

1-1-2014

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LAHBIB LATRACH

MOHAMED FARISSI

MOHAMMED MOURADI

BOUCHRA MAKOUDI

ABDELAZIZ BOUIZGAREN

See next page for additional authors

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LATRACH, LAHBIB; FARISSI, MOHAMED; MOURADI, MOHAMMED; MAKOUDI, BOUCHRA; BOUIZGAREN, ABDELAZIZ; and GHOULAM, CHERKI (2014) "Growth and nodulation of alfalfa-rhizobia symbiosis under salinity: electrolyte leakage, stomatal conductance, and chlorophyll fluorescence," *Turkish Journal of Agriculture and Forestry*. Vol. 38: No. 3, Article 3. <https://doi.org/10.3906/tar-1305-52>
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LAHBIB LATRACH, MOHAMED FARISSI, MOHAMMED MOURADI, BOUCHRA MAKOUDI, ABDELAZIZ BOUIZGAREN, and CHERKI GHOULAM

Growth and nodulation of alfalfa-rhizobia symbiosis under salinity: electrolyte leakage, stomatal conductance, and chlorophyll fluorescence

Lahbib LATRACH^{1,2,*}, Mohamed FARISSI^{1,2,**}, Mohammed MOURADI¹, Bouchra MAKOUZI¹,
Abdelaziz BOUIZGAREN², Cherki GHOUAM¹

¹Team of Plant Biotechnology and Agro-Physiology of Symbiosis, Faculty of Sciences and Techniques, Gueliz, Marrakesh, Morocco

²Unit of Plant Breeding, National Institute for Agronomic Research, Gueliz, Marrakesh, Morocco

Received: 14.05.2013 • Accepted: 14.10.2013 • Published Online: 14.03.2014 • Printed: 11.04.2014

Abstract: The present work was a study on the adverse effects of salinity on growth, nodulation, and some physiological parameters in 4 symbiotic combinations involving 2 Moroccan alfalfa (*Medicago sativa* L.) populations (Demnate and Tata) and 2 rhizobial strains (rhLAR 1 and rhLAR 4). The experiment was conducted in the greenhouse at 32/22 °C day/night, 50%–80% relative humidity, and a photoperiod of 16 h. The seedlings were separately inoculated with suspensions of 2 rhizobial strains and grown under 2 NaCl treatments, 0 mM (control) and 100 mM (salt stress), in plastic pots filled with sterile sand and peat at 9/10 and 1/10 ratios, respectively. The salt stress was applied for 5 weeks and some agro-physiological and biochemical parameters related to salt tolerance were assessed. The results showed that salinity significantly reduced the height of plants, their dry biomass, and nodulation. This constraint has also negatively affected the relative water content of leaves, the membrane permeability, the stomatal conductance, the maximum quantum yield of photosystem II, and the chlorophyll contents. Comparison among the symbiotic combinations tested showed that their behavior was significantly different. Plants inoculated with rhizobial strain rhLAR 4 were more tolerant to saline conditions. Their tolerance was associated with the maintaining of adequate levels in terms of physiological and biochemical parameters studied.

Key words: Alfalfa, growth, nodulation, salinity, tolerance

1. Introduction

Alfalfa (*Medicago sativa* L.) is the most cultivated forage legume in the world due to its high nutritional quality, high protein content, and effects on soil fertility (Huyghe, 2003). Indeed, alfalfa is a crop that has a very favorable influence on soil fertility by contributing to the incorporation of nitrogen in pastoral ecosystems with beneficial economic impact, helping to reduce or limit the use of chemical fertilizers by nitrogen-fixing symbiosis involving rhizobial strains. In Morocco, local populations of this species are widely used in traditional agro-ecosystems, oases, and mountains. They strongly contribute to socioeconomic development of local families as a principal source for livestock nutrition. However, the environmental constraints of arid and semiarid ecosystems constitute limiting factors for plant growth and productivity and affect the symbiotic nitrogen fixation. In fact, salinity is one of the most widespread problems for symbiotic nitrogen fixation (Krouma, 2009; Öğütçü et al., 2010; Faghire et al., 2011) and restricts the development of the extension of legume cultivation (Faghire et al., 2011; Farissi et al., 2011,

2013). The high salinity in the soil affects the initiation, development, and function of nodules (Saadallah et al., 2001). Symbiotic nitrogen fixation was found to be more affected by salt than by plant growth (Rao et al., 2002; Faghire et al., 2011). Generally, nodular activity (i.e. nitrogen fixation) is less affected by salt than nodulation (i.e. organ initiation and meristematic growth). Thus, the infection process seems to be the most sensitive to salt (Payakapong et al., 2006). Besides, this constraint affects many physiological and biochemical processes governing plant growth and development. Indeed, the presence of salt in a rooting medium affects nutrient uptakes, activities of many enzymes, membrane permeability of cells, amount of nitrogen fixed, chlorophyll contents, and water status (Ghoulam et al., 2002; Tatar et al., 2010; Farissi et al., 2013; Kaya et al., 2013).

The most important strategies employed in the last few years to reduce the effects of salt stress on legume production have focused on a selection of host genotypes that are tolerant to high-salt conditions. An increase of rhizobial bacteria tolerance might constitute another

* These authors contributed equally to this work.

** Correspondence: farissimohamed@gmail.com

approach to improve plant productivity under symbiosis (Kenenil et al., 2010). Thus, the selection of salt-tolerant alfalfa-rhizobia combinations could be a promising way to improve alfalfa production in salt-affected soils. Accurate selection requires an understanding of the mechanisms involved in salt tolerance. Understanding these mechanisms will be helpful to enhance the productivity of the crop in these areas adversely affected by this environmental constraint (Farissi et al., 2013). In this context, the aim of the present study is to assess the effect of salt stress on growth and nodulation in symbiotic associations involving 2 Moroccan alfalfa (*Medicago sativa* L.) populations and 2 rhizobial strains isolated from southeastern Morocco, characterized by drastic environmental conditions. Research was focused on some parameters associated with salt tolerance, such as relative water content, electrolyte leakage, stomatal conductance, maximum quantum yield of photosystem II, and chlorophyll contents. Subsequently, the symbiotic combinations more adapted to salinity stress were determined.

2. Materials and methods

2.1. Biological material and experimental treatments

This study was carried out on 2 Moroccan alfalfa (*Medicago sativa* L.) populations, Tata (T) and Demnate (D), originating from a southwestern oasis and the High Atlas mountains of Morocco, where they have been cultivated for many centuries and are still widely used by farmers in these traditional agro-ecosystems (Farissi et al., 2011). Continuous natural and human selection has led, with time, to their adaptation to the local habitats, with distinction in the agro-morphological characteristics of the landraces (Farissi et al., 2011). The seeds were supplied by the National Institute of Agronomic Research (INRA, Marrakech) and the experiment was conducted in a greenhouse at 32/22 °C day/night, with 50%–80% relative humidity and a photoperiod of 16 h. The seeds were surface-sterilized with sodium hypochlorite (5%) for 5 min, rinsed 4 times with sterile deionized water, and germinated in plastic pots (20 cm diameter × 30 cm depth) filled with sterile sand and peat at 9/10 and 1/10 ratios, respectively. After germination, the number of seedlings was adjusted to 10 per pot; pots were separately inoculated with the suspensions of 2 rhizobial strains, rhLAR 1 and rhLAR 4 (10^8 cells/mL), that were isolated from nodules of *Medicago sativa* grown in saline soils of the southeastern region of Morocco (Latrach et al., 2012). These local strains were previously subjected to infectivity testing under aseptic conditions (Latrach et al., 2012). N-free nutrient solution (Baragz et al., 2013) was added once a week. Each pot (10 seedlings) was considered as 1 replicate with 2 pots per treatment per combination. Six days after inoculation, the salt treatment was started

using 300 mL of solution with NaCl concentrations of 0 mM (control) and 100 mM NaCl. To avoid osmotic shock, NaCl concentrations were increased gradually by 25 mM every 2 days until the desired concentration (100 mM NaCl). The stress was applied for 5 weeks and then some agro-physiological and biochemical parameters associated with salt stress were assessed.

2.2. Dry weight and plant height measurements

For dry weight (DW) determination, shoots, roots, and nodules were separated and dried at 70 °C for 48 h. DW was then weighed. For standardizing data, the results were expressed as the relative reduction of dry biomass in comparison to the control (0 mM), using the following formula (Ghoulam et al., 2002):

$$\text{Relative reduction (\%)} = [(1 - (\text{stressed} / \text{optimal irrigation}))] \times 100.$$

The plant height allowed the assessment of the effect of salt treatment on the growth of air-stressed plants compared with the control. It was measured on 5 plants per pot.

2.3. Relative water content

Relative water content (RWC) was estimated by recording the turgid weight (TW) of 0.1 g of fresh leaflet samples by keeping them in water for 4 h, followed by drying in a hot-air oven until a constant weight was achieved (DW). RWC was defined as follows (Ghoulam et al., 2002): $\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100$.

2.4. Membrane permeability (electrolyte leakage)

Electrolyte leakage (EL) was used to determine the membrane stability. It was assessed as described by Lutts et al. (1996) using young leaves. Samples were washed 3 times with deionized water to remove surface-adhered electrolytes. They were then placed in closed vials containing 10 mL of deionized water and incubated at 25 °C on a rotary shaker for 24 h; subsequently, electrical conductivity of the solution (C_1) was determined. Samples were then autoclaved at 120 °C for 20 min and the last electrical conductivity (C_2) was obtained following equilibration at 25 °C. The electrolyte leakage was defined as follows: $\text{electrolyte leakage (\%)} = (C_1 / C_2) \times 100$.

2.5. Stomatal conductance

Stomatal conductance was measured on healthy leaves using a porometer (Leaf Porometer Version 5.0, Decagon Devices, Inc., USA) at a temperature of 25 ± 1 °C and a relative humidity of $55 \pm 5\%$.

2.6. Chlorophyll fluorescence measurement

Chlorophyll fluorescence was measured using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, UK) after 20 min of dark adaptation. Chlorophyll fluorescence was estimated by the Fv/Fm ratio, which represents the maximum quantum yield of photosystem II. It was calculated as $\text{Fv/Fm} = (\text{Fm} - \text{Fo}) / \text{Fm}$, where

Fm and Fo are maximal and minimal fluorescence of dark-adapted leaves, respectively, and Fv is variable fluorescence (Jifon and Syvertsen, 2003).

2.7. Total chlorophyll content

Total chlorophyll was determined as described by Farissi et al. (2013). It was extracted with acetone in a mortar, using a proportion of 200 mg of fresh leaf tissue and 5 mL of acetone (80%, v/v). Chlorophyll concentration was measured after centrifugation (10 min at $5000 \times g$) as absorbance (OD) of the supernatant measured at 663 and 645 nm. Total chlorophyll was determined using the following formula (Farissi et al., 2013):

$$\text{total Chl} = 8.02 \text{ OD}_{663} + 20.20 \text{ OD}_{645}$$

2.8. Statistical analysis

The statistical analysis was performed using SPSS 10.0. It concerned a 2-way analysis of variance (ANOVA II) and the Student–Newman–Keuls grouping test. Three replicates per combination per treatment were executed. The calculated means and standard errors were reported.

3. Results

3.1. Effect on growth

Results in Table 1 show the effect of salt stress on plant growth parameters of alfalfa populations under symbiotic nitrogen fixation conditions. The effects of saline treatment and symbiotic combination on the plant heights were clearly significant ($P < 0.001$). The interaction effect (salinity \times symbiotic combination) was also significant ($P < 0.01$). In fact, compared to control plants (0 mM NaCl), the application of salt stress induced a depressive effect on the height of plants whichever strain was used for inoculation.

The strongest reductions were noted in symbiotic combinations D-rhLAR 1 and T-rhLAR 1 (40% and 35%, respectively), while inoculation with rhizobial strain rhLAR 4 caused reductions of 21% and 19% for D-rhLAR 4 and T-rhLAR 4, respectively. As well as the height, the dry biomasses of shoots, roots, and nodules were significantly decreased by salt treatment with significant differences between the symbiotic combinations tested (Table 1). In general, comparisons among the symbiotic combinations indicated that the reductions were relatively weak in symbiotic interactions involving the 2 populations and rhizobial strain rhLAR 4. Indeed, the reductions in shoot dry biomass were 24%, 21%, 32%, and 22% for T-rhLAR 1, T-rhLAR 4, D-rhLAR 1, and D-rhLAR 4, respectively. The reductions in root dry biomass were 29%, 20%, 39%, and 24%, while reductions of 35%, 17%, 52%, and 24% were noted in nodular dry mass of the same symbiotic combinations, respectively.

3.2. Relative water content

The effect of salt stress on RWC is demonstrated in Figure 1. Under normal conditions (without NaCl treatment), the symbiotic combinations showed RWC values that ranged from 73% to 83% (Figure 1). The application of salt treatment caused significant reductions ($P < 0.001$; Table 2) in RWCs of all symbiotic combinations with significant differences in their behavior ($P < 0.05$; Table 2). The reduction was more important in D-rhLAR 1 and T-rhLAR 1 (19% and 17%, respectively), while the reductions were 14% and 16% in T-rhLAR 4 and D-rhLAR 4, respectively. The interaction effect (salinity \times symbiotic combination) was not significant ($P > 0.05$; Table 2).

Table 1. Salinity response in plant height, shoot, root and nodular dry weights in 4 symbiotic combinations involving 2 Moroccan alfalfa populations (Demnate and Tata) and 2 rhizobial strains (rhLAR 1 and rhLAR 4). Results are expressed as reduction percentage of control (0 mM NaCl).

Symbiotic combination	Reduction percentage of control (%)			
	Plant height	Shoot dry weight	Root dry weight	Nodular dry weight
T-rhLAR 1	35	24	29	35
T- rhLAR 4	19	21	20	17
D-rhLAR 1	40	32	39	52
D- rhLAR 4	21	22	24	24
Salinity	140.48***	103.78***	16.82**	28.40***
Symbiotic combination	13.73***	9.25**	23.96***	42.83***
Salinity \times combination	7.29**	2.95 NS	7.74**	4.16*

*: Significant at 0.05 probability level; **: significant at 0.01 probability level; ***: significant at 0.001 probability level; NS: not significant at 0.05.

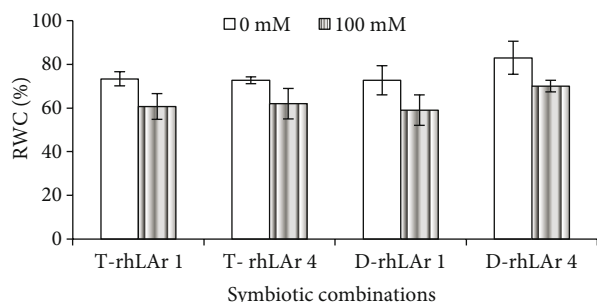


Figure 1. Salt treatment response of the relative water content (RWC) in 4 symbiotic combinations involving 2 Moroccan alfalfa populations (Demnate and Tata) and 2 rhizobial strains (rhLAR 1 and rhLAR 4). Values are means of 3 replicates and bars are standard errors.

3.3. Membrane permeability

Without salt treatment, we observed low electrolyte leakage percentages that did not exceed 15% in any symbiotic combination (Figure 2). The presence of 100 mM NaCl in rooting medium caused an increase in EL in all symbiotic combinations tested. The comparison among these combinations indicated that their EL percentages were significantly different ($P < 0.001$; Table 2). Indeed, the lowest values were recorded under inoculation with strain rhLAR 4, while the greatest value was recorded when the plants were inoculated with rhizobial strain rhLAR 1; the recorded values were 42% and 36% for strains D-rhLAR 1 and T-rhLAR 1, respectively.

3.4. Stomatal conductance

For stomatal conductance (Figure 3), the average values recorded without salt treatment were between 14 and 16 $\text{mmol m}^{-2} \text{s}^{-1}$. Treatment with salt induced a significant decrease in stomatal conductance ($P < 0.001$; Table 2). The behavior of the tested symbiotic combinations was

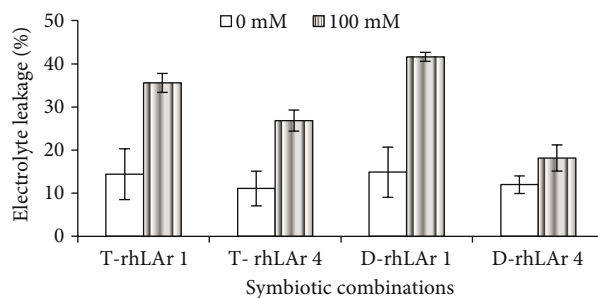


Figure 2. Salt treatment response of leaf electrolyte leakage in 4 symbiotic combinations involving 2 Moroccan alfalfa populations (Demnate and Tata) and 2 rhizobial strains (rhLAR 1 and rhLAR 4). Values are means of 3 replicates and bars are standard errors.

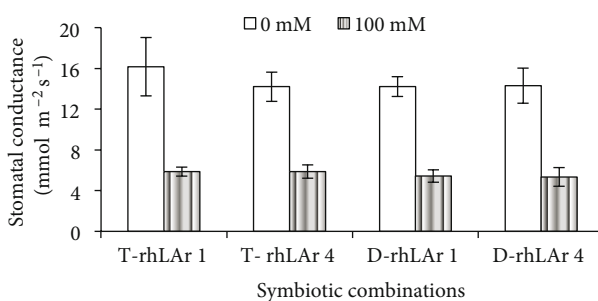


Figure 3. Salt treatment response of stomatal conductance in 4 symbiotic combinations involving 2 Moroccan alfalfa populations (Demnate and Tata) and 2 rhizobial strains (rhLAR 1 and rhLAR 4). Values are means of 3 replicates and bars are standard errors.

statistically similar according to the Student–Newman–Keuls test ($P > 0.05$; $P = 0.77$), and the average values recorded around 6 $\text{mmol m}^{-2} \text{s}^{-1}$ with the reductions varied between 57% and 64% compared with control conditions (0 mM). The interaction effect was not significant ($P > 0.05$; Table 2).

Table 2. Two-way analysis of variance (ANOVA II) of salt treatment and symbiotic combination effects and their interaction (salinity \times symbiotic combination) for the studied parameters.

Dependent variables	Independent variables		
	Salinity	Symbiotic combination	S \times SC
Relative water content	29.60***	4.60*	0.08 NS
Electrolyte leakage	132.47***	15.23***	8.32**
Stomatal conductance	246.53***	0.98 NS	0.54 NS
Fv/Fm	297.83***	92.94***	22.10***
Chlorophyll contents	229.04***	1.49 NS	21.33***

*: Significant at 0.05 probability level; **: significant at 0.01 probability level; ***: significant at 0.001 probability level; NS: not significant at 0.05.

3.5. Chlorophyll fluorescence

Without salt treatment, all symbiotic combinations developed the same behavior in terms of the maximum quantum yield of photosystem II (Fv/Fm); the average values were around 0.8 (Figure 4). However, the application of salt stress negatively and significantly ($P < 0.001$; Table 2) affected the activity of photosystem II by the reduction of the maximum quantum yield. Under this constraint, comparison among the tested symbiotic combination revealed that the values recorded for the Fv/Fm ratio were relatively important under inoculation with rhLAR 4 (0.74 and 0.73 in T-rhLAR 4 and D-rhLAR 4, respectively).

3.6. Total chlorophyll content

Figure 5 indicates the effect of salt stress on total chlorophyll contents. Results showed that salt stress induced a significant decrease in total chlorophyll of plants ($P < 0.001$; Table 2). Under this constraint, the grouping test of Student–Newman–Keuls revealed 2 distinct groups. The first showed higher contents of total chlorophyll and corresponded to T-rhLAR 4, D-rhLAR 4, and T-rhLAR 1 symbiotic combinations ($P > 0.05$; $P = 0.29$) with contents ranging between 7.2 and 9.5 mg FM⁻¹.

4. Discussion

The symbiotic interactions between 2 rhizobial strains and 2 alfalfa populations under salt conditions were analyzed. Many parameters related to salt stress were assessed, which included plant growth, nodulation, relative water content, membrane permeability, stomatal conductance, chlorophyll fluorescence, and chlorophyll contents. The results showed that salt reduced plant growth and nodulation in all symbiotic combinations that developed significant differences in their behavior. The symbiotic combinations involving the 2 alfalfa populations and strain rhLAR 4 were found more tolerant to salt stress. Saadallah et al. (2001) reported that the reduction in growth of some *Medicago* populations subjected to salt was related to a

decrease in leaf area, as well as inhibition of the initiation and the development of nodules. Indeed, our data showed that application of salt treatment decreased the biomass of nodules. Tejera et al. (2004) and Fahmi et al. (2011) noted that salt stress inhibits nodule formation by the inhibition of the initial steps of rhizobia-legume symbiosis. In *Phaseolus vulgaris* L., the concentration of 50 mM of NaCl caused a decrease of growth (Gama et al., 2007). The sensitivity of symbiotic plants was associated with an excessive accumulation of Na⁺ and Cl⁻ ions in leaves and overloading of nodules with toxic Na⁺ and Cl⁻ ions (Drevon et al., 2001). Tajini et al. (2012) observed a significant correlation between nodule biomass and nitrogen content in *Phaseolus vulgaris*-rhizobia symbiosis. Reduction of N₂-fixing activity by salt stress is usually attributed to a reduction in respiration of the nodules (Kenenil et al., 2010). Additionally, the salt-induced distortions in nodule structure could be the reason for the decline in the N₂ fixation rate by legumes subjected to salt stress (Fahmi et al., 2011). The depressive effect of salt stress on N₂ fixation by legumes is directly related to the salt-induced decline in dry weight and N content in the shoot (Zahran, 2001).

The presence of salt in the rooting medium reduced the water available to the plant and created an osmotic stress that makes the photosynthetic electron transport inactive. In fact, we have noted a significant decrease in the RWC of plants. Similarly, the increase in the osmotic potential by salinity causes leakage of Na⁺ ions from the cytosol, which inactivates both the electron transport in photosynthesis and respiration (Radhouane, 2009). The lack of water and salinity are factors that limit stomatal conductance and photosynthetic capacity (Radhouane, 2009). In fact, the significant decrease in stomatal conductance by salt treatment was marked in the present work. Additionally, the Fv/Fm ratio, a parameter commonly known as maximum quantum yield of primary photochemistry or maximal relative electron transport rate of photosystem II

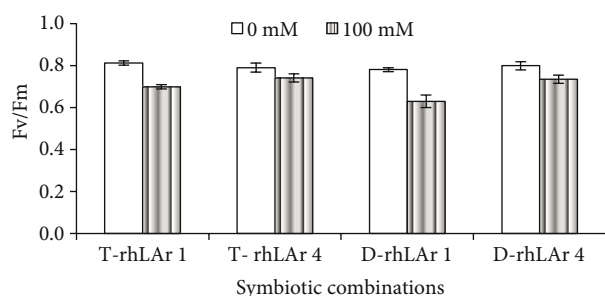


Figure 4. Effect of salt treatment on the maximum quantum yield of photosystem II (Fv/Fm) in 4 symbiotic combinations involving 2 Moroccan alfalfa populations (Demnate and Tata) and 2 rhizobial strains (rhLAR 1 and rhLAR 4). Values are means of 3 replicates and bars are standard errors.

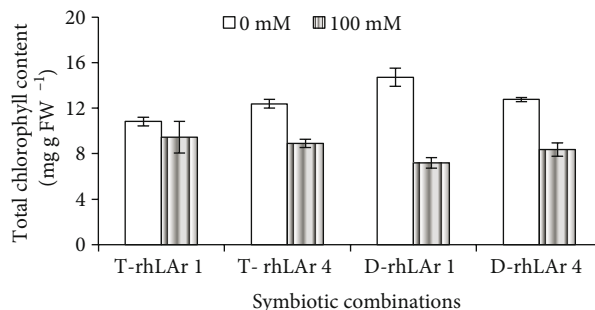


Figure 5. Salt treatment response of total chlorophyll content in 4 symbiotic combinations involving 2 Moroccan alfalfa populations (Demnate and Tata) and 2 rhizobial strains (rhLAR 1 and rhLAR 4). Values are means of 3 replicates and bars are standard errors.

(Waldhoff et al., 2002), and total chlorophyll content were found to be decreased under saline conditions.

Gama et al. (2007) mentioned that the decline in photosynthesis observed with increasing salinity could be attributed to stomatal factors and the reduction in plant growth is associated to the reduction in photosynthesis. During salt stress, the concentration of CO₂ in chloroplasts decreases because of a reduction in stomatal conductance. Percival et al. (2003) mentioned that the leaf chlorophyll fluorescence responses to increasing salinity were manifested by reduced Fv/Fo and Fv/Fm. The reduction in Fv/Fm due to salinity stress is possibly related to the damage of chlorophyll under saline conditions (Ganieva et al., 1998). Indeed, our data indicated that reduction in photosystem II efficiency was associated with decreasing total chlorophyll content. Decreasing chlorophyll content with increasing salinity in rooting medium could be related to increasing the activity of the chlorophyll-

degrading enzyme chlorophyllase (Jamil et al., 2007), the destruction of the chloroplast structure, and the instability of pigment protein complexes (Singh and Dubey, 1995). Consequently, the decreases in chlorophyll contents and photosystem II activity have adverse effects on plant growth (Nasir Khan et al., 2007).

Based on this research, we conclude that the presence of NaCl in the rooting medium significantly affected the growth and nodulation ability in all symbiotic combinations tested with significant differences in their behavior. The adverse effects of salinity on growth and nodulation were associated with perturbation in physiological and biochemical parameters analyzed. Decreases in RWCs and disturbance in membrane permeability evaluated by electrolyte leakage percentage were noted. The saline constraint negatively affected the stomatal conductance, the maximum quantum yield of photosystem II, and the total chlorophyll content.

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