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Leaf anatomy during leaf development of photoautotrophically *in vitro*-grown tobacco plants as affected by growth irradiance

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Abstract

Tobacco (*Nicotiana tabacum* L.) plants were cultured *in vitro* photoautotrophically at three levels of irradiance (PAR 400 - 700 nm): low (LI, 60 µmol m^{-2} s^{-1}), middle (MI, 180 µmol m^{-2} s^{-1}) and high (HI, 270 µmol m^{-2} s^{-1}). Anatomy of the fourth leaf from bottom was followed during leaf development. In HI and MI plants, leaf area expansion started earlier as compared to LI plants, and both HI and MI plants developed some adaptations of sun species: leaves were thicker with higher proportion of palisade parenchyma to spongy parenchyma tissue. Furthermore, in HI and MI plants palisade and spongy parenchyma cells were larger and relative abundance of chloroplasts in parenchyma cells measured as chloroplasts cross-sectional area in the cell was lower than in LI plants. During leaf growth, chloroplasts cross-sectional area in both palisade and spongy parenchyma cells in all treatments considerably decreased and finally it occupied only about 5 to 8 % of the cell cross-sectional area. Thus, leaf anatomy of photoautotrophically *in vitro* cultured plants showed a similar response to growth irradiance as *in vivo* grown plants, however, the formation of chloroplasts and therefore of photosynthetic apparatus was strongly impaired.

Additional key words: chloroplast, epidermis, growth irradiance, leaf thickness, leaf ontogeny, palisade parenchyma, spongy parenchyma.

Introduction

Conventionally *in vitro* cultured plants usually have altered leaf anatomy, including raised and ring-shaped stomata, poorly differentiated palisade parenchyma, large intercellular spaces in the mesophyll, reduced thickness of cuticle and epicuticular wax development (see Pospíšilová *et al*. 1999, Hazarika 2006 for reviews, Casanova *et al.* 2008). These modifications may be consequences of low irradiance (usually 30 - 60 µmol m^{-2} s^{-1}), low CO_2 concentration during the photoperiod, sucrose in the medium, and high relative humidity in the vessels.

Leaf anatomy of *in vitro* cultured plants is usually strongly modified by irradiance, similarly as it is known in *in vivo* conditions (Björkman 1981, Tichá 1985). In leaves of *in vitro* grown *Liquidambar styraciflua*, higher irradiance was associated with thicker leaves, more compact mesophyll and larger cells (Lee *et al*. 1988). An increase in irradiance stimulated the development of photosynthetic tissues also in leaflets of *Gardenia jasminoides* (Serret and Trillas 2000). In *Actinidia deliciosa*, an increase of irradiance resulted in a corresponding increase of the relative size of the leaf mesophyll cells and in a decrease of chloroplasts numbers and starch grains (Dimassi-Theriou and Bosabalidis 1997). Furthermore, growth of *Solanum tuberosum* plants under photo-autotrophic conditions, where both irradiance and CO_2 concentration were increased and sucrose in the medium eliminated, lead to thicker leaves and a more typical palisade parenchyma layer (Zobayed *et al*. 1999).

Photoautotrophic *in vitro* cultivation at higher irradiance and CO_2 concentration promotes growth and

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Abbreviations: DAE - days after emergence; HI - high irradiance; LI - low irradiance; MI - middle irradiance; PP/SP - palisade parenchyma to spongy parenchyma ratio.

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net photosynthetic rate (Kozai *et al*. 1991, Tichá 1996, Seon *et al*. 2000), however, high irradiance can cause photoinhibition (Tichá *et al*. 1998, Kadleček *et al*. 2003). Moreover, information about changes in photosynthetic performance and leaf structure during leaf development in *in vitro* plants is scarce. *Nicotiana tabacum* plants grown photoautotrophically at high irradiance exhibited symptoms, that usually accompany leaf senescence, very early during leaf area expansion (Radochová and Tichá 2008). Chlorophylls started to degrade (with preferential degradation of chlorophyll *a*), both maximum and actual photochemical efficiency of photosystem 2 was decreased and non-photochemical quenching of chlorophyll *a* fluorescence (NPQ) was increased. Nevertheless, net photosynthetic rate did not decrease consistently with decrease in chlorophyll content, but showed the typical ontogenetic course with gradual increase, reaching its maximum before full leaf area expansion and than followed by a decline (Radochová and Tichá 2008). Thus, in these plants photosynthetic performance and chlorophyll content in leaves was impaired and ontogenetic courses were changed. Therefore the aim of this study was to investigate, if such changes in photosynthetic performance and chlorophyll content are reflected as well on leaf structural level.

Materials and methods

Nodal cuttings of tobacco (*Nicotiana tabacum* L. cv. Samsun) were cultured *in vitro* on agar solidified Murashige and Skoog medium (*M5519*, *Sigma*, St. Louis, USA) photoautotrophically (without sucrose) at day/night temperatures of 25/18 °C, 16-h photoperiod, CO_2-enriched air (800 μmol mol^{-1}) and at three different levels of irradiance (PAR 400-700 nm) (60 μmol m^{-2} s^{-1}, LI; 180 μmol m^{-2} s^{-1}, MI and 270 μmol m^{-2} s^{-1}, HI). Carbonate/bicarbonate buffers were added into the growth chambers and enhanced CO_2 supply was ensured by covering the culture vessels with highly gas-permeable closures (*Suncaps*, *Sigma*) (Tichá 1996).

All measurements were made on the fourth leaves from bottom of the plants (five plants from each irradiance) at each sampling time. Leaf age was measured as the number of days after the leaf emerged on the plant apex. The fourth leaf emerged on the 11th to the 12th day after the experiment was started and measurements were made at the 5th, 9th, 13th, 17th and 23rd days after emergence (DAE).

Small segments (about 2.0 mm^2) from the middle of the leaf blade half 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. Semithin sections (about 1.0 µm thick) were cut on a *Reichert OmU2* (Vienna, Austria) ultramicrotome and stained with 1 % solution of toluidine blue in 1 % sodium tetraborate.

Three cross-sections from each leaf sample were captured by the light microscope (*Olympus BX 40*, Tokyo, Japan) equipped with an one-chip TV camera (*COHU*, San Diego, CA, USA) and evaluated using image analysis system *LUCIA G*, *version 3.52* (*Laboratory Imaging*, Prague, Czech Republic).

Thickness of the leaf and of each cell layer inside the leaf was measured three times in each cross-section. Palisade parenchyma to spongy parenchyma thickness (PP/SP) ratio was calculated.

Proportions of each leaf tissues (*i.e.* leaf epidermis, palisade and spongy parenchyma tissue) and intercellular spaces in the leaf were calculated as the ratio of tissue cross-sectional area to leaf cross-sectional area in the leaf cross-section.

From each leaf cross-section five cells of palisade parenchyma and five cells of spongy parenchyma were used to evaluate the relative size of the cell, expressed as the cell cross-sectional area. In the same cells, chloroplasts cross-sectional area per cell section was used to express chloroplast proportion in mesophyll cell.

Leaf area was determined by scanning leaf images and applying a computer program (*LUCIA G, v. 3.52*).

Two-way *ANOVA* test was used to analyze the data. Significant differences between means were detected by Student-Newman-Keuls Multiple Range Test using *BMDP Statistical Software* (University of California, Los Angeles, USA). Different letters in tables and figures indicate significant differences at $P = 0.05$.

Results and discussion

Fourth leaf in all treatments emerged from the apical bud 11th to 12th day after cultivation was started. The rapid leaf area expansion started earlier in HI and MI plants. Therefore, high irradiance caused acceleration of leaf development, as it was already described for many plant species in *in vivo* conditions (Wild and Wolf 1980, Smith and Longstreth 1994, Behera and Choudhury 2001, Yano and Terashima 2004). As the result of an accelerated rate of leaf area expansion in HI and MI plants a more differentiated tissue with larger intercellular spaces in the youngest (5 DAE) leaves as compared to LI ones was found (Fig. 1). During further leaf growth this difference disappeared.

During 23 d of leaf development leaf area expansion was not fully finished, with exception of HI plants (Table 1). Due to the different rate of leaf area expansion

Table 1. Thickness of different tissues during development of the 4th leaf in photoautotrophically *in vitro*-grown *Nicotiana tabacum* plants at low (LI, 60 µmol m^{-2} s^{-1}), middle (MI, 180 µmol m^{-2} s^{-1}) and high (HI, 270 µmol m^{-2} s^{-1}) irradiances. Different letters behind the mean values indicate significant differences at $P = 0.05$. The first letters indicate the evaluation of ontogenetic changes and the second letters indicate the evaluation of irradiance effect in respective treatment.

Thickness [µm]	Treatments	5 d	9 d	13 d	17 d	23 d
Leaf	LI	110.48 a,a	133.90 b,a	169.00 c,a	191.06 d,a	200.84 e,a
	MI	134.90 a,b	146.32 b,b	196.27 c,b	228.67 d,b	251.81 e,b
	HI	143.96 a,c	173.36 b,c	203.10 c,b	233.37 d,b	243.65 e,b
Adaxial epidermis	LI	13.66 a,a	16.48 b,a	20.19 c,a	23.64 d,a	22.87 d,a
	MI	15.48 a,b	19.09 b,a	22.66 c,a	24.68 c,a	24.30 c,a
	HI	19.02 a,c	23.63 b,b	23.55 b,a	24.77 b,a	25.78 b,a
Palisade parenchyma	LI	25.05 a,a	33.80 b,a	45.51 c,a	51.63 d,a	59.87 e,a
	MI	32.45 a,b	40.06 b,b	57.66 c,b	74.14 d,b	76.52 d,b
	HI	36.95 a,c	48.60 b,c	63.88 c,c	77.11 d,b	80.26 d,b
Spongy parenchyma	LI	57.70 a,a	69.18 b,a	85.22 c,a	94.29 d,a	100.60 e,a
	MI	71.98 a,b	70.64 a,a	97.87 b,b	107.25 c,b	127.45 d,c
	HI	72.39 a,b	83.14 b,b	95.73 c,b	110.15 d,b	115.69 d,b
Abaxial epidermis	LI	14.08 a,a	14.44 a,a	18.07 b,a	21.50 c,a	17.50 b,a
	MI	14.99 a,a	16.53 a,b	18.08 b,a	22.60 c,a	23.54 c,b
	HI	15.61 a,a	17.98 a,b	19.93 b,a	21.33 b,a	21.93 b,b
PP/SP	LI	0.44 a,a	0.49 b,a	0.54 c,a	0.56 c,a	0.61 d,a
	MI	0.45 a,a	0.57 b,b	0.60 b,a	0.72 c,b	0.62 b,a
	HI	0.52 a,b	0.59 a,b	0.69 b,b	0.72 b,b	0.71 b,b

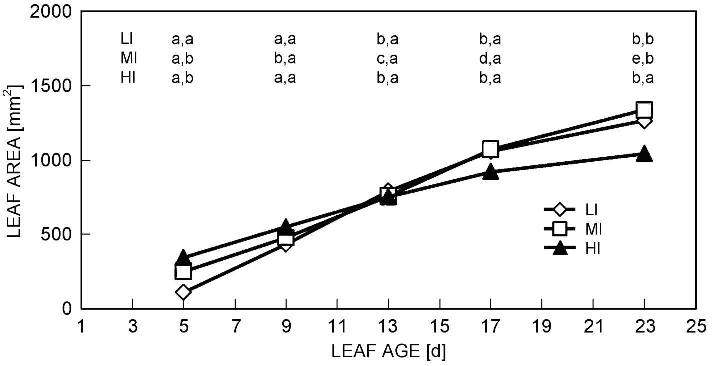

Fig. 1. Leaf area during development of the 4th leaf in photoautotrophically *in vitro*-grown *Nicotiana tabacum* plants at low (LI, 60 µmol m^{-2} s^{-1}), middle (MI, 180 µmol m^{-2} s^{-1}) and high (HI, 270 µmol m^{-2} s^{-1}) irradiances. Different letters in the upper part of the chart indicate significant differences at $P = 0.05$. The first letters indicate the evaluation of ontogenetic changes and the second letters indicate the evaluation of irradiance effect in respective treatment.

the area of the 4th leaf was the same in all treatments 13 DAE. The lowest rate of leaf area expansion had HI plants, which also tended to have the smallest leaves. Plants grown at HI were also smaller and more compact, what is a typical response for sun plants (Björkman 1981). The changes in leaf structure during leaf area expansion in all the treatments were evident (Fig. 2).

Leaf thickness (Table 1, Fig. 2) markedly increased during leaf development in all treatments. Leaves were the thinnest in LI plants, while differences between MI and HI plants gradually disappeared.

Because the both epidermes were relatively thin, the differences in leaf thickness were mainly due to the different thickness of leaf mesophyll (Table 1, Fig. 2). Adaxial epidermis was thicker in HI leaves, but the difference disappeared early and no difference in abaxial epidermis thickness was found. Both palisade and spongy parenchyma tissues were thicker in MI and HI leaves and increased significantly during leaf growth. Palisade parenchyma to spongy parenchyma ratio (PP/SP) was the highest in HI leaves and due to a more pronounced increase of palisade parenchyma thickness, the ratio PP/SP increased, too. Thus, higher irradiance stimulated the relative development of the palisade layer with regard to the spongy parenchyma layer although the leaves of HI or MI plants did not develop additional layers of palisade parenchyma. Leaves of both MI and HI plants have developed some characteristics typical for sun leaves of *in vivo* plants (Wild and Wolf 1980, Björkman 1981, Rundel *et al.* 1998, Ishida *et al.* 1999, Yano and Terashima 2004).

Table 2. Proportion of tissues in the 4th leaf (measured as the cross-sectional area of a tissue in the leaf cross-section) and proportion of intercellular spaces during leaf development. All details as in Table 1.

Proportion of a leaf tissue [%]	Treatments	5 d	9 d	13 d	17 d	23 d
Adaxial epidermis	LI	12.17a,a	12.19 a,a	11.91 a,a	12.33 a,b	11.82 a,b
	MI	11.21 b,a	12.74 b,a	12.07 b,a	12.09 b.b	10.06 a,a
	HI	13.25 b,b	13.25 b,a	12.15b,a	10.33 a,a	10.80 a,a
Palisade parenchyma	LI	19.51 b,a	19.38 b,a	17.47 a,a	16.93 a,a	20.35 b,a
	MI	20.08 a,a	17.74 a,a	17.96 a,a	20.09 a,b	18.86 a,a
	HI	19.82 a,a	18.74 a,a	17.24 a,a	20.14 a,b	19.65 a,a
Spongy parenchyma	LI	42.15 b,b	37.21 b,c	27.70 a,a	26.57 a,a	26.22 a,a
	MI	41.66 c,b	32.24 c,b	27.38 b,a	23.62 a,a	27.21 b,a
	HI	34.94 b,a	27.71 a,a	27.18 a,a	25.42 a,a	25.15 a,a
Abaxial epidermis	LI	12.44 c,b	10.92 b,a	11.00 b,b	11.87 b,b	9.07 a,a
	MI	11.42 b,a	11.03 b,a	9.60 a,a	10.22 a,a	9.23 a,a
	HI	11.33 c,a	10.55 b,a	9.70 a,a	9.27 a,a	8.94 a,a
PP/SP	LI	0.47 a,a	0.53 a,a	0.64 b,a	0.66 b,a	0.81 c,a
	MI	0.48 a,a	0.55 a,a	0.67 b,a	0.87 d,b	0.72 c,a
	HI	0.59 a,b	0.69 a,b	0.64 a,a	0.80 b,b	0.80 b,a
Intercellular airspaces	LI	13.72 a,a	20.30 b,a	31.93 c,a	32.29 c,a	32.55 c,a
	MI	15.62 a,a	26.25 b,b	33.00 c,a	33.98 c,a	34.64 c,a
	HI	20.66 a,b	29.76 b,b	33.72 b,a	34.84 b,a	35.45 b,a

Although the increase in leaf tissue thickness during leaf development was considerable and the effect of irradiance on mesophyll cell thickness was clearly visible, the proportion of leaf tissues in the leaf (measured as the cross-sectional area of a tissue in the leaf cross-section) was more stable, irrespective of growth irradiance, indicating proportional growth of all tissues (Table 2). This stability was evident especially on the leaves 13 DAE, when leaves of all treatments had approximately same leaf area.

The only exception was spongy parenchyma, the proportion of which considerably decreased, together with a considerable increase in the proportion of leaf intercellular spaces. Leaf intercellular spaces enlarged rapidly during early development from 13 - 20 % (5 DAE) to 35 % (13 DAE) and then they were maintained during further leaf growth. Intercellular spaces were present in both palisade and spongy parenchyma cells, however, palisade parenchyma cells looked more compact. Interestingly, irradiance had no effect on intercellular space proportion, although a decrease in proportion of intercellular spaces at high irradiance is usually found in *in vivo* plants (Tichá 1985, Smith and Longstreth 1994) and was described also for *in vitro* plants (Lee *et al.* 1988, Serret and Trillas 2000).

Large intercellular spaces could be the consequence of high relative humidity inside the culture vessels. Torre *et al.* (2003) observed enlarged intercellular spaces in leaves of *Rosa* × *hybrida* grown at high relative humidity in greenhouse. Large intercellular spaces were present also in *in vitro* cultured *Dianthus caryophyllus* plants grown at lower ventilation rate (Majada *et al.* 2000).

During leaf development, relative size of both palisade and spongy parenchyma cells increased considerably (Table 3). Both types of parenchyma cells were larger in MI and HI leaves, as it was found for some other *in vivo* (Wild and Wolf 1980, Kubínová 1991) and *in vitro* grown plants (Lee *et al.* 1988, Dimassi-Theriou and Bosabalidis 1997).

The relative abundance of chloroplasts (measured as chloroplasts cross-sectional area per cell section) in palisade parenchyma cells (Table 3) was higher than in spongy parenchyma cells. This is in agreement with the fact, that the palisade parenchyma is thought to be the main photosynthetic tissue (Mauseth 1988). In the youngest leaves chloroplasts formed a layer covering inner side of the cell wall, but during further leaf development this covering was not maintained. Chloroplasts were more often situated along the cell walls exposed to internal leaf atmosphere what facilitates the inward diffusion of CO_2 (Psaras *et al.* 1996). In palisade parenchyma cells of MI and HI leaves chloroplasts moved from periclinal to anticlinal cell walls (Fig. 2). This phenomenon, called chloroplast avoidance movement, serves as protective mechanism against high irradiance and decreases the amount of absorbed radiant energy by chloroplasts, thus reducing their photodamage (Kasahara *et al.* 2002).

During leaf growth, chloroplasts relative abundance in both palisade and spongy parenchyma cells (Table 3) decreased significantly − in all leaves of all plants from 17 - 33 % (5 DAE) to 5 - 8 % (23 DAE). Proportion of chloroplasts in mesophyll cells was the highest in LI plants, while the differences between HI and MI plants get smaller. Hence, although the cells of HI and MI plants were larger, they had lower proportion of chloroplasts and thus the development of chloroplasts was more strongly limited at these conditions. Usually, in sun and shade leaves of *in vivo* plants proportion of the cell volume occupied by the chloroplasts should be similar (Butterfass 1979).

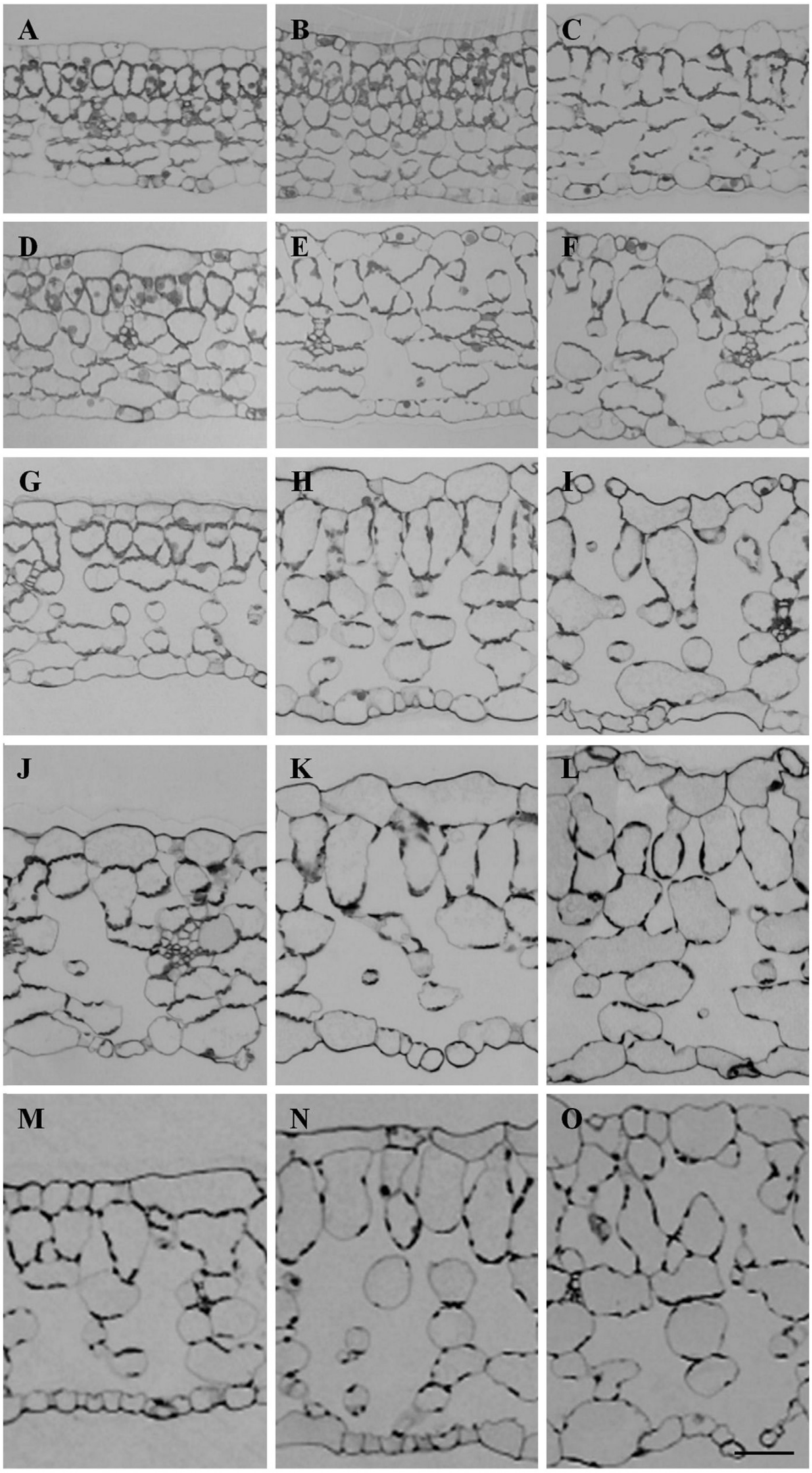

Fig. 2. Leaf cross-sections of photoautotrophically *in vitro*-grown *Nicotiana tabacum* plants during development of the 4th leaf at low (LI, 60 µmol m^{-2} s^{-1} , *A,D,G,J,M*), middle (MI, 180 µmol m^{-2} s^{-1}, *B,E,H,K,N*) and high (HI, 270 µmol m^{-2} s^{-1}, *C,F,I,L,O*) irradiances. Cross-sections were made at leaf age 5 d (*A-C*), 9 d (*D-F*), 13 d (*G-I*), 17 d (*J-L*) and 23 d (*M-O*). *Bar* represents 50 µm and is valid for all images.

Table 3. Cross-sectional area of leaf mesophyll cells [μm^2] (measured in the leaf cross-section) and proportion of chloroplasts (chlp) [%] (measured as chloroplast cross-sectional area per cell section) during leaf development. All details as in Table 1.

Variable	Treatments	5 d	9 d	13 d	17 d	23 d
Palisade parenchyma cell area	LI	328.8 a,a	587.7 b,a	1144.7 c,a	1493.5 d,a	2279.5 e,a
	MI	418.6 a,b	921.4 b,b	1471.7 c,b	2098.2 d,b	2659.4 e,b
	HI	662.4 a,c	1262.7 b,c	1915.9 c,c	2011.2 c,b	2595.2 d,b
Spongy parenchyma cell area	LI	448.7 a,a	757.4 b,a	1170.6 c,a	1528.4 d,a	1751.2 e,a
	MI	589.4 a,b	885.5 b,b	1390.2 c,b	1722.9 d,b	2436.1 e,c
	HI	737.4 a,c	1147.4 b,c	1567.4 c,c	1793.7 d,b	2153.9 e,b
Palisade parenchyma cell chlp	LI	32.6 e,c	25.4 d,c	14.8 c,c	10.6 b,c	8.1 a,b
	MI	29.0 d,b	18.4 c,b	9.5 b,b	8.4 b,b	5.7 a,a
	HI	22.4 e,a	11.7 d,a	8.3 c,a	7.2 b,a	5.5 a,a
Spongy parenchyma cell chlp	LI	23.3 d,c	17.3 c,c	11.6 b,b	7.9 a,a	7.1 a,b
	MI	21.2 e,b	14.6 d,b	8.8 c,a	7.4 b,a	6.0 a,a
	HI	17.7 e,a	11.7 d,a	8.7 c,a	7.0 b,a	5.5 a,a

At 23 DAE about 5.5 - 6 % of cross-sectional area in both palisade and spongy parenchyma cells in HI and MI plants and 7 - 8 % in LI plants were covered by chloroplasts. These values are indeed very low compared with data published previously for *in vivo* plants (Woodrow *et al*. 1984, Ellis and Leech 1985), who described that in mature vacuolated mesophyll cells chloroplasts may occupy about 20 % of total cell volume. Moreover, this decrease in relative abundance of chloroplasts in mesophyll cells was, at least in HI and MI plants, in accordance with an early degradation of chlorophyll *a*+*b* content (Radochová and Tichá 2008).

Both the loss of chloroplasts and degradation of chlorophylls are processes that in *in vivo* plants are usually linked with leaf senescence (Ford and Shibles 1981, Wardley *et al*. 1984). As reported by other authors (Chanemougasoundharam *et al*. 2004, Lucchesini *et al*. 2006) conventional *in vitro* culture conditions can speed up the senescence processes. However, in this study, *Nicotiana tabacum* plants were not cultured conventionally, but photoautotrophically and at enhanced CO_2 concentration. Previously, Tichá (1996) described, that the improved CO_2 supply to these plants resulted in a significantly increased dry matter accumulation and leaf area production. Moreover, improved CO_2 supply was clearly more effective in photoautotrophically cultured plants. Further, though photosynthesis was down-regulated at HI and MI plants, typical ontogenetic course of P_N was not changed (Radochová and Tichá 2008) and did not correspond with either the degradation of chlorophylls or the decline in chloroplast proportion in mesophyll cells.

Decreasing chloroplast cross-sectional area in both palisade and spongy parenchyma cells during leaf area expansion in plants of all treatments could be caused by the rapid growth of these cells without sufficient rate of chloroplast replication and/or enlargement. That means that either division or expansion of chloroplasts (or both of them) in developing leaves of *Nicotiana tabacum* plants grown *in vitro* photoautotrophically were disturbed.

We can only speculate, why plants grown *in vitro* photoautotrophically and at enhanced CO_2 concentration and irradiance were limited in chloroplast development. Because chloroplast proportion was lower in both palisade and spongy parenchyma cells of HI and MI plants than in LI ones, it suggests that photoinhibition can be involved here. In fact, Tichá *et al*. (1998) described occurrence of photoinhibition in *Nicotiana tabacum* plants grown photoautotrophically already at irradiance of 200 μmol m^{-2} s^{-1}. The development of chloroplast was, nevertheless, strongly limited also in leaves of LI plants, where photoinhibition could not be expected. Possibly, plants cultured *in vitro* without sucrose lacked a source of metabolites and energy for the plant development. Indeed, the inducing effect of sucrose on chloroplast formation in *in vitro* cultured plants was previously described (Serret and Trillas 2000).

High irradiance during *in vitro* cultivation accelerated leaf development of photoautotrophically *in vitro* grown *Nicotiana tabacum* plants, and lead to some typical adaptations known in sun leaves of *in vivo* plants. However, large intercellular spaces were present irrespective of growth irradiance. Relative abundance of chloroplasts in both palisade and spongy parenchyma cells considerably decreased during leaf development indicating that photoautotrophic *in vitro* cultivation could be limiting for chloroplast formation and consequently for development of the photosynthetic apparatus.

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