

Effects of salinity on growth, proteins and antioxidant enzymes in three *Acanthophyllum* species of different ploidy levels

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Abstract

The effects of salinity on some growth parameters, protein content and antioxidant enzymes were studied in three *Acanthophyllum* species of different ploidy levels including *A. laxiusculum* Shiman-Czeika (diploid species with $2n = 30$), *A. sordidum* Bunge ex Boiss. (tetraploid species with $2n = 60$) and *A. glandulosum* Bunge ex Boiss. (hexaploid species with $2n = 90$). Seedlings of the species were subjected to NaCl stress (50, 100, 150 and 200 mM) for 40 days. Salinity affected the growth parameters and caused a reduction in germination percentage, relative growth rate (RGR) and relative water content (RWC) with a greater reduction in *A. laxiusculum*. However, salinity stress caused only slight decrease in RGR and RWC of *A. glandulosum* and *A. sordidum*. Protein content in both *A. laxiusculum* and *A. sordidum* increased up to 150 mM NaCl, but this increase in *A. glandulosum* occurred at 150 and 200 mM NaCl. *A. laxiusculum* exhibited a decrease in peroxidase (POX) and polyphenol oxidase (PPO) under NaCl stress; while *A. glandulosum* showed a remarkable increase in POX and PPO between 50 to 200 mM NaCl. In *A. sordidum*, POX and PPO activities increased at 50 mM NaCl and then decreased at higher salinities. The obtained results showed that the differences in the antioxidant enzyme activities of seedling may, at least in part explain the greater tolerance of *A. glandulosum* comparing to *A. sordidum* and *A. laxiusculum*. According to our results, *A. glandulosum* (hexaploid species) showed a better protection mechanism against salinity induced oxidative damage than *A. sordidum* (tetraploid species).

Keywords: *Acanthophyllum*, Antioxidative enzymes, Salt stress, Seed germination, Ploidy level.

1. Introduction

Because of severe damages of salinity on plant growth and development (Sairam & Tyagi 2004), considerable attempts have been made in discovering physiological and biochemical processes contributing in adaptation to salinity in plant (Ashraf & Harris 2004; Sreenivasulu *et al.*, 1999). Some of these processes include osmotic adjustment by accumulation of compatible solutes (such as proline, polyols, amino acids and proteins) and regulatory mechanism for ion transport (Neto *et al.*, 2006; Ashraf & Harris 2004; Agarwal & Pandey, 2004). However, excess salt concentrations cause enhanced generation of reactive oxygen species (ROS) in plants (Muscolo *et al.*, 2003; Kim *et al.*, 2005). These ROS can seriously cause oxidative damages by disrupting of lipids, proteins and nucleic acids (Ghorbanli *et al.*, 2004). In contrast, plants are equipped with a diverse array of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and polyphenol oxidase (PPO) against oxidative

damages by ROS (Vaidyanathan *et al.*, 2003; Agarwal & Pandey, 2004; Mittova *et al.*, 2004). Moreover, increasing in the POX enzyme activity in responses to salinity is reported (Neto *et al.*, 2006; Sreenivasulu *et al.*, 1999; Siegel 1993). *Acanthophyllum* is a genus with a total of 61 species in the world. Of these 33 species occur in Iran of which 23 species are endemic (Ghaffari 2004). Besides their obvious interest as naturally salt-tolerant plants, these species show considerable economical and medicinal potentials. Many species of *Acanthophyllum* are interesting as potential sources of different bioactive substances such as saponins and polysaccharides that could be used for medicinal purposes (e.g. Gaidi *et al.*, 2004). According to important role of ploidy in *Acanthophyllum* evolution and speciation (Ghaffari 2004), the main objectives of this work were to examine whether the ploidy level is involved in salinity tolerance and antioxidative responses. Therefore, we evaluated the effect of salt stress on growth parameters, proteins and antioxidant

enzymes in three species of *Acanthophyllum* with different ploidy levels.

2. Materials and Methods

Seeds of three *Acanthophyllum* species including *A. laxiusculum* Shiman-Czeika (diploid species with $2n = 30$), *A. sordidum* Bunge ex Boiss. (tetraploid species with $2n = 60$) and *A. glandulosum* Bunge ex Boiss. (hexaploid species with $2n = 90$) were collected from various regions of Tehran, Iran. Taxonomic and locality data of the materials used for the studies are listed in Tab. 1. Seeds were surface sterilized with 90% ethanol for 1 min and followed by washing with distilled water and germinated on MS medium (Murashige & Skoog 1962) supplemented by different concentrations of NaCl (50, 100, 150 and 200 mM) for 40 days. MS medium without NaCl was used as control. Three seedlings from each treatment were taken to measure seed germination. Relative growth rate (RGR) was calculated according to Beadle (1993). Relative water content (RWC) was calculated according to equation $RWC (\%) = [(FW-DW)/(TW-DW)] \times 100$, where FW is fresh weight, DW is dry weight and TW is turgid weight, according to Meloni et al., (2004). Plant growth was estimated by measuring seedling dry weight after drying the plant materials at 70 °C for 48 h. The degree of stress was assessed according to Hsiao (1973), in which a loss of 8%–10% RWC was set as a mild stress, a loss of 10%–20% moderate stress, above 20% severe stress. For extraction of crude proteins for protein determination and enzyme assay, leaf samples were extracted by 50 mM Tris-HCl buffer (pH 6.8). The extract was centrifuged at $16\,000 \times g$ for 30 min at 4 °C and the resulting supernatant was used as the crude extract. Protein content was

evaluated by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. Electrophoretic patterns of protein were identified by SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) using 12% acrylamide gels. The gels were stained with Coomassie Brilliant Blue R 250. Peroxidase (POX; E.C. 1.11.1.7) activity was measured according to the method of Abeles and Biles (1991). The assay mixture consisted of 4 cm³ of 0.2 M acetate buffer (pH 4.8), 0.4 cm³ H₂O₂ (3 %), 0.2 cm³ 20 mM benzidine and 0.05 cm³ enzyme extract. The absorbance was recorded at 530 nm. Polyphenol oxidase (PPO; E.C. 1.10.3.1) activity was estimated following the method of Raymond et al., (1993) by measuring the increase in absorbance at 430 nm. The reaction mixture contained 2.5 cm³ 200 mM sodium phosphate buffer (pH 6.8), 0.2 cm³ pyrogallol 20 mM and 0.1 cm³ enzyme extract. The temperature of the reaction mixture was 40 °C. Non denaturing polyacrylamide gel electrophoresis (PAGE) was performed according to modified method of Davis (1964) with a 10 % acrylamide gel at 4 °C. The electrophoretic run was carried out with 120 mV for 2 h. Electrophoretic pattern of POX was obtained by staining the gels by benzidine according to Van Loon (1971). The gels immersed in 0.2 M acetate buffer (pH 4.8) containing 3 % H₂O₂ and 4 % benzidine in 50 % methanol at room temperature until the brown color appeared. The data determined in triplicate were analyzed by analysis of variance (ANOVA) using SPSS (version 9.05). The significance of differences was determined according to Duncan's Multiple Range Test (DMRT). *P* values < 0.05 are considered to be significant.

Table 1- Taxonomic and locality data of *Acanthophyllum* species used for the analyses.

<i>Species</i>	<i>Locality</i>	<i>Distribution</i>
<i>A. laxiusculum</i> Shiman-Czeika	Tehran: Garmsar; Behbar	Iran, Afghanistan, Turkmenistan
<i>A. sordidum</i> Bunge ex Boiss.	Tehran: 60 Km toward Goum	Iran, Afghanistan, Pakistan, Turkmenistan
<i>A. glandulosum</i> Bunge ex	Tehran: Damavand, 40 Km toward	Iran, Afghanistan , Turkmenistan,

3. Results and discussion

NaCl stress caused a significant decrease in germination percentage, with a greater reduction in *A. laxiusculum*, so that at 200 mM NaCl approximately no germination was observed (Fig.

1A). These results are similar to Agarwal and Pandey (2004) in *Cassia angustifolia*. The relative growth rate (RGR) in three *Acanthophyllum* species subjected to salinity stress, based on leaf dry weight, is showed in Figure 1B. In spite of the reduction in

RGR which observed in all the examined *Acanthophyllum* species, the reduction in growth rate varied significantly among the species. Our results showed that *A. laxiusculum* and *A. sordidum* growth was decreased with increasing of salinity, but reduction was more pronounced in *A. laxiusculum* than that of *A. sordidum*, so that *A. laxiusculum* growth approximately inhibited at 200 mM NaCl. However, growth rate of *A. glandulosum* reduced slightly under high NaCl concentrations

that can reflecting the greater salt tolerance of *A. glandulosum* compared to *A. sordidum* and *A. laxiusculum*. Similar results were reported in *Oryza sativa* (Dionisio-Sese & Tobita, 1998). Our results showed that NaCl salinity significantly affected the seedling growth parameters (germination percentage, RGR and RWC) in *A. laxiusculum*. According to these results it can be concluded that *A. laxiusculum* is the most sensitive species to salinity than two other species.

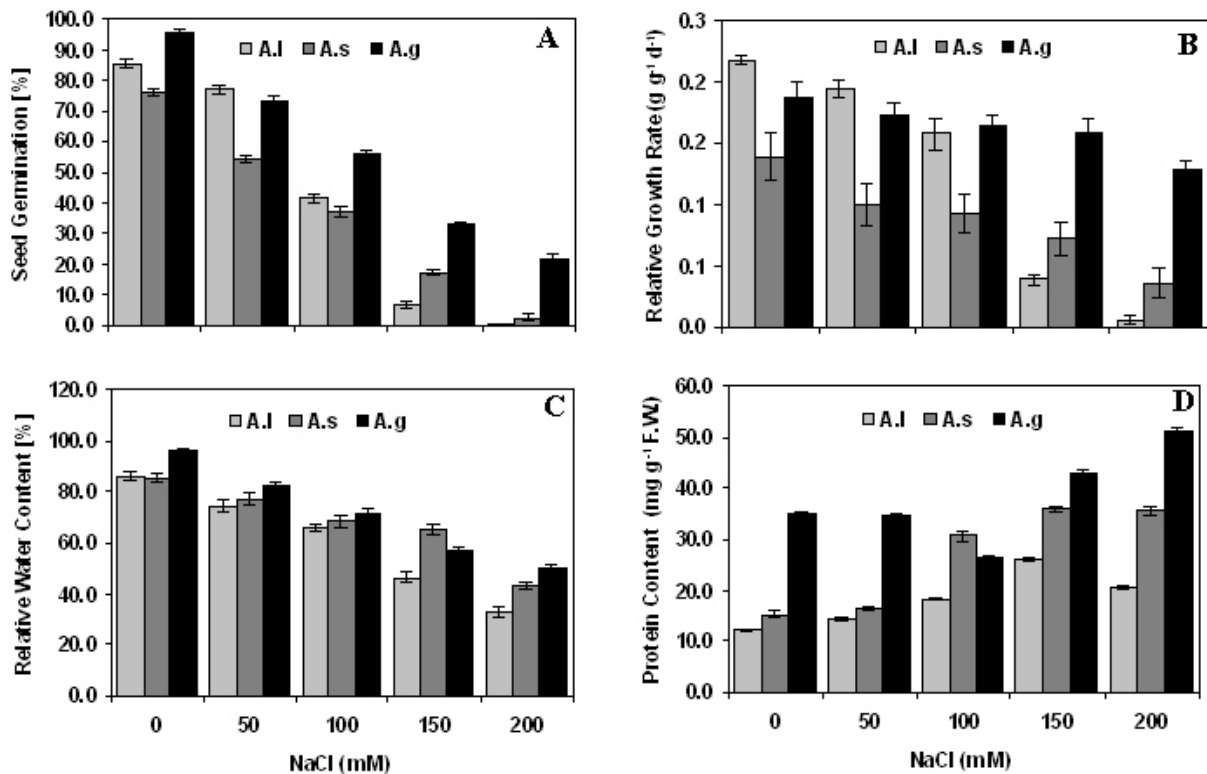


Figure 1- Effect of NaCl stress on seed germination (A), relative growth rate (B), relative water content (C) and protein content (D) in seedlings of three *Acanthophyllum* species. Data represents the average of experiment with three replicates. Vertical bars indicate means \pm S.

The fluctuation of RWC in seedlings of *Acanthophyllum* species under salinity was shown in Figure 1C. Seedling of *A. sordidum* at 200 mM NaCl were able to grown, maintain RWC, although in *A. laxiusculum*, the RWC of seedlings gradually reduced with an increase in NaCl concentration. The variation in RWC was used as a measure of stress. In our experiment, only in *A. sordidum*, 50 and 100 mM NaCl induced mild and moderate stresses, respectively. While in other two species, these concentrations of NaCl induced moderate and severe stresses, respectively. Thus according to the RWC analysis (Hsiao 1973), *A. sordidum* and *A.*

laxiusculum are the most tolerant and sensitive species, respectively. NaCl increased significantly the protein content of *A. laxiusculum* and *A. sordidum* in respect to that of control. NaCl at 150 mM and higher increased also the protein content of *A. glandulosum* significantly (Fig. D). The sensitivity of this parameter to salt treatment suggests that protein biosynthesis was modified. Protein content in seedling of *glandulosum* (hexaploid species with $2n = 90$) at control condition and under salinity stress was higher than that of two other species. Moreover, protein content in *A. sordidum* (tetraploid species with $2n = 60$) was

also higher than that of *A. laxiusculum* (diploid species with $2n = 30$). A higher content of soluble proteins has been observed in salt tolerant than in salt sensitive cultivars of plants such as barley, sunflower, finger millet, and rice (Ashraf & Harris 2004). According to our results, NaCl generally increased protein content in seedling of all the species. In agreement to present result, protein content in seedlings of *Nicotiana tabacum* (Niknam *et al.*, 2004) and *Trigonella foenum-graecum* increased under NaCl stress (Niknam *et al.*, 2006). Contrary to our findings, Levine *et al.*, (1990) reported that salt stress causes inhibition of growth and development, reduction in photosynthesis, respiration, and protein synthesis and disturbs nucleic acid metabolism. Responses of antioxidant enzyme activities in seedlings of three *Acanthophyllum* species to salt stress were shown in Figure 2. Figure 2A described the effects of increasing concentrations of NaCl on POX activities. *A. laxiusculum* exhibited a significant and continuous decline in POX activity with increasing of salt stress. The tetraploid species, *A. sordidum*, showed an increase in POX activity only at low salinity (50 mM) and then POX activity decreased with increasing of NaCl concentrations. However, POX activity in 100 mM NaCl was also higher than that of control. In *A. glandulosum*, a hexaploid species, POX activity declined at 50 mM NaCl and then increased at higher salinities. Moreover, POX activity under salinity, except at 50 mM NaCl was higher than that of control. PPO activities under NaCl treatments showed somewhat similar trends to that of POX (Fig. 2B). *A. laxiusculum* exhibited a continuous decrease in PPO activity at all concentrations of NaCl. A significant increase in PPO activity was observed for *A. sordidum* at 50 mM NaCl. With increasing of salinity, PPO activity in *A. sordidum* declined significantly. In contrast to that of *A. sordidum*, PPO activity in *A. glandulosum* decreased at 50 mM NaCl and then increased with increasing of salinity levels (Fig. 2B). Thus, the decline in POX and PPO activities under salinity could be the cause of salinity in-tolerance in *A. glandulosum*. Stress-induced variation of antioxidant is dependent of the severity and duration of the treatment and also the species and age of the plant. Differences in protective enzyme activities are known for a number of species. A large number of studies on various species indicated that salt stress

alters the amount and the activities of the enzymes involved in ROS scavenging (Gosset *et al.*, 1994; Olmos *et al.*, 1994; Meneguzzo *et al.*, 1999). In tolerant plant species, POX activity was found to be higher, providing protection against the oxidative stress (Scalet *et al.*, 1995). Increase in POX activity was reported in fox-tail millet (Sreenivasulu *et al.*, 1999) and cucumber (El-Baz *et al.*, 2003). The POX and PPO are the two important enzymes responsible for oxidation of phenolic compounds (Sheen & Calvert, 1969). In our experiment, *A. glandulosum* PPO activity declined initially, and then increased to higher than the control level. Increasing in PPO activity was also reported in *Cassia angustifolia* (Agarwal & Pandey, 2004). Therefore POX and PPO may play an important role in antioxidant response of plants against salt stress. Differences in the number and intensities of antioxidative enzymes isoforms were observed in non-denaturing polyacrylamide gel electrophoresis (PAGE). As shown in Figure 3A, only one prominent POX band was detected in *A. laxiusculum*. According to our results, in *A. sordidum* and *A. glandulosum* two and five isozymes with different intensities were detected, respectively. As well as POX activity assays, POX isozymes showed a reducing and increasing patterns in high concentrations of salinity in *A. sordidum* and *A. glandulosum*, respectively (Fig. 3B and Fig. 3C). Thus, it can be concluded that the diversity and content of isoforms could be involved in salinity tolerance of the species. The protein patterns of the three *Acanthophyllum* species grown at different levels of NaCl obtained by SDS-PAGE method are shown in Figure 4. In general, several bands were disappeared in *A. laxiusculum*, while in *A. glandulosum* some protein bands appeared or their intensity increased under salinity treatments. For example, major bands with MW 60, 55 and 45, kDa in *A. laxiusculum* disappeared at high levels of NaCl concentrations. Also, in *A. sordidum* two protein bands about with MW 70 kDa disappeared and instead, two protein bands with MW 55 and 45 kDa were induced by salt stress. In contrast, several protein bands were appeared in *A. glandulosum*. For example, three protein bands with MW 60, 25 and 14.2 kDa were induced under salinity compared to control. These results are in agreement with the finding of El-Baz *et al.*, (2003) and El-Baky *et al.*, (2003).

Disappearance of the protein bands may be

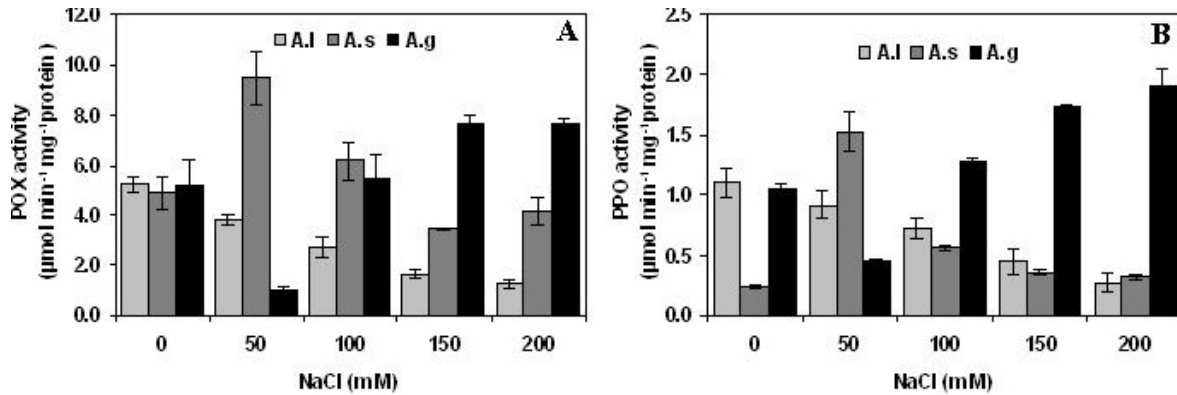


Figure 2- Effect of increasing salinity on the activity of POX (A) and PPO (B) in the seedlings of three *Acanthophyllum* species: *A. laxiusculum*, *A. sordidum* and *A. glandulosum*. Data represents the average of experiments with three replicates. Vertical bars indicate means \pm SE.

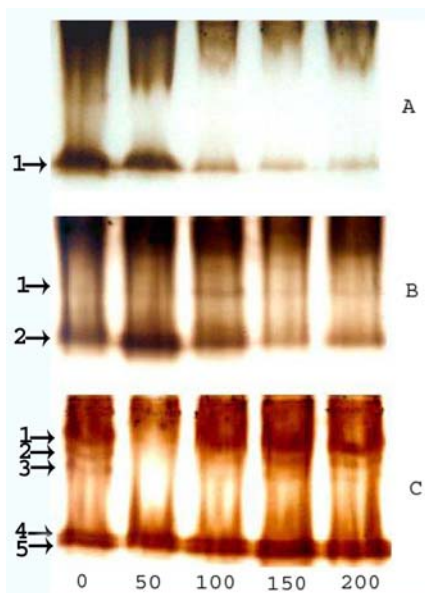


Figure 3- Activity staining of POX on nondenaturing gels in seedlings of three *Acanthophyllum* species under different concentrations of NaCl; *A. laxiusculum* (A), *A. sordidum* (B) and *A. glandulosum* (B).

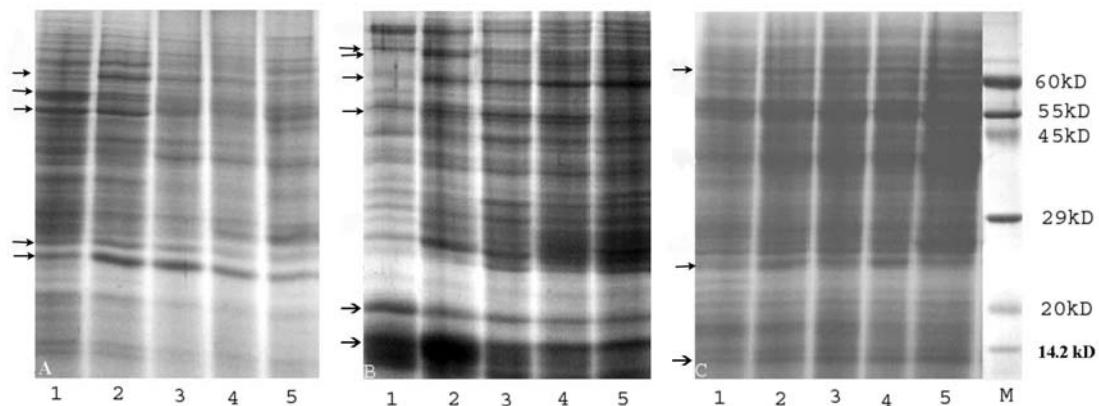


Figure 4- SDS-PAGE resolved band pattern of proteins of seedling of three species of *Acanthophyllum*: *A. laxiusculum* (A), *A. sordidum* (B) and *A. glandulosum* (B) at 0 (1), 50 (2), 100 (3), 150 (4) and 200 (5) mM NaCl. Molecular marker (M) proteins are indicated on the right side of the figure. Arrows indicate the new or effected bands in seedlings.

interpreted as the “turning off” of protein synthetic genetic machinery in response to salt. It is more likely, however, that the “disappeared” proteins in response to stress are a result of their denaturation. Depressed protein synthesis and acceleration and its degradation in plants in response to salt stress have been reported by number of workers (Chandershekhar *et al.*, 1986). Sousa *et al.*, (2004) reported that cowpea seedlings subjected to NaCl stress showed increased concentration of 9 proteins, decreased concentration of one and *de novo* synthesis of one 21.2 kDa protein. However no such studies on protein expression under salinity appear to have been conducted. According to our results *A. glandulosum* (hexaploid species) showed a better protection mechanism against salinity induced oxidative damage than *A. sordidum* (tetraploid species). Moreover, with regard to the role of polyploidy in the evolution and speciation of the *Acanthophyllum* genus, higher ability for salt tolerance observed in *A. glandulosum*, might have been due to the greater polyploidy level in this species compare to the *A. sordidum*. There are many

reports that indicated, in *Acanthophyllum* genus, polyploidy appear to have played an important role in its evolution and speciation (Ghaffari 2004). Therefore, higher tolerance of *A. glandulosum* to salinity than other species can be related to high ploidy level of this species. In agreement with our results, hexaploid bread wheat is considerably more salt-tolerant than tetraploid durum wheat (Munns *et al.*, 2003). The present study demonstrated that, oxidative stress may play an important role in salt-stress *Acanthophyllum* plants and that the greater protection of *A. glandulosum* seedlings from salt-induced oxidative damage results from the maintenance and / or increase of the activity of antioxidant enzymes. In conclusion, salinity tolerance of *A. glandulosum* is associated with higher POX and PPO activities than in *A. sordidum* and *A. laxiusculum*. *A. glandulosum* exhibited better protection from oxidative damage under salt stress. Moreover, the higher salt tolerance of *A. glandulosum* could be attributed to its higher ploidy level.

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