BRIEF COMMUNICATION

Excess irradiance causes early symptoms of senescence during leaf expansion in photoautotrophically *in vitro* grown tobacco plants

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Abstract

Photosynthetic parameters, growth, and pigment contents were determined during expansion of the fourth leaf of *in vitro* photoautotrophically cultured *Nicotiana tabacum* L. plants at three irradiances [photosynthetically active radiation (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}]. During leaf expansion, several symptoms usually accompanying leaf senescence appeared very early in HI and then in MI plants. Symptoms of senescence in developing leaves were: decreasing chlorophyll (Chl) *a+b* content and Chl *a/b* ratio, decreasing both maximum (F_V/F_M) and actual (Φ_{PS2}) photochemical efficiency of photosystem 2, and increasing non-photochemical quenching. Nevertheless, net photosynthetic oxygen evolution rate (P_N) did not decrease consistently with decrease in Chl content, but exhibited a typical ontogenetic course with gradual increase. P_N reached its maximum before full leaf expansion and then tended to decline. Thus excess irradiance during *in vitro* cultivation did not cause early start of leaf senescence, but impaired photosynthetic performance and Chl content in leaves and changed their typical ontogenetic course.

Additional key words: chlorophyll *a* fluorescence; leaf area; leaf dry mass; leaf ontogeny; net oxygen evolution rate; *Nicotiana*; photosynthesis.

In vitro plants are usually grown under high relative humidity, low irradiance (usually 50–60 μmol m^{-2} s^{-1}), and low CO_2 concentration. These conditions may result in low photosynthetic activity of the plants, therefore the cultivation media are often supplemented by saccharides as carbon and energy sources (*e.g.* Pospíšilová *et al*. 1992, Hazarika 2006). This may cause bacterial and fungal contamination of the saccharose containing media and changes during transfer of plants from *in vitro* culture to soil (Preece and Sutter 1991). Current research concentrates on the development of photoautotrophic systems in which the sugar content in the medium is reduced or eliminated and CO_2 concentration and irradiance are increased (Kozai 1991, Düring and Harst 1996, Nguyen *et al.* 1999, Sha Valli Khan *et al.* 2003, Teixeira Da Silva *et al.* 2006). However, application of high irradiance during cultivation is limited by susceptibility to photoinhibition of both photoautotrophically (Tichá *et al.* 1998, Kadleček *et al.* 2003) and photomixotrophically (Serret *et al.* 1996, 2001) *in vitro* grown plants.

Chronic photoinhibition was found in *in vitro* photoautotrophically cultured plants already at irradiance of 200 μmol m^{-2} s^{-1} (Tichá *et al.* 1998) and was demonstrated as decreased efficiency and/or decreased maximum rate of photosynthesis, decreased Chl *a+b* content and Chl *a/b* ratio, increased content and de-epoxidation state of xanthophyll cycle pigments, decreased both maximum and actual photochemical efficiency of photosystem 2 (PS2), and increased non-photochemical quenching (NPQ) (Tichá *et al.* 1998, Kadleček *et al.* 2003).

The symptoms of photoinhibition mentioned above are similar to the symptoms of leaf senescence (Yoo *et al.*

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2003). Because the leaves develop at a higher rate when growing at high irradiance (Smith and Longstreth 1994, Behera and Choudhury 2001), the changes in photosynthetic performance and pigment contents of photoinhibited plants may be influenced also by the temporal shift in leaf ontogeny. This is why we checked if photoinhibition shifts leaf ontogeny and/or ontogenetic courses of photosynthesis and Chl content.

Nodal cuttings of tobacco (*Nicotiana tabacum* L. cv. Samsun) were cultured *in vitro* on solidified Murashige-Skoog medium (M5519, *Sigma*) photoautotrophically (without saccharose) at 25/18 °C day/night temperatures, 16-h photoperiod, CO_2-enriched air (800 µmol mol^{-1}), and at three different irradiances (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}). All measurements were made on the fourth leaves from the bottom of the plants (three to five plants from each irradiance) at each sampling time. Leaf age was measured as the number of days after the leaf emerged from the apical bud (DAE). The fourth leaf emerged 11–12 d after the experiment was started and measurements were made at 4th leaf age of 5, 9, 13, 17, and 23 DAE.

Chl was extracted from leaf discs (50 mm^2) with N,N´-dimethylformamide and determined spectrophotometrically (spectrophotometer *HEλIOSα*, *Unicam*, Cambridge, UK) using the equations of Wellburn (1994). Photosynthetic net oxygen evolution rate (P_N) was measured with a Clark type leaf-disc oxygen electrode (*LD2*, *Hansatech*, King´s Lynn, UK) at irradiances of 60, 180, and 270 µmol m^{-2} s^{-1}, and at 25 °C in CO_2-enriched air. Irradiation was provided by the "white light" source *LS2* (*Hansatech*, King´s Lynn, UK) equipped with neutral density filters. Incident photon flux density was measured with a *LI-189* quantum-meter (*LI-COR*, Lincoln, NE, USA). Chl *a* fluorescence emission from the upper surface of the leaves was measured in modulated irradiation with a pulse amplitude modulation fluorometer (*PAM*, *Walz*, Effeltrich, Germany) simultaneously with O_2 evolution: the tip of the fibre optic of the *PAM* was inserted at an angle of 45° in the top water jacket of the *LD2* chamber. The minimum Chl fluorescence yield in dark (F_0), the steady state Chl fluorescence yield in the light (F_s), and the maximum Chl fluorescence yield in darkened (F_M) and light-adapted (F_M') leaves were measured. The quantum fluence rate of the "actinic light" was 60, 180, and 270 µmol m^{-2} s^{-1}, respectively. The flash (1 s) of saturating radiation (for calculation of F_M and F_M') was 2 600 µmol m^{-2} s^{-1}. The maximum photochemical efficiency of PS2 (F_V/F_M) in 20-min-dark-adapted leaves, actual photochemical efficiency of PS2 [$\Phi_{PS2} = 1 - (F_S/F_M')$] and non-photochemical quenching (derived from Stern-Volmer equation) [NPQ = $(F_M - F_M')/F_M'$] were calculated.

Leaf area was determined by scanning leaf images and applying a computer program (*LUCIA G*, version *3.52*, *Laboratory Imaging*, Prague, Czech Republic). For estimation of dry mass, leaves were dried for 24 h at 80 °C.

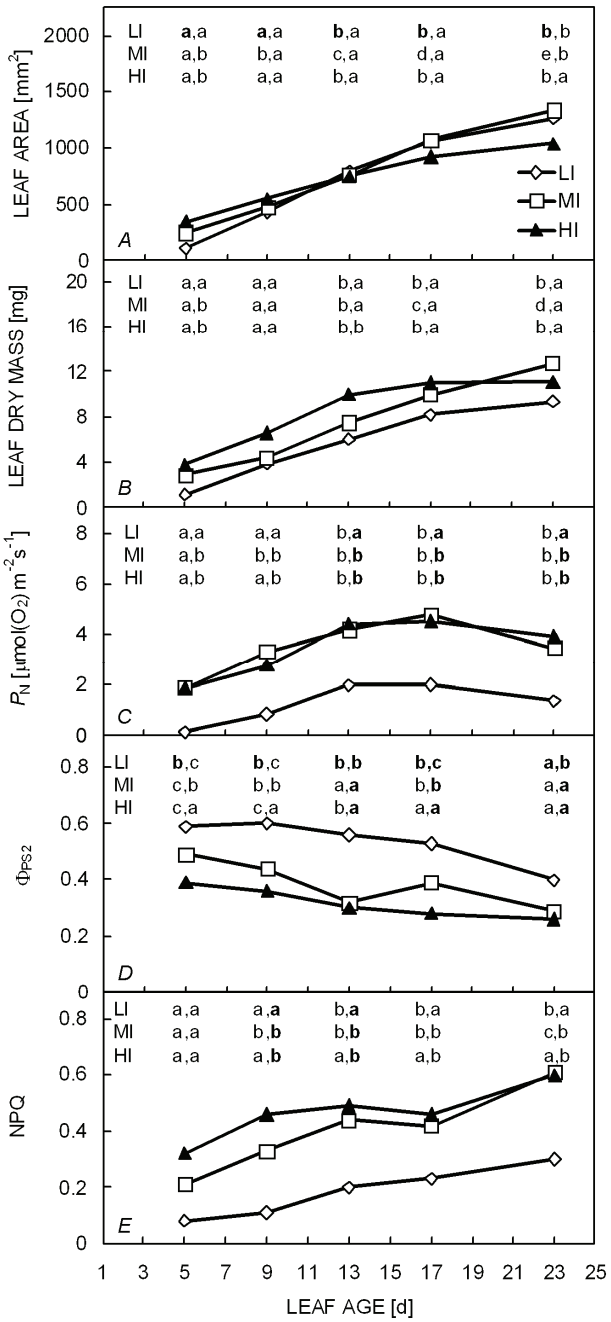

Fig. 1. Leaf area (*A*), leaf dry mass (*B*), net oxygen evolution rate, P_N (*C*), actual photochemical efficiency of photosystem 2 [$1 - (F_S/F_M')$] (*D*), and non-photochemical quenching, NPQ [$(F_M - F_M')/F_M'$] (*E*) during expansion of the 4th leaf in photoautotrophically *in vitro*-grown *Nicotiana 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}) irradiance. Different letters in the upper part of the chart indicate significant differences at p=0.05 and p=0.01 (bold). The first letters indicate the evaluation of ontogenetic changes and the second letters indicate the evaluation of irradiance effect in the respective treatment.

Table 1. Chlorophyll (Chl) *a+b* content [mg m^{-2}], Chl *a/b* ratio, maximum photochemical efficiency of photosystem 2 (F_V/F_M), minimum Chl fluorescence yield in dark (F_0), and the maximum Chl fluorescence yield in darkened (F_M) leaves during expansion 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}) irradiance. Different letters behind the mean values (n = 9–15) indicate significant differences at p=0.05 and p=0.01 (bold). The first letters indicate the evaluation of ontogenetic changes and the second letters indicate the evaluation of irradiance effect in the respective treatment.

Variable	Treatment	Age [d] 5	9	13	17	23
Chl *a+b*	LI	223.1 c,a	227.9 c,**b**	218.4 c,c	180.4 b,**b**	139.8 a,**b**
	MI	254.9 d,a	202.1 c,**a**	171.2 b,b	164.2 b,**b**	119.4 a,**a**
	HI	230.7 d,a	192.3 c,**a**	147.1 b,a	121.4 a,**a**	111.3 a,**a**
Chl *a/b*	LI	2.9 b,a	2.8 b,b	2.7 b,**b**	2.6 b,c	2.3 a,a
	MI	2.9 b,a	2.6 b,a	2.3 a,**a**	2.4 a,b	2.2 a,a
	HI	2.9 c,a	2.7 c,a	2.4 b,**a**	2.1 a,a	2.2 a,a
F_V/F_M	LI	0.65 b,a	0.70 b,a	0.69 b,b	0.67 b,**b**	0.58 b,b
	MI	0.71 b,a	0.66 b,a	0.55 a,a	0.61 a,**b**	0.54 a,b
	HI	0.68 b,a	0.65 b,a	0.58 b,a	0.50 a,**a**	0.47 a,a
F_0	LI	10.7 a,a	10.9 a,a	12.5 a,a	14.9 b,a	22.6 **c**,a
	MI	11.8 **a**,a	12.4 **a**,a	15.6 **a**,a	15.5 **a**,a	22.7 **b**,a
	HI	12.1 **a**,a	11.9 **a**,a	14.4 **a**,a	17.0 **a**,a	22.2 **b**,a
F_M	LI	30.5 a,a	36.4 b,a	40.0 b,b	45.9 c,b	53.7 **d**,c
	MI	42.9 b,a	36.3 b,a	34.7 a,a	40.5b,b	48.3 b,b
	HI	39.0 a,a	34.5 a,a	34.5 a,a	34.1 a,a	41.8 a,a

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, USA). Data are presented as means of at least 3 replicates.

The length of the delay between emergence and the start of rapid leaf expansion was longest in LI leaves, and both the LI and MI leaves had a more rapid rate of expansion and finally larger leaf area than HI leaves (Fig. 1*A*). Twenty three DAE, leaf area enlargement ceased only in HI plants and tended to be lower in LI plants, and it increased almost linearly in MI plants. Leaf dry mass increased more rapidly in HI and MI leaves and the increase ceased first in HI plants (17 DAE) (Fig. 1*B*).

Chl *a+b* content per leaf area unit (Table 1) in the leaves of MI and HI plants decreased during leaf expansion already from the 5th DAE as compared with LI plants, where the decrease was considerably delayed and started as late as 17 DAE. Thus, with the exception of the youngest leaves, Chl *a+b* content was the highest in LI plants. When measured on whole leaf area basis, Chl *a+b* content first increased. A decrease in Chl *a+b* content per leaf started 17 DAE in HI and 23 DAE in leaves of both MI and LI plants—but still during the period of rapid leaf area expansion (data not shown). The Chl *a/b* (Table 1) was the highest in the youngest leaves (5 DAE) and declined during leaf expansion consistently with Chl *a+b* content. Due to the more pronounced decline, Chl *a/b* ratio was lower in HI and MI leaves. Leaf development is usually accompanied by an increase in Chl content and photochemical activities during leaf expansion, which remain stable at leaf maturity; it is followed by a decline during leaf senescence (Šesták *et al.* 1985). However, such a typical ontogenetic course for the 4th leaf was partly observed only in plants growing *in vitro* photoautotrophically under LI, which developed more slowly than MI and HI plants. In these plants, no increase in Chl content per unit of leaf area and in photochemical efficiency was found, although first measurement was done very early (*i.e.* on the 5th d) after the leaf emerged from the apical bud. Within 23 d of leaf development, leaf area expansion (Fig. 1*A*) (as basic parameter of leaf development) and leaf dry mass accumulation (Fig. 1*B*) of the 4th leaf were finished only in HI plants, although leaves of all the treatments already exhibited symptoms usually accompanying senescence.

The gradual decrease in Chl *a/b* during leaf expansion was more pronounced in leaves of HI and MI plants and lead to lower values of the ratio in these treatments. Decreasing Chl *a/b* was found in many plant species (Šantrůček *et al.* 1991, Young *et al.* 1991, Lu and Zhang 1998a, Behera and Choudhury 2001, Yoo *et al.* 2003), during leaf development, suggesting preferential loss of Chl *a*. Low Chl *a/b* ratio is a typical adaptation in leaves expanding in shade because it reflects higher amount of light-harvesting complex 2 (LHC2). As the function of LHC2 is efficient harvesting of photon energy, it is less necessary for HI and MI plants and can therefore increase their photoinhibition. Because the major target of photooxidative damage is PS2 (Niyogi 1999) and in this photosystem higher stability of LHC2 than of PS2 core

during Chl degradation was found (Šantrůček *et al.* 1991), it is possible that the lower Chl *a/b* in leaves of HI and MI plants was caused by slower degradation of LHC2 than of PS2 core. During leaf area expansion, Chl *a+b* content decreased if calculated per unit of leaf area or per whole leaf in all the treatments, though the decrease on the whole leaf started later. Accelerated degradation of Chl during expansion of leaves of HI and MI plants was probably caused by photooxidation of pigments under excess of irradiance (Powles 1984). Indeed, leaves of HI plants were nearly chlorotic before leaf area was fully expanded. Similar changes in content of Chl *a+b* during plant ontogeny were found formerly in photoautrophically, but not photomixotrophically cultured *Nicotiana* plants (Tichá *et al.* 1998), suggesting a stimulation of Chl synthesis by saccharose. The loss of Chl is a phenomenon typical for leaf senescence (Guo and Gan 2005) and could be used as its indicator (Yoo *et al.* 2003).

P_N measured at growth irradiance (Fig. 1*C*) did not correlate with Chl content, but increased during leaf expansion, reached maximum between the 13 and 17 DAE (*i.e.* before full leaf area expansion) in all treatments, and then tended to decline. P_N was the lowest in LI plants and no differences were found between MI and HI plants. Therefore, photosynthetic activity was photon energy-saturated already at MI and a further increase to HI during *in vitro* culture did not enhance P_N.

Φ_{PS2} (Fig. 1*D*) decreased during leaf expansion and the decrease started earlier in leaves of MI and HI plants. It was delayed in LI plants, leading to the highest values in LI plants. F_V/F_M (Table 1) in the youngest leaves (5 DAE) was similar for all the treatments, ranging between 0.65–0.71. During leaf expansion F_V/F_M decreased in all treatments with the most pronounced decline in HI plants, where F_V/F_M gradually decreased to a value of 0.47. In LI plants, F_V/F_M was more stable and the decrease was delayed. F_M values decreased slightly during early leaf expansion in HI and MI plants (till 9 and 13 DAE, respectively) and then slightly increased, while F_M increased significantly in LI plants. F_0 values markedly increased during leaf expansion in all the treatments, but the most pronounced increase was found in LI plants. NPQ was significantly higher in MI and HI plants and increased during leaf expansion in all the irradiance treatments (Fig. 1*E*).

Symptoms usually accompanying senescence appeared earlier in leaves of HI and MI plants what is in agreement with the fact that growth at HI causes acceleration of leaf development (Smith and Longstreth 1994, Behera and Choudhury 2001). Symptoms included gradual decrease in Chl *a+b* content per leaf area unit and Chl *a/b* (Table 1), a decrease in both F_V/F_M (Table 1) and Φ_{PS2} (Fig. 1*D*), and an increase in NPQ (Fig. 1*E*). The decrease in both F_V/F_M and Φ_{PS2} was more rapid in HI and MI than LI plants. A decrease in Φ_{PS2} is a frequently observed indicator of photoinhibition (Krause and Weis 1991) and/or leaf senescence (Jiang *et al.* 1993, Lu and Zhang 1998b, Lu *et al*. 2003) and this decrease was matched by increasing NPQ. The increase in NPQ has been recognized as being associated with the xanthophyll pigment cycle that photo-protects the PS2 by the dissipation of excess absorbed photon energy (Demmig-Adams 1990).

Typical values for F_V/F_M lay in the range 0.75–0.85 for non-stressed plants grown *ex vitro* (Demmig and Björkman 1987). In photoautotrophically *in vitro* grown tobacco plants this ratio was generally lower, even for LI plants. Low F_V/F_M during *in vitro* culture was reported also by Serret *et al.* (2001) and may result from incomplete development of the photosynthetic apparatus. Decreasing F_V/F_M could be attributed rather to photoinhibition than to senescence, although some decrease in this ratio during leaf development was found in several plant species (Šesták and Šiffel 1997).

Photosynthetic activity during leaf ontogeny surprisingly did not correlate with the decrease in total Chl content and photochemical efficiency, as it is frequently seen in *ex vitro* plants (Šesták 1985, Gratani and Ghia 2002, Lu *et al.* 2003, Yoo *et al.* 2003). In contrast, in our experiments P_N in all the treatments exhibited the typical ontogenetic course (cf. Rawson and Craven 1975, Tichá *et al.* 1985): an increase in the young leaf, a maximum before full leaf expansion, and then a decline. These results indicate that the ontogenetic changes in P_N were independent on the ontogenetic changes in Chl content, as it was found for photosynthesis at high irradiance (Björkman 1981).

The higher P_N for both MI and HI plants than for LI plants could be explained by a better penetration of the incident radiation into the leaf with lower Chl content. However, when compared at HI (an irradiance already saturating P_N), P_N did not differ between treatments, indicating photoinhibition of MI and HI plants.

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