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Prolonged exposure of tobacco to a low oxygen atmosphere to suppress photorespiration decreases net photosynthesis and results in changes in plant morphology and chloroplast structure

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Abstract

Air-grown tobacco (*Nicotiana tabacum* L.) plants were transferred for one week into a low oxygen atmosphere (2 kPa O_2, LO) to study both immediate and long-term effects of the suppression of photorespiration on net photosynthetic rate (P_N), plant morphology, and chloroplast ultrastructure. The P_N and the leaf conductance for CO_2 increased upon exposure of attached tobacco leaves to LO. These results may suggest that under LO, external CO_2 is used to consume the radiant energy normally utilized in photorespiration by net CO_2 assimilation at the expense of an increased rate of transpiration. The increase in the coefficient of nonphotochemical fluorescence quenching indicates that under LO, (surplus) radiant energy is also dissipated as heat. Prolonged LO-treatment of tobacco resulted in a decrease in the P_N (measured in air) and in a reduction in the number of starch grains in the chloroplasts. Concomitantly, large lipid globuli appeared in the chloroplasts and the distance between the thylakoids forming the grana decreased. These changes in the ultrastructure of chloroplasts may have contributed to the decline in the P_N. The LO-treated plants were considerably smaller than the control plants maintained in air. This appears to have resulted from a reduction in the rate of leaf area expansion at the expense of an

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Abbreviations: g, leaf conductance for CO_2; LO, low oxygen atmosphere; P_N, rate of net photosynthetic CO_2 uptake; 3-PGA, 3-phosphoglycerate; PS, photosystem; q_{NP}, coefficient of non-photochemical fluorescence quenching; q_P, coefficient of photochemical fluorescence quenching; R_D, rate of dark respiration.
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increase in the specific mass of the leaves. This long-term response to LO-treatment may allow the plants to conserve water.

Additional key words: chlorophyll fluorescence quenching; gas exchange; leaf anatomy; *Nicotiana tabacum*; oxygen partial pressure; respiration rate; stomatal conductance.

Introduction

In plant leaves, the plastidic enzyme ribulose-1,5-bisphosphate carboxylase/-oxygenase catalyzes both the carboxylation and oxygenation of ribulose-1,5-bisphosphate. Carboxylation results in the formation of two molecules of 3-phosphoglycerate (3-PGA) while oxygenation produces one molecule of 3-PGA and one of glycollate phosphate (Vácha 1995). The 3-PGA is utilized within the photosynthetic carbon reduction cycle to produce sugars. Glycollate phosphate is metabolized *via* the photosynthetic carbon oxidation cycle. The operation of this pathway results in the uptake of oxygen and in the release of both CO_2 and ammonium. This process is designated photorespiration (Leegood *et al.* 1995). In leaves of C_3-plants, the photorespiratory CO_2 is in part reassimilated and in part lost to the atmosphere (Gerbaud and André 1987) while the ammonium is reassimilated during the photorespiratory nitrogen cycle (Häusler *et al.* 1994).

The rate at which CO_2 and ammonium are released during photorespiration at ambient CO_2 partial pressure in the leaves of C_3-plants is about 30 % of P_N (Gerbaud and André 1987, Biehler and Fock 1996). The major environmental cues affecting the rate of photorespiration are the ratio of CO_2 to O_2 and temperature (Chen and Spreitzer 1992). Exposure of attached leaves to LO (2 kPa O_2) results in the suppression of photorespiration and in the concomitant increase in P_N (Stuhlfauth *et al.* 1990). The increased P_N is, however, not maintained during prolonged incubation of whole plants in LO (Ishii and Schmid 1983).

The physiological function of photorespiration is controversial (Aro *et al.* 1993, Long *et al.* 1994, Brestic *et al.* 1995) although there is now strong evidence that photorespiration may protect C_3-plants from photooxidation (Osmond and Grace 1995, Heber *et al.* 1996, Kozaki and Takeba 1996). Prolonged exposure of whole plants to a LO may, therefore, involve the risk of photooxidative damage to the chloroplasts.

Information about the repercussions of LO on plant growth which include a strong reduction in seed production (Musgrave and Strain 1988, Crispi *et al.* 1996) has to be taken into account in the design of plant culture systems operating at sub-ambient oxygen partial pressure. Plant culture at LO/low pressure is envisioned as part of a life support system during long-duration space missions to regenerate air and water and to produce food (Kuang *et al.* 1998). In the present study, we examined short- and long-term effects of LO on tobacco P_N, leaf conductance, chlorophyll (Chl) fluorescence quenching, and on both plant morphology and chloroplast ultrastructure.

Materials and methods

Plants: *Nicotiana tabacum* L. cv. Xanthi (INRA, Versailles, France) was grown for four weeks in garden soil in a greenhouse under "white light" from fluorescent lamps providing a photosynthetically active irradiance of 250 μmol(photon) m^{-2} s^{-1} for 16 h each day. The temperature was 25 °C during the day and 18 °C during the night. The plants were supplied daily with a complete nutrient solution containing 11 mM nitrate (Deng *et al.* 1989).

Exposure of tobacco to low oxygen partial pressure: Four-week-old tobacco plants were transferred from the greenhouse into a gas-proof plexiglass chamber (500 × 250 × 300 mm). Air was continuously passed through the chamber at a flow rate of 5.6 cm^3 s^{-1}. The irradiance and the temperature in the chamber were identical to those in the greenhouse. After an adaptation period of 24 h, the air stream was replaced by a gas stream of 5.6 cm^3 s^{-1} containing 35 Pa CO_2, 2 kPa O_2, and balance N_2 (*Linde*, Bielefeld, Germany). Control plants were kept in an identical plexiglass chamber under an air stream of 5.6 cm^3 s^{-1}. The experiment was repeated three times with a total of 24 LO-treated plants.

Gas exchange measurements: An open gas-exchange system (Biehler *et al.* 1997) equipped with an infrared gas analyzer (*UNOR, Maihak*, Hamburg, Germany) and a humidity sensor (*Vaisala HMP 125, Driesen & Kern*, Tangstedt, Germany) was used to simultaneously measure the CO_2- and H_2O-gas exchange of attached tobacco leaves at steady-state photosynthesis. Air or a gas stream containing 35 Pa CO_2, 2 kPa O_2, and balance N_2 (*Linde*) was passed through the thermostated leaf cuvette at a flow rate of 19.4 cm^3 s^{-1}. The humidity of the gas stream was kept constant at 70 % relative humidity (25 °C) by means of a humidifying-condensing system installed between the gas reservoir and the leaf cuvette. The leaf temperature was measured with a Cu-constantan thermocouple on the lower side of the leaf. Actinic radiation was applied at a fluence rate of 250 μmol(photon) m^{-2} s^{-1}.

Chlorophyll fluorescence analysis: Maximal (F_m), variable (F_v), variable at full reduction of Q_A (F'_m), and minimal fluorescence (F_0) at full oxidation of Q_A were measured with a pulse amplitude modulation fluorometer (*Walz*, Effeltrich, Germany) according to Schreiber *et al.* (1986). F_0 was induced by exposing dark-adapted (60 min) leaves to a weak [0.1 μmol(photon) m^{-2} s^{-1}] modulated measuring beam. For the determination of F_m, a flash (700 ms) of saturating radiation [4500 μmol(photon) m^{-2} s^{-1}; *Schott KL 1500* projector; *Walz*] was applied. The radiation pulses were transferred by fibre optics to the leaf with an angle of 45° to the upper side. F_v was monitored following the onset of irradiation with actinic radiation. F'_m was recorded at steady-state photosynthesis by exposing the leaf to pulses of saturating radiation every 20 s during the measurement. The coefficients of both photochemical fluorescence quenching (q_P) and non-photochemical fluorescence quenching (q_{NP}) were calculated according to van Kooten and Snel (1990).

Tissue processing for light and electron microscopic inspection: Leaf specimens were fixed for 60 min in 50 mM sodium phosphate buffer (pH 7.2) containing 2.5 % (v/v) glutaraldehyde, and post-fixed for 120 min in 50 mM sodium phosphate buffer (pH

7.2) supplemented with 2 % (v/v) osmium tetroxide. After fixation, the leaf sections were washed with 50 mM Na-phosphate buffer (pH 7.2), dehydrated through a graded acetone/water series, and embedded in *TRANSMIT EM* resin (TAAB). For light microscopy, thin (0.5-1.0 μm) leaf sections were cut with a glass knife on a *Reichert Ultracut* ultramicrotome and stained with 1 % (m/v) toluidine blue prior to examination in an *Olympus BH* photomicroscope. For electron microscopy, ultrathin sections (silver-grey interference colours) were cut with a diamond knife on a *Reichert Ultracut* ultramicrotome and collected on uncoated copper grids (200 mesh). After staining for 15 s with 5 % (m/v) $KMnO_4$ and for 30 s with 2 % (m/v) lead citrate, the leaf specimens were examined with a *Hitachi H 500* electron microscope at 75 kV.

Results and discussion

As expected from previous studies (Stuhlfauth *et al.* 1990, Scheuermann *et al.* 1991, Chen and Spreitzer 1992), the transfer of air-grown tobacco plants into LO (2 kPa O_2) resulted in an immediate and strong increase in P_N (Table 1). The rate of photorespiration is about 30 % of the rate of P_N (Gerbaud and André 1987, Biehler and Fock 1996). The 40 % increase in P_N of tobacco leaves under LO can, therefore, be accounted for by the suppression of the photorespiratory loss of CO_2 to the atmosphere and the absence of internal reassimilation of photorespiratory CO_2 under these conditions.

Table 1. Photosynthetic performance of tobacco leaves during low oxygen-treatment. The rates of net CO_2 uptake (P_N; μmol m^{-2} s^{-1}) and dark respiration [R_D; μmol(CO_2) m^{-2} s^{-1}], the leaf conductance for CO_2 (g; cm s^{-1}), and the coefficients of photochemical (q_P) and nonphotochemical (q_{NP}) fluorescence quenching by attached leaves of tobacco grown in air, and 1 h or 3 d after the transfer into a low-oxygen atmosphere (2 kPa O_2). The photon fluence rate was 250 μmol m^{-2} s^{-1} and the leaf temperature was 23 °C. Steady-state photosynthesis was reached *ca.* 30 min after the onset of illumination. R_D was measured in darkness 30 min after the light was switched off. The standard deviation is < ± 5 % for each given value representing the average of four measurements. [a] measured in air; n.d. - not determined.

	Air [22 kPa(O_2)]	2 kPa(O_2) for 1 h	2 kPa(O_2) for 3 d
P_N	6.10	9.90	4.70[a]
R_D	0.27	0.31	0.30
g	0.17	0.28	0.24
q_P	0.71	0.93	n.d.
q_{NP}	0.32	0.61	n.d.

The partial closure of stomata under low O_2 would reduce the increased P_N to the normal rate and allow the conservation of water. However, the increase in g upon exposure of tobacco leaves to LO (Table 1) suggests that the increase in the P_N may also be correlated with the partial opening of stomata under these conditions. The

partial opening of stomata inevitably results in an increase in water loss due to transpiration under LO (Scheuermann *et al.* 1991). This consequence of LO-treatment may explain why wilting of tobacco leaves occurs when the oxygen partial pressure is reduced below 2 kPa (Ishii and Schmid 1983).

If the concept of a photoprotective role of photorespiration (Heber *et al.* 1996, Kozaki *et al.* 1996) is valid, then survival of plants in LO would require responses to compensate for the radiant energy dissipation normally achieved by photorespiration. One possibility to explain the increase in g at the expense of an increased rate of transpiration upon transfer of air-grown tobacco into LO is that external CO_2 is used to consume the (surplus) radiant energy normally utilized in photorespiration by P_N. It may, therefore, be assumed that under LO photoprotection by radiant energy dissipation is more important than the conservation of water (Stuhlfauth *et al.* 1990). It can be speculated that the reduction in the ambient O_2 partial pressure is perceived within the guard cells. Alternatively, however, the change in the oxygen partial pressure may be sensed in the mesophyll and then communicated to the stomata.

An additional or alternative possibility to provide photoprotection under LO may be the reversible decrease in the efficiency of radiant energy utilization by photosystem 2 (PS2; Scheuermann *et al.* 1991). This down-regulation of PS2 can be accomplished by the thermal dissipation of (surplus) radiant energy within the antennae of PS2 (Stuhlfauth *et al.* 1990). The coefficient of nonphotochemical fluorescence quenching (q_{NP}), reflecting the portion of total excitation energy that is dissipated nonphotochemically (Krause and Weis 1991), increased upon transfer of tobacco plants into LO (Table 1). Although the significance of this calculated parameter should not be overstated, the increase in q_{NP} may suggest that in tobacco leaves exposed to LO, (surplus) radiant energy not utilized in photorespiration is, at least in part, dissipated as heat.

Taken together, our results seem to support the importance of photorespiration in radiant energy dissipation. This presumption is further supported by an apparent light-avoidance reaction of the plants. One day after the transfer of tobacco plants grown in air into an LO-atmosphere, the orientation of leaves relative to the radiation source had changed from the initial more or less horizontal and thus fully light-exposed position to an almost vertical and, therefore, less light-exposed position (values not shown). However, we have not analyzed graded responses, *i.e.*, the magnitude of this apparent light-avoidance reaction in relation to the photon fluence rate and, therefore, we cannot conclusively correlate this response of tobacco to suppression of photorespiration with a function in light-avoidance. Nevertheless, the observed change in the leaf position relative to the radiation source may have reduced the absorption of radiant energy and, consequently, the potential for the accumulation of (surplus) reducing equivalents that cannot be used in photorespiration under LO.

The initially increased rate of P_N was not maintained during prolonged LO-treatment of whole tobacco plants. Three days after the transfer of whole tobacco plants into LO, the P_N (measured in air) by attached leaves was lower than the initial P_N in air (Table 1). This is not due to stomatal closure because g remained fairly unchanged (Table 1). The rate of dark respiration (R_D) was unaffected by the LO-

treatment (Table 1). This result suggests that the decrease in P_N is due to damage to the photosynthetic and not to the respiratory apparatus. Damage to the photosynthetic apparatus as a cause of the decline in P_N during LO-treatment is also supported by the observation that P_N did not completely recover within three days after the transfer of the LO-treated plants into air (values not shown).

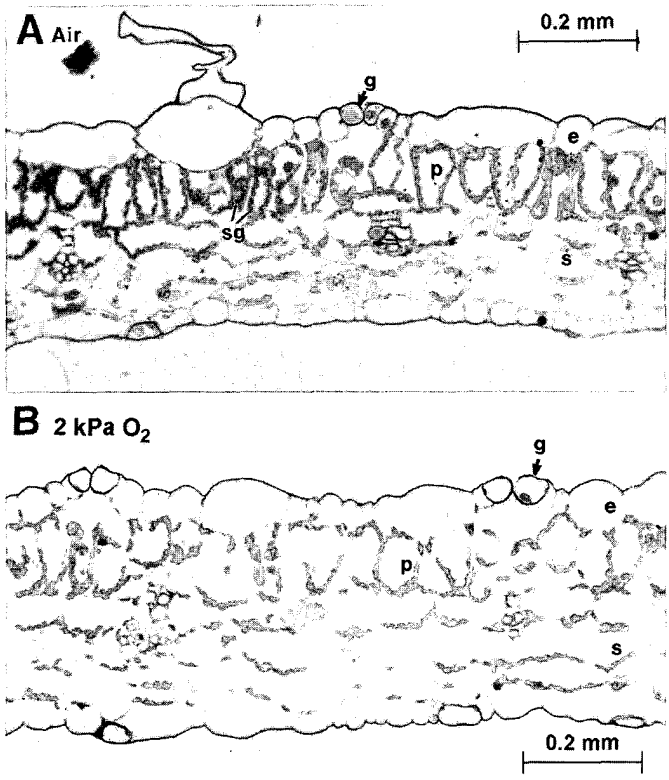

Fig. 1. Leaf anatomy of air-grown or low oxygen-treated tobacco plants. The light-micrographs show thin leaf sections of representative tobacco plants grown in air for 5 weeks (*A*) or grown in air for 4 weeks and then exposed to a low oxygen atmosphere (2 kPa O_2) for 3 d (*B*). The letters indicate starch grains (*sg*), guard cells (*g*) or cells of the epidermis (*e*), the palisade parenchyma (*p*) or the spongy tissue (*s*).

The coefficient of photochemical fluorescence quenching (q_P), reflecting the reoxidation of Q_A, the primary quinone acceptor of PS2 (Krause and Weis 1991), was higher in LO than in air (Table 1). This result suggests that Q_A was more oxidized under LO than in air. Prolonged suppression of photorespiration in LO, therefore, results in a high potential for photooxidation despite radiant energy dissipation as heat during nonphotochemical fluorescence quenching or by using external CO_2 in P_N. A gradual onset of photooxidation may, at least in part, explain why the initially increased P_N by tobacco leaves was not maintained during prolonged LO-treatment (Table 1). If this was the case, then radiant-energy

dissipation within the light reactions by non-photochemical fluorescence quenching and within the dark reaction by P_N is not effective enough to entirely compensate for the lack of photorespiratory energy consumption under LO.

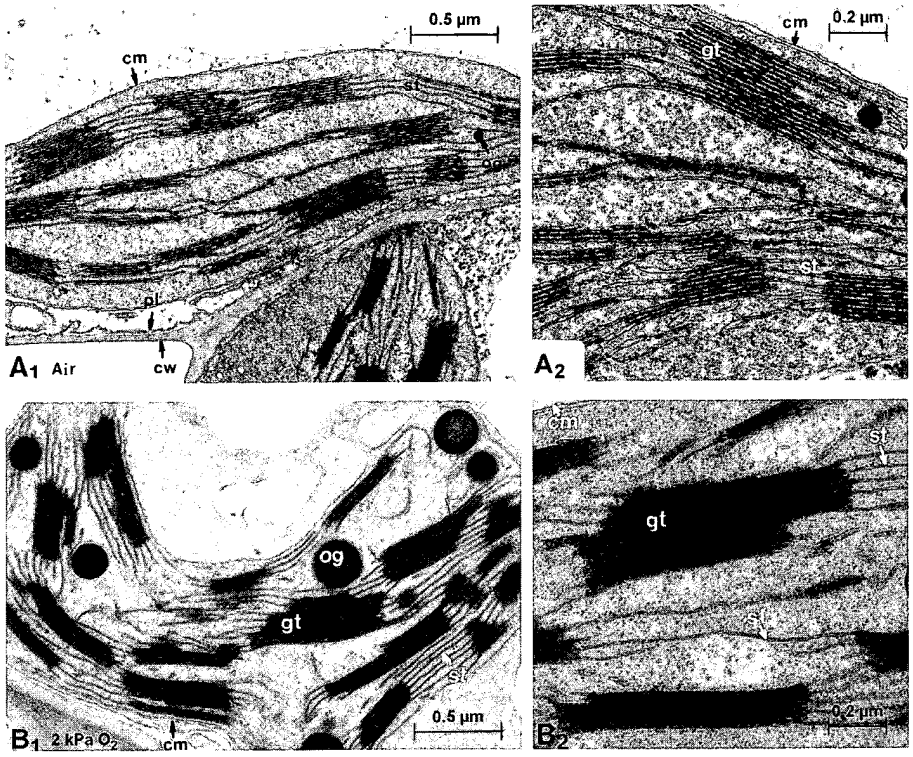

Fig. 2. Chloroplast ultrastructure of air-grown or low oxygen-treated tobacco plants. The electron-micrographs of ultrathin leaf sections show a representative chloroplast (A_1, B_1) or a section of a chloroplast (A_2, B_2) of tobacco plants grown in air for 5 weeks (*A*) or grown in air for 4 weeks and then exposed to a low oxygen atmosphere (2 kPa O_2) for 3 d (*B*). The letters indicate the cell wall (*cw*), chloroplast membrane (*cm*), grana thylakoids (*gt*), osmiophilic lipid globuli (*og*), the plasma membrane (*pl*) or stroma thylakoids (*st*).

The decline in P_N during prolonged LO-treatment is paralleled by pronounced changes in the ultrastructure of the chloroplasts. The number of starch grains decreased (Fig. 1) while large osmiophilic (lipid) globules appeared (Fig. 2). These structural changes are reminiscent of those seen in the chloroplast structure during leaf senescence (Schwabe and Kulkarni 1987). The appearance of the large lipid globules may be due to an accumulation of lipids as a consequence of partial thylakoid breakdown or have resulted from an accumulation of lipids not utilized in *de novo* membrane formation. The physiological significance of the apparent

decrease in the distance between the grana thylakoid membranes following exposure to LO (Fig. 2) is not known. Nevertheless, the changes seen in the ultrastructure of tobacco chloroplasts during prolonged LO-treatment (Fig. 2) may have contributed, at least in part, to the decline in P_N (Table 1).

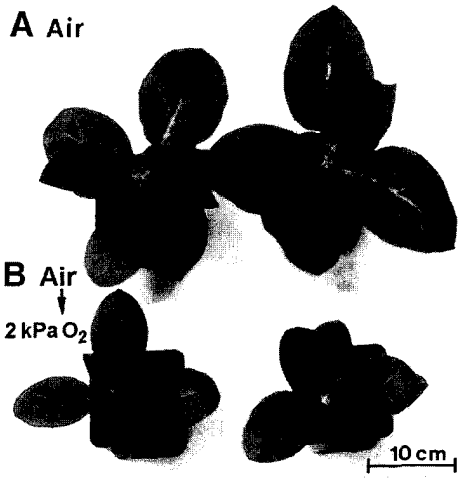

Fig. 3. Phenotype of air-grown or low oxygen-treated tobacco plants. Comparison of five-week-old tobacco plants raised in air (*A*) and tobacco plants grown in air for 4 weeks and then exposed to a low oxygen atmosphere (2 kPa O_2) for one week (*B*).

The prolonged LO-treatment of whole tobacco plants not only had repercussions on the ultrastructure of chloroplasts but also on the plant morphology. Tobacco plants that had been exposed for one week to LO were significantly smaller than the control plants maintained in air (Fig. 3). It is attractive to attribute the apparent reduction in plant growth to the decline in P_N during the LO-treatment. The reduction in the rate of leaf area expansion under LO is, however, also correlated with an increase in the specific mass of the leaves (Ishii and Schmid 1983) and, apparently, in leaf thickness (Fig. 1).

The increase in specific leaf mass at the expense of a decrease in leaf area is expected to result in a reduction in transpiratory water loss and may, therefore, represent a long-term acclimation of the plants to LO (which results in increased g). It is, however, also possible that the incubation of whole tobacco plants under LO may have, with time, disturbed root metabolism. The rate of root respiration is about 50 % lower at 2 kPa O_2 than in air (Salglio *et al.* 1983). In turn, root growth and/or the rate of water uptake may have been reduced. The resulting decrease in the availability of water during prolonged LO-treatment may then have been compensated for by a reduction in transpiratory water loss *via* the observed reduction in the total leaf area.

To summarize, our results support the importance of photorespiration in radiant energy dissipation. In addition, we have shown that, with time, LO-treatment results in pronounced and potentially detrimental changes in both the chloroplast

ultrastructure and the plant morphology. A forthcoming study will attempt to define the minimal O_2 partial pressure required under LO to avoid these repercussions on the growth of tobacco. This information may then be considered in the concept of a life support system for space flights based on plant culture at subambient O_2 concentration and low pressure.

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