have been mentioned. Stomatal density of *in vitro* leaves was found to be higher in *Vaccinium corymbosum* (Noé and Bonini 1996) and *Betula uber* (Jamison and Renfroe 1998) but lower in *Prunus insititia* (Brainerd *et al.* 1981) as compared with that of *ex vitro* ones. Stomata of *in vitro* grown plantlets were often found to be ring shaped, raised and widely open whereas during *ex vitro* acclimatization stomatal shape changed in a more elliptical and stomata were sunken (*e.g.*, Wetzstein and Sommer 1983).

Stomatal morphology in photoautotrophically and photomixotrophically grown *in vitro* tobacco plantlets under two irradiances was studied by Tichá *et al.* (1997). Stomatal density was enhanced in leaves developing at higher growth irradiance, and this effect was more evident in upper leaves. Furthermore, growth and photosynthetic performance of the photoautotrophically and photomixotrophically grown *in vitro* plantlets were heavily affected by irradiance and the presence of sugar in the medium (Tichá *et al.* 1998).

The aim of this study was to follow sets of the above mentioned plantlets during transfer to soil and acclimatization to *ex vitro* environment, *i.e.*, how the differently *in vitro* pretreated plantlets will respond to the identical conditions for all the plants after transfer. Changes in stomatal density, form and sizes on both the adaxial and abaxial leaf epidermes during acclimatization in parallel with photosynthetic parameters (Kadleček 1997, Kadleček *et al.* 1998) were studied. Since the trends in stomatal parameters during acclimatization were very similar in plants having different pretreatments *in vitro*, as an example the data for originally photomixotrophically grown plants under higher irradiance are presented here.

Nodal cuttings of tobacco (*Nicotiana tabacum* L. cv. Samsun) were grown *in vitro* on the Murashige and Skoog solid medium with 3 % sucrose under irradiance of 200 μmol m^{-2} s^{-1}, day/night temperatures 25/18 °C and photoperiod 16 h. After 35 d plantlets were potted into soil and transferred to the greenhouse (irradiance 30 to 90 μmol m^{-2} s^{-1} and temperature 18 to 26 °C at 15:00) and after further 20 d to open air (irradiance 200 to 1400 μmol m^{-2} s^{-1} and temperature 24 to 31 °C at 15:00; Kadleček 1997, Kadleček *et al.* 1998). The fourth leaves from bottom in *in vitro* grown plantlets, and the tenth leaves from bottom in plants 11 and 45 d after transfer were analysed. Microrelief preparations of both leaf surfaces were made and analysed using an image analysis system *LUCIA G*, version *3.52* (*Laboratory Imaging*, Praha, Czech Republic). By this method more precise quantitative data in an easier and quicker way were got than it is possible by classical microscopy. On each leaf side five measurement frames of known area from the middle of leaf half were examined. Binary images of stomata were made for calculating stomatal density (number of stomata inside measurement frame where stomata touching left and bottom lines were excluded, whereas stomata touching top and right lines were included, and related to leaf area unit), length as MaxFeret (maximal value of the set of Feret's diameters), width as MinFeret (minimal value of the set of Feret's diameters) and elongation (ratio of MaxFeret to MinFeret). Stomatal area represented area of both guard cells including pore. Leaf area was determined by a stereological method on leaf copies. Total stomatal numbers were calculated by multiplying stomatal densities and leaf areas.

Tobacco has amphistomatous leaves. Stomatal density on the abaxial leaf side was kept higher than on the adaxial one (Fig. 1). The relatively high stomatal density typical for *in vitro* grown tobacco plantlets was decreased after *ex vitro* transfer (Fig. 1, Table 1). The decrease in stomatal density in tobacco leaves after transfer

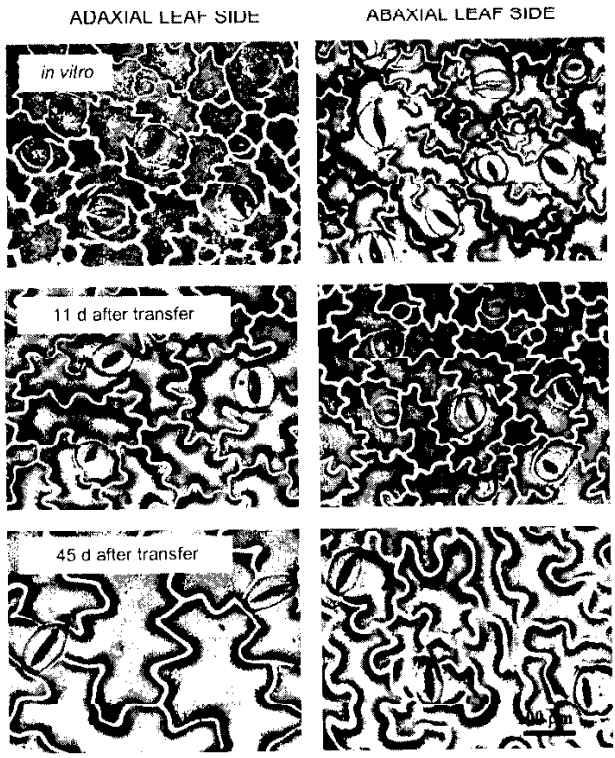

Fig. 1. Stomatal morphology on adaxial and abaxial leaf sides in *in vitro* grown tobacco plantlets, and in plants 11 d after transfer (greenhouse) and 45 d after transfer (open air).

was accompanied by an increase in stomatal sizes and area of the whole stomatal complex (Table 1). This compensation effect was found in *in vivo* plants (for review see Tichá 1982) as well as in *in vitro Nicotiana tabacum* plantlets (Tichá *et al.* 1997). A similar decrease in stomatal density during acclimatization to *ex vitro* environment was found in *Liquidambar styraciflua* (Wetzstein and Sommer 1983) and in *Vaccinium corymbosum* (Noé and Bonini 1996). On the other hand, in *Rhododendron* ssp. plants, stomatal density increased (Waldenmaier and Schmidt 1990). In *Prunus serotina* stomatal density increased from the first to the fourth week but decreased during the following four weeks of acclimatization, and stomatal length changed in the opposite way (Drew *et al.* 1992).

Table 1. Stomatal characteristics in *in vitro* grown tobacco plantlets and in plants 11 d after transfer (greenhouse) and 45 d after transfer (open air). Means ± SE of three plants.

Parameter		Adaxial leaf side	Abaxial leaf side
Stomatal density [mm^{-2}]	*in vitro* plantlets	104.0 ± 4.3	156.6 ± 10.6
	plants 11 d after transfer	52.4 ± 1.6	124.4 ± 5.1
	plants 45 d after transfer	24.2 ± 2.3	41.1 ± 3.3
Stomatal length [μm]	*in vitro* plantlets	36.7 ± 0.5	34.0 ± 0.4
	plants 11 d after transfer	31.9 ± 0.7	30.5 ± 0.5
	plants 45 d after transfer	43.7 ± 0.7	46.0 ± 0.6
Stomatal width [μm]	*in vitro* plantlets	29.7 ± 0.4	29.2 ± 0.3
	plants 11 d after transfer	25.4 ± 0.5	24.6 ± 0.3
	plants 45 d after transfer	29.0 ± 0.5	31.3 ± 0.4
Stomatal area [μm^2]	*in vitro* plantlets	867 ± 21	798 ± 17
	plants 11 d after transfer	642 ± 26	597 ± 17
	plants 45 d after transfer	981 ± 27	1117 ± 26
Stomatal elongation	*in vitro* plantlets	1.23 ± 0.01	1.16 ± 0.01
	plants 11 d after transfer	1.25 ± 0.02	1.24 ± 0.01
	plants 45 d after transfer	1.52 ± 0.02	1.47 ± 0.02

Total numbers of stomata per whole leaf, *i.e.*, on both leaf surfaces together, were nearly four times higher 45 d after *ex vitro* transfer as compared to those of *in vitro* plantlets (Table 2). The main reason for this was the enormous enlargement in leaf area after transfer (*cf.* also our data on total stomatal numbers in Pospíšilová *et al.* 1998).

Table 2. Leaf area, total number of stomata per leaf, and ratio adaxial/abaxial stomatal density in *in vitro* grown tobacco plantlets and in plants 11 d after transfer (greenhouse) and 45 d after transfer (open air). Means ± SE of three plants.

	Leaf area [mm^2]	Number of stomata [leaf^{-1}]	Stomatal ratio adaxial/abaxial
In vitro plantlets	1 292 ± 132	336 786 ± 34 529	0.70 ± 0.04
Plants 11 d after transfer	2 210 ± 140	302 275 ± 26 376	0.43 ± 0.02
Plants 45 d after transfer	19 008 ± 5308	1 240 652 ± 346 426	0.60 ± 0.05

Leaves from *in vitro* grown tobacco plantlets showed still new developing stomata, and therefore a large heterogeneity in stomatal sizes on both leaf sides was observed (Fig. 1). Plantlet leaves had guard cells forming more or less a circular ring but in leaves of *ex vitro* transferred plants (45 d after transfer) no more dividing stomata were found and the outer shape of the both guard cells was elliptical, stomata were prolongated (Fig. 1). This was best manifested by the stomatal elongation which increased on both leaf sides (Table 1). Similar changes in stomatal shape during

acclimatization were observed by Wetzstein and Sommer (1983) in *Liquidambar styraciflua*, by Marin *et al.* (1988) in *Prunus cerasus* and by Noé and Bonini (1996) in *Vaccinium corymbosum*.

In conclusion, during acclimatization of *in vitro* grown tobacco plantlets after transfer to soil and open air important changes in stomatal shape, sizes and density were found. Whereas stomatal characteristics in *in vitro* grown plantlets resemble those of young, intensively growing plants, in plants after transfer and after a period of acclimatization stomatal features correspond to those found in fully differentiated mature leaves *in vivo*.

References

Brainerd, K.E., Fuchigami, L.H., Kwiatkowski, S., Clark, C.S.: Leaf anatomy and water stress of aseptically cultured "Pixy" plum grown under different environments. - HortScience **16:** 173-175, 1981.

Drew, A.P., Kavanagh, K.L., Maynard, C.A.: Acclimatizing micropropagated black cherry by comparison with half-sib seedlings. - Physiol. Plant. **86:** 459-464, 1992.

Jamison, J.A., Renfroe, M.H.: Micropropagation of *Betula uber* (Ashe) Fernald. - *In Vitro* cell. dev. Biol. - Plant **34:** 147-151, 1998.

Kadleček, P.: [Effect of pretreatment by irradiance and exogenous saccharose under *in vitro* conditions on photosynthesis and growth of tobacco (*Nicotiana tabacum* L.) plants during acclimatization after transfer to soil.] - Diploma work, Charles University, Department of Plant Physiology, Praha 1997. [In Czech.]

Kadleček, P., Tichá, I., Čapková, V., Schäfer, C.: Acclimatization of micropropagated tobacco plantlets. - In: Garab, G. (ed.): Photosynthesis: Mechanisms and Effects. Vol. V. Pp. 3853-3856. Kluwer Academic Publishers, Dordrecht - Boston - London 1998.

Kozai, T.: Micropropagation under photoautotrophic conditions. - In: Debergh, P.C., Zimmerman, R.H. (ed.): Micropropagation. Technology and Application. Pp. 447-469. Kluwer Academic Publishers, Dordrecht - Boston - London 1991.

Marin, J.A., Gella, R., Herrero, M.: Stomatal structure and functioning as a response to environmental changes in acclimatized micropropagated *Prunus cerasus* L. - Ann. Bot. **62:** 663-670, 1988.

Noé, N., Bonini, L.: Leaf anatomy of highbush blueberry grown *in vitro* and during acclimatization to *ex vitro* conditions. - Biol. Plant. **38:** 19-25, 1996.

Pospíšilová, J., Čatský, J., Šesták, Z.: Photosynthesis in plants cultivated *in vitro*. - In: Pessarakli, M. (ed.): Handbook of Photosynthesis. Pp. 525-540. Marcel Dekker, New York - Basel - Hong Kong 1997.

Pospíšilová, J., Wilhelmová, N., Synková, H., Čatský, J., Krebs, D., Tichá, I., Hanáčková, B., Snopek, J.: Acclimation of tobacco plantlets to *ex vitro* conditions as affected by application of abscisic acid. - J. exp. Bot. **49:** 863-869, 1998.

Preece, J.E., Sutter, E.G.: Acclimatization of micropropagated plants to the 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.: Photosynthetic characteristics during ontogenesis of leaves 7. Stomata density and sizes. - Photosynthetica **16:** 375-471, 1982.

Tichá, I., Čáp, F., Pacovská, D., Hofman, P., Haisel, D., Čapková, V., Schäfer, C.: Culture on sugar medium enhances photosynthetic capacity and high light resistance of plantlets grown *in vitro*. - Physiol. Plant. **102:** 155-162, 1998.

Tichá, I., Obermajer, P., Snopek, J.: Stomata density and sizes in *in vitro* grown tobacco plantlets. - Acta Fac. Rer. nat. Univ. Comenianae, Physiol. Plant. **29:** 101-107, 1997.

Waldenmaier, S., Schmidt, G.: Histologische Unterschiede zwischen *in-vitro*- und *ex-vitro*-Blättern bei der Abhärtung von *Rhododendron*. - Gartenbauwissenschaft **55:** 49-54, 1990.

Wetzstein, H.Y., Sommer, H.E.: Scanning electron microscopy of *in vitro*-cultured *Liquidambar styraciflua* plantlets during acclimatization. - J. amer. Soc. hort. Sci. **108:** 473-480, 1983.