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Differential sensitivity of stomatal and non-stomatal components to NaCl or Na_2SO_4 salinity in horsegram, *Macrotyloma uniflorum* (Lam.)

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Abstract

$^{14}CO_2$ assimilation rate (P), leaf diffusive conductance (g_s), photosynthetic electron flow, and activities of enzymes of Calvin cycle were studied in a horsegram [*Macrotyloma uniflorum* (Lam.)] in response to salinity induced by NaCl or Na_2SO_4. A significant reduction in P and g_s by both salt treatments was registered. Na_2SO_4 caused a greater reduction in g_s than the NaCl salinity. Studies with isolated chloroplasts confirmed a greater sensitivity to NaCl than to Na_2SO_4. Salinity inhibited the photosynthetic electron transport. The activity of ribulose-1,5-bisphosphate carboxylase (E.C.4.1.1.39) was under salinity inhibited more than the activities of other three enzymes of the Calvin cycle, ribulose-5-phosphate kinase (E.C.2.7.1.19), ribose-5-phosphate isomerase (E.C.5.3.16), and NADP-glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.13). These inhibitions lead to a reduced capacity for ribulose-1,5-bisphosphate regeneration. Isolated chloroplasts extracted from salt stressed plants and supplemented with the substrates of Calvin cycle could elevate P, but the P was always lower than in the controls. Decreased P in horsegram exposed to high salinity can be attributed to both stomatal and non-stomatal components, however, the sensitivity to the salt source, NaCl or Na_2SO_4, was different.

Additional key words: CO_2 fixation; NADP-glyceraldehyde-3-phosphate dehydrogenase; photosystem 2 activity; ribose-5-phosphate isomerase; ribulose-1,5-bisphosphate carboxylase; ribulose-5-phosphate kinase; salt stress; stomatal conductance.

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Abbreviations: ATP - adenosine triphosphate; DCPIP - 2,6-dichlorophenolindophenol, DTT - dithiothreitol; g_s - diffusive leaf conductance; P - CO_2 fixation rate; 3-PGA - 3-phosphoglycerate; PS - photosystem; R-5-P - ribose-5-phosphate; RuBP - ribulose-1,5-bisphosphate; RuBPC - ribulose-1,5-bisphosphate carboxylase.
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Introduction

Salinity is one of the main environmental constraints which limit photosynthesis and consequently productivity in crop plants. Limitation of photosynthesis can be due (*1*) to reduced leaf diffusive conductance (Walker *et al.* 1982, Plaut *et al.* 1990), and (*2*) to changes in the leaf's photosynthetic machinery and changes in the efficiency with which this machinery operates (Seemann and Critchley 1985, Seemann and Sharkey 1986). Photosynthesis and RuBP carboxylase activity are usually positively correlated in plants grown under various environmental stresses. Hence we analysed the response of stomatal and non-stomatal factors to different saline conditions induced by NaCl or Na_2SO_4 in horsegram.

Materials and methods

Plants: Seeds of horsegram [*Macrotyloma uniflorum* (Lam.) cv. VZM 1] were surface sterilized with 0.1 % $HgCl_2$ solution for 30 s, washed repeatedly with water, and germinated in Petri dishes lined with filter paper. To induce salt stress, 100 eq m^{-3} of NaCl or Na_2SO_4 in half-strength Hoagland nutrient medium was used. The salts were replenished by changing the solution daily. Medium without salts served as control. The treatments were maintained under a constant irradiance of 150 W m^{-2} and temperature of 27±2 °C. After 7 d, the first pair of fully expanded leaves was harvested and processed for enzymic determination.

g_s was measured using a diffusion porometer (*Delta T, Mark II*). Conversion factors suggested by Šesták *et al.* (1971) were used to calculate the leaf conductance for CO_2.

CO_2 fixation by leaves: An intact leaf of 7-d-old seedlings (1.0-1.5 cm^2 leaf area) was inserted through a slot in the top of a glass jar (200 cm^3) with a small vial in it containing 2.5 cm^3 of 10 mM $Na^{14}CO_3$. $^{14}CO_2$ was then released by injecting 0.1 M HCl into the vial. After 10 min exposure to $^{14}CO_2$, the leaf was homogenized once with 80 % ethanol (v/v). After centrifugation, the pellets were washed with 60 % and then 40 % ethanol and twice with boiling water. The extracts were pooled and concentrated by evaporating *in vacuo*. Aliquots (0.1 cm^3) of the extracts were placed on flat steel planchets and evaporated. The radioactivity was measured using a gas flow proportional counting system.

Isolated chloroplasts: Prechilled leaves were cut into pieces, immersed in an isolation medium containing 50 mM HEPES buffer (pH 7.5), 0.33 M sorbitol, 1 mM $MgCl_2$, and 0.01 M HCl, and ground in a mortar with pestle. The homogenate was strained through a 4-layered muslin cloth and centrifuged at 600×*g* for 5 min. The green supernatant was then centrifuged at 1000×*g* for 10 min, and the sedimented chloroplasts were suspended in the isolation medium and again centrifuged at 1000×*g* for 10 min. The sediment containing washed chloroplasts was resuspended in the same buffer before use. All of the above steps were performed at 2 °C.

Chlorophyll was determined after extraction in 80 % acetone (v/v) spectrophotometrically according to Arnon (1949). DCPIP reduction in isolated chloroplasts was measured using the method of Sudhasundari and Raghavendra (1990). The photosynthetic $^{14}CO_2$ fixation by chloroplasts isolated from both the control and stressed plants was studied with (50 μM each) or without addition of substrates, RuBP, 3-PGA, ATP, R-5-P, and R-5-P+ATP in the assay medium by the method described by Walker (1971) and Gibbs *et al.* (1967).

Photosynthetic enzymes were extracted by adopting the method of Veeranjaneyulu (1978). The leaves were extracted (0.5 g cm^{-3}) by grinding with 50 mM Tris-HCl buffer (pH 7.8) containing 5 mM DTT, 2 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The extract filtered through 4-layered muslin cloth was centrifuged at 20 000×*g* for 15 min. The cleared supernatant was passed through a *Sephadex G-50* column which was pre-equilibrated with same extraction buffer. Active fractions were collected and used for the assay of enzymes.

RuBPC (E.C.4.1.1.39) activity was measured by observing the incorporation of radioactive bicarbonate into acid-stable products. The reaction mixture of 3 cm^3 contained 50 mM Tris-HCl buffer (pH 7.8), 10 mM $MgCl_2$, 3 mM DTT, 20 mM $NaH^{14}CO_3$, 0.5 mM RuBP, and the enzyme. After preincubation for 5 min the reaction was started by the addition of RuBP and after 5 min stopped by adding 1.0 cm^3 4 M HCl. An aliquot was examined for incorporated radioactivity.

Ribulose-5-phosphate kinase (E.C.2.7.1.19) activity was determined according to Graham *et al.* (1970), by coupling the reaction with RuBPC. The reaction medium of 3 cm^3 containing the enzyme, 50 mM Tris-HCl buffer (pH 8.2), 8 mM DTT, 20 mM $MgCl_2$, 1 mM K_2HPO_4, 1 mM KF, 5 mM R-5-P, 15 mM $NaH^{14}CO_3$, and 0.05 unit R-5-P isomerase was mixed. After 10 min, 0.15 unit RuBPC (*Sigma*) was added. After further 10 min (to enable the activation of RuBPC) the reaction was started by adding 5 mM ATP and stopped after 5 min by adding 1 cm^3 of 4 M HCl. The radioactivity in the reaction mixture was determined.

R-5-P isomerase (E.C.5.3.1.6) activity was measured following the incorporated radioactivity in the presence of ribulose-5-phosphate kinase and RuBPC. Ribulose-5-phosphate formed by enzyme action was converted into RuBP and carboxylated, resulting in the formation of acid-stable ^{14}C-labelled products. The mixture of 3 cm^3 contained 50 mM Tris-HCl buffer (pH 8.0), 5 mM DTT, 15 mM $NaH^{14}CO_3$, 0.15 unit RuBPC, and 5 mM ATP. The reaction was coupled with the endogenous ribulose-5-phosphate kinase which was in excess. The enzyme activity was determined similarly to that of the R-5-P kinase.

NADP-glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.2.1.13) activity was determined spectrophotometrically by observing the reduction of NADP at 340 nm according to Graham *et al.* (1970). Chlorides were extracted with hot distilled water and determined by titration against $AgNO_3$

Results

The g_s decreased as a result of submitting to NaCl or Na_2SO_4 salinity (Table 1), but the degree of inhibition varied greatly between the salt sources. Na_2SO_4 decreased it to a greater extent (42 % decrease) than NaCl (27 % inhibition). A significant inhibition of *P* by leaves subjected to both salt treatments was observed, though there was no striking difference in the magnitude of inhibition between the salinity source (34 % decrease by NaCl and 31 % by Na_2SO_4) (Table 1).

Table 1. Leaf conductance, g_s [cm s^{-1}], CO_2 fixation, *P* [mmol m^{-2} s^{-1}], DCPIP reduction [mmol kg^{-1}(Chl) s^{-1}], activities of photosynthetic enzymes [mol kg^{-1}(Chl) s^{-1}], and chloride content [g^{-1} kg^{-1}(f.m.)] in 7-d-old control and NaCl or Na_2SO_4 stressed seedlings of horsegram. Means ± SE; $n = 6$. Mean values in a row followed by the same letter are not significantly different ($p<0.05$) as per Duncan's multiple range (DMR) test.

Parameter	Control	NaCl	Na_2SO_4
g_s	0.27 ± 0.04c	0.19 ± 0.02b	0.16 ± 0.02a
P in leaves	58.2 ± 2.4b	38.8 ± 1.8a	40.4 ± 1.9a
P in chloroplasts	760.0 ± 65.3c	245.0 ± 36.0b	90.0 ± 16.0a
DCPIP reduction	19.3 ± 0.5c	10.8 ± 0.3a	14.2 ± 0.4b
RuBP carboxylase	312.9 ± 17.5c	186.0 ± 8.5a	230.3 ± 9.3b
R-5-P kinase	925.9 ± 29.5b	630.5 ± 21.0a	702.1 ± 26.0a
R-5-P isomerase	492.4 ± 17.5c	268.4 ± 16.0a	316.3 ± 13.0b
NADP-glyceraldehyde-3-P-dehydrogenase	763.8 ± 23.0b	480.3 ± 18.5a	508.4 ± 16.0a
Chlorides	8.9 ± 0.6a	24.5 ± 1.6b	9.0 ± 0.8a

The *P* and PS2 electron flow in isolated chloroplasts were also significantly reduced by both salts. NaCl salinity reduced *P* by 45 % whereas Na_2SO_4 by 35 %, the related differences in electron flow were 44 and 27 % (Table 1).

The RuBPC activity was significantly inhibited by both kinds of salinity. NaCl stress caused a greater inhibition (38 % loss) than the Na_2SO_4 stress (27 % inhibition); the difference was statistically significant (Table 1). The activities of ribulose-5-phosphate kinase, R-5-P isomerase and NADP-glyceraldehyde-3-phosphate dehydrogenase were also significantly lowered by both stresses, but the difference between them was significant only for R-5-P isomerase (Table 1).

When RuBP, 3-PGA, ATP, R-5-P and R-5-P+ATP were supplemented to the isolated chloroplasts extracted from NaCl or Na_2SO_4 stressed seedlings, the *P* was elevated, however, it was always lower than in the control (Table 2). The RuBP or R-5-P+ATP addition lead to a greater rise in *P* of isolated chloroplasts than when R-5-P, ATP or 3-PGA were individually added. A significant difference between the salt treatments was observed, and always NaCl was more toxic than Na_2SO_4. A greater accumulation of chloride resulted from NaCl than Na_2SO_4 salinity (Table 1).

Table 2. Influence of additions of Calvin cycle substrates and ATP (50 μM) on $^{14}CO_2$ fixation [mol kg^{-1}(Chl) s^{-1}] by chloroplasts isolated from leaves of 7-d-old control and salt stressed seedlings of horsegram. Means ± SE, n = 6. Mean values in a row followed by the same letter are not significantly different (p<0.05) as per DMR test.

Substrate	Control	NaCl	Na_2SO_4
No addition	76.0 ± 6.5c	37.2 ± 4.4a	48.7 ± 6.0b
Ribulose-1,5-bisphosphate	79.3 ± 5.2c	52.0 ± 3.3a	62.3 ± 4.1b
3-phosphoglycerate	78.4 ± 6.2c	47.2 ± 3.0a	63.7 ± 4.3b
ATP	75.0 ± 4.7c	46.5 ± 3.2a	52.0 ± 3.7b
Ribose-5-phosphate	76.4 ± 5.2c	42.0 ± 2.1a	56.0 ± 4.4b
Ribose-5-phosphate + ATP	80.0 ± 4.8c	51.6 ± 4.1a	59.2 ± 3.9b

Discussion

Salinity usually affects P and g_s to a similar extent (Lakshmi *et al.* 1996). In our experiments, P and g_s declined under both NaCl or Na_2SO_4. However, g_s was inhibited more by Na_2SO_4 salinity compared to NaCl. The NaCl salinity was probably more toxic to the biochemical processes of photosynthesis than Na_2SO_4 salinity in this study. Different responses of these processes to the salt source were supported by the responses of enzymes and PS2 activity.

PS2 is the primary target of photoinhibitory damage. We found a significant inhibition of electron transport by both salt treatments as evidenced by the DCPIP reduction, and NaCl was more inhibitory. An inhibition of PS2 activity under salinity has been documented in mangrove and pea by Ball and Anderson (1986), in rice by Singh and Dubey (1995), and in *Aster* and pea by Wignarajah and Baker (1981), but Brugnoli and Lauteri (1991) have shown salt-insensitivity of PS2 in *Gossypium hirsutum* L. and *Phaseolus vulgaris* L. Murata *et al.* (1992) and Murota *et al.* (1994) suggest that the target of salinity action is the dissociation of 23-kDa polypeptide: this polypeptide is extrinsically bound to the reaction centre proteins of PS2 at the lumenal surface of thylakoid membranes and is important in the evolution of oxygen.

The RuBPC activity was significantly inhibited by both salts, but NaCl salinity was more toxic. A salt-associated reduction in the specific activity of RuBPC has been also reported by Taleisnik (1987) in *Lycopersicon pennellii*, both *in vivo* and *in vitro*. There is rather a reduction in the apparent efficiency of RuBPC than in the concentration of enzyme (Seemann and Critchley 1985). High Cl^- concentration in the chloroplast cytoplasm in their experiments with *Phaseolus vulgaris* was responsible for the reduced efficiency of RuBPC. We found a remarkable increase in chloride accumulation in the tissue during both salt treatments, but the accumulation of chloride under NaCl salinity was far greater than under the Na_2SO_4 treatment. This finding corroborates with the hypothesis of Seemann and Critchley (1985). Though our study did not provide specific localisation of high Cl^-, we suppose that the Cl^- concentration reached a toxic level in the chloroplast cytoplasm.

The higher decrease in activities of Calvin cycle enzymes found under NaCl

salinity than with Na_2SO_4 resulted in a decrease in the substrates of the cycle and ultimately led to the reduced RuBP pool.

Decreased *P* produces less 3-phosphoglycerate and therefore less RuBP. Inadequate availability of ATP decreases the capacity for RuBP regeneration and thus for the net CO_2 assimilation rate (Jacob and Lawlor 1992). A reduced availability of ATP under saline conditions is responsible for decrease in RuBP regeneration capacity (Seemann and Sharkey 1986). We presented an indirect evidence of reduced availability of ATP, as shown by the decrease in DCPIP reduction. Also the studies with isolated chloroplasts extracted from seedlings subjected to NaCl or Na_2SO_4 strongly supported the view of reduced RuBP pool and other Calvin cycle intermediates. When RuBP or R-5-P+ATP were added, the *P* was elevated more than when ATP, R-5-P or 3-PGA were added individually. Here also NaCl seemed to be more toxic, because the rise in *P* was lower than under Na_2SO_4 treatment. There was an enhancement in *P* when the above substrates were added. however, *P* never reached the control value, indicating the operation of some other limitations at different level. Certainly, this is a supporting evidence for the presence of low amounts of substrates of Calvin cycle, relative to the enzymic activities during salinity.

In halophytes, Na^+ and Cl^- concentrations are lower in cytoplasm than in the vacuole (Robinson *et al.* 1983, Storey *et al.* 1983). In glycophytes, the nature and degree of intracellular compartmentation of ions in the leaves are unclear. Nevertheless, the sensitivity of many glycophytic species to salt may be a consequence of a failure to keep the ions out of cytoplasm (Flowers *et al.* 1977, Seemann and Critchley 1985).

We may conclude that the salinity (NaCl or Na_2SO_4) induced decrease in CO_2 fixation is due to both stomatal and non-stomatal limitations, however, with a differential sensitivity to the salt source. The differential sensitivity of *P* to the salt source can be attributed, at least in part, to a direct chloride effect.

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