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Photorespiration rate in spinach leaves under moderate NaCl stress

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Abstract

We analyzed the effect of NaCl stress on photorespiration of spinach leaves by calculating the rate of carboxylation/oxygenation of ribulose-1,5-bisphosphate carboxylase/oxygenase, and by measuring the content of amino acids produced through photorespiration. After 20 d of NaCl stress the carboxylation rate was reduced while the oxygenation rate was not affected. The contents of serine, glycine, and alanine increased relevantly. The amount of glutamine also increased after 20 d but the amount of glutamate did not. Hence photorespiration may be stimulated under moderate NaCl stress. A relevant electron transport rate was observed under CO_2-free air, which may indicate refixation of photorespiratory CO_2. When NaCl accumulation proceeded for more than 20 d, photosynthesis was reduced and the content of photorespiratory amino acids started to decrease, but the oxygenation rate did not change.

Additional key words: amino acids; carboxylation rate; electron transport rate; oxygenation rate; photosynthetic rate; *Spinacia oleracea*.

Introduction

Salinity is the most important environmental stress and may severely limit plant production. When plants are exposed to excess of salt they suffer both an osmotic stress by limiting the absorption of water from the soil, and a ionic stress resulting from the high concentration of potentially toxic salt ions. The osmotic stress reduces plant water potential mimicking the effect of water stress and affecting many important physiological processes such as stomatal opening and photosynthesis. When salt concentration in the tissue is low, diffusion resistances to CO_2 at stomata and in the mesophyll may be the only factor to limit photosynthesis of plants, while

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Abbreviations: J_f - electron transport rate driving photosynthesis and photorespiration measured by fluorescence; P_N - net photosynthetic rate; R_D - respiration rate in the dark; RuBPCO - ribulose-1,5-bisphosphate carboxylase/oxygenase; v_c - velocity of carboxylation; v_o - velocity of oxygenation.

ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) content and activity are unchanged (Delfine *et al.* 1998). Under these conditions, oxygenation of RuBP is favoured over carboxylation. The photosynthetic electron transport rate generally down-regulates following the inhibition of photosynthesis and photorespiration (Loreto *et al.* 1994). If photosynthesis limitation is only related to CO_2 diffusion, however, the electron transport may continue to feed other processes; mainly photorespiration, and, to a much smaller extent, direct O_2 photoreduction, or alternative electron sinks (Loreto *et al.* 1994). The oxygenation rate which feeds photorespiration can be estimated by knowing the electron transport, photosynthetic, and dark respiration rates (Di Marco *et al.* 1994).

Many amino acids (*e.g.*, glycine, serine, and alanine) are produced by photorespiration. Photorespiratory NH_3 is released by mitochondria at a flux comparable to the velocity of oxygenation. Such amount of NH_3 must be re-assimilated in the chloroplasts by a cycle involving glutamate and glutamine, two amino acids localized in chloroplast stroma and cytosol (Winter *et al.* 1994) where they may also act as intermediates of other metabolic processes. Yet, the rapid turn over of photorespiration intermediates, makes unlikely their regulation by processes other than photorespiration (Sivak *et al.* 1988, Leegood *et al.* 1995). On the other hand, the content of photorespiratory amino acids in the leaf may change because of changes in RuBPCO oxygenation rate. An accumulation of these amino acids may be also observed when the oxygenation rate is not matched by the rate of any other enzymatic reaction which is necessary to regenerate glycerate in the photorespiratory cycle (Leegood *et al.* 1995).

Amino acids, especially proline, may also accumulate during osmotic adjustments (Singh *et al.* 1973). They lower the water potential of cell sap without affecting cell metabolism (Ahmad *et al.* 1979). This has been considered a common way to protect the structure of macromolecules in stressed cells (Schobert 1977, Yancey *et al.* 1982, Low 1985).

We studied how the exposure from moderate to high salt stress influences photorespiration in spinach plants by concurrently measuring photosynthetic electron transport and photosynthetic rate under photorespiratory and non-photorespiratory conditions and the content of the main amino acids produced by photorespiration.

Materials and methods

Plant and experimental conditions: Two groups of 20 spinach (*Spinacia oleracea* L. cv. Matador) plants were grown in 3 000 cm^3 pots containing a mixture of soil, peat, and sand (1 : 1 : 1). All plants grew in a greenhouse under the same temperature and irradiance. Temperatures varied between 20/30 °C (night/day) and irradiance did not exceed 1000 $\mu mol(quantum)\ m^{-2}\ s^{-1}$. When five to six leaves were fully expanded, control plants grew under optimal water conditions by restoring daily the water lost through evapotranspiration. NaCl-stressed plants were irrigated with water containing 1 % (m/v) of NaCl. Measurements of gas exchange and fluorescence were made simultaneously after 13 and 20 d of exposure to NaCl. Photosynthesis inhibition was found after 20 d and at this stage we started to measure amino acid contents. Two further measurements of gas exchange, fluorescence, and amino acids were made after 30 and 40 d. The two youngest, fully expanded leaves were used for gas exchange and fluorescence measurements. Part of each leaf was then frozen in liquid nitrogen and used for the determination of amino acids and NaCl content.

Na^+ accumulation: Leaf content of Na^+ was measured 13, 20, 30, and 40 d after starting the irrigation with saline water. Leaves were dried at 65 °C for one day. Sodium was extracted from 150 mg of dry mass in a 10 cm^3 mixture of HNO_3, $HClO_4$, and distilled water (1 : 5 : 2.5). The solution was exposed for 12 h at 100 °C, diluted to 25 cm^3 with 100 mol m^{-3} HCl, and analyzed by atomic emission spectrometry (*I.C.P. Plasma 40, Perkin Elmer*, UK).

Gas exchange and fluorescence measurements, electron transport rate determination, and calculation of kinetic parameters: The gas exchange system described by Loreto *et al.* (1992) was used to determine leaf net photosynthetic rate (P_N) and dark respiration rate (R_D) with the following modifications: the infrared gas analyzer was a *LiCor 6262* (*Li-Cor*, Lincoln, USA). The leaf cuvette was small (4.9 cm^2), and made with an aluminum body and two glass windows to allow irradiation of both parts of the leaf. In this experiment only the upper part of the leaf was irradiated. The leaf was thermostated by circulating water in the carved body. Irradiation was supplied by an optic fiber ring connected to a *KL1500* (*Schott*, Mainz, Germany) light source. All gas exchange and fluorescence measurements were made at a leaf temperature of 25 °C and an irradiance of 800 μmol(quantum) m^{-2} s^{-1}.

Fluorescence was measured by inserting the terminal end of a polyfurcated optic fiber inside the fiber ring normal to the leaf plane. This fiber was used to supply the weak and red measuring radiation and the saturating (10 000 μmol m^{-2} s^{-1}) pulses of "white light" as well as to detect the emitted leaf fluorescence as described by Loreto *et al.* (1992).

The electron transport rate driving photosynthesis and photorespiration (J_f) was measured by fluorescence as previously described (Loreto *et al.* 1992, 1994). Measurements were done under ambient air composition (350 μmol mol^{-1} CO_2 and 210 mmol mol^{-1} O_2), under CO_2-free atmosphere, and under CO_2-free and low O_2 (20 mmol mol^{-1}) atmosphere. For the correct measurement of J_f it is necessary to know leaf absorptance. It was measured as described in Massacci *et al.* (1995) on six leaves for each group of plants by using a *LiCor 1800* portable spectroradiometer and a *LiCor 1800-12* integrating sphere (*LiCor*, Lincoln, USA), and did not change significantly throughout the experimental period.

The oxygenation and carboxylation velocity (v_o and v_c, respectively) and their ratio were calculated by combining the two equations:

$$P_N + R_D = v_c - 0.5\ v_o \qquad (1)$$

$$J_f = 4\ v_c + 4\ v_o \qquad (2)$$

The second equation is a slightly modified version of the equation reported by Di Marco *et al.* (1994). It assumes that both CO_2 and O_2 are reduced by four electrons and that no other process requiring relevant electron transport exists when photosynthesis and photorespiration are present.

Amino acids content: A leaf disk (3.2 cm^2 corresponding to 90 to 100 mg of fresh mass) was ground with sand in liquid nitrogen, and 2 cm^3 of cold 80 % ethanol was added to the frozen powder. After 10 min the solution was centrifuged and the supernatant was utilized for the amino acid determination.

All amino acids except proline were determined by HPLC (*MT2* system *Kontron Instruments*, UK) after derivatization with *o*-phthaldialdehyde (OPA). An aliquot (0.1 cm^3) of the extract was derivatized with 0.05 cm^3 of OPA for 1 min. Twenty mm^3 of the mixture was then injected and eluted at a flow rate of 1 cm^3 min^{-1} at 30 °C and in gradient condition. The derivatized amino acids were separated on a reverse phase 5 μm, 250×4.6 mm, *C18* ultrasphere column (*Beckman*, Fullertone, USA). Solvent A was 50 mM sodium acetate adjusted to pH 7 with acetic acid and 1 % tetrahydrofuran, solvent B was 100 % methanol.

The eluted amino acid-OPA derivatives were detected by fluorescence spectrophotometry (*Kontron SFM 25*) at an excitation wavelength of 340 nm, and an emission wavelength of 455 nm.

Proline was determined by HPLC as stable and fluorescent P-FMOC-carbamate, using FMOC-Cl solution as the derivatization reagent. The extract (0.1 cm^3) was added to 0.2 cm^3 borate buffer (0.2 M boric acid adjusted to pH 8.5 with 30 % KOH solution). OPA (0.04 cm^3) was added to remove the influence of other amino acids and the solution was mixed for 1 min. Then 0.02 cm^3 of FMOC (3 mM in acetone) was added and mixed for 3 min. The excess of FMOC was removed by adding 0.02 cm^3 heptylamine reagent. Proline was eluted on a reverse phase 5 μm, 150×4.6 mm, *C8* column (*Beckman*) in isocratic condition and at flow rate of 8.33 mm^3 s^{-1}. Solvent was a mixture of 20 mM acetate buffer, pH 7.2 (60 %), acetonitrile (20 %), and methanol (20 %). The eluted proline-FMOC-carbamate was detected by fluorescence spectrophotometry (*Kontron*) at an excitation wavelength of 265 nm and an emission wavelength of 315 nm.

Results

The P_N of NaCl-stressed leaves was significantly lower than in controls 20 d after starting the salt treatment (Fig. 1*A*). At this stage, Na^+ started to accumulate in the leaf (Fig. 1*B*). P_N further dropped when Na^+-accumulation proceeded over the experimental period.

After 20 d, the J_f under ambient conditions was lower in NaCl-stressed than in control leaves (Fig. 2). This rate depends on the carboxylation and oxygenation velocity (Eq. 2). However, the reduction of J_f reflected only a reduction of v_c while v_o was not affected (Table 1). As a consequence, the v_o/v_c ratio increased because of NaCl stress. Increasing salt accumulation at 30 and 40 d caused a further increase of the v_o/v_c ratio in NaCl-stressed leaves.

After 30 d, the controls also showed a significant decrease of v_c with respect to the previous sampling. However, at this time a similar decrease of v_o also occurred. As a consequence, the v_o/v_c ratio of controls did not change over time.

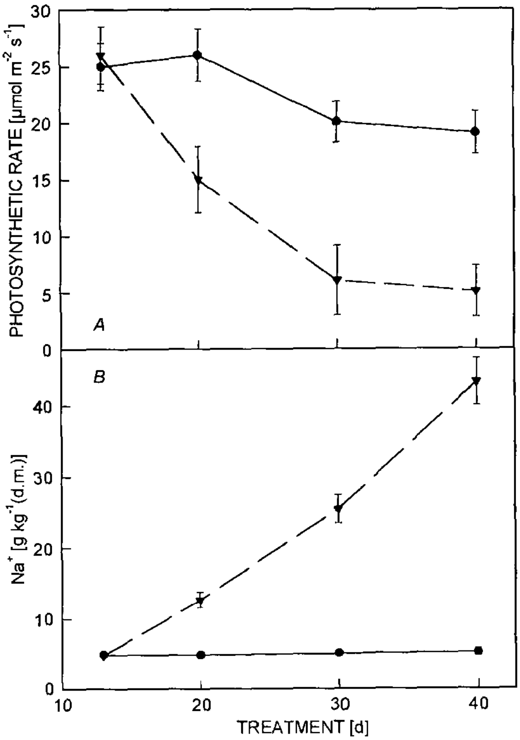

Fig. 1. Photosynthetic rate (*A*) and Na^{+} accumulation (*B*) in controls (●) and NaCl-stressed (▲) leaves of spinach over the experimental period. Bars represent the S.E. (*n* = 6). When bars are not shown, S.E. is lower than symbol size.

When CO_2 was removed from the air, the electron transport rate dropped to about 30 µmol m^{-2} s^{-1} in controls but was more than three times higher in NaCl-stressed leaves (Fig. 2). Only when O_2 was lowered to 20 mmol mol^{-1} and photorespiration was inhibited, the electron transport rate of both controls and NaCl-stressed leaves was almost completely suppressed.

After 20 d, a significant increase of proline, glycine, serine, alanine, and glutamine and a decrease of glutamate was found in NaCl-stressed leaves with respect to controls (Table 2). After 30 d, proline content of NaCl-stressed leaves was even higher then after 20 d, while the content of the other amino acids started to decrease. Glycine content, in particular, dropped dramatically. However, glycine, serine, and alanine contents were still significantly higher in NaCl-stressed leaves than in controls while glutamine content of NaCl-stressed leaves was lower than in controls. After 40 d, a significant decrease of amino acid content occurred both in control and NaCl-stressed leaves. The amounts of glycine and serine were still significantly higher in NaCl-stressed leaves than in controls. Interestingly, 40 d after beginning the treatment, controls showed an increase of glutamine at the expenses of glutamate.

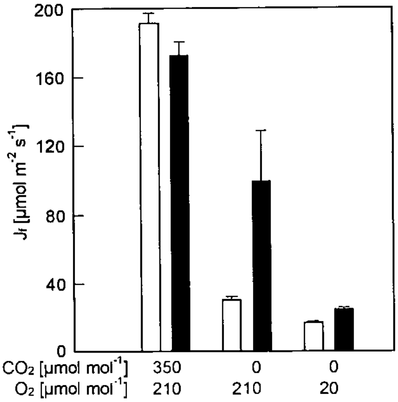

Fig. 2. Electron transport rate measured by fluorescence (J_f) in leaves of control (*open columns*) and NaCl-stressed (*closed columns*) spinach after 20 d of NaCl stress. Leaves were exposed to different CO_2 and O_2 concentrations as shown in the abscissa axis.

Table 1. Oxygenation rate, v_o [μmol m^{-2} s^{-1}], carboxylation rate, v_c [μmol m^{-2} s^{-1}], and their ratio (v_o/v_c) in control and NaCl-stressed leaves (C and SS, respectively) during the experimental period. Different letters represent mean significant difference at $p < 0.05$ ($n = 5$).

Treatment [d]	v_o C	 SS	v_c C	 SS	v_o/v_c C	 SS
20	11^{ab}	14^{a}	33^{a}	22^{b}	0.33^{c}	0.66^{b}
30	7^{b}	12^{a}	22^{b}	10^{c}	0.31^{c}	1.20^{a}
40	7^{b}	14^{a}	22^{b}	10^{c}	0.31^{c}	1.40^{a}

Table 2. Contents of amino acids [μmol kg^{-1}(f.m.)] in the leaves of control and NaCl-stressed plants. Different letters represent mean significant difference at $p < 0.05$ ($n = 4$).

Time	Proline		Glycine		Serine		Alanine		Glutamate		Glutamine	
[d]	C	SS	C	SS	C	SS	C	SS	C	SS	C	SS
20	52^{d}	393^{b}	127^{c}	2034^{a}	119^{d}	554^{a}	44^{b}	82^{a}	171^{a}	75^{c}	95^{b}	190^{a}
30	55^{d}	578^{a}	113^{c}	447^{b}	101^{d}	368^{b}	51^{b}	91^{a}	174^{a}	45^{d}	98^{b}	69^{c}
40	45^{de}	254^{c}	74^{d}	164^{c}	62^{c}	176^{c}	61^{b}	32^{bc}	130^{h}	57^{cd}	162^{a}	80^{b}

Discussion

Photosynthesis is inhibited by Na^+ accumulation in leaves. The calculated oxygenation rate, however, does not decline, irrespective of the amount of Na^+ accumulated in the leaf over the experimental period. On the contrary, the carboxylation rate is affected by Na^+ accumulation and by plant aging. The interaction between leaf aging and stress effect makes it generally impossible to separate their relative effects on v_c, but NaCl was the only stress limiting v_c 20 d after beginning the treatment. The increase of the v_o/v_c ratio after 20 d of treatment therefore reflects the insensitivity of v_o to salt stress. Delfine *et al.* (1998) showed that photosynthesis inhibition after 20 d was caused only by increasing diffusive resistances to CO_2, while RuBPCO was not affected. Under these conditions, the oxygenation rate is not inhibited but can even be stimulated.

If the electron transport is not totally used to drive photosynthesis and photorespiration, then the calculation of v_o and v_c is not correct (see Eq. 2). The presence of electron sinks other than photosynthesis and photorespiration (*e.g.*, direct O_2 photoreduction) has been suggested when photosynthesis was artificially poisoned (Loreto *et al.* 1994) or inhibited by low temperature (Massacci *et al.* 1995) and water stress (Loreto *et al.* 1995, Biehler and Fock 1996). To check if they are also important when a salt stress occurs, both photosynthesis and photorespiration must be inhibited. When CO_2 was removed from the air, J_f was strongly inhibited in controls while it remained fairly high in NaCl-stressed leaves. However, when also O_2 was lowered to an extent which could not sustain photorespiration, J_f was very low in both control and NaCl-stressed leaves. This indicated that the electron transport was almost fully used to drive photosynthesis and photorespiration and that alternative electron sinks were negligible. The conspicuous electron transport revealed by fluorescence in NaCl-stressed leaves when only CO_2 was removed may be necessary to recycle the CO_2 formed by photorespiration in the leaf and which can build up to a high concentration particularly when gas exchange between leaf and air is reduced by diffusion resistances (Delfine *et al.* 1998).

There was an increasing content of amino acids produced by photorespiration in NaCl-stressed leaves. After 20 d, the amounts of glycine and serine were dramatically enhanced with respect to controls. The content of alanine, which has also pyruvate as a precursor and may be in part formed during photorespiration by a transamination between serine and pyruvate, also increased in 20-d NaCl-stressed leaves with respect to controls. Finally, the content of glutamine, the first amino acid formed by recycling of photorespiratory NH_3 in the chloroplast, was higher than in controls. We interpret the accumulation of photorespiratory intermediates as a further indication that the photorespiratory flux is stimulated under moderate salt stress. However, the extremely high content of some amino acids is difficult to explain with increasing photorespiration only. It may indicate that a limitation of the photorespiratory flux also occurs, temporarily increasing the content of photorespiratory intermediates before the limitation point. The high content of glutamine in NaCl-stressed leaves suggests that the limitation may occur after ammonia is refixed in the chloroplasts by glutamine synthetase.

After 30 d of Na^+ accumulation, the amounts of serine, glycine, and alanine of stressed leaves decreased with respect to the amount measured after 20 d. However, the content of these amino acids was still higher than in controls. On the other hand, glutamine content in NaCl-stressed leaves was lower than in controls. After 40 d of salt accumulation, the content of photorespiratory amino acids was generally lower in

NaCl-stressed leaves than in controls. The progressive reduction of photorespiration intermediates was not caused by changes of oxygenation rate (Table 1). Glyoxylate accumulation would reduce the activation state of RuBPCO but this, in turn, would reduce both photosynthesis and photorespiration (Leegood *et al.* 1995). On the other hand, the accumulation of glycollate-2-phosphate would inhibit the regeneration of RuBP without directly affecting RuBPCO characteristics (Anderson 1981). Another possibility is that a reduced NH_3 refixation by glutamine synthetase occurred when salt accumulated for more than 20 d. This would limit the flux through the photorespiratory pathway and would affect first the content of glutamine. Mutants with low activity of glutamine synthetase do not change RuBPCO characteristics significantly when exposed to ambient conditions (Häusler *et al.* 1994).

The ratio between glutamate and glutamine of NaCl-stressed leaves was lower than in controls after 20 d of stress. A similar shift toward a higher content of glutamine was observed in control leaves after 40 d. It may be caused by increasing ammonia concentrations (Lawyer *et al.* 1981). We surmise that an accumulation of ammonia naturally occurs in aging plants and that this accumulation occurs earlier in NaCl-stressed leaves. If NH_3 is not refixed, its accumulation may feed back on photosynthesis (Blackwell *et al.* 1988, Sivak *et al.* 1988). The feedback may be exerted directly on carbon fixation enzymes (Campbell and Ogren 1990). However, several other studies have shown that photosynthesis is not directly inhibited by ammonia accumulation (reviewed in Leegood *et al.* 1995).

Photorespiration is an important component of the nitrogen cycle in plants (Keys *et al.* 1978). The low content of photorespiratory amino acids after 40 d of salt stress may be also attributed to aging, which impairs nitrogen assimilation both in controls and stressed leaves. A reduction of the enzymes of the NH_3-reassimilatory cycle, glutamine synthetase, and ferredoxin-dependent glutamate synthase decreases protein synthesis (Häusler *et al.* 1994). However, contents of amino acids which are not directly involved in photorespiration (leucine, isoleucine, lysine, and valine) increased after 40 d of salt stress (values not shown). Protein degradation, also caused by plant aging (Sexton and Woolhouse 1984), may have caused the increase in contents of these amino acids.

Proline was accumulated in NaCl-stressed leaves. Its content in NaCl-stressed leaves was 7 and 11 fold higher than in controls after 20 and 30 d of stress, respectively. Proline in part derives from glutamate cyclization and changes of leaf content may also be related to changes in photorespiratory flux. However, the increase of proline under stress conditions is widely reported (Hasson and Poljakoff-Mayber 1983, Lea 1993). Proline is likely to accumulate in water and salt stress as a compatible osmolyte and may have a rather non-specific protective effect against osmotic stresses (Maggio *et al.* 1997).

In conclusion, *in vivo* measurements of gas exchange and fluorescence and quantitative measurements of amino acids suggest that the photorespiration is not impaired or is even stimulated when photosynthesis of spinach leaves is inhibited by moderate salt stress. However, the high accumulation of some of the amino acids intermediate in the photorespiratory cycle may indicate a limitation of the photorespiratory flux.

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