BIOLOGIA PLANTARUM 42 (3): 463-468, 1999

BRIEF COMMUNICATION

Photosynthetic pigments and gas exchange of *in vitro* grown tobacco plants as affected by CO_2 supply

D. HAISEL, J. POSPÍŠILOVÁ, H. SYNKOVÁ, J. ČATSKÝ, N. WILHELMOVÁ, and Š. PLZÁKOVÁ

Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Na Karlovce 1a, CZ-160 00 Praha 6, Czech Republic

Abstract

Contents and functioning of photosynthetic pigments and gas exchange of *Nicotiana tabacum* L. leaves were studied in platlets cultivated *in vitro* under different CO_2 supply. The plantlets were grown for six weeks either in glass vessels tightly closed with aluminium foil (G-plants) or in polycarbonate *Magenta GA-7* vessels covered with closures with microporous vents (M-plants). M-plants (better supplied with CO_2) had higher contents of chlorophyll (Chl) *a*, Chl *b*, and β-carotene, higher photochemical activities of photosystem 2 and whole electron transport chain, and lower contents of xanthophyll cycle pigments. Differences in Chl *a* fluorescence kinetic parameters between G-plants and M-plants were not statistically significant. M-plants had higher net photosynthetic rate, and lower transpiration rate and stomatal conductance than G-plants.

Additional key words: carotenoids, chlorophyll content, chlorophyll fluorescence, net photosynthetic rate, *Nicotiana tabacum*, stomatal conductance, xanthophyll cycle pigments.

For the successful cultivation of plants *in vitro*, not only medium composition but also irradiance and composition of gaseous atmosphere are important. In fairly

Received 18 June 1999, *accepted* 2 July 1999

Abbreviations: Car - carotenoids; Chl - chlorophyll; g_{ad} - adaxial stomatal conductance; g_{ab} - abaxial stomatal conductance; DEPS - degree of de-epoxidation; ETC - electron transport chain; F_m - maximum fluorescence; F_v - variable fluorescence; P_N - net photosynthetic rate, PS2 - photosystem 2; q_N - non-photochemical quenching; q_P - photochemical quenching; Φ_{II} - quantum yield of PS2.

Acknowledgements: The authors thank Mrs. L. Hávová, L. Kolčabová and Ing. D. Procházková for skilled assistance. The authors acknowledge the financial support of the Grant Agency of the Charles University (100/1998/B BIO/PřF).

Fax: (+420) 2 24310113; e-mail: pospisilova@ueb.cas.cz

air-tight cultivation vessels air humidity is extremely high, air turbulence is low, and inflow of CO_2 and outflow of secondary plant products are limited. These conditions induce the formation of plantlets of abnormal morphology, anatomy, and physiology. Photosynthetic rate and in consequence biomass production in *in vitro* grown plants are often limited by low irradiance and low CO_2 concentration during the light period. Therefore the vessel closures have to fulfil controversial requirements: to be sufficiently tight to prevent bacterial and fungal contamination and to be sufficiently loose to enable gas exchange. The increased growth and vigour of many plant species by improved CO_2 supply has been shown in many reviews (*e.g.*, Kozai 1991, Kozai *et al.* 1992, Pospíšilová *et al.* 1992, 1997, Aitken-Christie *et al.* 1994, Buddendorf-Joosten and Woltering 1994, Desjardins 1995), and original papers (from recent, *e.g.*, Solárová *et al.* 1996, Tichá 1996, Murphy *et al.* 1998). However, previous experiments showed that elevated CO_2 concentration during acclimation *ex vitro* promoted more effectively the growth of plantlets grown *in vitro* at lower than at higher CO_2 concentration (Solárová and Pospíšilová 1997). The aim of this paper was to explain this by studying in detail photosynthetic pigments and gas exchange in leaves of *in vitro* grown tobacco plantlets affected by different CO_2 supply to cultivation vessels.

Tobacco (*Nicotiana tabacum* L., cv. Petit Havana SR1) were sown on the agar gelled Murashige and Skoog (1962) medium containing 20 g dm^{-3} saccharose in Petri dishes. After 2 weeks, plantlets were transferred into two types of cultivation vessels: glass vessels (volume 113 cm^3) covered with aluminium foil and polycarbonate *Magenta GA-7* vessels (volume 288 cm^3) covered with closures with vents (diameter 10 mm; microporous polypropylene mebrane, pore size 0.22 μm, *Sigma*, St. Louis, USA). Plants were grown at a 16-h photoperiod, and day/night temperature of 25/20 °C. Temperature inside vessels reached 27 °C during the light period. Irradiance (400-700 nm) at the leaf level inside glass vessels was 90 - 100 and inside *Magenta* boxes 110 - 120 μmol m^{-2} s^{-1}. Relative humidity inside both types of vessels was more than 90 %; droplets of liquid water frequently appeared on their walls. CO_2 concentration in cultivation chamber was 350 μmol mol^{-1}, but inside cultivation vessels it was rather low during the light period (for detail, see Solárová 1989). The exchange of CO_2 and water vapour between vessels and surrounding air was two fold higher in *Magenta* boxes than in glass vessels (for detail, see Solárová *et al.* 1996).

Contents of photosynthetic pigments were determined in acetone extracts of leaf discs by HPLC (*Spectra-Physics*, San Jose, USA) using a reverse phase column (*Sepharon SGX C18*, 5 μm particle size, 150 × 3 mm, *Tessek*, Prague, Czech Republic). The solvent system was acetonitrile:methanol:water (80:12:6) followed by 100 % methanol, and the gradient was run from 8 to 12 min. The flow rate was 1 cm^3 min^{-1}, the detection wavelength 445 nm. Photochemical activities of the whole electron transport chain and of photosystem 2 were measured as oxygen evolution using an oxygen electrode (*Hansatech*, King's Lynn, UK). Final chlorophyll (Chl) concentration was 50 g m^{-3}. Ferricyanide (FeCy) to a final concentration of 6.25 mM was added prior to the measurements, 1,4-phenylene diamine (PD) to a final concentration of 0.5 mM was added during the measurements. The measurements were done at 25 °C and

irradiance of 1 200 μmol m^{-2} s^{-1} (for detail see Pospíšilová *et al.* 1998). Chl *a* fluorescence characteristics of the adaxial surface of attached leaves were measured after a 15-min dark period with the *PAM Chlorophyll Fluorometer* (*Walz*, Effeltrich, Germany) at room temperature and ambient CO_2 concentration. Measuring irradiance was 0.35 μmol m^{-2} s^{-1}, actinic irradiance 200 μmol m^{-2} s^{-1}, and 700-ms saturated flashes of "white light" (2 500 μmol m^{-2} s^{-1}) were applied at 300 s intervals. Data sampling, control and calculation were served by the *DA 100 Data Acquisition System* (*Walz*, Effeltrich, Germany) (for detail, see Pospíšilová *et al.* 1998). Net photosynthetic rate was determined as CO_2 influx in a PC-assisted closed gas exchange system with an infra-red gas analyser *Infralyt IV* (*Junkalor*, Dessau, Germany) in a CO_2 concentration range from 100 to 3 000 mg m^{-3}, leaf temperature 20 °C, and saturating irradiance of 860 μmol m^{-2} s^{-1}. Diffusive conductances of abaxial and adaxial epidermes for water vapour were measured by a diffusion porometer *Delta-T* type *Mk3* (*Delta-T Devices*, Kingston upon Thames, UK) at a temperature of 25 °C, irradiance of 860 μmol m^{-2} s^{-1}, and air humidity of 60 - 70 %. Under the same conditions, water loss curves were measured gravimetrically on leaves originally fully saturated with water. Experiments were made twice. In each experimental set 10 to 20 plants were used for determination of each parameter. Values were evaluated by *ANOVA* or Student *t*-test.

After six weeks, characteristics of leaf photosynthesis and water relations were determined. On the first sight, tobacco plantlets in *Magenta* boxes grew more quickly than plantlets in glass vessels. At the same time they had more and larger leaves. This is in agreement with previous experiments showing higher biomass accumulation in potato and carnation plantlets grown in *Magenta* boxes than in glass vessels (Solárová *et al.* 1996). However, the medium in *Magenta* boxes dried more quickly and therefore upper part of tobacco shoots had to be subcultured on a new medium after three weeks. Nevertheless, they have similar number of leaves (6 to 8) and total leaf area (about 38 cm^2) as plantlets grown in glass vessels without subculturing.

Table 1. Contents of chlorophyll (Chl) *a*, Chl *b*, β-carotene, light-harvesting carotenoids lutein and neoxanthin (Lu + Ne), and xanthophyll cycle pigments violaxanthin + antheraxanthin + zeaxanthin (Vi + An + Ze) [μg cm^{-2}], and degree of their de-epoxidation [DEPS = (Ze + 0.5 An) / (Ze + An + Vi)] in leaves of G-plants and M-plants. Means of 7 independent measurements ± SE. Statistically significant differences ($P < 0.05$) are marked by different letters.

Pigments	G-plants	M-plants		G-plants	M-plants
Chlorophyll *a*	7.92 ± 0.30a	10.78 ± 0.43b	Chl *a*/*b*	2.60	2.71
Chlorophyll *b*	3.05 ± 0.16a	3.98 ± 0.17b	Chl/β-carotene	40.62	41.00
β-carotene	0.27 ± 0.02a	0.36 ± 0.02b	Chl/xanthophylls	11.92	16.77
Lutein	0.42 ± 0.02a	0.45 ± 0.02a	Chl/Lu + Ne	16.88	21.39
Neoxanthin	0.23 ± 0.02a	0.24 ± 0.01a	Chl/Vi + An + Ze	40.63	77.68
Violaxanthin	0.17 ± 0.01b	0.13 ± 0.01a	Vi + An + Ze	0.27	0.19
Antheraxanthin	0.02 ± 0.00a	0.01 ± 0.00a	DEPS	0.33	0.29
Zeaxanthin	0.08 ± 0.01b	0.05 ± 0.00a			

Photosynthetic pigment contents were higher (Chl *a* by about 36 %, Chl *b* 30 %, β-carotene 33 %, but lutein only 7 %) in leaves of plantlets grown in *Magenta* boxes (M-plants) that in leaves of plantlets grown in glass vessels (G-plants). The differences might be caused by better CO_2 supply combined with slightly higher irradiance of M-plants. This corresponded also with the higher Chl *a*/*b* ratio in M-plants, which meant that smaller light-harvesting complexes containing Chl *b* were needed for energy capture. The contents of lutein and neoxanthin were not significantly different, but contents of xanthophyll cycle pigments (Vi + An + Ze) were lower by 30 % in M plants than in G-plants. Even if also DEPS was slightly higher in G-plants than in M-plants its value did not signal any photoinhibition (Table 1).

Table 2. Chlorophyll *a* fluorescence kinetic parameters [variable to maximum fluorescence ratio (F_v/F_m), quantum yield of PS2 (Φ_{II}), photochemical quenching (q_P), non-photochemical quenching (q_N), and ratio of variable fluorescence decrease (Rfd)], and photochemical activities of the whole electron transport chain (ETC; $H_2O \rightarrow FeCy$) and photosystem 2 (PS2; $H_2O \rightarrow PD$) measured as oxygen evolution [nmol(O_2) g^{-1}(Chl) s^{-1}] of G-plants and M-plants. Means ± SE. Statistically significant differences ($P < 0.05$) are marked by different letters.

Parameter	G-plants	M-plants	Parameter	G-plants	M-plants
F_v/F_m	0.80 ± 0.03a	0.83 ± 0.02a	ETC	1.37 ± 0.13a	1.57 ± 0.19b
Φ_{II}	0.66 + 0.06a	0.68 + 0.06a	PS2	2.11 ± 0.28a	2.46 ± 0.14b
q_P	0.86 ± 0.02a	0.82 ± 0.07a			
q_N	0.24 + 0.05a	0.20 ± 0.04a			
Rfd	2.07 ± 0.56a	2.20 ± 0.65a			

In both plant types also the values of maximal photochemical efficiency of open photosystem 2 centres (F_v/F_m) indicated that no photodamage occurred. Despite the fact that the differences in Chl *a* fluorescence kinetic parameters between G-plants and M-plants (Table 2) were not statistically significant, the tendency to higher photochemical efficiency, actual quantum yield of PS2 (Φ_{II}), and potential photosynthetic activity (Rfd) in M plants can suggest the higher capacity of their photosynthetic apparatus. The main difference in the regulation of light utilization by PS2 between M- and G-plants was observed in the development of higher radiationless energy dissipation in PS2 (q_N) in G-plants. Moderately higher values of q_N corresponded also with higher values of DEPS observed there as xanthophyll cycle closely correlates with q_N (Demmig-Adams 1990). In contrary, M-plants exhibited a decrease in photochemical quenching, *i.e.*, higher fraction of closed PS2 traps, which represents a PS2 population that is likely to be damaged by chronic overexcitation and changes in D1 protein (Aro *et al.* 1993). As F_v/F_m was maintained or was even higher, the rapid turnover of the D1 protein is probably mechanism responsible for photoprotection in M-plants. The photochemical activities of both ETC and PS 2 were significantly higher in M-plants compared to G-plants (Table 2).

Stomatal conductance (g_s) of *in vitro* grown tobacco plantlets was high which was in agreement with our previous results and data from literature (for recent review see, *e.g.* Pospíšilová *et al.* 1997). The high g_s and high transpiration rate was the cause why plantlets taken out from cultivation vessels wilted rapidly (Fig. 1). Therefore lower abaxial (g_{ad}) and especially adaxial (g_{ab}) stomatal conductance in M-plants than in G-plants are their important features (Table 1). The comparison of water loss curves showed that also cuticular conductance was lower in M-plants than in G-plants (Fig. 1).

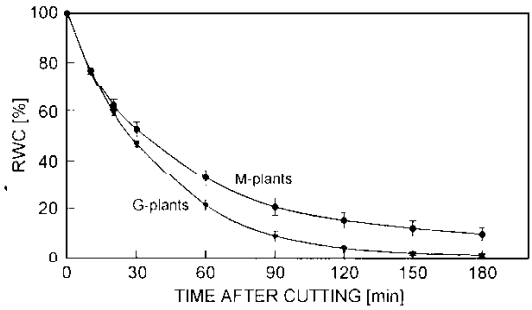

Fig. 1. Transpiration curves (changes in relative water content of detached leaves) of G-plants and M-plants.

Table 3. Stomatal conductances of adaxial and abaxial epidermes [cm s^{-1}], and net photosynthetic rate [μg m^{-2} s^{-1}] of leaves of G-plants or M-plants. P_N was measured under CO_2 concentration 600 or 1 200 mg m^{-3}. For other conditions see Materials and methods. Means ± SE, n = 18. Statistically significant differences ($P < 0.05$) between G- and M-plants are marked with different letters and were found in all parameters.

	Stomatal conductance adaxial	abaxial	Photosynthetic rate 600 mg m^{-3}	1 200 mg m^{-3}
G-plants	1.17 ± 0.07a	0.94 ± 0.04a	312.6 ± 24.4a	478.2 ± 48.6a
M-plants	0.80 ± 0.05b	0.72 ± 0.05b	171.3 ± 28.5b	624.4 ± 48.5b

In spite of lower g_s in M-plants than in G-plants, net photosynthetic rate (P_N) of M-plants was higher than that in G-plants (Table 3). However, g_s in all *in vitro*-grown plants was so high that it was not possible to suppose stomatal limitation of P_N. When ambient CO_2 concentration was doubled P_N was markedly increased but similar differences were found between G- and M-plants.

Our results showed positive effect of improved CO_2 supply in less tightly closed vessels on all measured characteristics of leaf photosynthesis and water relations. Because the *in vitro* growth can affect acclimation of plantlets to *ex vitro* conditions (for review, see Pospíšilová *et al.* 1999), the effect of CO_2 concentration after *ex vitro* transfer of G-plants and M-plants on these parameters will be the subject of following paper.

References

Aitken-Christie, J., Kozai, T., Smith, M.A.L.: Automation and Environmental Control in Plant Tissue Culture. - Kluwer Academic Publishers, Dordrecht - Boston - London 1994.

Aro, E.-M., McCaffery, S., Anderson, J.M.: Photoinhibition and D1 protein degradation in peas acclimated to different growth irradiances. - Plant Physiol. **103**: 835-843, 1993.

Buddendorf-Joosten, J.M.C., Woltering, E.J.: Components of the gaseous environment and their effects on plant growth and development *in vitro*. - Plant Growth Regul. **15**: 1-16, 1994.

Demmig-Adams, B.: Carotenoids and photoprotection in plants: a role for the xantophyll zeaxanthin. - Biochim. biophys. Acta **1020**: 1-24, 1990.

Desjardins, Y.: Photosynthesis *in vitro* - on the factors regulating CO_2 assimilation in micropropagation systems. - Acta Hort. **393**: 45-61, 1995.

Kozai, T.: Autotrophic micropropagation. - In: Bajaj, Y.P.S. (ed.): Biotechnology in Agriculture and Forestry. Vol. 17. Pp. 313-343. Springer-Verlag, New York 1991.

Kozai, T., Fujiwara, K., Hayashi, M., Aitken-Christie, J.: The *in vitro* environment and its control in micropropagation. - In: Kurata, K., Kozai, T.: Transplant Production Systems. Pp. 247-282. Kluwer Academic Publishers, Dordrecht - Boston - London 1992.

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

Murphy, K.P., Santamaria, J.M., Davies, W.J., Lumsden, P.J.: Ventilation of culture vessels. I. Increased growth *in vitro* and survival *ex vitro* of *Delphinium*. - J. hort. Sci. Biotechnol. **73**: 725-729, 1998.

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., Solárová, J., Čatský, J.: Photosynthetic responses to stresses during *in vitro* cultivation. - Photosynthetica **26**: 3-18, 1992.

Pospíšilová, J., Tichá, I., Kadleček, P., Haisel, D., Plzáková, Š.: Acclimatization of micropropagated plants to *ex vitro* conditions. - Biol. Plant. **42**: 481-497, 1999.

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.

Solárová J.: Photosynthesis of plant regenerants. Diurnal variation in CO_2 concentration in cultivation vessels resulting from plantlet photosynthetic activity. - Photosynthetica **23**: 130-135, 1989.

Solárová, J., Pospíšilová, J.: Effect of carbon dioxide enrichment during *in vitro* cultivation and acclimation to *ex vitro* conditions. - Biol. Plant. **39**: 23-30, 1997.

Solárová, J., Součková, D., Ullmann, J., Pospíšilová, J.: *In vitro* culture: environmental conditions and plantlet growth as affected by vessel type and stopper material. - Zahradnictví **23**: 51-58, 1996.

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