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SOYBEAN PLANTS WITH SYMBIOTIC N_2 FIXATION ARE MORE RESISTANT TO SALT STRESS THAN NITRATE-FED PLANTS

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Summary: Salinity is one of the major abiotic stress factors limiting plant growth and productivity. In the present study, the relationship between the source of N_2 nutrition (biological N_2 fixation or nitrate assimilation) and the resistance to salt stress in soybean plants grown as hydroponics in a growth chamber under controlled conditions was assessed. Part of the plants were inoculated by adding 10^8 cells ml^{-1} suspension of *Bradyrhizobium japonicum* strain 273 (nitrogen-fixing plants). The rest plants received N_2 from the nutrient solution (nitrate-fed plants). Salt treatment was applied during the vegetative stage of growth (14 days from the onset of germination) by adding NaCl into the nutrient solution at concentrations of 40 mM and 80 mM. Analyses were performed on the 4th trifoliate leaf 14 days after salt treatment. Our results showed that compared with nitrate-fed plants, the nitrogen-fixing soybean plants had better water status, less affected electrolyte leakage indicating better membrane integrity, enhanced dry biomass, higher net photosynthetic rate, transpiration rate and stomatal conductance. The addition of 80mM NaCl increased the activity of glycolate oxidase (GO, E.C. 1.1.3.1) in leaves of nitrate-fed plants thus indicating enhanced photorespiration. In contrast, the enzyme activity measured in leaves of nitrogen-fixing soybean plants was found to decrease. Furthermore, when compared with the respective controls, the inhibition of both pigment and fresh biomass accumulation was less pronounced in the nitrogen-fixing plants. Thus, our results suggest that soybean plants with symbiotic N_2 fixation are more resistant to salt stress and therefore, can be better adapted to high salinity than nitrate-fed plants.

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Keywords: nitrate fed soybean plants; nitrogen-fixing soybean plants; salt stress; photosynthesis; glycolate oxidase activity.

INTRODUCTION

Saline soils can be found at different altitudes, from below sea level (e.g. around the Dead Sea) to mountains rising above 5000 m, such as the Tibetan Plateau or the Rocky Mountains. Of nearly 160 million ha of cultivated land under irrigation worldwide, about one-third is already affected by salt, which

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makes salinity a major constraint to food production (Manchanda and Garg, 2008). Drought and salinity are the major abiotic stress factors limiting plant growth and productivity (Kalinina et al., 2012). In general, biological and physiological effects induced by salinity include decreased water potential, ion imbalance and toxicity, reduction in CO_2 assimilation rate and increased accumulation of reactive oxygen species (ROS) leading to oxidative stress (Sheetal et al., 2013). Soil salinity is the major cause inducing biochemical and physiological changes in rice plants leading to growth inhibition and yield reduction (Yang et al., 2008).

Plant growth inhibition under conditions of salinity stress is due to ionic and osmotic effects triggered by excess salinity in the soil solution. The ionic effects are the consequence of increased uptake of Na^+ ions which can substitute for K^+ ions in plant cells, thereby leading to disturbances mainly in photosynthesis and carbohydrate metabolism. In parallel with the ionic effects, high salt concentrations in soils increase the osmotic potential of the soil solution thus leading to water stress effects (Hsiao, 1973). The reduced water uptake by roots is accompanied also by limited uptake of major ions necessary for normal plant growth including nitrate and ammonium ions.

Photosynthesis is a major metabolic process in plants. Keeping photosynthesis at a good rate helps maintaining plant growth under salinity stress (Mguis et al., 2013). The decrease in photosynthesis in salt stressed plants could be due to stomatal or/and non-stomatal limitation (Dubey, 2005). Salinity effects on photosynthesis vary substantially among plant species. It was reported that salt stress drastically reduced CO_2 exchange rate (CER) in barley leaves, but had little effect on leaf CER in wheat (Rawson, 1986).

Most bean plants depend on either biological N_2-fixation or nitrate and/or ammonia assimilation in order to meet all of the plant's nitrogen needs (Youssef and Sprent, 1983). The growth of bean plants with symbiotic N_2 fixation is often limited under adverse climatic conditions, more specifically drought and salinity (Zahran, 1999). Salinity is one of the major abiotic stress factors affecting the yield of bean plants in dry and semi-arid areas especially when plants are dependent on symbiotic N_2 fixation for N_2 supply (Drevon et al., 2001). Soil salinity can limit plant productivity due to the negative effect on host plant growth, the symbiotic growth of root tubers, N_2-fixing bacteria as well as the N_2-fixation capacity (Jebara et al., 2010). Unlike the host plant, rhizobia can survive in conditions of high salt concentrations demonstrating distinct changes in their salt tolerance: some strains are inhibited in the presence of 100 mM NaCl while others like *Rhizobium meliloti* and *R. fredii* can grow at NaCl concentrations above 300 mM.

Photosynthesis depends on leaf chlorophyll content and stomatal conductance, thereby correlating linearly with leaf N_2 content (Dingkuhn et al., 1992). Nitrogen nutrition is a major factor controlling transpiration in plants because of differences in specific leaf area and leaf conductance. Transpiration was found to be dependent on the source of N_2 nutrition, the reduction being more marked in nitrate-fed pea plants when compared with nitrogen-fixing plants subjected to water deficit (Frechilla et al., 2000). These authors showed also that under water deficit conditions pea plants with symbiotic N_2 fixation had higher efficiency for CO_2 assimilation than nitrate-fed plants. These results showed that the type of N_2 nutrition can determine the degree of sensitivity of plants towards drought stress.

The aim of the present work was to assess how the source of N_2 nutrition (nitrate assimilation or biological N_2-fixation) could affect plant resistance to salinity stress. More specifically, we examined the response of nitrate -fed and nitrogen -fixing soybean plants to high salinity based on the changes in leaf water status, electrolyte leakage, photosynthetic performance as well as the activity of glycolate oxidase - a key enzyme in photorespiration.

MATERIALS AND METHODS

Plant material and experimental scheme

Soybean plants (*Glycine max* L., cultivar Pavlikeni 101) were used as a model system. Seeds were surface sterilized with 70% ethanol and washed afterwards several times in distilled water. Then, the seeds were let to germinate on a moist filter paper at 24°C in the dark. Three-days-old seedlings were inoculated by adding 10^8 cells ml^{-1} suspension of *Bradyrhizobium japonicum* strain 273. After that they were transferred into plastic vessels and further grown in well aerated Hellriegel nutrient solution containing macroelements (1mM $MgSO_4$ $7H_2O$; 1mM KH_2PO_4, 3mM $Ca(NO_3)_2$) and microelements according to Hoagland and Arnon (1950). The plants were grown in a growth chamber at a photon flux density (PPD) of 90 µmol m^{-2} sec^{-1}, a 16/8 h light/dark photoperiod, and temperature 25±1°C. Nitrogen in the nutrient solution was kept equal to the full dose until the growth stage of fully expanded second trifoliate leaf, i.e. up to nodule formation and beginning of effective nitrogen fixation. The control N_2-fixing plants formed 55 nodules per plant while those subjected to 40mM and 80 mM NaCl formed 33 and 21 nodules per plant, respectively. Soybean plants were subjected to salinity during the vegetative stage of growth (14 days from the onset of germination) by adding NaCl into the nutrient solution at two concentrations: 40 mM and 80 mM. The control plants were grown in nutrient solution without NaCl. All analyses were performed 14 days after salt treatment (14-28 d) on the 4th trifoliate leaf of 28-days-old plants. Each value represents the mean ± SE of two different experiments with two replicates.

Electrolyte leakage

Membrane integrity damage was monitored by measuring the electrolyte leakage. Leaves were cut, rinsed in distilled water and placed in a test tube with 15 ml of distilled water at 24°C for 24 h. The initial conductivity of the solution (spontaneous leakage of ions due to membrane damage) and total ionic conductivity after boiling the sample for 20 min were measured using a conductivity meter (type 102/1, Radelkis, Budapest, Hungary). Percent leakage was expressed as described previously (Pinhero and Fletcher, 1994) using the following formula:

$$\text{Electrolyte leakage (\%)} = \frac{\text{Initial conductivity}}{\text{Total conductity}} \times 100$$

Relative Water Content

Relative water content (RWC) was measured as described by Morgan (1986). RWC values were calculated according to the formula:

RWC (%) = (initial fresh mass − dry mass/full turgid mass – dry mass) × 100.

Leaf pigments

Leaf pigments were extracted in 80% (v/v) acetone and determined according to the method of Arnon (1949). Calculations were made using Mackinney's coefficients (1941).

Dry weight

Dry weight was determined after drying the plant material at 105°C until constant weight.

Photosynthetic parameters

Net carbon dioxide (CO_2) assimilation rate was measured with a portable infrared gas-analyzer (Li 6400, Li-Cor, Lincoln, USA) at a quantum flux density of 1200 μmol m^{-2} s^{-1} PAR at 25°C. Statistical analysis of data was performed using Systat 7.0.

Glycolate oxidase activity assay

Leaf tissue was ground in a mortar on ice at a ratio of 1 g fresh mass to 5 ml cold extraction medium containing 0.33 M sorbitol, 0.05 M HEPES-NaOH, 0.002 M KNO_3, 0.002 M EDTA, 0.001 M $MgCl_2$, 0.0005 M K_2HPO_4, 0.02 M NaCl and 0.2 M Na-isoascorbate, pH 7.6. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000×*g* for 15 min. The supernatant was used directly for the enzyme assay.

Glycolate oxidase (GO, E.C. 1.1.3.1) activity was measured as described by Kolesnikov (1962). For enzyme extraction, 25 mg of leaves were ground with 20 ml 1:15 M K/Na phosphate buffer, pH 8.0, and centrifuged at 20,000×*g* for 15 min. 0.5 ml of 0.1 M Na-glycolate was added to 5 ml of extract and incubated for 10 min at 25°C. Extracts were precipitated with 3% TCA and developed a color reaction with 0.3% phenylhydrazine hydrochloride and 1.5% $K_3Fe(CN)_6$. The amount of glyoxylic acid was measured specrophotometrically at 530 nm (Specol 11, Carl Zeiss, Jena, Germany).

Soluble protein content was determined by the method of Bradford (1976).

RESULTS

Changes in fresh and dry biomass

Our results showed that control nitrate-fed soybean plants accumulated greater fresh and dry biomass when compared with nitrogen-fixing control plants (Fig. 1A, B). Treatment with 40 mM NaCl increased the fresh and dry biomass in both nitrate-fed and nitrogen-fixing soybean plants. In contrast, treatment with 80 mM NaCl reduced both the fresh and dry biomass with the exception of dry biomass in the nitrogen-fixing plants which was slightly increased (by 11%) compared with the control (Fig. 1B). As a result, leaf dry biomass in the nitrogen-fixing plants was higher than in the nitrate-fed plants grown at the same salt concentration (80 mM NaCl) (Fig. 1B).

Changes in RWC and electrolyte leakage

Salt stress affected negatively RWC of the soybean plants (Fig. 1C). Our results showed a reduction in RWC in both

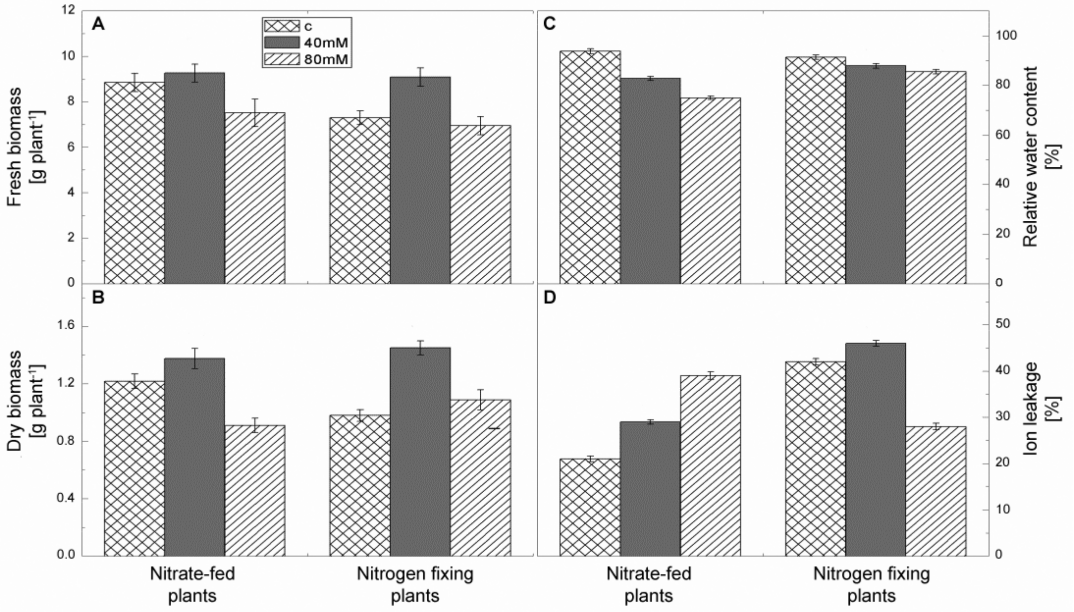

Figure 1. Changes in fresh bomass (A), dry biomass (B), relative water content (C) and electrolyte leakage (D) in nitrate-fed and nitrogen-fixing soybean plants subjected to NaCl stress. Salt treatment was applied during the vegetative stage of growth (14 days from the onset of germination) by adding NaCl into the nutrient solution at concentrations of 40 mM and 80 mM. Analyses were performed on the 4th trifoliate leaf 14 days after salt treatment.

types of test plants, the effect being less pronounced in the nitrogen-fixing plants at both test salt concentrations (40 mM and 80 mM NaCl).

Treatment with 40 mM NaCl increased the electrolyte leakage in leaves of both the nitrate-fed and nitrogen-fixing soybean plants indicating increased cell membrane permeability (Fig. 1D). Treatment with the high salt concentration (80 mM NaCl), however, caused opposite effects in the two types of test plants. While the membrane permeability in the nitrate-fed plants was found to increase by 85%, it was reduced by 33% in the nitrogen-fixing soybean plants compared with the respective controls (Fig. 1D). As a result, electrolyte leakage in leaves of the nitrate-fed plants was higher (by 39%) when compared with the nitrogen-fixing plants grown at the same salt concentration (80 mM NaCl).

Photosynthetic parameters

Chlorophyll content measured in leaves of control nitrate-fed soybean plants was higher (by 95%) compared with nitrogen-fixing plants (Fig. 2A). Salt treatment caused a decrease in total chlorophyll content, the effect being stronger at the high NaCl concentration (80 mM). Further, our results showed that when compared with the respective controls, the decrease in chlorophyll content (by 56%) in leaves of nitrate-fed soybean plants grown in the presence of 80mM Na Cl was stronger than in the nitrogen-fixing plants (by 23%).

The changes in carotenoid content followed the same trend as total chlorophyll content (Fig. 2B). The content of carotenoids measured in leaves of control nitrate-fed soybean plants was higher (by 62%) compared with nitrogen-

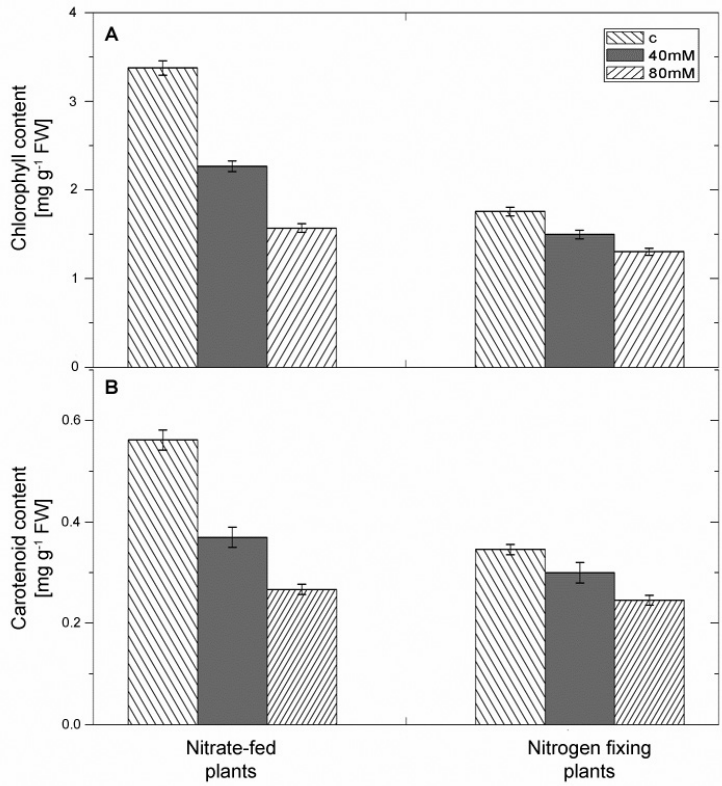

Figure 2. Chlorophyll content (A) and carotenoid content (B) in nitrate-fed and nitrogen-fixing soybean plants subjected to NaCl stress. Salt treatment was applied during the vegetative stage of growth (14 days from the onset of germination) by adding NaCl into the nutrient solution at concentrations of 40 mM and 80 mM. Analyses were performed on the 4th trifoliate leaf 14 days after salt treatment.

fixing plants. Treatment with 80mM NaCl reduced carotenoid content in both nitrate-fed and nitrogen-fixing soybean plants compared with controls, but the inhibitory effect in the nitrate-fed plants (by 52%) was stronger compared with the nitrogen-fixing plants (by 29%).

Net photosynthetic rate measured in leaves of both control nitrate-fed and nitrate-fixing soybean plants was similar (Fig. 3A). Salt treatment inhibited significantly the net photosynthetic rate compared with the respective controls and the inhibition in the nitrate-fed soybean plants (by 60%) measured upon treatment with 80mM NaCl was higher than in the nitrate-fixing soybean plants (by 32%). As a result, the net photosynthetic rate in the nitrogen-fixing plants was higher (by 28%) than in the nitrate-fed plants grown at the same salt concentration (80 mM NaCl). The comparative analysis showed also that while in the nitrate-fed soybean plants the transpitation rate and stomatal conductance decreased progressively with increasing NaCl concentration, in the nitrogen-fixing plants the values for transpiration rate and stomatal conductance were higher at 80 mM NaCl compared with the low (40 mM) salt concentration (Fig. 3C, D). Treatment with 80mM NaCl drastically reduced both transpiration rate (by 68%) and stomatal conductance (by 72%) in the

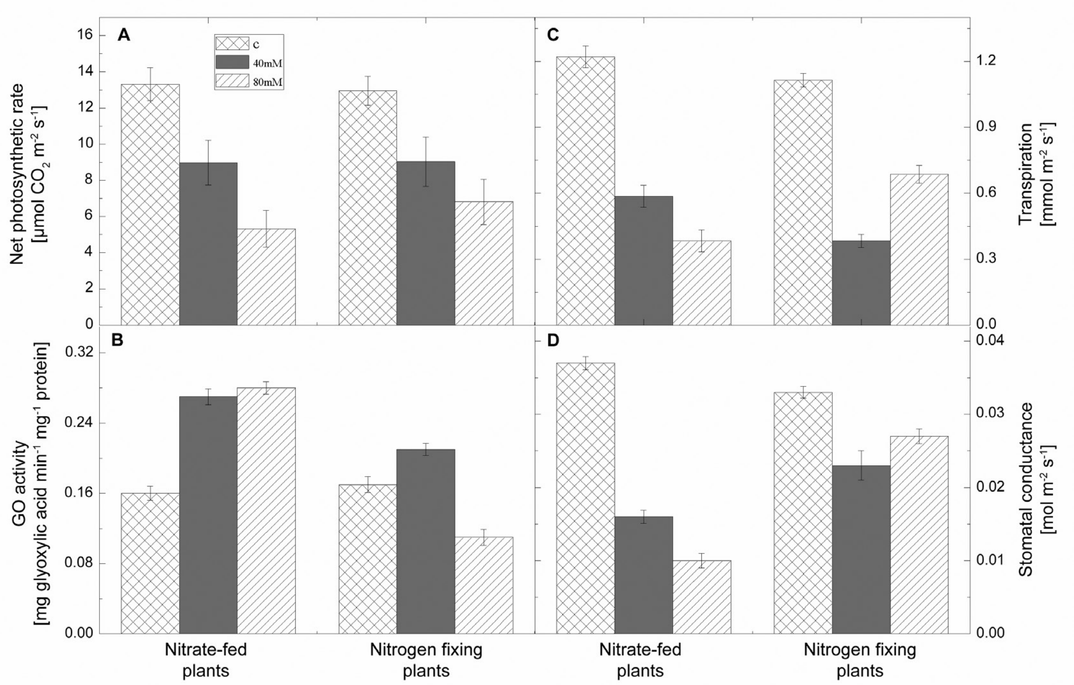

Figure 3. Changes in net photosynthetic rate (A), glycolate oxidase activity (B), transpiration rate (C) and stomatal conductance (D) in nitrate-fed and nitrogen-fixing soybean plants subjected to NaCl stress. Salt treatment was applied during the vegetative stage of growth (14 days from the onset of germination) by adding NaCl into the nutrient solution at concentrations of 40 mM and 80 mM. Analyses were performed on the 4th trifoliate leaf 14 days after salt treatment.

nitrate-fed plants whereas in the nitrogen-fixing plants these two parameters were less reduced compared with controls (by 38% and 18%, respectively). As a result, in accordance with the higher net photosynthetic rate, the nitrogen-fixing plants had higher transpiration rate and stomatal conductance when compared with the nitrogen-fed plants grown in the presence of 80mM NaCl.

Activity of glycolate oxidase (GO)

GO activities measured in leaves of control nitrate-fed and nitrogen-fixing soybean plants were similar (Fig. 3B). The addition of 80mM NaCl increased GO activity in the nitrate-fed plants by 75% compared with the control. In contrast, the enzyme activity was found to decrease by 48% in the nitrogen-fixing soybean plants at the same salt concentration thus indicating decreased photorespiration in these plants. As a result, the enzyme activity measured in the nitrate-fed soybean plants at the high salt concentration was almost 2.5-fold higher than in the nitrogen-fixing plants.

DISCUSSION

The relative water content gives information about of the relationship between cell water status and metabolism (Lawlor and Cornic, 2002). The osmotic effect produced by salinity can disturb plant water balance and inhibit plant growth by inducing stomata closure and inhibition of photosynthesis (Hernandez and Almansa, 2002). The increased concentrations of Na and Cl ions in plant tissues could lead to a toxic effect thereby inhibiting photosynthesis and respiration. Our results showed that salt stress decreased RWC to a lesser extent in the nitrogen-fixing soybean plants compared with the nitrate-fed plants, thus indicating better water status in the nitrogen-fixing soybean plants even at the high salt concentration (80 mM) (Fig. 1C). This could be a reason for more intensive metabolic processes in these plants compared with nitrate-fed plants in response to salinity stress.

It is known that salinity affects both plant water status and plant growth (Khan and Panda, 2008). Leaf size and leaf biomass appear to be the most strongly affected morphometric parameters by water and salt stress compared with other adverse environmental factors (Boyer, 1982). Our results showed that treatment with 80 mM NaCl reduced both fresh and dry biomass only in the nitrate-fed plants compared with the control (Fig. 1A, B). On the other hand, leaf fresh biomass in the nitrogen-fixing plants remained almost unaffected and dry biomass was even slightly increased (by 11%) compared with the control (Fig. 1A, B). Our results are in accordance with data obtained with rice plants grown under salinity stress (Amirjani, 2011). Thus, it could be suggested that the nitrogen-fixing soybean plants were more resistant to salt stress compared with the nitrate-fed plants.

Electrolyte leakage is a marker of cell membrane integrity. Our results showed that electrolyte leakage was affected by both the salt concentration in the nutrient solution and the source of N_2 nutrition (Fig. 1D). The electrolyte leakage in the nitrate-fed plants grown at 80 mM NaCl was increased by 85%, thus indicating increased cell membrane permeability. In contrast, it was reduced by 33% in the nitrogen-fixing soybean plants (Fig. 1D). As a result, electrolyte leakage in leaves of the nitrate-fed plants was higher (by 39%) than in the nitrogen-fixing plants grown at the same salt concentration (80 mM NaCl), thus indicating the ability of the nitrogen-fixing plants to maintain cell membrane integrity under salinity stress. This result confirmed again the higher capacity of the nitrogen-fixing soybean plants to cope with salt stress compared with the nitrate-fed plants. Our results are in accordance with data reported by Pinhero et al. (1999) showing that freezing of leaves from different maize cultivars changed electrolyte leakage differently: the changes were significant in the less resistant cultivars and insignificant in the resistant cultivars. Similar results were obtained also with nitrate-fed and nitrogen-fixing soybean plants subjected to water deficit (Kirova and Ignatov, 2004).

Sage and Pearcy (1987) suggested the existence of a direct correlation between leaf photosynthetic capacity and the content of N_2, the latter being used predominantly for the synthesis of the photosynthetic machinery components. The changes in the photosynthetic parameters observed in our study confirm the above statement. The content of chlorophyll measured in leaves of control nitrate-fed soybean plants was higher (by 95%) compared with nitrogen-fixing plants (Fig. 2A). These results could be explained by the lower leaf N_2 content in the nitrogen-fixing soybean plants as N_2 fixation had not yet reached the maximum efficiency at this stage of development. On the other hand, plants which received N_2 from the nutrient solution showed much higher chlorophyll content obviously due to the enhanced leaf N_2 content. Besides, the comparative analysis showed also that treatment with 80mM Na Cl caused a stronger decrease in chlorophyll content (by 56%) in leaves of nitrate-fed soybean plants compared with nitrogen-fixing plants (by 23%), thus suggesting accelerated senescence in the nitrate-fed plants in response to salt stress. Our results are in agreement with data on the effects of salinity stress in mung bean (Zayed and Zeid, 2006). Similar results were obtained also by Shangguan et al. (2000) with winter wheat cultivated at different levels of N_2 content and water availability in the soil.

The changes in carotenoid content followed the same trend as total chlorophyll content (Fig. 2B). Treatment with 80mM NaCl reduced carotenoid content to a lesser extent in the nitrogen-fixing plants (by 29%) compared with the nitrate-fed plants (by 52%). The ability of the nitrogen-fixing soybean plants to keep the content of these antioxidants at a higher level compared with the nitrate-fed plants when exposed to high salt concentrations indicates higher resistance of these plants to salinity stress.

Similar to pigment content, salt treatment inhibited significantly the net photosynthetic rate and the degree of inhibition in the nitrogen-fixing soybean plants exposed to 80mM NaCl was lower than in nitrate-fed soybean plants (Fig. 3A). The higher photosynthetic rate in the nitrogen-fixing plants could be also due to the better water status based on the less decreased RWC compared with the nitrate-fed plants (Fig. 1C). In addition, the nitrogen-fixing plants had higher transpiration rate and stomatal conductance when compared with the nitrate-fed plants (Fig. 3C, D).

Glycolate oxidase is a key enzyme in photorespiration, catalizing the oxidation of glycolate to glyoxylate (Sahu et al., 2010). GO activity is a good indicator of photorespiration rate (Peterhansel and Maurino, 2011). Despite the considerable loss of assimilated carbon, photorespiration protects the photosynthetic system from excess light damage using large amounts of excess light energy (Wilhelm and Selmar, 2011). Photorespiration consumes the excess O_2 and releases sufficient CO_2 which would support photosynthesis when stomatal conductance is reduced and carbon assimilation is limited under stress conditions. Our results showed that the changes in the activity of GO were opposite in the two types of test plants (Fig. 3B). While GO activity in the nitrate-fed plants upon treatment with 80mM NaCl increased by almost 80%, it was reduced by 48% in the nitrogen-fixing soybean plants compared with the controls. Our results suggest that photorespiration in the salt-treated nitrate-fed plants was strongly enhanced, but in the nitrogen-fixing soybean plants it was reduced. The observed higher activity of GO in the salt-treated nitrate-fed plants could be accounted for by enhanced energy dissipation through photorespiration in response to salt stress. Furthermore, the decreased photorespiration in the nitrogen-fixing plants together with the less affected net photosynthetic rate could be an indicator of higher resistance of these plants under salinity conditions. Our results are in accordance with data reported by Frechilla et al. (2000) with nitrate-fed and nitrogen-fixing pea plants subjected to water deficit. These authors showed that the nitrogen-fixing pea plants had higher efficiency for CO_2 assimilation in conditions of water deficit than the nitrate-fed plants.

In conclusion, the present investigation showed that the source of N_2 nutrition affected plant resistance to salinity stress. The nitrogen-fixing soybean plants had better water status, less affected electrolyte leakage indicating better membrane integrity, enhanced dry biomass, higher net photosynthetic rate, transpiration rate and stomatal conductance, as well as reduced photorespiration compared with the nitrate-fed soybean plants upon treatment with 80 mM NaCl. Thus, our results suggest that soybean plants with symbiotic N_2 fixation are more resistant to salt stress and therefore, can be better adapted to high salinity than nitrate-fed plants.

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