SEEDS GERMINATION AND SHOOT GROWTH RESPONSES OF A THREATENED POACEAE (*CENCHRUS CILIARIS* L.) TO INCREASING SALT STRESS

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RÉSUMÉ.— *Réponse à un stress salin croissant de la germination des graines et de la croissance des pousses d'une poacée menacée,* Cenchrus ciliaris *L.— Cenchrus ciliaris* L. (Cenchrus cilié) est une poacée pérenne cataloguée parmi les espèces menacées dans les régions arides de la Tunisie. Dans le présent travail, l'implication de la salinisation des sols dans la rareté de cette espèce est évaluée par l'étude de la germination des graines et la croissance de la partie aérienne sous stress NaCl (0, 50, 100, 200 et 300 mM). Nos résultats montrent qu'au cours d'un stress salin modéré (50 et 100 mM NaCl), *C. ciliaris* manifeste une bonne capacité germinative (CG), un temps moyen de germination adéquat (TMG) et une reprise de la germination (13 %) après le transfert de semences à l'eau distillée. La production de biomasse de la partie aérienne est réduite de 50 % pour les traitements 50 et 100 mM NaCl par rapport au témoin, sans toutefois induire l'oxydation des lipides, ni la déshydratation des tissus. L'induction de l'activité gaïacol peroxydase (GPX, EC. 1.11.1.7) semble être efficace dans la lutte contre le stress oxydatif éventuellement provoqué par les doses 50 et 100 mM NaCl. Pour un stress salin sévère (200 et 300 mM NaCl), on constate une augmentation de la dormance des graines, une diminution de la CG, une augmentation du TMG et une faible reprise de la germination après transfert à l'eau distillée. Bien que la production de biomasse aérienne se maintienne à 50 % par rapport au témoin, on a mesuré une augmentation des teneurs en malonydialdehyde (MDA), suggérant que la GPX n'est plus efficace pour lutter contre l'oxydation des lipides membranaires. Il semble qu'une salinisation du sol supérieure à 100 mM NaCl peut contribuer à la raréfaction de *C. ciliaris* par une diminution de la capacité germinative et de la croissance et de l'implantation des jeunes plants.

SUMMARY.— *Cenchrus ciliaris* L. (Buffel Grass), a perennial Poaceae, is a threatened species in arid regions of Tunisia. In the present work, involvement of soil salinization on its scarcity is evaluated through studying seeds germination and shoot growth ability under NaCl stress (0, 50, 100, 200 and 300 mM). Our results showed that at moderate stress including 50 and 100 mM NaCl, *C. ciliaris* sustained sufficient germination capacity (GC), adequate germination mean time (GMT) and recovery aptitude (13 %) after transferring seeds to distilled water. Shoot growth was reduced to 50 % by 50 and 100 mM NaCl treatments relative to control, inducing neither lipid oxidation nor tissues dehydration. Salt-induced stimulation of gaïacol peroxidase (GPX, EC. 1.11.1.7) activity seemed to be efficient against oxidative stress. Severe stress, including 200 and 300 mM NaCl, lengthened seeds dormancy, decreased GC and germination rate (increasing GMT) with low germination recovery. While plant growth was not severely affected, increasing malonydialdehyde (MDA) production indicated that 200 and 300 mM NaCl provoked lipid oxidation and that GPX could no longer overcome oxidative stress. It seems that soil salinization with doses greater than 100 mM NaCl may contribute to *C. ciliaris* scarcity by lowering germination capacity and seedlings growth and establishment.

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Increasing soil salinization led to a progressive loss of crop and plant biodiversity in many regions, mainly in arid and semi-arid countries where salt water is largely used for irrigation. A gradual degradation of Poaceae flora is observed in all arid natural ecosystems of Tunisia (Ferchichi, 1999). The warning of scarcity or even extinction of some species has been reported (M'Seddi *et al.,* 2002; Minif *et al.,* 2005). For instance, *Cenchrus ciliaris* L. (Buffel Grass) is a seriously endangered species in arid regions of Tunisia (Minif *et al.,* 2005). It is very palatable mainly by sheep and goats and has a high nutritional value at all phenological stages; it is consumed even after drying and deemed to increase milk production in sheep (Neffati & Akrimi, 1991). Besides its pastoral importance, *C. ciliaris* is an excellent fixing soil plant. Gyasi-Agyei *et al.* (2005) argued that this species plays an important role in protecting soil against erosion thanks to its root fascicle with many sub-horizontal roots in the soil surface.

Currently, the threat to *C. ciliaris* was attributed to overgrazing and prolonged drought period. In Australia, development disruption of this species was attributed to low germination rate and the prolongation of seed dormancy (Bhattaraia *et al.,* 2008). However, the hypothesis of involvement of other abiotic factors in the scarcity of *C. ciliaris* is not rejected. Specifically, the progressive salinization of several sites in arid zone of Tunisia due to uncontrolled irrigation may cause degradation of *C. ciliaris* cover. Irrigation water generally comes from wells with salinity ranging between 4 and 7 g/L due to overexploitation or intrusion of brackish water or seawater. The concentration of dissolved salts in irrigation water tends to increase in soil under evaporation during the dry season.

In our study area, cultivated or spontaneous vegetation is exposed to salt stress originating from saline irrigation water and natural saline charge in soil. The region is arid to semi-arid with a Mediterranean climate, characterized by irregular rainfall and a severe summer drought. Salt excess in soil inhibits growth and reduces yield of many plant species. It is usually assumed that plant growth inhibition under salt-stress is associated with altered water relations (osmotic effects), specific ion effects (excess or deficiency) or energy availability (carbohydrates) (Munns, 1993). Salt stress affects plant establishment at germination stage (Sosa *et al.,* 2005; Gorai & Neffati, 2007), growth activity and cellular levels through osmotic and ionic stress (Hasegawa *et al.,* 2000; Murphy & Duraka*,* 2003).

In this paper, we attempt to evaluate the involvement of soil salinization on *C. ciliaris* scarcity through studying the effect of increasing NaCl doses on seeds germination and shoots growth.

MATERIALS AND METHODS

GERMINATION EXPERIMENTS

Cenchrus ciliaris seeds were harvested from plants that grew spontaneously in the South-East of Tunisia (33° 29' 93» N, 10° 38' 70» E). Seeds were sterilized with H_2O_2 2.5 % for 5 min and subsequently washed with distilled water before being used in the germination experiments to avoid fungus attack (Gulzar *et al.,* 2001). Seeds were germinated in Petri dishes containing two disks of Whatman No. 1 filter papers with 5 mL of distilled water (0) or saline solutions (50, 100, 200 and 300 mM NaCl). A completely randomized design was used in the germination tests at room temperature. For each treatment, three replicates of 25 seeds each were used. During 20 days, the germinated seeds were counted and removed every 2 days (El-Keblawy & Al-Rawai, 2005). A seed was considered to have germinated when the emerging radical elongated to 2 mm. Germination was considered complete when no further germination occurred in 2 successive days.

Three characteristics of germination were determined: germination capacity (GC) which is the germination percentage calculated as GC = (Σ n)*100/N, with (Σ n) as the cumulative number of germinated seeds and N as the total number of seeds. The delay of germination is the number of days to first germination. The mean time to germinate (MTG) was estimated according to the formula: MTG = Σ (ni x di)/N, where n is the number of seeds germinated at day i, d is the incubation period in days and N the total number of germinated seeds in the treatment (Brenchley & Probert, 1998).

The recovery percentage was determined by the following formula: (a–b) x 100/(c–b), where a is the total number of seeds germinating after being transferred to distilled water, b is the total number of seeds germinating in saline solution, and c is the total number of seeds (Tlig *et al.,* 2008).

GROWTH EXPERIMENTS

Seeds of *Cenchrus ciliaris* were sown in plastic pots (5 plants / pot) at a depth of 1 cm and 2 cm spaced. Culture was conducted in a glass greenhouse at a temperature of 25 °C and a photoperiod of 10 h (natural light). Seedlings

were regularly (every 2 days) irrigated with rainwater. After a month of culture on control medium, the different salt treatments (0, 50, 100, 200, 300 mM NaCl) were applied for a week. At harvest, shoots fresh weigh (FW) was rapidly determined and then samples were placed in an oven set at 65 °C for 48 hours for the dry weigh (DW) determination.

The water content (WC) was calculated by the following formula: WC (%) = (FW-DW)*100/FW.

METABOLITES ASSAYS

Chlorophyll and soluble protein contents

Chlorophyll (Chl) contents were determined by the method of Arnon (1949). The absorbance of a sample was read at 460 nm, 645 nm and 663 nm, then contents of Chl a, Chl b and carotenoïds (cart) were calculated using the formulas of MacKinney (1941). Soluble protein was determined using a commercially kit (Coomassie Protein assay reagent, Bio-Rad).

Malonydialdehyde (MDA) contents

The level of lipid peroxidation in leaves and roots was assessed in terms of malonydialdehyde (MDA) content by thiobarbituric acid (TBA), as recommended by Heath & Parcker (1968), with minor modifications following Dhindsa *et al.* (1981). Fresh samples were homogenized in trichloroacetic acid (TCA) (0.1 % p/v). The homogenate was then centrifuged at 8000 g for 15 min. The supernatant (1 ml) was then precipitated with 4 mL 20 % TCA containing TBA (0.5 % p/v). The mixture was heated in a water bath shaker at 95 °C for 30 min and quickly cooled in an ice bath. Samples were centrifuged at 8000 g for 10 min, then the absorbance was measured at 532 nm and the value for non-specific absorption at 600 nm was subtracted. The MDA content was calculated using its extinction coefficient $\varepsilon = 155 mM^{-1} cm^{-1}$.

Proline contents

Proline was determined by the method of Bates *et al.* (1973). Plant tissue (0.5 g) was homogenized with 5 mL of 3 % aqueous sulfosalicylic acid and then the homogenate was centrifuged at 14 000 g for 2 min. Two millilitres of acid ninhydrin and 2 mL of glacial acetic acid were added into 2 mL of the homogenate in a test tube. The mixture was then incubated at 100 °C for 1 h, after which the reaction was stopped by placing the test tube in an ice bath. Four millilitres of toluene were added to each test tube and vortexed for 15-20 s. The organic and inorganic phases were separated, and the absorbance at 520 nm of the organic toluene phase containing the chromophore was used to quantity the amount of proline.

ENZYMES ASSAYS

Gaïacol peroxydase activity (GPX)

Gaïacol peroxidase activity (GPX) was assayed in a solution containing gaïacol (0.05 % v/v), H_2O_2 (10 mM), and sodium phosphate buffer (50 mM, pH 7.0) (Cakmak, 1994). The increase in absorbance was measured at 470 nm and the activity was calculated using an extinction coefficient $\varepsilon = 25.2 mM^{-1} cm^{-1}$. Gaïacol peroxidase activity was expressed as µmol of gaïacol consumed g^{-1} FW min^{-1}.

STATISTICAL ANALYSIS

The data presented in this work are the average of at least three replicates per treatment and means ± SD, are given in the figures. Each experiment was conducted in duplicate. The differences between means were calculated using Tukey test at $P < 0.05$ level by a Statistica 6.0 software.

RESULTS

GERMINATION

Seeds germination is a primordial step that determines plant establishment and sustainment, particularly in saline soils. Salt excess in soil adversely affected germination by decreasing water potential which altered seeds imbibitions and consequently reduced the germination capacity.

The germination capacity (GC) is the percentage of germinated seeds ratioed to the total number of seeds. Far from salt treatment, less than the half (40 %) of *C. ciliaris* seeds were able to germinate and about 60 % of seeds were dormant (Tab. I).

TABLE I

Changes in germination parameters of Cenchrus ciliaris *seeds treated with increasing NaCl doses (0, 50, 100, 200 and 300 mM). Each point is the average of three replications ± SD. Means sharing at least one same letter are not significantly different according to Tukey test at P < 0.05*

	Germination (%)	Mean Time to Germinate (MTG)	Delay of germination (day)	Recovery of Germination (%)
Control	40.00a	9.67a	04	-
50 mM	37.34a	12.80b	04	06.93a
100 mM	30.00b	16.00bc	06	12.52b
200 mM	5.33c	23.25cd	14	12.17b
300 mM	3.40d	22.13d	20	04.00c

Increasing salt concentration in Petri dishes led to a progressive decrease in GC of *C. ciliaris* seeds. This decrease became significant at 100 mM NaCl (at $P < 0.05$), while 50 mM treatment did not affect the CG (Tab. I). The GC was reduced compared to control by about 25 %, 85 % and 90 % at 100, 200 and 300 mM NaCl treatments respectively (Tab. I). Besides, *C. ciliaris* seeds germination was delayed by severe stat stress. Seed germination was observed only after 14 and 20 days at 200 and 300 mM NaCl treatments, respectively (Tab. I).

MEAN TIME TO GERMINATION (MTG)

To better understand the effect of salinity on the *C. ciliaris* seeds germination, we determined the mean time to germinate (MTG). Increasing salt concentration in the external medium resulted in a gradual increase of MTG (Tab. I). The MTG was twofold higher than control, reflecting a 50 % reduction in the germination rate at 200 and 300 mM NaCl treatments.

RECOVERY PERCENTAGE OF GERMINATION

To determine the recovery percentage of germination, seeds previously treated with different doses of NaCl were transferred to distilled water. Our results showed that un-germinated seeds partially recovered at all salt treatments (Tab. I). The highest recovery percentage (12.52 %) was recorded at 100 mM NaCl treatment (Tab. I). The recovery percentage did not exceed 6 % for the highest salt treatments.

BIOMASS AND WATER CONTENT

Irrespective of salt dose, shoot fresh weight (FW) and dry weight (DW) of *C. ciliaris* were reduced to 50 % compared to control (Fig. 1A, B). Growth reduction was not accentuated by increasing salt concentration in the external medium. This may be related to an unchanged number of leaves at all salt treatments (unpublished results).

Calculation of the relative water content showed that *C. ciliaris* shoot sustained adequate hydration, even at high salt stress (Fig. 1C).

CHLOROPHYLL

Increasing the dose of NaCl in the external medium was associated with a gradual drop in total chlorophyll (Chl) content (Fig. 1D). This decrease was related to a decrease in Chl a and carotenoïds (Carot) contents by salt stress. When plants were treated with 300 mM NaCl, the levels of Chl a and Carot were respectively reduced by 35 % and 45 % compared to the control (Fig. 1D).

SOLUBLE PROTEIN CONTENT

Soluble protein contents were not significantly affected by all NaCl treatment (Fig. 2A). The decrease in soluble protein contents was approximately 50 % compared to the control (Fig. 2A).

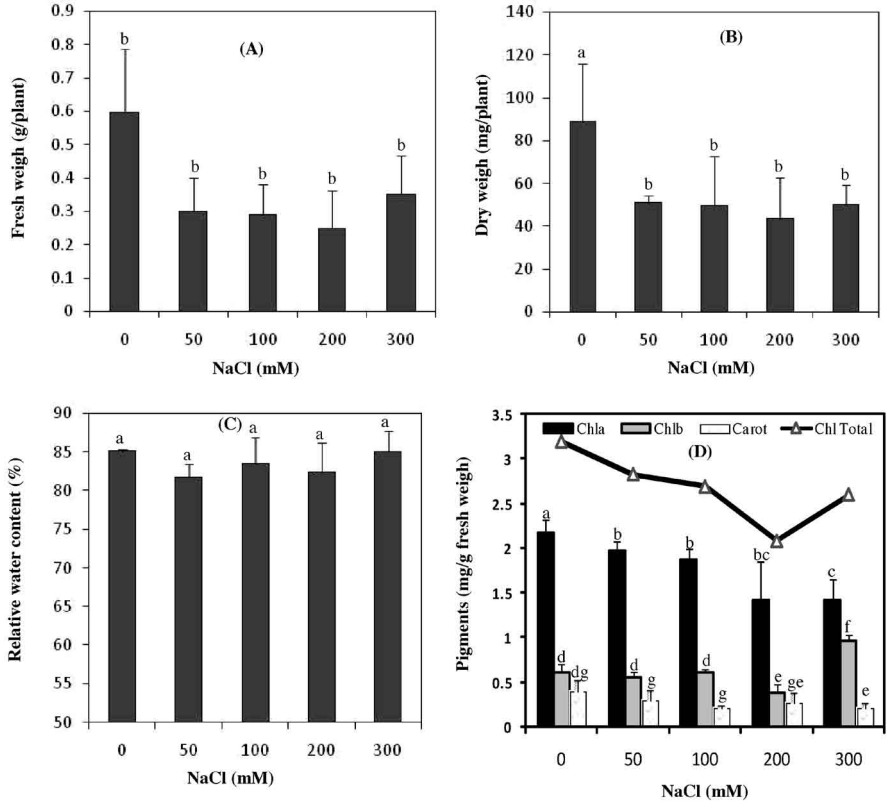

Figure 1.— Changes in (A) fresh weigh (FW), (B) dry weigh (DW), (C) relative water content (RWC) and (D) pigments contents (Chl, chlorophyll and carot, carotenoïds) in the shoots of *Cenchrus ciliaris* treated with increasing NaCl doses (0, 50, 100, 200 and 300 mM) during 07 days. Each point is the average of three replications ± SD. Means sharing at least one same letter are not significantly different according to Tukey test at $P < 0.05$.

PROLINE CONTENT

Proline (Pro) contents in the shoots showed control values when *C. ciliaris* was treated with 50 mM NaCl (Fig. 2B). Beyond this dose, Pro contents reached values about 2 times higher than control (Fig. 2B).

The products of lipid peroxidation which react with acid thiobarbiturique are termed TBARS (Thiobarbituric acid reacting substances) and are characteristic of aldehydes, mainly malonydialdehyde (MDA), products of monohydroperoxydation and secondary oxidation of lipids. The content of MDA is considered as a marker to estimate the extent of oxidation of membrane lipids and damage caused by stress. According to figure 2C, it appeared that 50 mM and 100 mM NaCl treatments did not induce membrane lipids oxidation in the shoots of *C. ciliaris*. The production of MDA significantly increased by 45 % and 20 % relative to control at 200 mM and 300 mM NaCl treatments, respectively (Fig. 2C).

PEROXIDASE ACTIVITY

In response to stress conditions, free radicals generation is generally accelerated in plant tissues. These radicals i.e., hydrogen peroxide (H_2O_2), provoke harmful damage to biological systems (protein, lipid, DNA) by the formation of other organic peroxides propagated through

typical radical chain reaction. One of the mechanisms that scavenge H_2O_2 is peroxidases. These enzymes can use a wide range of electron donors, e.g. gaïacol; therefore, they are referred as gaïacol peroxidase (GPX) (Hegedus *et al.,* 2001). The initial step in the catalytic mechanism of a peroxidase is heterolysis of the oxygen-oxygen bond of hydrogen peroxide.

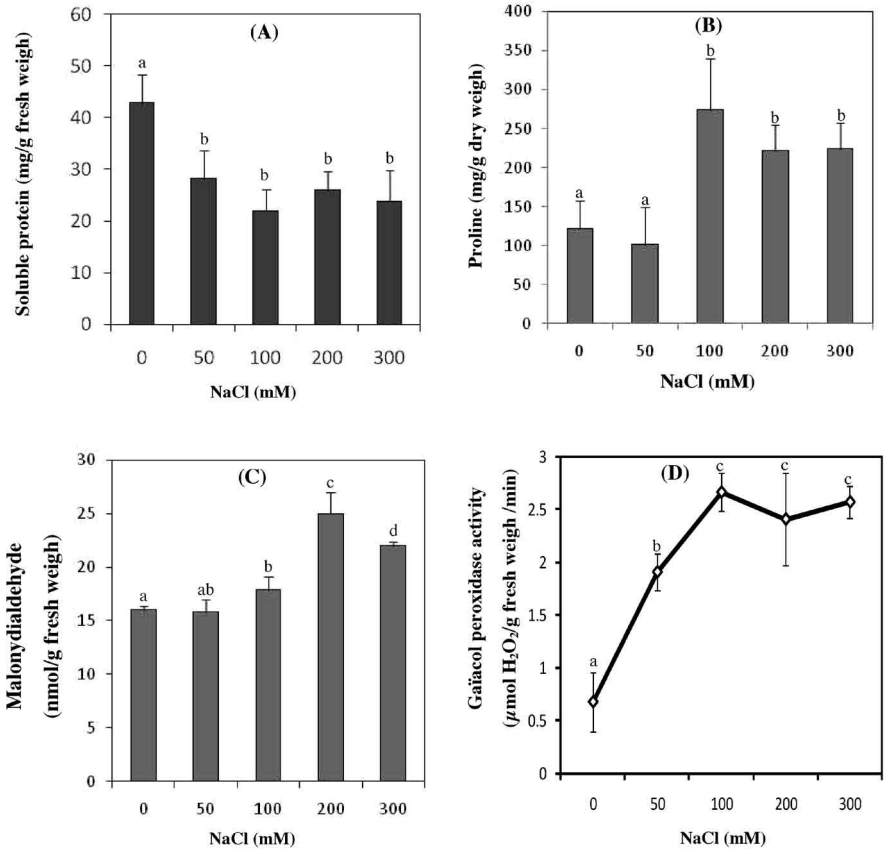

Figure 2.— Changes in (A) soluble protein (mg/g fresh weigh), (B) proline (mg/g dry weigh), (C) malonydialdehyde (nmol/g fresh weigh) and (D) gaïacol peroxidase activity (µmol H_{202}/g fresh weigh /min) in the shoots of *Cenchrus ciliaris* treated with increasing NaCl doses (0, 50, 100, 200 and 300 mM) during 07 days. Each point is the average of three replications ± SD. Means sharing at least one same letter are not significantly different according to Tukey test at $P < 0.05$. Antioxidant activity: malonydialdehyde content (MDA)

Our results showed that GPX activity was rapidly stimulated in *C. ciliaris* shoots following exposure to salt stress (Fig. 2D). This stimulation was proportional to the increasing salt stress in the medium until 100 mM dose. For this treatment, GPX activity was approximately 5 times greater than control (Fig. 2D). Then, GPX activity was unchanged despite the increase of NaCl concentration in the medium (200 and 300 mM).

DISCUSSION

Studying changes in germination parameters, we stated that even at control conditions dormancy is the limiting factor for *C. ciliaris* establishment; about 60 % of seeds were dormant (Tab. I). Germination is one of the most salt-sensitive plant growth stages and is seve-

rely inhibited with increasing salinity both in glycophytes and halophytes (Sosa *et al.,* 2005). Here, imbibitions of seeds with NaCl-enriched solutions lowered germination capacity (GC), delayed germination and slowed germination rate (Tab. I). Decreasing germination capacity by salt was already reported either for halophytes (Gorai & Neffati, 2007). This decrease is due to both osmotic and toxic effects of NaCl (Katambe *et al.,* 1998; Song *et al.,* 2005). High salt concentrations (200 and 300 mM NaCl) lowered water potential which makes difficult seed imbibitions (Francios *et al.,* 1986). Besides, salt ions may eventually inhibit metabolic pathways involved in the remobilization of reserves during seed germination (Dirik, 2000).

Nevertheless, germination of *C. ciliaris* seeds was not totally abolished even at highest salt treatment (Tab. I). Further, transferring seeds to distilled water was associated with an early recovery of germination (Tab. I). Indeed, even the seeds of halophytes (i.e *Reaumuria vermiculata*) were unable to germinate under similar conditions of salinity (Gorai & Neffati, 2007). Tlig *et al.* (2008) showed that the germination of *Diplotaxis harra* seeds was completely abolished in the presence of 200 and 300 mM NaCl.

Our findings indicate that under salinization, a fraction (6 to 12 %) of *C. ciliaris* seeds can survive in soil to form a «seed stock» which will germinate when optimal conditions are met (rain during fresh season). The larger fraction (more than 85 %) included un-germinated seeds that NaCl stress lengthened dormancy and/or provoked their mortality by inducing irreversible damages.

Notwithstanding, *C. ciliaris* succeeded to germinate at moderate salinity (50 and 100 mM NaCl); we have no available data about its response to salinity at vegetative growth stage. We found that this Poaceae showed a glycophytic behaviour displayed by reduced biomass production (Fig. 1A, B), chlorophyll (Fig. 1D) and soluble protein contents (Fig. 2A). Remarkably, shoot growth dropped to 50 % with sustain of sufficient hydration relative to control despite increasing salt stress from 50 to 300 mM NaCl (Fig. 1A, B). It appears that *C. ciliaris* can manage the excess of salt in its environment to limit the adverse effects that severe NaCl dose may cause. The reduced growth activity is a strategy that allows plant to minimize water and mineral nutrition under limiting conditions. Depending on the species, this physiological adaptation may be a "survival state" until the return of favourable conditions. This state is likely to be regulated by physiological and biochemical mechanisms developed by plants under salt stress. According to our results, maintaining such balance would be linked to an accumulation of Pro (Fig. 2B) and the unchanged chlorophyll (Fig. 1D) and soluble proteins (Fig. 2A) in shoots despite increasing external salt concentration. Also, the decline in Chl a levels was apparently balanced by an increase in that of Chl b when plants were treated with 200 mM NaCl (Fig. 1D). The accumulation of Pro is one of the symptoms commonly reported in plants under abiotic stress conditions, although its precise role still remains a controversial subject. Cytoplasmic accumulation of this Pro is thought to be involved in osmotic adjustment of stressed tissues (Delauney & Verma, 1993). Proline is also considered to be involved in the protection of enzymes (Solomon *et al.,* 1994) and cellular structures (Van Rensburg *et al.,* 1993) and to act as a free radical scavenger (Prasad & Saradhi, 1995). According to Bellinger & Lahrer (1987), the Pro increase is a protective response of plants to all the factors that lead to a decrease in water of the cytoplasm. These authors showed that the synthesis of Pro may be involved in the regulation of cytoplasmic pH and membrane proteins stabilization. Pro biosynthesis could be associated with the regulation of cytosolic pH or with the production of $NADP^+$ for the stimulation of the pentose phosphate pathway (Hagedorn & Phang, 1986). Ueda *et al.* (2007) suggested that free Pro presumably behaved as a component of cell wall synthesis in the apical region of barley roots under salt stress.

According to the MDA contents (Fig. 2C), *C. ciliaris* succeeded to maintain membrane integrity up to 100 mM NaCl treatment. This situation suggests that this plant would have a system ensuring the integrity of membrane lipids under conditions of moderate salt stress. This is usually the function of antioxidant enzymes that protect cells against free radicals generated by the salt stress. The peroxidases are among the antioxidant enzymes involved in the elimination of hydrogen peroxide which is reputed cytotoxic (Gara *et al.,* 2003; Mittler, 2002).

The gaïacol peroxidase activity (GPX) was tested *in vitro* on leaf extracts of *C. ciliaris* under different salt treatments. Our results showed that increasing salinity was associated with a progressive stimulation of GPX activity until the dose 100 mM NaCl. Thereafter, GPX activity was maintained at a constant level even when the plants were treated with 200 and 300 mM NaCl (Fig. 2D). The concomitant GPX stimulation (Fig. 2D) and the lack of MDA production until the dose 100 mM NaCl (Fig. 2C) suggested that GPX protects leaf tissue and ensures cell membrane integrity by neutralizing H_2O_2 generated under salt stress. Indeed, Liu & Huang (2000) found that MDA production is tightly controlled by peroxidases activities. Esfandiari *et al.* (2007) showed that in two wheat varieties, tolerance to salt stress is closely related to the elimination of reactive oxygen species (ROS) by peroxidase, catalase and superoxide dismutase. The increase in GPX activity was due to salt-induced expression of peroxidases isoforms (Abd El-Baky *et al.,* 2003) and this expression probably reaches its maximum beyond 100 mM NaCl treatment (Fig. 2D).

CONCLUSION

We can conclude that *C. ciliaris* is a tolerant Poacae at moderate salinity (50 and 100 mM NaCl). This tolerance was related to an ability of seeds to germinate even at high doses of salt, maintain of water status and sufficient antioxidative system. However, at severe NaCl doses (200 and 300 mM NaCl), salt-sensitivity traits appeared (low germination, lipid peroxidation), indicating that soil salinization may contribute to *C. ciliaris* scarcity in the South-East of Tunisia.

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