Plant Physiology, March 1999, Vol. 119, pp. 1101–1106, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Restrictions to Carbon Dioxide Conductance and Photosynthesis in Spinach Leaves Recovering from Salt Stress

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Salt accumulation in spinach (*Spinacia oleracea* L.) leaves first inhibits photosynthesis by decreasing stomatal and mesophyll conductances to CO_2 diffusion and then impairs ribulose-1,5-bisphosphate carboxylase/oxygenase (S. Delfine, A. Alvino, M. Zacchini, F. Loreto [1998] Aust J Plant Physiol 25: 395–402). We measured gas exchange and fluorescence in spinach recovering from salt accumulation. When a 21-d salt accumulation was reversed by 2 weeks of salt-free irrigation (rewatering), stomatal and mesophyll conductances and photosynthesis partially recovered. For the first time, to our knowledge, it is shown that a reduction of mesophyll conductance can be reversed and that this may influence photosynthesis. Photosynthesis and conductances did not recover when salt drainage was restricted and Na content in the leaves was greater than 3% of the dry matter. Incomplete recovery of photosynthesis in rewatered and control leaves may be attributed to an age-related reduction of conductances. Biochemical properties were not affected by the 21-d salt accumulation. However, ribulose-1,5-bisphosphate carboxylase/oxygenase activity and content were reduced by a 36- to 50-d salt accumulation. Photochemical efficiency was reduced only in 50-d salt-stressed leaves because of a decrease in the fraction of open photosystem II centers. A reduction in chlorophyll content and an increase in the chlorophyll *a/b* ratio were observed in 43- and 50-d salt-stressed leaves. Low chlorophyll affects light absorptance but is unlikely to change light partitioning between photosystems.

Environmental stresses such as drought (Lauteri et al., 1997), salt stress (Bongi and Loreto, 1989), and leaf aging (Loreto et al., 1994) reduce conductance to CO_2 diffusion in the leaf mesophyll (mesophyll conductance). No information exists about possible increases of mesophyll conductance, such as when the stresses are alleviated. One obstacle to the investigation of this possibility is that mesophyll conductance reduction is frequently associated with the impairment of biochemical and photochemical characteristics of the leaf. The former is generally permanent, whereas the latter may recover slowly. However, it was recently shown that low salt accumulation (leaf Na concentration less than 15 mg g^{-1}) primarily affects the conductance to CO_2 diffusion in spinach (*Spinacia oleracea* L.) leaves (Delfine et al., 1998). A coordinate reduction in stomatal and mesophyll conductance decreased the chloroplast CO_2 concentration of salt-stressed spinach. This, in turn, caused an inhibition of photosynthesis that was not associated with changes in biochemical or photochemical capacity when salt accumulation in the leaves was two to three times that of the controls.

Mesophyll conductance reduction is also frequently associated with changes in leaf anatomy (Longstreth and Nobel, 1979; Bongi and Loreto, 1989; Evans et al., 1994; Syvertsen et al., 1995). This is likely to be a permanent effect, at least when leaf thickness is involved. However, low salt accumulation did not increase but slightly decreased the thickness of spinach leaves (Delfine et al., 1998). On the other hand, salt accumulation caused a 25% reduction of the intercellular spaces in the mesophyll of spinach leaves with respect to the controls. This could have caused a more tortuous path for CO_2 directed toward the chloroplast and was suggested to be responsible for the observed photosynthesis reduction associated with low mesophyll conductance in salt-stressed leaves (Delfine et al., 1998).

The objectives of this work were to understand, under conditions that do not affect relevantly the biochemical and photochemical capacity of salt-stressed leaves, and are not able to change leaf anatomy significantly: (a) whether the reduction of mesophyll conductance can be reversed by alleviating the salt stress, and (b) how important changes in mesophyll conductance are in determining photosynthesis limitation.

MATERIALS AND METHODS

Plant Material and Experimental Conditions

Four groups of 30 spinach (*Spinacia oleracea* L. cv Matador) plants were grown in 3-dm^3 pots containing a mixture of soil, peat, and sand (1:1:1). When five to six leaves were fully expanded, the first group of plants (control) was grown under optimal water conditions by daily restoring the water lost through evapotranspiration. Evapotranspiration was estimated by weighing the pots daily. The second group of plants (salt stressed) was irrigated for 50 d with saline water (containing 1% [w/v] NaCl) when evapotranspiration was restored. The third group of plants (rewatered) was irrigated with saline water for only 21 d. The plants were then provided with 150% of the evapotranspi-

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Abbreviations: Φ_{exc}, excitation energy capture by open PSII centers; qP, photochemical quenching.

ration losses by irrigation with salt-free water. The restitution of more water than that evapotranspired allowed for the drainage of part of the accumulated salt. The fourth group of plants (rewatered plus bag) was subjected to the salt treatment for 21 d as were salt-stressed leaves and then irrigated with salt-free water as were control leaves. Salt drainage was completely restricted by wrapping the soil with a plastic bag placed in the pot. All plants were grown in a greenhouse under the same temperature and light regimes.

Measurements of gas exchange and chlorophyll fluorescence were simultaneously taken on the same leaf of all of the groups after 22, 36, 43, and 50 d of exposure to salt. The last fully expanded leaf at the 22-d sampling was used. In conjunction with gas-exchange measurements, leaf discs (2.5 cm^2) were cut from ontogenetically similar leaves of each group of replicates, frozen in liquid nitrogen, and used to determine salt accumulation, Rubisco content and activity, chlorophyll *a* and *b* amount, and chlorophyll *a/b* ratio.

Salt Accumulation

Five leaf discs per treatment were dried for 1 d at 65°C. Sodium was extracted from 150 mg of dry mass taken from each disc in a 10-cm^3 mixture of HNO_3, $HClO_4$, and distilled water (1:5:2.5). The solution was kept 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).

Measurements of Photosynthesis and Conductances

The gas-exchange system described by Delfine et al. (1998) was used to determine leaf photosynthesis, respiration in the dark, and stomatal conductance. All gas-exchange and fluorescence measurements used to calculate photosynthesis and conductances were taken on five different leaves for each treatment, at a leaf temperature of 25°C and a light intensity of 1200 μmol quanta m^{-2} s^{-1}. The actinic light was supplied by a round illuminator made with optic fibers and placed 2.5 cm above the leaf cuvette. Fluorescence was measured with a PAM 101 fluorimeter (Walz, Effeltrich, Germany). The terminal end of a polyfurcated optic fiber was inserted in the round illuminator normal to the leaf plane with the tip reaching the cuvette surface. This fiber was used to supply weak red measuring light and 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).

Mesophyll conductance was measured by comparing the electron-transport rate driving photosynthesis and photorespiration measured by gas exchange and fluorescence, as described previously (Loreto et al., 1992, 1994; Delfine et al., 1998). Measurements were taken under ambient air composition (350 μmol mol^{-1} CO_2 and 210 mmol mol^{-1} O_2). Measurements under a CO_2-free atmosphere, and under a CO_2-free and low-O_2 (20 mmol mol^{-1}) atmosphere, were used to determine if the estimate of electron transport by the two methods was correct (under nonphotorespiratory conditions the same electron-transport rate should be obtained with the two methods) and to check for the presence of alternative electron sinks.

Rubisco Measurements

A leaf disc was ground in a chilled mortar with 30 mg of polyvinylpolypyrrolidone, quartz sand, and 2 cm^3 of extraction buffer (100 mM Bicine, pH 8.0, 10 M $MgCl_2$, 5 mM DTT, 1 mM EDTA, and 0.02% [w/v] BSA). The solution was centrifuged at 10,000*g* for 10 s. A fraction of the supernatant was used to determine radiometrically the total carboxylase activity of Rubisco (Di Marco and Tricoli, 1983). The assay was conducted at 25°C in vials containing 0.5 cm^3 of CO_2-free extraction buffer, 20 mM $NaHCO_3$, and 10 mm^3 of leaf extract. After 9 min of incubation the radioactive substrate (8.3 kBq with 0.2 μmol in 10 mm^3 of extraction buffer) was added. One minute later the reaction was started by adding ribulose-1,5-bisphosphate to bring the reaction mixture to 1 mM and was stopped after 1 additional min by adding 100 μL of 1 M HCl. The acidified mixture was evaporated to dryness and radioactivity in the residues was measured in a scintillation counter (Packard Instrument Company, Downers Grove, IL).

Another fraction of the supernatant was denatured at 95°C for 5 min in 20% (w/v) SDS, 20% (w/v) β-mercaptoethanol, and 200 mM Tris-HCl, pH 6.8. Rubisco content was determined on the denatured solution by SDS-PAGE using a 14% acrylamide gel. Gels were stained with Coomassie brilliant blue R-250, destained, and scanned at 550 nm using a Dual-Wavelength Flying Spot Scanner in the transmission mode (model CS-9000, Shimadzu, Tokyo, Japan). Five replicates were performed per treatment.

Measurements of PSII Quantum Yield and Its Components

The quantum yield of PSII was measured simultaneously with gas-exchange measurements. The fluorescence apparatus described previously was used to measure the quantum yield of PSII in 12-h dark-adapted leaves (F_v/F_m), where F_v is variable and F_m is maximal fluorescence. The leaves were then exposed to ambient CO_2 and O_2 concentrations and to a light-intensity saturating photosynthesis (1200 μmol photons m^{-2} s^{-1}). When photosynthesis was steady, the quantum yield of PSII in the light, $\Delta F/F_m$ (Genty et al., 1989), was measured. $\Delta F/F_m$ was then partitioned into its two components: qP and Φ_{exc}. Φ_{exc} is given by the F_v/F_m ratio in the light ($F_{v'}/F_{m'}$; Harbinson et al., 1989; Genty and Harbinson, 1990). Photochemical quenching was calculated according to the protocol described by Van Kooten and Snel (1990), and the fluorescence nomenclature reported in that paper was followed.

Determination of Chlorophyll Content and Leaf Absorptance

Discs of 2.5 cm^2, cut from the last fully expanded leaf close to the midvein, were ground to a fine powder in liquid N_2 and extracted with 2 mL of 80% acetone (v/v). The homogenate was centrifuged at 10,000*g* at 5°C for 10

min, and the supernatant was separated and used for a chlorophyll assay. Four replicates of individual samples were analyzed. The amounts of chlorophyll *a* and *b* were determined spectrophotometrically, by reading the absorbance at 663.6 and 646.6 nm. The chlorophyll content was calculated by using the extinction coefficients and the equations given by Porra et al. (1989).

Leaf absorptance may change as salt stress develops. It was measured as described elsewhere (Massacci et al., 1995) on six leaves for each group of plants using a Li-Cor 1800 portable spectroradiometer and a Li-Cor 1800–12 integrating sphere (Li-Cor, Lincoln, NE). Measurements of light absorption were necessary to correct the calculation of the electron-transport rate by fluorescence (Loreto et al., 1992).

RESULTS

Sodium accumulated in salt-stressed leaves (Fig. 1). After 22 d Na contribution to dry matter was more than 2% in the leaves of all of the plants irrigated with saline water. Salt-stressed leaves continued to accumulate salt throughout the experimental period. After 50 d Na was about 7% of the dry matter of these leaves. Leaves of rewatered and rewatered-plus-bag plants showed a further accumulation of salt at the 36-d sampling, after which Na as a percentage of dry matter started to decrease. This depletion was more evident in rewatered plants without the plastic bag.

All leaves showed a similar Rubisco content at the 22- and 36-d samplings (Fig. 2a). Subsequently, Rubisco content decreased in salt-stressed leaves and, to a lesser extent, in rewatered-plus-bag leaves. The Rubisco content of rewatered plants did not change significantly during the experimental period. After 50 d the Rubisco content of rewatered leaves was intermediate between those of controls and salt-stressed leaves.

Rubisco activity was similar in all of the leaves at d 22 (Fig. 2b). It then decreased significantly in salt-stressed leaves and, to a lesser extent, in the two rewatered groups. Rubisco activity also started to decrease in control leaves at d 50.

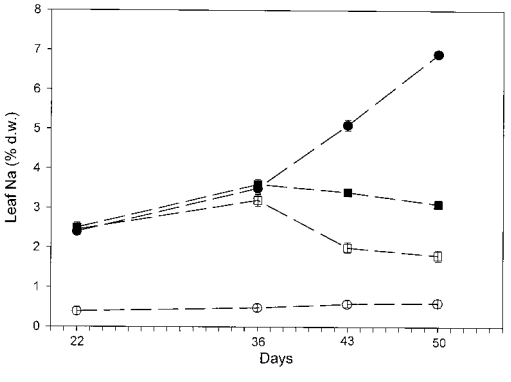

Figure 1. Accumulation of salt (percent of dry weight, d.w.) during the four sampling dates in control (○), salt-stressed (●), rewatered (□), and rewatered-plus-bag (■) spinach leaves. Means ± SE of five samples are shown. When error bars are not visible, SE is smaller than the symbol size.

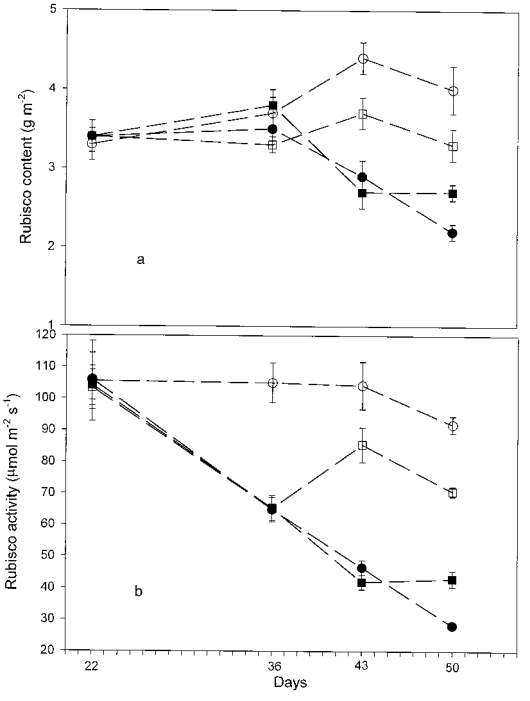

Figure 2. Rubisco content (a) and activity (b) of control (○), salt-stressed (●), rewatered (□), and rewatered-plus-bag (■) spinach leaves during the experimental period. Means ± SE of five samples are shown.

The photosynthesis of plants irrigated with saline water was lower than that of controls at 22 d (Fig. 3a). It decreased further in salt-stressed and rewatered-plus-bag leaves, reaching very low values at the 50-d sampling. The photosynthesis of rewatered leaves was highly inhibited at the 36-d sampling, then started to recover. The photosynthesis of controls was stable during the first two samplings, then decreased relevantly and was similar to that of rewatered leaves at the 50-d sampling.

At the 22-d sampling, stomatal and mesophyll conductances were very low in the leaves of plants irrigated with saline water with respect to controls (Fig. 3, b and c). The subsequent samplings indicated that the two conductances became almost negligible in salt-stressed and rewatered-plus-bag leaves. On the contrary, the conductances of the rewatered leaves increased with time and were significantly higher at the 50-d sampling than at the 22-d sampling. The conductances of the control leaves were high at the 22-d sampling but then decreased with time. At the 50-d sampling the conductances of controls were similar to those of rewatered leaves.

The quantum yield of PSII, as indicated by F_v/F_m in the dark, was not affected by salt accumulation after 22 d but was significantly lower in salt-stressed leaves than in controls after 50 d (Table I). When analyzing the components of PSII yield and the efficiency of energy dissipation in leaves exposed to stress, we observed that both qP and, to a greater extent, Φ_{exc} were affected by the stress after 22 d. After 43 and 50 d the inhibition of the two components was even stronger. These samplings revealed a further 70% reduction of Φ_{exc} with respect to the 22-d sampling,

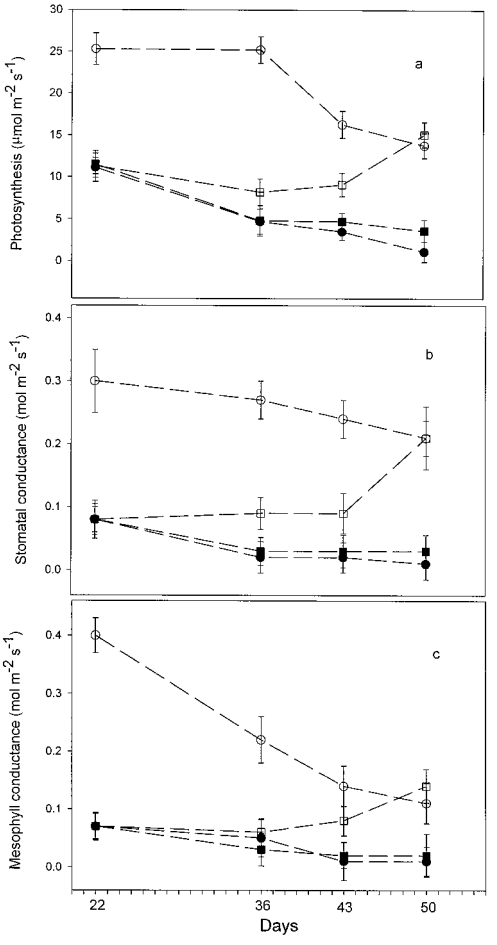

Figure 3. Photosynthesis (a), stomatal conductance (b), and mesophyll conductance (c) of the same control (○), salt-stressed (●), rewatered (□), and rewatered-plus-bag (■) spinach leaves during the experimental period. Means ± SE of five samples are shown.

whereas qP was reduced by only 30%. However, the qP and Φ_{exc} of rewatered and control leaves were similar at the 43- and 50-d samplings. In the case of rewatered-plus-bag leaves neither component recovered with respect to salt-stressed leaves.

The chlorophyll characteristics of leaves were also measured at the 22-, 43-, and 50-d samplings (Table II). No differences were found between controls and salt-stressed leaves at the 22-d sampling. At the 43-d sampling a strong reduction of total chlorophyll content and an increase of the chlorophyll *a/b* ratio were found in salt-stressed and rewatered-plus-bag leaves with respect to controls. Rewatered leaves, however, showed a chlorophyll content and a chlorophyll *a/b* ratio similar to controls. Chlorophyll reduction induced a relevant reduction of the absorbed light. Light absorption was 0.86 ± 0.50 in controls and was not significantly lower in rewatered leaves. However, it was 0.62 ± 0.10 in salt-stressed leaves at the 43-d sampling.

DISCUSSION

The level of salt accumulation in spinach plants at the 22-d sampling did not impair the biochemical (Fig. 2) and photochemical (Table I) characteristics of the leaves. As already shown by Delfine et al. (1998), however, photosynthesis was substantially reduced by the low accumulation of salt, probably because of the reduced conductance to CO_2 diffusion at the stomata and in the mesophyll of salt-stressed leaves (Fig. 3). In fact, the concentration of CO_2 in the chloroplasts, calculated by summing stomatal and mesophyll resistances (Loreto et al., 1994) from the data in Figure 3, averaged 100 ppm in all leaves exposed to salt stress, about 100% less than in controls. This CO_2 concentration in the chloroplasts of salt-stressed leaves consistently sustains a photosynthesis of about 10 μmol m^{-2} s^{-1} (Delfine et al., 1998). Higher photosynthetic rates would require an increase of Rubisco activity, whereas lower photosynthetic rates would indicate a reduction of Rubisco characteristics in salt-stressed leaves.

By measuring the physiological parameters on the same leaves during a mild salt stress and the subsequent recovery, we attempted to determine whether the inhibition of photosynthesis and conductances is reversible. At the 43- and 50-d samplings, salt accumulation was successfully interrupted and partially reverted in the leaves of rewatered plants (Fig. 1). Rewatering was less effective when plants were maintained in a plastic bag, probably because of minimum salt leakage. Corresponding with recovery from salt accumulation, photosynthesis was less inhibited in rewatered than in salt-stressed leaves and, at the 50-d sampling, was even slightly higher than at the 22-d sampling. An inhibition of photosynthesis in control leaves occurred after the 36-d sampling and was also associated with a reduction of the conductances, although it was not mirrored by changes in Rubisco content and activity.

At the 50-d sampling, photosynthesis and conductances of control and rewatered leaves were similar. Because of their effect on CO_2 uptake, the average chloroplast CO_2 concentration of both controls and rewatered leaves was about 150 ppm, a value still 20% to 25% lower than that found in 22-d-old controls. We interpret this as an indication that the salt-induced inhibition of conductances was recovered but that the age-related inhibition of conductances (Loreto et al., 1994; Fig. 3), perhaps associated with a slight reduction of Rubisco activity (Fig. 2), did not allow a higher photosynthetic rate in either controls or rewatered leaves. The slightly higher amount of salt accumulated by rewatered-plus-bag plants surprisingly did not allow a recovery of photosynthesis and conductances. On the contrary, these leaves, as well as those from the salt-stressed group, also showed a progressive inhibition of Rubisco content and activity, and a very low rate of photosynthesis, by the end of the experiment. It is possible that a threshold exists and that the inhibition of biochemical characteristics does not occur and a recovery of photosynthesis and conductances is allowed only when Na accumulation is maintained at less than 3% of the dry matter, as in the case of rewatered plants.

A 22-d salt accumulation did not significantly change the quantum yield of dark-adapted leaves (Table I). We therefore confirm that leaf photochemistry is rather resistant to salt stress (Brugnoli and Lauteri, 1991; Brugnoli and Björkman, 1992). However, the photochemical efficiency of salt-

Table I. *Quantum yield of PSII in dark-adapted leaves, F_v/F_m, qP, and Φ_{exc} of controls, salt-stressed, rewatered, and rewatered-plus-bag (C, SS, R, RB, respectively) spinach leaves at the 22-, 43-, and 50-d samplings*

The photochemical parameters were measured on the same five leaves from different plants for each treatment. Means and mean separation between columns (Tukey's test, 5% level of confidence) are reported. Leaves were exposed to ambient CO_2 and O_2 concentration and to 1200 μmol photons m^{-2} s^{-1}.

Leaf Type	Days								
	F_v/F_m			qP			Φ_{exc}		
	22	43	50	22	43	50	22	43	50
C	0.79_a	0.76_a	0.77_a	0.63_a	0.53_a	0.50_a	0.56_a	0.45_a	0.53_a
SS	0.77_a	0.73_{ab}	0.70_b	0.54_b	0.17_d	0.17_b	0.41_b	0.29_{bc}	0.29_{bc}
R	0.77_a	0.72_{ab}	0.76_a	0.55_b	0.46_{ab}	0.47_a	0.38_b	0.45_a	0.50_a
RB	0.77_a	0.72_{ab}	0.70_b	0.50_b	0.33_c	0.20_b	0.43_b	0.36_b	0.36_b

stressed and rewatered-plus-bag leaves was reduced after a 50-d salt accumulation, indicating that high salt concentrations also started to affect leaf photochemistry.

The quantum yield of PSII in the light results from the fraction of open centers that perform photochemistry (i.e. qP) and the quantum efficiency of the Φ_{exc} (Harbinson et al., 1989; Genty and Harbinson, 1990). Salt stress reduces both components of PSII yield after 22 d (Table I). This is a down-regulation of leaf photochemistry to match the reduced carbon acquisition under low salt accumulation (Delfine et al., 1998).

In 43- to 50-d salt-stressed and rewatered-plus-bag leaves the two components of PSII yield were reduced further. However, this additional reduction was only 30% in the case of Φ_{exc} and 70% in the case of qP. Therefore, the fraction of open PSII centers appears to be the most sensitive component of PSII yield to the stress. In 43- to 50-d controls and rewatered leaves both components of the PSII yield were similar, showing that neither one is permanently affected by the stress.

Delfine et al. (1998) reported no change in the chlorophyll content of 20-d salt-stressed spinach leaves. We reached a similar conclusion but found that chlorophyll content was highly reduced in 43- and 50-d salt-stressed leaves. A low chlorophyll content may cause a relevant reduction of light absorption by leaves (Evans, 1996). It was found that salt-stressed leaves absorbed about 60%, whereas controls and rewatered leaves absorbed more than 80% of the incident light. Thus, the optical properties of the leaf are impaired only when salt accumulation is high. This change in absorbance may cause relevant errors in the estimation of the electron-transport rate and mesophyll conductance (Harley et al., 1992; Laisk and Loreto, 1996), which were therefore taken into account when calculating these parameters on heavily stressed leaves.

The strong decrease of chlorophyll content was associated with a 2-fold increase of the chlorophyll *a/b* ratio with respect to controls in salt-stressed and rewatered-plus-bag leaves at the 43-d sampling. A high chlorophyll *a/b* ratio also indicates that the ratio between PSII/PSI content changes in stressed leaves (Anderson, 1986). However, it has been shown that by altering the chlorophyll ratio the light distribution between photosystems does not change (Evans, 1986). Variations in light partitioning between photosystems across species were estimated by using a combined fluorescence-gas-exchange approach (Laisk and Loreto, 1996). By using this method, however, Delfine et al. (1998) did not find differences in the light distribution between photosystems of controls and salt-stressed (20 d) spinach leaves. Besides confirming their data, we did not find changes in the fraction of light distributed to PSII in rewatered leaves with respect to controls. At the 50-d sampling about 46% of the light was estimated to be distributed to PSII in both rewatered and control leaves (data not shown). The fact that light distribution is unaffected by salt accumulation reduces the uncertainties in the calculation of mesophyll conductance (Laisk and Loreto, 1996). Moreover, the calculation of mesophyll conductance is quite insensitive to changes in the light distribution when photosynthesis and the photosynthetic electron transport are

Table II. *Chlorophyll a and b content and chlorophyll a/b ratio of control, salt-stressed, rewatered, and rewatered-plus-bag (C, SS, R, RB, respectively) spinach leaves at the 22-, 43-, and 50-d samplings*

Measurements were taken on four different leaves from different plants for each treatment. Means and mean separation between rows (Tukey's test, 5% level of confidence) are reported.

Days	Chlorophyll *a* and *b*				Chlorophyll *a/b*			
	C	SS	R	RB	C	SS	R	RB
	gm^{-2}							
22	0.41_a	0.39_a	0.37_a	0.42_a	3.57_a	4.08_a	3.77_a	3.69_a
43	0.39_a	0.13_b	0.35_a	0.15_b	4.38_a	7.29_b	5.36_{ab}	6.76_b
50	0.33_a	0.15_b	0.28_a	0.22_{ab}	3.31_a	5.58_b	3.39_a	3.85_{ab}

low because of their inhibition by salt accumulation. In this case, errors in the estimation of light partitioning between photosystems are not expected to significantly change the calculated conductance.

Reduced mesophyll conductance under low salt accumulation in the leaves was not accompanied by anatomical changes other than a lower proportion of intercellular spaces. This was suggested to restrict carbon flow toward the chloroplasts (Delfine et al., 1998). In fact, a relationship between leaf anatomy and mesophyll conductance has been observed frequently (Loreto et al., 1992; Evans et al., 1994; Syvertsen et al., 1995). We did not observe differences in the leaf thickness of rewatered and salt-stressed leaves (data not shown). The observed recovery of mesophyll conductance in rewatered leaves may indicate that the intercellular spaces of these leaves were again reorganized to increase porosity and favor carbon acquisition. Recent experiments using helium to decrease diffusive resistances, however, suggest that mesophyll resistances are limited to the liquid phase (B. Genty, personal communication). In this case, mesophyll conductance may be affected by the probably low osmotic potential of the liquid phase in salt-stressed leaves. This hypothesis needs to be tested.

ACKNOWLEDGMENTS

We thank Dr. Edgardo Volterra for measuring salt accumulation in the leaves by atomic emission spectrometry. F.L. is grateful to Professor T.D. Sharkey for critical reading of an earlier version of the paper. We thank Carla Ticconi for proofreading the paper.

Received September 3, 1998; accepted December 10, 1998.

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