BRIEF COMMUNICATION

Leaf structure of tobacco *in vitro* grown plantlets as affected by saccharose and irradiance

B. RADOCHOVÁ, A. VIČÁNKOVÁ, J. KUTÍK and I. TICHÁ

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

Abstract

Tobacco plantlets were cultured *in vitro* under high (200 μmol $m^{-2} s^{-1}$) or low (60 μmol $m^{-2} s^{-1}$) irradiance with or without saccharose in the medium. Light microscopy and image analysis were used to evaluate the effect of these culture conditions on leaf anatomy. Addition of saccharose resulted in thicker leaves (all leaf layers) and larger mesophyll cells under both growth irradiances. Various irradiance affected leaf anatomy differently when plantlets had been cultivated in presence or absence of saccharose in the medium. While under high irradiance in presence of saccharose leaf thickness and number of chloroplasts per cell section were increased, plantlets grown under high irradiance in absence of saccharose had thinner leaves and less chloroplasts per cell section. The changes were more pronounced in palisade parenchyma layer.

Additional key words: epidermis, leaf anatomy, *Nicotiana tabacum*, spongy parenchyma layer.

Plants cultured *in vitro* grow usually under very different conditions as compared to plants grown in open air. Low irradiance and high relative humidity in culture vessels strongly alter their anatomy, including reduction in the palisade parenchyma layer with extensive mesophyll intercellular spaces (Donnelly and Vidaver 1984, Smith *et al.* 1986), reduced cuticle and wax development (Johansson *et al.* 1992) and raised stomata often with diminished function (Wetzstein and Sommer 1983, Blanke and Belcher 1989, Johansson *et al.* 1992). It was stated earlier (Lee *et al.* 1988, Capellades *et al.* 1990) that irradiance influences leaf anatomy and morphology during *in vitro* culture similarly as it is known in *in vivo* grown plants (Björkmann 1981). Furthermore, leaf anatomy of *in vitro* grown plantlets was modified by irradiance to a higher extent as compared to plants grown *in vivo* (Lee *et al.* 1988). However, all the studies mentioned above were made on conventionally *in vitro* grown plantlets, *e.g.*, supported by saccharose addition to the medium. Saccharose has been found to have a positive effect on net photosynthetic rate in *Nicotiana tabacum* and *Flaveria bidentis* plantlets (Furbank *et al.* 1997), and absence of saccharose has been found to cause photoinhibition in *Nicotiana tabacum* plantlets at high growth irradiance (Tichá *et al.* 1998). However, effect of irradiance on leaf anatomy in the presence or absence of saccharose has been studied only rarely (in *Actinidia deliciosa*, Dimassi-Theriou and Bosabalidis 1997). The aim of the present investigation was to study leaf structure in tobacco plantlets under two irradiances in the presence or absence of saccharose in the medium.

Tobacco (*Nicotiana tabacum* L. cv. Samsun) nodal cuttings were cultivated in baby food jars on solidified Murashige-Skoog medium (Murashige and Skoog 1962) photoautotrophically (without saccharose) or photomixotrophically (with 3 % saccharose) at 25/18 °C day/night temperatures, 16-h photoperiod, under two irradiances (PAR): 200 μmol $m^{-2} s^{-1}$ (high irradiance, HL), and 60 μmol $m^{-2} s^{-1}$ (low irradiance, LL). Vessels were covered with gas permeable closures (*Suncaps*, *Sigma*) and CO_2 concentration in the cultivation chambers was increased by adding beakers filled with carbonate/bicarbonate buffers (Tichá 1996).

Received 9 June 2000, *accepted* 12 July 2000.
Acknowledgement: This work was supported by the Grant Agency of Charles University (Project 96/1998).
Fax: (+420) 2 21953306, e-mail: barbora@natur.cuni.cz

Segments from third or fourth leaf blades of 20-days-old plantlets were fixed in 5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 2 h, rinsed three times in buffer, post-fixed with 2 % OsO_4 in the same buffer for 2 h and then dehydrated through an ethanol series and embedded into Spurr's epoxy resin (Spurr 1969). Semithin sections (about 1.0 μm thick) were cut on a *Reichert OmU2* (Austria) ultramicrotome and stained with 1 % solution of toluidine blue in 1 % sodium tetraborate.

In each treatment, leaves from three plantlets were used for the assessments. Five cross-sections from each leaf were captured from light microscope (*Olympus BX 40*, Tokyo, Japan) to computer by one-chip TV camera (*COHU*, San Diego, USA) and evaluated using image analysis system *LUCIA G*, version *3.52* (*Laboratory Imaging*, Prague, Czech Republic). Thickness of the leaf and of each leaf layer was measured five times on each cross-section. Palisade parenchyma to spongy parenchyma thickness (PP/SP) ratio was calculated. From each cross-section five cells of palisade and five cells of spongy parenchyma were used to evaluate relative size of the cell, measured as cell cross-sectional area. In the same cells number of chloroplasts per cell section was counted.

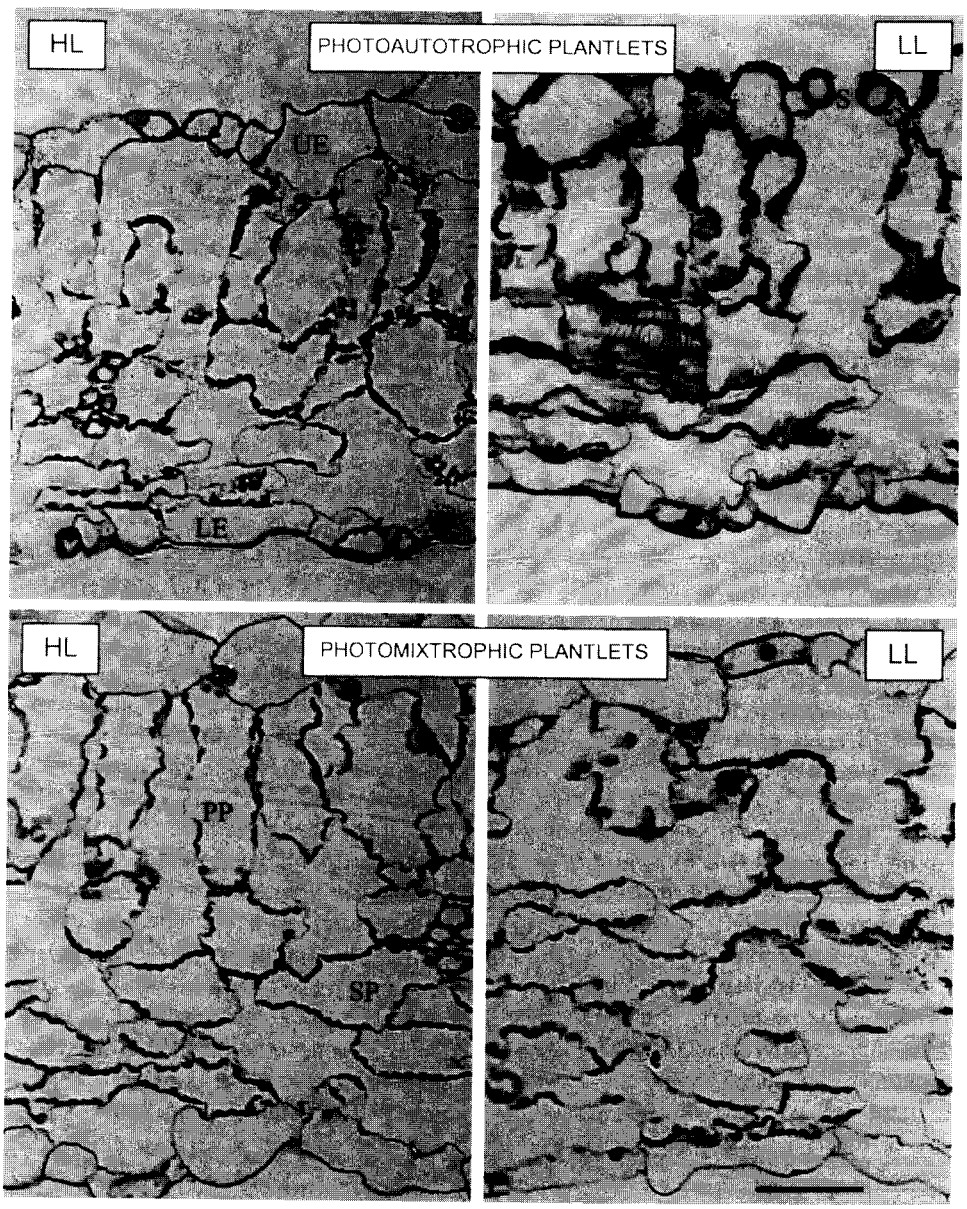

Fig. 1. Leaf cross-sections of *in vitro* tobacco plantlets grown photoautotrophically or photomixotrophically under high (HL) or low (LL) irradiances. UE - upper epidermis, LE - lower epidermis, S - stoma, PP - palisade parenchyma, SP - spongy parenchyma. *Bar* = 50 μm valid for all four images.

Photomixotrophically grown plantlets under both growth irradiances had thicker, better developed leaves with larger mesophyll cells than plantlets grown photoautotrophically (Fig. 1). Furthermore, presence or absence of saccharose in the medium strongly altered response of plantlets to HL and LL. As compared to photoautotrophically grown plantlets, photomixotrophically grown plantlets showed some typical changes in leaf anatomy, described previously for both *in vitro* (Lee *et al.* 1988, Dimassi-Theriou and Bosabalidis 1997) and *in vivo* (Wild and Wolf 1980, Smith and Longstreth 1994) grown plants and known as "sun" and "shade" response. HL plantlets had slightly thicker leaves, well developed and more compact palisade parenchyma layer (Fig. 1, Table 1). PP/SP ratio was much higher due to more developed palisade parenchyma and less developed spongy parenchyma. Both palisade and spongy cells were smaller and in palisade parenchyma cells chloroplasts did not occur along the upper periclines. Number of chloroplasts per cell section was higher in both palisade and spongy parenchyma and this trend was more expressed in spongy parenchyma layer (Table 1).

In photoautotrophically grown plantlets response to HL showed a quite different picture (Fig. 1). All leaf layers were thinner including palisade parenchyma resulting in thinner leaves and only slightly enhanced PP/SP ratio as compared to LL plantlets (Table 1). Palisade parenchyma was also less compact and worse developed (Fig. 1). Cells of palisade and spongy parenchyma were smaller than in LL plantlets and the differences were more pronounced than in plantlets grown photomixotrophically. As in 3 % HL plantlets, chloroplasts in palisade parenchyma cells of 0 % HL plantlets occurred only along the anticlines. In HL plantlets number of chloroplasts per cell section was reduced more in palisade parenchyma cells than in spongy parenchyma ones.

Our results seem to be in agreement with those previously described by Tichá *et al.* (1998) who stated that plantlets grown *in vitro* photoautotrophically are more prone to photoinhibition. Decrease in number of chloroplasts per cell section in plantlets grown photoautotrophically under high irradiance was reported also by Dimassi-Theriou and Bosabalidis (1997) for *Actinidia deliciosa*. Our results, furthermore, suggest higher effect of irradiance on palisade parenchyma layer. Possibly, higher amount of irradiance received by the cell situated nearer to the upper leaf surface could explain the higher degree of injury of photosynthetic apparatus.

Table 1. Leaf structure of *in vitro* photoautotrophically and photomixotrophically grown tobacco plantlets under different irradiances (HL - 200 μmol m^{-2} s^{-1} and LL - 60 μmol m^{-2} s^{-1}). Means ± SE; **n* = 15, ***n* = 75.

Parameter		Photoautotrophic plantlets		Photomixotrophic plantlets	
		HL	LL	HL	LL
Leaf thickness [μm]*		178.5 ± 4.47	190.4 ± 2.84	237.7 ± 3.60	234.7 ± 4.26
Upper epidermis thickness [μm]*		24.9 ± 0.62	27.4 ± 0.80	29.8 ± 0.81	28.4 ± 0.47
Palisade parenchyma thickness [μm*]		59.8 ± 1.71	61.1 ± 0.82	78.0 ± 1.78	67.6 ± 1.29
Spongy parenchyma thickness [μm]*		77.0 ± 2.68	82.8 ± 1.80	104.1 ± 1.50	113.5 ± 2.44
Lower epidermis thickness [μm]*		16.8 ± 0.33	19.0 ± 0.25	25.8 ± 0.50	25.3 ± 0.56
PP/SP ratio [%]*		77.6	73.8	74.9	59.6
Cell cross-sectional area [μm^2]**	palisade	1412.3 ± 74.57	1667.7 ± 50.48	1743.1 ± 60.49	1866.8 ± 75.29
	spongy	1051.3 ± 49.20	1475.7 ± 57.35	1492.3 ± 50.27	1677.7 ± 76.32
Number of chloroplasts**	palisade	13.3 ± 0.40	19.9 ± 1.41	19.5 ± 0.45	18.6 ± 0.54
	spongy	13.6 ± 0.39	16.1 ± 0.43	17.3 ± 0.54	15.9 ± 0.47

References

Blanke, M.M., Belcher, A.R.: Stomata of apple leaves cultured *in vitro*. - Plant Cell Tissue Organ Cult. **19**: 85-89, 1989.

Björkman, O.: Responses to different quantum flux densities. - In: Lange, O.L., Nobel, P.S., Osmond, C.B., Ziegler, H. (ed.): Encyclopedia of Plant Physiology, New Series. Vol. 12A. Physiological Plant Ecology I. Pp. 57-107. Springer-Verlag, Berlin - Heidelberg - New York 1981.

Capellades, M., Fontarnau, R., Carulla, C., Debergh, P.: Environment influences anatomy of stomata and epidermal cells in tissue-cultured *Rosa multiflora*. - J. amer. Soc. hort. Sci. **115**: 141-145, 1990.

Dimassi-Theriou, K., Bosabalidis, A.M.: Effects of light, magnesium and sucrose on leaf anatomy, photosynthesis, starch and total sugar accumulation, in kiwifruit cultured *in vitro*. - Plant Cell Tissue Organ Cult. **47**: 127-134, 1997.

Donnelly, D.J., Vidaver, W.E.: Leaf anatomy of red raspberry transferred from culture to soil. - J. amer. Soc. hort. Sci. **109**: 172-176, 1984.

Furbank, R.T., Pritchard, J., Jenkins, C.L.D.: Effects of exogenous sucrose feeding on photosynthesis in the C_3 plant tobacco and the C_4 plant *Flaveria bidentis*. - Aust. J. Plant Physiol. **24**: 291-299, 1997.

Johansson, M., Kronestedt-Robards, E.C., Robards, A.W.: Rose leaf structure in relation to different stages of micropropagation. - Protoplasma **166**: 165-176, 1992.

Lee, N., Wetzstein, H.Y., Sommer, H.E.: Quantum flux density effects on the anatomy and surface morphology of *in vitro*- and *in vivo*-developed sweetgum leaves. - J. amer. Soc. hort. Sci. **113**: 167-171, 1988.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Smith, J.E., Longstreth, D.J.: Leaf expansion and carbon assimilation in cotton leaves grown at two photosynthetic photon flux densities. - Amer. J. Bot. **81**: 711-717, 1994.

Smith, M.A.L., Palta, J.P., McCown, B.H.: Comparative anatomy and physiology of microcultured, seedling, and greenhouse-grown Asian white birch. - J. amer. Soc. hort. Sci. **111**: 437-442, 1986.

Spurr, A.R.: A low-viscosity epoxy resin embedding medium for electron microscopy. - J. Ultrastruct. Res. **26**: 31-43, 1969.

Tichá, I.: Optimization of photoautotrophic tobacco *in vitro* culture: effect of suncaps closures on plantlet growth. - Photosynthetica **32**: 475-479, 1996.

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.

Wetzstein, H.Y., Sommer, H.E.: Scanning electron microscopy of *in vitro*-cultured *Liquidambar styraciflua* plantlets during acclimatization. - J. amer. Soc. hort. Sci. **108**: 475-480, 1983.

Wild, A., Wolf, G.: The effect of different light intensities on the frequency and size of stomata, the size of cells, the number, size and chlorophyll content of chloroplasts in the mesophyll and the guard cells during the ontogeny of primary leaves of *Sinapis alba*. - Z. Pflanzenphysiol. **97**: 325-342, 1980.