Insertion profiles in stomatal density and sizes in *Nicotiana tabacum* L. plantlets

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Abstract

Tobacco (*Nicotiana tabacum* L. cv. Samsun) plantlets were cultured *in vitro* on Murashige-Skoog medium photoautotrophically (without sucrose) or photomixotrophically (with 3 % sucrose) under two irradiances [70 or 230 μmol m^{-2} s^{-1}]. Significant differences in stomatal density and sizes in leaves of different insertion levels (3rd, 5th and 7th leaves from bottom) in photomixotrophic plantlets but not in photoautotrophic ones were found after 35 d of culture. Stomatal density was higher in upper leaves and on abaxial leaf side. Higher irradiance enhanced stomatal density in photoautotrophic plantlets. Stomatal sizes decreased with leaf insertion level but no significant differences between leaf sides were found. Abaxial stomata were more circular than the adaxial ones. In photomixotrophic plantlets stomata tended to be more elongated in the 3rd and the 5th leaves, whereas stomatal elongation in photoautotrophic plantlets was similar in all leaves.

Additional key words: irradiance, photoautotrophic and photomixotrophic growth, plant heterogeneity, stomatal elongation and shape, sucrose.

Introduction

Heterogeneity in stomatal anatomy and photosynthetic characteristics in leaves of different insertion levels, along leaf blade, and in leaves of different age are well known in field grown plants (for reviews see, *e.g.*, Tichá 1982, Tichá 1985, Tichá *et al.* 1985, Čatský and Šesták 1997, Weyers and Lawson 1997). Differences in leaf structure in leaves of different insertion levels were first systematically studied by Zalenskiĭ (1904). He found that upper leaves have a more xeromorphic structure than lower ones on the stem and explained this by the fact that upper leaves are relatively less supplied with water. This relationship has been later on reformulated by Maximov (1929) as the so-called Zalenskiĭ law: „Anatomical structure of leaves on the stem is the function of their distance from the root system". Zalenskiĭ presented altogether three reasons that may cause changes in anatomical structure in leaves of different insertion levels: *1*) genetic predetermination of leaf primordium, *2*) different water supply of leaves, and *3*) different microclimatic conditions that influence the leaf during its growth on the plant. In the field or glasshouse all these factors are important.

In vitro conditions are very specific - the microclimate gradients are minimized as compared to *ex vitro* environment. This means that two factors thought to cause differences among leaves of different insertion levels are minimized when plantlets are grown *in vitro* and the main reason of changes could be therefore genetic predetermination of leaves. The plantlets cultivated *in vitro* are usually grown under very low irradiance (30 - 70 μmol m^{-2} s^{-1}) with sugars and phytohormones in the medium (Lee *et al.* 1988, Sutter 1988, Shackel *et al.* 1990, Ziv and Ariel 1994, Pierik 1997). In cultivation vessels almost 100 % relative humidity is achieved, but sometimes plantlets are not well supplied with carbon dioxide. CO_2 supply can be improved if gas-semipermeable closures (*Suncaps*, *Sigma*; Tichá 1996) are used.

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Abbreviations: HL - high irradiance; LL - low irradiance.

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Ontogenetic changes in leaf morphology and photosynthesis are sometimes substituted by changes in characteristics with leaf insertion level but this is not the same (*e.g.*, review Tichá 1982): upper leaves on the plant are younger and lower leaves are older as well but they originate from different leaf primordia.

Differences in stomatal characteristics in leaves of different insertion levels, during leaf ontogeny and in different parts of a single leaf blade are well known in *ex vitro* plants but only several facts are available about differences in *in vitro* cultured plantlets (Tichá *et al.* 1997, Zacchini *et al.* 1997). The aim of this study was to find out whether there exist any insertion profiles in stomatal characteristics in leaves of *in vitro* grown plantlets (where the plantlets are small and only minimal gradients in the controlled microclimate are present).

Materials and methods

Tobacco (*Nicotiana tabacum* L. cv. Samsun) plantlets were cultured from nodal cuttings for 35 d on Murashige-Skoog medium (Murashige and Skoog 1962) photoautotrophically (without sucrose) or photomixotrophically (with 3 % sucrose) under low (70 μmol m^{-2} s^{-1}) or high irradiance (230 μmol m^{-2} s^{-1}). Plantlets were cultured at day/night temperature 25/18 °C with 16-h photoperiod. Culture vessels were covered with highly gas-permeable closures (*Suncaps, Sigma)* and elevated CO_2 concentration in the culture chamber was achieved by adding beakers filled with 2 M carbonate/bicarbonate buffers (Tichá 1996).

Negative nail-varnish replicas from both leaf sides of the 3rd, 5th and 7th leaves from the bottom of the plantlets were prepared. Three plantlets for each combination of sucrose and irradiance were taken. Ten images from each leaf side were captured from light microscope (*Olympus BX 40,* Tokyo, Japan) to PC by one-chip TV camera (*COHU,* San Diego, USA) and studied by digital image processing and analysis (*Lucia G,* version 3.52, *Laboratory Imaging*, Prague, Czech Republic). Stomatal density, length and width were measured for each image. Stomatal elongation was counted as the ratio of stomatal length and stomatal width. Leaf area was measured by image analysis and the total number of stomata per leaf was estimated by multiplying the sum of stomatal densities on upper and lower leaf surfaces by leaf area.

GLM analysis of variance for statistics and Tukey-Kramer test for multiple comparisons were used. Stomatal density and sizes did not have normal distribution, therefore logarithms of the original values for statistical tests were used. For elongation only trends were shown and this characteristic was not statistically evaluated.

Results and discussion

Parts of adaxial and abaxial leaf epidermes of the 3rd, 5th and 7th leaves in tobacco plantlets cultured under high irradiance with sucrose in the medium are shown on Fig. 1. Significant differences in stomatal density (Fig. 2) and sizes (Fig. 3, Tables 1, 3) in tobacco leaves of different insertion levels in photomixotrophic plantlets

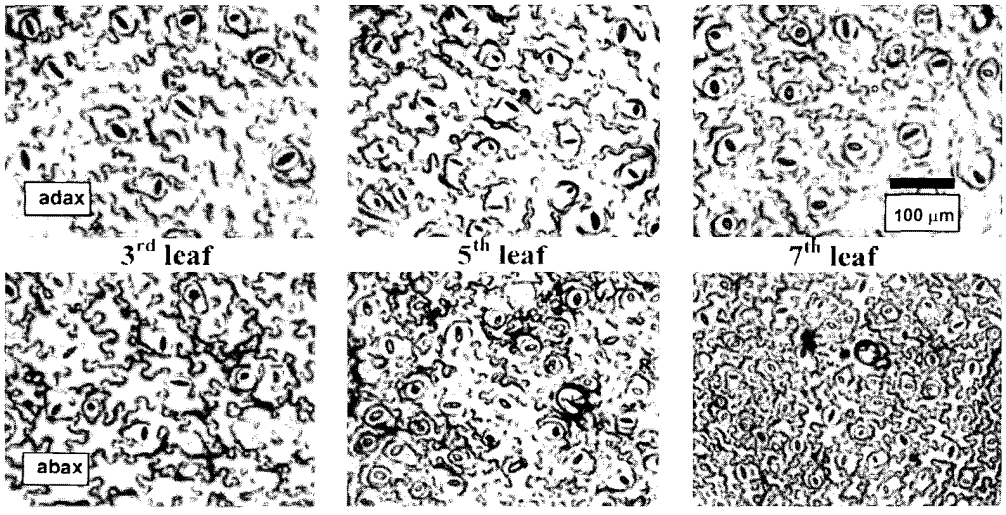

Fig. 1. Adaxial (upper row) and abaxial (lower row) leaf epidermes of the 3rd, 5th and 7th leaves from bottom in tobacco plantlets cultured *in vitro* on MS medium with 3 % sucrose under high irradiance (HL - 230 μmol m^{-2} s^{-1}).

but not in photoautotrophic ones were found. Stomatal density on both leaf sides in photomixotrophic plantlets increased whereas stomatal length decreased from the 3rd to the 7th leaves (Figs. 1, 2, 3). Similarly, stomatal width decreased towards the upper leaves (Table 1). These results are in agreement with the compensation effect (the higher stomatal density the smaller stomatal sizes). Compensation effect is well known in *ex vitro* plants (for reviews see Tichá 1982, Weyers and Lawson 1997; and papers by Slavík 1963, Wild and Wolf 1980, Drew *et al.* 1992, Furukawa 1992, 1997) and was found also in some *in vitro* cultured plantlets (Lee *et al.* 1988, Zacchini *et al.* 1997).

Higher irradiance enhanced stomatal density but the effect of sucrose in the medium was much stronger (Table 3). Similar results were obtained also by Tichá *et al.* (1997) in the 2nd and 4th leaves of *in vitro* grown tobacco and Lee *et al.* (1988) in *Liquidambar styraciflua* cultured *in vitro*. Furthermore, changes in stomatal density and sizes in tobacco during transfer from *in vitro* to *ex vitro* conditions have been analyzed (Wetzstein and Sommer 1983, Noé and Bonini 1996, Pospíšilová *et al.*

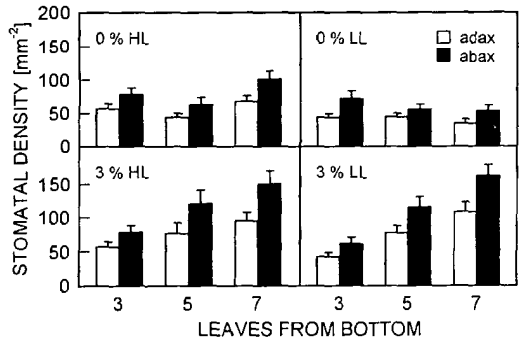

Fig. 2. Stomatal density on the upper (adax) and lower (abax) leaf sides in tobacco *in vitro* plantlets cultured photoautotrophically (0 % sucrose) or photomixotrophically (3 % sucrose) under high (HL - 230 μmol m^{-2} s^{-1}) or low (LL - 70 μmol m^{-2} s^{-1}) irradiance. Means of 30 measurements ± standard deviation.

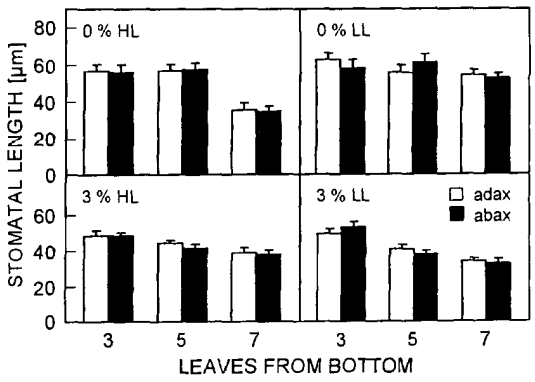

Fig. 3. Stomatal length on the upper (adax) and lower (abax) leaf sides in tobacco *in vitro* plantlets cultured at 0 or 3 % sucrose under high (HL) or low (LL) irradiance. The values are means of 30 measurements ± standard deviation.

Table 1. Stomatal width in leaves of different insertion levels of tobacco plantlets cultured under high (230 μmol m^{-2} s^{-1}, HL) or low (70 μmol m^{-2} s^{-1}, LL) irradiance on medium without sucrose (0 %) or with 3 % of sucrose (3 %). Means from 30 measurements ± standard deviation.

Plantlets	Leaf side	3rd leaf	5th leaf	7th leaf
0 % HL	adaxial	50.3 ± 2.8	49.9 ± 3.1	30.6 ± 3.3
	abaxial	49.9 ± 4.3	51.4 ± 3.1	31.7 ± 2.4
3 % HL	adaxial	36.1 ± 1.9	35.5 ± 1.8	32.4 ± 2.3
	abaxial	40.1 ± 2.1	36.4 ± 2.1	33.4 ± 2.3
0 % LL	adaxial	54.4 ± 3.4	48.0 ± 3.8	44.3 ± 2.1
	abaxial	50.5 ± 3.7	52.8 ± 3.8	46.2 ± 1.9
3 % LL	adaxial	37.7 ± 1.5	32.7 ± 2.2	29.9 ± 1.5
	abaxial	45.2 ± 2.5	32.8 ± 1.9	28.1 ± 2.2

1998, Tichá *et al.* 1999) and analogous conclusions were achieved.

Differences in stomatal density between upper and lower leaf sides of *in vitro* grown tobacco were found: significantly higher stomatal density appeared on abaxial (lower) leaf surfaces. Similar results were obtained in plants *in vivo, e.g.*, in *Nicotiana tabacum* (Turner and Begg 1973), *Pueraria lobata* (De Pereira-Netto *et al.* 1999) and *Wollemia nobilis* (Burrows and Bullock 1999). Sometimes no differences or higher stomatal numbers in adaxial (upper) leaf side were found (*Nicotiana glutinosa* - Glater *et al.* 1962, *Commelina communis* - Weyers and Meidner 1990).

Table 2. Stomatal elongation in leaves of different insertion levels of tobacco plantlets cultured under high (230 μmol m^{-2} s^{-1}, HL) or low (70 μmol m^{-2} s^{-1}, LL) irradiance on medium without sucrose (0 %) or with 3 % of sucrose (3 %). Means from 30 measurements ± standard deviation.

Plantlets	Leaf side	3rd leaf	5th leaf	7th leaf
0 % HL	adaxial	1.129±0.034	1.146±0.030	1.165±0.034
	abaxial	1.123±0.028	1.119±0.023	1.111±0.024
3 % HL	adaxial	1.372±0.071	1.260±0.051	1.203±0.031
	abaxial	1.226±0.051	1.152±0.033	1.146±0.029
0 % LL	adaxial	1.156±0.048	1.156±0.042	1.220±0.056
	abaxial	1.147±0.030	1.157±0.042	1.139±0.027
3 % LL	adaxial	1.322±0.060	1.246±0.060	1.149±0.027
	abaxial	1.180±0.056	1.156±0.034	1.168±0.034

The ratio of stomatal density on the upper and lower leaf sides (adax/abax) was similar in all studied leaves and variants of tobacco plantlets (± 0.70). No differences between leaf sides were found in stomatal length and stomatal width. In our experiments stomatal density was always higher on the abaxial leaf side in plantlets grown under both irradiances on medium with or without sucrose. This is also consistent with the findings of

Pospíšilová *et al.* (1998). Higher abaxial density is also known in field or greenhouse grown plants (*Nicotiana glutinosa* - Glater *et al.* 1962, *N. tabacum* - Slavík 1963, *Sinapis alba* - Wild and Wolf 1980). The ratio of the number of stomata on the adaxial and abaxial leaf sides may be influenced not only by the species studied but also by environmental factors.

Elongation of stomata was different in photoautotrophic and photomixotrophic plantlets (Table 2). In photoautotrophic ones no differences among leaves of different insertion levels were found. In photomixotrophic plantlets stomata were more elongated in the 3rd and 5th leaves and tended to be more circular with increasing leaf insertion level. These trends in stomatal shape are opposite to those found by Zacchini *et al.* (1997) in fruit trees cultured *in vitro*.

Table 3. Statistical evaluation of stomatal density and length on adaxial and abaxial sides in leaves of different insertion level of tobacco plantlets cultured under high (HL) or low (LL) irradiance on medium without sucrose (0 %) or with 3 % of sucrose (3 %). Tukey-Kramer test was used for multiple comparisons. Different characters indicate statistically significant difference (5 % significance level) separately for stomatal density and stomatal length.

	Density			Length		
	3rd leaf	5th leaf	7th leaf	3rd leaf	5th leaf	7th leaf
Adaxial	a	a,b	b,c	a	a	b
Abaxial	b,c	c	d	a	a	b
0 %	a	a	a	a	a	b
3 %	a	b	c	a	b,c	c
HL	a,b	b,c	b,c	a,b	b,c	c,d
LL	a,b,c	c	d	a,b,c	a,b,c	d

Total number of stomata per leaf increased with leaf insertion level in plants grown under low irradiance, whereas in plants cultivated under high irradiance no differences among leaves were found (data not shown).

In conclusion, presence of sucrose in the medium seemed to be an important factor in plant *in vitro* culture: it enables appearance of insertion profiles in stomatal density and sizes. As was stated in our previous studies with the same experimental material, photosynthetic and photochemical efficiencies and especially growth in photoautotrophic plantlets were negatively affected by the high irradiance applied and symptoms of photoinhibition were found (Tichá *et al.* 1998). A further finding is now that these high-light-stressed plantlets are not able to display insertion profiles in stomatal density and length. On the contrary, in photomixotrophic plantlets no symptoms of photoinhibition were observed under HL treatment (it indicates that several defensive mechanisms are active in 3 % HL plantlets), and insertion profiles in stomatal characteristics appeared.

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