Variations in leaf gas exchange, chlorophyll fluorescence and membrane potential of *Medicago sativa* root cortex cells exposed to increased salinity: The role of the antioxidant potential in salt tolerance

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Abstract: Salinity is one of the most serious agricultural problems that adversely affects growth and productivity of pasture crops such as alfalfa. In this study, the effects of salinity on some ecophysiological and biochemical criteria associated with salt tolerance were assessed in two Moroccan alfalfa (*Medicago sativa* L.) populations, *Taf 1* and *Tata.* The experiment was conducted in a hydro-aeroponic system containing nutrient solutions, with the addition of NaCl at concentrations of 100 and 200 mM. The salt stress was applied for a month. Several traits in relation to salt tolerance, such as plant dry biomass, relative water content, leaf gas exchange, chlorophyll fluorescence, nutrient uptake, lipid peroxidation and antioxidant enzymes, were analyzed at the end of the experiment. The membrane potential was measured in root cortex cells of plants grown with or without NaCl treatment during a week. The results indicated that under salt stress, plant growth and all of the studied physiological and biochemical traits were significantly decreased, except for malondialdehyde and H_2O_2 contents, which were found to be increased under salt stress. Depolarization of membrane root cortex cells with the increase in external NaCl concentration was noted, irrespective of the growth conditions. The *Tata* population was more tolerant to high salinity (200 mM NaCl) and its tolerance was associated with the ability of plants to maintain adequate levels of the studied parameters and their ability to overcome oxidative stress by the induction of antioxidant enzymes, such as guaiacol peroxidase, catalase and superoxide dismutase.

Key words: alfalfa; salinity; leaf exchange; membrane potential; antioxidant enzymes

Abbreviations: APX – ascorbate peroxidase; C_i – intercellular CO_2 concentration; CAT – catalase; Chl – chlorophyll; DW – dry weight; E – transpiration rate; F_v/F_m – chlorophyll fluorescence; g_s – stomatal conductance; GR – glutathione reductase; LSD – least significant difference; MDA – malondialdehyde; P_N – net photosynthesis; POD – guaiacol peroxidase; prot – protein; PPO – polyphenol oxidase; PS – photosystem; ROS – reactive oxygen species; RWC – relative water content; SOD – superoxide dismutase; TBA – thiobarbituric acid; TCA – trichloroacetic acid

INTRODUCTION

Increased salinity is a serious global problem. It is one of the major limiting factors in agriculture. In the Mediterranean region, particularly in dry areas, it adversely affects pasture crops such as alfalfa, by decreasing their growth and yield via osmotic stress and the injurious effects of increased concentrations of toxic Na^+ and Cl^- ions [1].

The most important strategies employed in the last few years to reduce the effects of salt stress on plant production have focused on a selection of tolerant genotypes to extreme salt conditions. This selection would permit the cultivation of crops on saline soils or the partial use of saline water in irrigation. These situations frequently occur today in many regions of the Mediterranean. Accurate selection re-

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quires a deep understanding of the various complex mechanisms implicated in salt tolerance. Therefore, plant tolerance to high salinity constitutes a decisive environmental research topic [2].

In general, exposure to salt stress engenders several common reactions in plants. These reactions lead to cellular dehydration with concomitant osmotic changes and the removal of water from the cytoplasm into the extracellular space, thereby reducing cytosolic and vacuolar volumes [3]. Plants respond to salt stress through ion (Na^+) and osmotic signals. An excess of Na^+ in the rooting medium can be detected by transmembrane protein receptors [4]. This excess causes changes in protein structure and membrane depolarization that can lead to the perception of ion toxicity. Suárez [5] and Netondo et al. [6] reported that photosynthetic activity decreases when plants are grown under saline conditions, leading to reduced growth and productivity. The reduction in photosynthesis under salinity can be attributed to a decrease in the chlorophyll content [7], stomatal conductance [8], PS II activity and CO_2 fixation [9]. Another consequence of exposure to this circumstance is the generation of ROS, which include hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical (HO) and singlet oxygen ($^1O_2$), and increased oxidative stress. This adversely affects cellular structures and whole plant metabolism [10]. To cope with the oxidative stress and cellular damage, plants have developed several nonenzymatic and enzymatic defense mechanisms [11]. Nonenzymatic antioxidant processes involve the binding or scavenging of ROS with β-carotenes, ascorbic acid, α-tocopherol, reduced glutathione, soluble sugars, glycine betaine and proline. Enzymatic processes involve reactions with SOD, GPX, APX, CAT, PPO and GR [1,3,12,13]. It has been reported that salt stress induces the activities of mitochondrial and chloroplast SOD and APX [14,15]. Little is known about the responses of these enzymes to salt stress. A higher level of antioxidants is considered a manifestation of the salt tolerance mechanism developed by plants to cope with the ROS. Previous studies reported that tolerant genotypes have higher levels of antioxidants in their tissues than sensitive ones [16-18]. Mhadhbi et al [19] reported that in tolerant genotypes of *Medicago truncatula* antioxidant gene-enzyme responses strongly contribute to defense against salinity stress, especially in tolerant genotypes. In this context, the aim of the present study was to evaluate salt stress effects on two Moroccan alfalfa populations with the hypothesis that salt tolerance may be related to some determinant mechanisms of ROS scavenging, PS II performance and the ability to exclude Na^+ toxic ions form the cells. The assessment focused on some agrophysiological and biochemical parameters related to salt tolerance, including plant growth, the RWC, leaf gas exchange, chlorophyll fluorescence, chlorophyll contents, membrane depolarization, nutrient concentrations, lipid peroxidation and hydrogen peroxide contents. The role of antioxidant enzymes in salt tolerance was also investigated and discussed.

MATERIALS AND METHODS

Plant material and growth conditions

Two Moroccan populations (*Tata* and *Taf 1*) of alfalfa (*M. sativa* L.) were used in this study. These populations originated from Moroccan oases where they have been cultivated for many centuries and are still widely used by farmers in traditional agroecosystems. Continuous natural and human selection have led to their adaptation to their local habitats, with distinct agromorphological characteristics of landraces [1,20,21]. Seeds from these populations were supplied by the National Institute for Agronomical Research (INRA-Marrakech). They were germinated in Petri dishes inside a growth cabinet with dim light, 70% air humidity and an ambient temperature of 25±1°C. After germination, young seedlings were transferred to a controlled environment chamber with day/night temperatures of 30/20±1°C, respectively, and a photoperiod of 16 h (18 000 lux). At the stage of the first trifoliate leaf, 12-day-old seedlings of similar size and shape were transferred to 3000-cm³ plastic containers (10 plants per container) containing Hoagland's nutrient solution. After one week, the plants underwent three NaCl treatments: 0 (control), 100 and 200 mM NaCl. Three containers with 10 plants each were used per alfalfa population per salt treatment. All the solutions were kept at a pH 6.5 and aerated throughout the whole experimental period. The amount of water was daily adjusted using distilled water and weekly renewed. After 30 days of salt treatment, the plants were subjected to different physiological measure-

ments and biochemical analyses and were harvested. Each container was considered as a replicate, with three replicates per treatment per population.

DW measurement

For DW determination, shoots and roots were dried in an oven at 70°C for 48 h and weighed. To standardize the data, the results were expressed as the relative reduction of yield in comparison to the control using the following formula [13]:

Relative reduction (%) = [(1− (salinized /control)]×100

RWC

RWC was estimated by recording the turgid weight (TW) of 0.1 g fresh leaflet (FW) samples by maintaining in water for 4 h, followed by drying in a hot air oven until a constant weight was achieved (DW). The RWC was calculated using the following formula [13]:

$$RWC = [(FW-DW) / (TW-DW)]×100$$

Chlorophyll fluorescence (Fv/Fm) measurement

The chlorophyll fluorescence was measured using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England) after 20 min of dark adaptation. Chlorophyll fluorescence was estimated by the F_v/F_m ratio = $(F_m−F_o)/F_m$, which represents the maximum quantum yield of PS II, where F_v is the varietal fluorescence of dark adapted alfalfa leaves and F_m and F_o are the maximal and minimal fluorescence respectively.

Measurement of photosynthetic pigments

Chl (a+b) was extracted with acetone in a mortar, using 200 mg of fresh leaf tissue and 5 mL of acetone (80%, v/v). The Chl concentration was measured by the method described by Arnon [22]. After centrifugation for 10 min at 5000 × g, the absorbance (OD) of the supernatant was measured at 663 and 645 nm. Chl (a+b) were determined using the following formula:

$$Chl(a+b)=8.02OD_{663}+20.20OD_{645}$$

Leaf gas exchange measurement

Photosynthetic activity was estimated by an infrared gas analyzer (LICOR 6400-40; Lincoln, Nebr. USA) after 30 days of treatment. P_N, C_i, g_s and E were determined at an ambient CO_2 concentration of 350 µmol mol^{-1}, temperature of 25±1°C, 60±5% relative humidity and a photon flux density of 1000 µmol m^{-2} s^{-1}.

Lipid peroxidation assessment

Lipid peroxidation was evaluated by malondialdehyde (MDA) accumulation according to the described method [23]. Leaves or roots (100 mg) were crushed in 1.5 mL of TBA at 0.1%. The homogenate was then centrifuged at 10000 × g for 10 min. To the obtained supernatant (1 mL), 1 mL 20% trichloroacetic acid containing 0.5% of TBA was added. The mixture was heated at 95°C for 30 min. The reaction was stopped by immersion of the reaction vessel into an ice bath, followed by centrifugation at 10000 × g for 10 min. The absorbance of the obtained supernatant was determined at 532 nm. Nonspecific absorbance at 600 nm was measured and subtracted from the 532 nm readings. The results were calculated using the molecular extinction coefficient of the complex MDA-TBA: 155 mM^{-1} cm^{-1} and expressed as nmol MDA g^{-1} FW.

Hydrogen peroxide content

The H_2O_2 content in roots and leaves of plants was determined as described [24]. Fresh samples (100 mg) of nodules or leaves were homogenized in an ice bath with 5 mL of 0.1 % TCA. The homogenate was centrifuged at 12000 × g for 15 min and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide, and the absorbance was measured at 390 nm. The H_2O_2 content was expressed as µmol H_2O_2 g^{-1} FW.

Antioxidant potential assessment

Leaves or roots (100 mg) were crushed in 1 mL of phosphate buffer (20 mM, pH 7). The homogenate was centrifuged at 15000 × g for 20 min at 4°C. The obtained supernatant was used for the determination of the POD (EC 1.11.1.7) enzymatic activity according

to [25]. The reaction mixture consisted of 200 μL of H_2O_2 at 0.3%, 300 μL of guaiacol at 20 mM, 2 mL of phosphate buffer (0.1 M, pH 6), 1 mL of distilled water and 10 μL of enzymatic extract. After 3 min, POD activity was determined at 470 nm against a control, where the enzymatic extract was replaced by distilled water. The activity of POD was calculated using the extinction coefficient of guaiacol, 26.6 mM^{-1} cm^{-1}, and expressed as μmol H_2O_2 min^{-1} mg^{-1} prot.

CAT activity

CAT (EC 1.11.1.6) activity was determined as described [26]. The plant material (100 mg of leaves or roots) was homogenized in 1.5 mL of Tris-HCl buffer (pH 8.5), including 2 mm EDTA and 10% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 16000 × g for 14 min at 4°C. The supernatant was used for activity determination by the addition of 250 μL enzyme extract to 2 mL of the assay mixture (50 mM Tris-HCl buffer pH 6.8, containing 5 mM H_2O_2). The reaction was stopped by adding 250 μL of 20% titanic tetrachloride in concentrated HCl (v/v) after 10 min at 20°C. A blank was prepared by the addition of 250 μL of 20% titanium tetrachloride at zero time to stop enzyme activity. Absorbance was read at 415 nm against water. CAT activity was determined by comparing the absorbance against a standard curve of H_2O_2 from 0.25 to 2.5 mM. CAT activity was expressed as μmol H_2O_2 min^{-1} mg^{-1} prot.

SOD activity

SOD (EC 1.15.1.1) activity was determined as described [27]. Fifty μL of crude enzymatic extract in phosphate buffer (20 mM, pH 7) was added to a solution containing 13 mM *L*-methionine, 75μM *p*-nitro blue tetrazolium chloride (NBT), 100 μM EDTA and 2 μM riboflavin in a 50-mM potassium phosphate buffer (pH 7.8). The reaction was performed in assay tubes upon illumination using a 30 W fluorescent lamp at 25°C for 15 min. The blue formazan produced by NBT photoreduction was spectrophotometrically measured at 620 nm. The blank solution had the same complete reaction mixture but was kept in the dark. One SOD unit of activity was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction in comparison to tubes without the plant extract. SOD activity was expressed as enzymatic U min^{-1} mg^{-1} prot.

Membrane potential measurements in root cortex cells

Alfalfa seedlings were grown with or without 200 mM NaCl for one week. The membrane potential in root cortex cells was measured as previously described [28] with slight modification. Five-cm root segments were fixed in a plexiglass chamber filled with a bath solution containing 1 mM $CaCl_2$, 2 mM 2-(N-morpholino) ethanesulfonic acid (MES) /Tris, pH 6 (background solution). To impale cortex cells, a microelectrode was placed at the root surface using a manually operated micromanipulator (Narishige, http://narishige-group. com) and a micro-elevator (IT6D CA1; Microcontrole, http://www.newport.com) was used to adjust the vertical position of the root chamber, allowing precise penetration of the microelectrode into a cortical cell. During impalement, the bathing solution was continuously refreshed. After stabilization of the membrane potential, the solution containing 1 mM $CaCl_2$, 2 mM Mes/Tris pH 6.0, and 100 mM NaCl was added and the membrane potentials were recorded.

Phosphorus (P) and nitrogen (N) contents

The tissue P content was calorimetrically determined using the molybdate blue method [29]. P concentration was measured by reading the absorbance at 820 nm after color development at 100ºC for 10 min. A standard curve was established with KH_2PO_4 solutions. To measure the N content, 0.5g of dry matter was used from every sample. N was measured according to the Tecator and Kejeldal method [30].

Statistical analysis

All statistical analyses were performed with IBM SPSS 21 software. Significant differences between the means were compared by the least significant difference (LSD) test (P<0.05).

RESULTS

Effect of salt stress on dry weight

The data in Table 1 indicate that the treatments with NaCl caused significant (P<0.001) reductions in shoot

and root dry biomass of the studied alfalfa populations in comparison with matching controls (0 mM NaCl). The reductions were more pronounced under the high salinity treatment (200 mM NaCl). All of the tested populations were affected to a significant (P<0.01) degree. The *Tata* population presented the lowest biomass reductions of 24.5% and 33.6% for roots and shoots, respectively, under high salinity (200 mM). However, *Taf 1* exhibited the highest reductions of 41.3% and 44.8%, respectively, under the same conditions. According to ANOVA II, the interaction effect (salinity×population) was significant.

Effect on the RWC

Salt treatment caused a significant (P<0.01) decrease in leaf RWC in the studied alfalfa populations (Table 1). This parameter gradually decreased with the increase in NaCl concentration in the rooting medium. From the control to the high NaCl treatment (200 mM), the variation of RWC values was more pronounced for *Taf 1*, ranging from 84.7% in the control to 76.5% in the 200 mM NaCl treatment. A less important variation was recorded in *Tata*, with 83.2% determined for the control and 80.6% for the 200 mM NaCl treatment. However, according to ANOVA, the population effect was not significant (P>0.05).

Effect on Chl fluorescence (F_v/F_m)

The results presented in Table 1 show that the F_v/F_m ratio was significantly (P<0.001) decreased in the leaves

of both populations subjected to salt stress. The *Tata* population displayed the lowest reduction (5.01%) under the high NaCl treatment. However, *Taf 1* exhibited a more pronounced reduction in value (17.4%) under the same conditions. No significant variation was detected in plants subjected to medium salt stress (100 mM NaCl). ANOVA II indicated that the population and interaction effects were significant (P<0.001; Table 1).

Effect on the total Chl content

Table 1 shows that salinity stress caused a significant decrease in total Chl contents (P<0.001). This reduction was more pronounced at higher NaCl concentrations. Again, the *Taf 1* population was the most negatively affected, possessing a Chl value of 4.02 mg g^{-1} FW, while *Tata* had 5 mg g^{-1} FW.

Effect on leaf gas exchange

The results presented in Table 2 indicate that all of the tested photosynthesis traits were significantly (P<0.001, Table 1) reduced in the two alfalfa genotypes subjected to both stress levels. Under high salinity, the measured traits were significantly decreased (by half) in the *Taf 1* population, as compared to the control (0 mM NaCl). Meanwhile, the reductions were relatively small in the *Tata* population. This population presented the highest P_n, g_s, C_i and E values (18.64 μmol CO_2 m^{-2} s^{-1}, 0.109 mmol H_2O m^{-2} s^{-1}, 97.20 μmol mol^{-1} and 4.63 mmol m^{-2} s^{-1}, respectively) under high salinity (200 mM NaCl).

Table 1. Effect of salt treatments (0, 100 and 200 mM NaCl) on shoot and root growth, RWC and quantum yield of PS II (Fv/Fm) in plants of two Moroccan alfalfa populations, *Taf 1* and *Tata*.

NaCl	Population	Growth reductions (%)		RWC (%)	F_v/F_m	Chl (a+b) mg. g FM^{-1}
		Roots	Shoots			
0 mM	*Taf 1*	-	-	84.7a	0.851a	6.04a
	Tata	-	-	83.2a	0.851a	6.28a
100 mM	*Taf 1*	9.48c	9.23c	82.7a	0.846a	5.74ab
	Tata	7.57c	6.14c	84.1a	0.868a	5.85ab
200 mM	*Taf 1*	41.32a	44.86a	76.5b	0.703c	4.02c
	Tata	24.54b	33.63b	80.6a	0.808b	4.99b
		F	F	F	F	F
Salinity		337.2***	190.6**	10.3**	73.3***	24.8***
Population		17.3**	27.9**	1.61^{NS}	30.56***	4.77*
S×P		5.61*	17.6**	2.37^{NS}	17.6***	1.8^{NS}

The values are means of three replicates. Mean comparisons were performed using the LSD test.

Table 2. Effect of salt treatments (0, 100 and 200 mM NaCl) on P_N, g_s, C_i and E in plants of two Moroccan alfalfa populations, *Taf 1* and *Tata*.

NaCl	Population	P_n μmol CO_2 m^{-2} s^{-1}	g_s mol H_2O m^{-2} s^{-1}	C_i μmol mol^{-1}	E mmol m^{-2} s^{-1}
0 mM	*Taf 1*	30.17a	0.165a	141.67a	6.75a
	Tata	29.57a	0.160a	144.45a	6.93a
100 mM	*Taf 1*	24.30b	0.120c	98.18c	4.72c
	Tata	25.93b	0.135b	120.97b	5.76b
200 mM	*Taf 1*	12.90d	0.079d	71.23d	3.37d
	Tata	18.64c	0.109c	97.20c	4.63c
		F	F	F	F
Salinity		159.01***	140.3***	159.8***	150.9***
Population		11.8**	21.4**	42.4***	36.9***
S×P		7.9**	7.9**	6.7*	5.8*

Values are means of three replicates. Mean comparisons were performed using the LSD test.

Effect on MDA and H_2O_2 contents

To investigate the oxidative damage caused by salt stress, we measured the H_2O_2 and MDA contents in roots and leaves (Table 3). Under normal conditions (0 mM NaCl), H_2O_2 and MDA contents of *Taf 1* and *Tata* were statistically similar. However, their levels were significantly increased in plant organs when salinity was applied (100 and 200 mM NaCl), with significant variations between them. The *Taf 1* population had the highest MDA and H_2O_2 concentrations (24.22 vs 34.88 nmol g^{-1} FM and 0.82 vs 1.18 μmol g^{-1} FM, respectively) in the roots and leaves under high salinity. Indeed, higher values were recorded for both traits in plant roots than in leaves. ANOVA indicates that the interaction effect was significant for the two parameters in both plant organs.

Table 3. Effect of salt treatment (0, 100 and 200 mM NaCl) on MDA and H_2O_2 accumulation in roots and leaves of two Moroccan alfalfa populations, *Taf 1* and *Tata*.

NaCl	Population	MDA nmol g^{-1} Roots	FM Leaves	H_2O_2 μmol g^{-1} Roots	FM Leaves
0 mM	*Taf 1*	9.87d	11.64d	0.35c	0.36c
	Tata	9.56d	12.47d	0.39c	0.39c
100 mM	*Taf 1*	17.42c	22.27c	0.47c	0.51c
	Tata	14.90c	20.10c	0.47c	0.47c
200 mM	*Taf 1*	32.04a	41.96a	1.08a	1.50a
	Tata	24.22b	34.88b	0.82b	1.18b
		F	F	F	F
Salinity		92.3***	271.5***	222.8***	290.3***
Population		10.01**	8.97*	9.68**	9.07*
S × P		3.94*	6.04*	15.28**	8.92**

Values are means of three replicates. Means comparison was performed using the LSD test.

Effect on antioxidant enzyme activities

Exposure to increased saline conditions significantly (P<0.001) increased POD, CAT and SOD activities in root and leaf parts of the two studied populations (Figs. 1, 2 and 3). Significant (P<0.05) variations were detected between the populations according to the LSD test. This was associated with increased NaCl concentrations in the rooting medium. The activity of POD was higher in roots than in the leaves, whereas the activities of CAT and SOD were higher in the leaves. *Tata* exhibited higher POD, CAT and SOD activities of (51.85, 6.85 μmol H_2O_2 min^{-1} mg^{-1} prot, and 5.50 U min^{-1} mg^{-1} prot, respectively) under salt stress than *Taf1* (Figs. 1A, 2A and 3A). In the leaves, the activities were 44.49, 8.11 μmol H_2O_2 min^{-1} mg^{-1} prot, and 6.80 U min^{-1} mg^{-1} prot, respectively, under the same conditions (Fig. 1B, 2B and 3B). The interaction effect was significant (P<0.05) for all of the considered enzymes in both organs.

Membrane potential in root cortex cells

The data in Fig. 4 ilustrates the membrane potential of root cortex cells of two alfalfa populations grown without NaCl stress (A) or under exposure to increased salinity (200 mM NaCl) after one week (B). In the absence of NaCl, the recorded membrane potential was about -140 mV (Fig. 4). The addition of 100 mM NaCl rapidly depolarized the membrane of cortex cells irrespective of the growth conditions (under or unexposed to increased salinity). The membrane potentials were, respectively, -19±4 mV and -17±2 mV for *Taf 1* and *Tata* when the
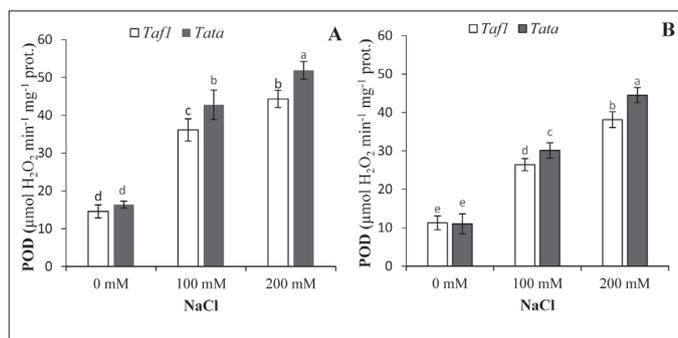
Fig. 1. Effect of salt treatment (0, 100 and 200 mM NaCl) on root (**A**) and shoot (**B**) POD activity in plants from two Moroccan alfalfa populations, *Taf 1* and *Tata*. The results are means of three replicates and the bars are standard errors. Mean comparison was performed using the LSD test.
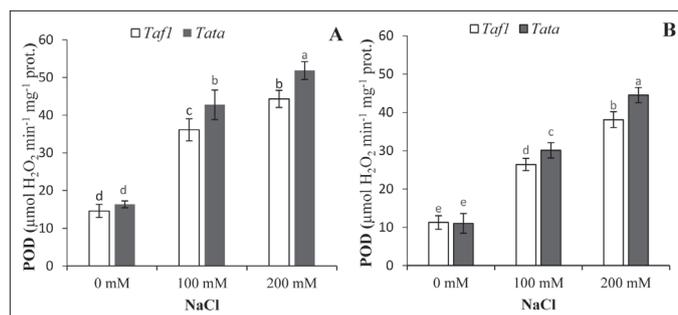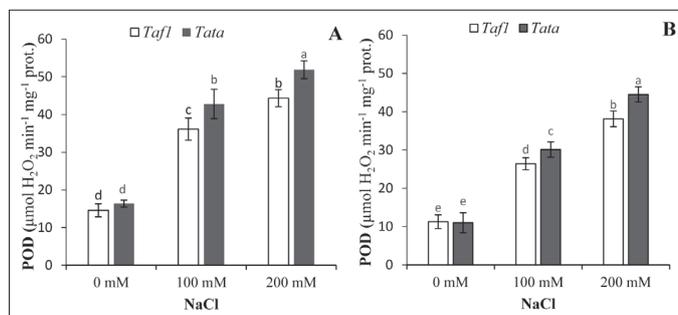

Fig. 2. Effect of salt treatment (0, 100 and 200 mM NaCl) on CAT activity in roots (**A**) and shoots (**B**) of plants from two Moroccan alfalfa populations, *Taf 1* and *Tata*. The results are means of three replicates and the bars are standard errors. Mean comparison was performed using the LSD test.

Fig. 3. Effect of salt treatment (0, 100 and 200 mM NaCl) on SOD activity in roots (**A**) and shoots (**B**) of plants from two Moroccan alfalfa populations, *Taf 1* and *Tata*. The results are means of three replicates and the bars are standard errors. Mean comparison was performed using the LSD test.

plants were grown without salt stress, and when exposed to increased salinity the values were -18±2.08 mV and -13±2 mV, respectively.

Effect on the N and P contents

The data reported in Fig. 5 show that under salt stress there was a significant (P<0.001) decrease in shoot P and N contents in the two alfalfa populations, with significant (P<0.01) variation between them with regard to the N content. The highest N value of 35.66 mg g^{-1} DW was displayed by the *Tata* population under 200 mM NaCl; this value did not exceed 27.13 mg g^{-1} DW in the *Taf 1* population under the same conditions.

The two populations exhibited high P contents under extreme salinity (Fig. 5). However, the *Tata* population had a higher P content of 4.60 mg g^{-1} DW in comparison to *Taf 1*, which presented only 3.38 mg g^{-1} DW under the same conditions. The interaction effect was not significant (P>0.05) for these nutritional elements.

DISCUSSION

In the present study, significant dry biomass reduction was found in the two studied alfalfa genotypes, *Tata* and *Taf 1*, subjected to salt stress. Reduction in plant growth by salt stress has been well documented [1,21,31], although different physiological processes have been mentioned to account for this reduction in different species. The salt tolerance of the two investigated alfalfa populations was associated with the ability of plants to maintain physiological and biochemical processes. Indeed, for almost all of the analyzed parameters in this experiment we found significant variations between the more and the less tolerant populations. Measurements of photosynthesis, chlorophyll fluorescence and chlorophyll content are often used in the evaluation of plant adaptation to different environmental stresses such as salinity and drought [32, 33]. The observed reduction in these parameters clearly reflected the decrease in plant growth. Reduction of P_N mainly resulted from reduced g_s [34]. Stomatal closure causes a decrease in
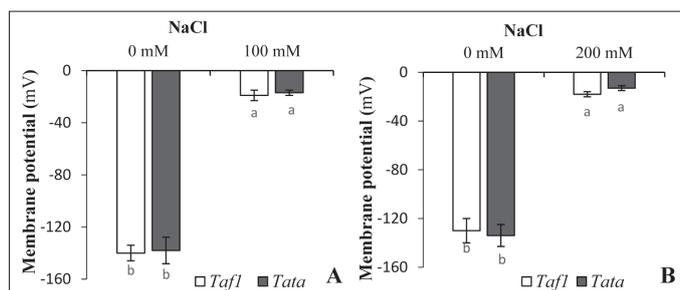
Fig. 4. Variation in membrane potentials in root cortical cells of plants from two Moroccan alfalfa populations, *Taf 1* and *Tata*, grown in the absence of NaCl (**A**) or exposed to 200 mM NaCl for one week (**B**). The resting potential was measured for roots perfused with 1 mM $CaCl_2$, 2mM Mes/Tris pH 6.0. Roots were exposed to the same perfusion solution supplemented with 100 mM NaCl to determine membrane depolarization. The results are means of three replicates and the bars are standard errors. Mean comparison was performed using the LSD test.
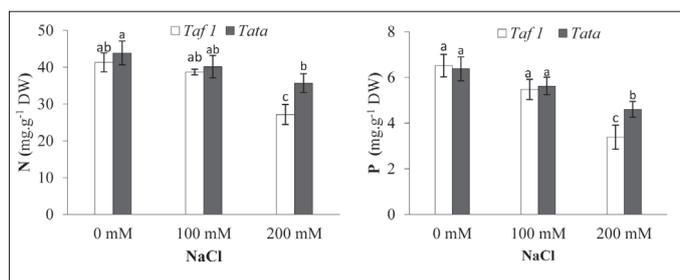

Fig. 5. The effect of salt treatment (0, 100 and 200 mM NaCl) on the N and P contents of plants from two Moroccan alfalfa populations, *Taf 1* and *Tata*. The results are means of three replicates and the bars are standard errors. Mean comparison was performed using the LSD test.

the intracellular concentration of CO_2 by limiting the entry of CO_2 into the leaves, so that less CO_2 is assimilated. Some studies have shown that inhibition of photosynthesis can also result from nonstomatal mechanisms, especially at high salt concentrations [6]. Our results for leaf gas exchange are consistent with those documented previously [8]. A measure of the integrity of the photosynthetic apparatus is the F_v/F_m ratio, a parameter commonly known as maximum quantum yield of primary photochemistry or maximal relative electron transport rate of PS II [35,36] and the total chlorophyll content. As previously mentioned [37], leaf chlorophyll fluorescence responses to increasing salinity were manifested by reduced F_v/F_o and F_v/F_m ratios. A similar observation was reported in [38]. The reduction in the F_v/F_m ratio due to salinity stress is possibly related to a reduction of chlorophyll levels under conditions of increased salinity [9]. Our data indicated that the reduction in PS II efficien-

cy was associated with a decrease in the total chlorophyll content. A decreasing chlorophyll content with increasing salinity of the rooting medium could be related to an increased activity of the chlorophyll degrading chlorophyllase [7], destruction of the chloroplast structure and a greater instability of pigment protein complexes [39]. All these effects reduce the carbon gain if plants are under salt stress and consequently, reduce plant growth [40,41].

The great reduction in plant biomass under conditions of increased salinity is paralleled by reductions in the N and P contents of plants. These elements are key nutrients that affect plant growth. Similar results were reported for *Acacia dealbata* Link. [42]; it was shown that a gradual decrease in the N contents accompanied an increase in the salinity. The decrease in plant total N under increased salinity can be attributed to the effect of the salinity on decreased biosynthesis of protein and/or the decrease in N fixation and/or inhibition in nitrate reductase activity [43].

NaCl treatment caused a sustained depolarization of cortex cells in both populations, whatever the growth conditions in terms of salinity. Membrane depolarization can, at best, provide a qualitative comparison of membrane conductance of Na^+ [44]. Similar results were previously reported in the root cortex of maize [45, 46].

Salt stress increases the formation of ROS through enhanced leakage of electrons to molecular oxygen [47]. ROS cause oxidative damage to different cellular components, including membrane lipids, protein and nucleic acids [48]. The present study showed that the salt stress caused a significant increase in MDA and H_2O_2 in roots and leaves of plants. Determination of the MDA concentration and, hence, the extent of membrane lipid peroxidation, is often used as a tool to assess the severity of oxidative stress caused by abiotic stressors [16, 49]. Similar data with regard to the accumulation of these components under conditions of increased salinity have been reported in many species, including *M. sativa* L. [3], *Arabidopsis thaliana* L. and *Cakile maritima* Scop. [50]. Plants have developed enzymatic process to protect cells from oxidative damage [11]. SOD is the primary ROS scavenger in plants subjected to salt stress, converting O_2^- to H_2O_2

and O$_2$, which leads to the activation of APX and GR that detoxify the remaining products of SOD activity in chloroplasts and the cytosol [18, 51]. CAT can also detoxify the H$_2$O$_2$ to H$_2$O, but it has a lower affinity to H$_2$O$_2$ in comparison to SOD [52]. Our data indicated that salt treatment caused the increase in SOD activity, followed by CAT and POD, especially in the *Tata* population, which seems to be more tolerant to salinity than *Taf1*. Similar results were noted in many plants, including wheat [53] and *M. sativa* L. [3, 16]. Wang et al. [54] reported that H$_2$O$_2$ and lipid peroxidation activate the antioxidant defense mechanisms. Hence, *Tata* plants that had high levels of antioxidant enzyme activities under increased salinity exhibited low H$_2$O$_2$ and MDA contents in their tissues. Our data indicate that antioxidant activities were higher in the roots than in the leaves. This may be explained by the sensitivity of the root system, as it is the first organ to be directly exposed to toxic ions, as well as osmotic stress in the soil [16,55].

CONCLUSION

Salt treatment caused a significant reduction in dry biomass of plants. Growth reduction was associated with perturbations in several physiological and biochemical parameters. Decreases were observed in the relative water content, leaf gas exchange, chlorophyll fluorescence, chlorophyll contents and nutrient concentrations. In contrast, MDA and H$_2$O$_2$ levels were higher under salt stress. The two alfalfa populations responded with different sensitivities to salt stress. The *Tata* population was more tolerant to increased salinity, and this marked tolerance was associated with the ability of plants to maintain adequate levels of the examined parameters and with the ability to overcome oxidative stress by induction of antioxidant enzymes.

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