

<http://www.pjbs.org>

**PJBS**

ISSN 1028-8880

**Pakistan  
Journal of Biological Sciences**

**ANSI***net*

Asian Network for Scientific Information  
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

## Antioxidative Responses in Calli of Two Populations of *Acanthophyllum laxiusculum* With and Without B-chromosomes under Salt Stress

<sup>1</sup>A.A. Meratan, <sup>2</sup>S.M. Ghaffari, <sup>3</sup>V. Niknam and <sup>2</sup>M. Salami

<sup>1</sup>Department of Agronomy and Plant Breeding,

Ramin University of Agricultural and Natural Resources, Ahwaz, Iran

<sup>2</sup>Institute of Biochemistry and Biophysics (IBB), University of Tehran,  
P.O. Box 13145-1384, Tehran, Iran

<sup>3</sup>School of Biology, University college of Science, University of Tehran,  
P.O. Box 14155-6455, Tehran, Iran

**Abstract:** Salinity is one of the most significant stresses that affect plant growth and agricultural production. Considerable efforts have therefore been made to investigate how plants respond to salt stress. To investigate the responses of *Acanthophyllum* to salinity stress, changes on lipid peroxidation, H<sub>2</sub>O<sub>2</sub> content and the activity of antioxidant enzymes in calli of two *Acanthophyllum laxiusculum* variants, without (variant A) and with (variant B) B-chromosomes, were analyzed. Under salinity stress, lipid peroxidation and H<sub>2</sub>O<sub>2</sub> content decreased in variant A compared to variant B. Moreover, variant A produced a higher amount of proline under salt stress than variant B. In calli of variant A, salinity stress preferentially enhanced the activities of the superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2), whereas it decreased their activity in variant B. Guaiacol peroxidase (GPX, EC 1.11.1.7) activity decreased with increasing NaCl concentrations in variant A but its activity increased in variant B. After native polyacrylamide gel electrophoresis (PAGE) analysis, calli of both A and B variants had only Mn-SOD and Fe-SOD isoforms. Expression of the isoforms mentioned above, showed marked increase in variant A; while in variant B salinity caused a significant reduction as compared to control. Moreover, changes in the CAT and GPX isoforms pattern of treated plants were in accordance with the activity changes in time. These results suggest that variant A was able to induce better antioxidative responses against salt stress than variant B.

**Key words:** *Acanthophyllum*, antioxidant enzymes, B-chromosome, lipid peroxidation, salinity

### INTRODUCTION

It has been well-documented that during stress conditions including salinity, the production of Reactive Oxygen Species (ROS) increases. ROS are capable of unlimited oxidation of various cellular components and can lead to oxidative damages of the cell (Dionisio-Sese and Tobita, 1998; Sairam and Srivastava, 2002; Mittler, 2002; Juan *et al.*, 2005). To remove ROS, plants have evolved specific strategies including both enzymatic and non-enzymatic antioxidant mechanisms (Ashraf, 2009; Demiral and Turkan, 2005). The most important antioxidant enzymes that participate in the scavenging of ROS are Superoxide Dismutase (SOD), Catalase (CAT), Guaiacol Peroxidase (GPX) Ascorbate Peroxidase (APX), Polyphenol Oxidase (PPO) and Glutathione Reductase (GR) (Niknam *et al.*, 2011; Turkan and Demiral, 2009; Meratan *et al.*, 2008;

De Azevedo-Neto *et al.*, 2006; Demiral and Turkan, 2005; Koca *et al.*, 2007; Mittler, 2002). SOD is a major scavenger of ROS and catalyzes the conversion of superoxide radicals into oxygen and H<sub>2</sub>O<sub>2</sub> (De Azevedo-Neto *et al.*, 2006). H<sub>2</sub>O<sub>2</sub> is then removed by CAT and several classes of peroxidases. CAT dismutates the H<sub>2</sub>O<sub>2</sub> into water and molecular oxygen and is apparently absent in the chloroplast (Sudhakar *et al.*, 2001). Peroxidases are distributed throughout the cell and catalyze the reduction of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O. APX which is considered to be the most important plant peroxidase in H<sub>2</sub>O<sub>2</sub> detoxification, uses ascorbate as the electron donor in the first step of the ascorbate-glutathione cycle; while GPX, removes H<sub>2</sub>O<sub>2</sub> by oxidation of co-substrate such as phenolic compounds and/or ascorbate (De Azevedo-Neto *et al.*, 2006). GR, a flavoenzyme found in the chloroplast, cytosol and mitochondria, catalyzes the last and rate-limiting step of the Halliwell-Asada enzymatic pathway

(De Azevedo-Neto *et al.*, 2006; Mittler, 2002; Reddy *et al.*, 2004; Koca *et al.*, 2007). Owing to lipid membrane damage induction under salinity stress, MDA content which is a secondary end product of polyunsaturated fatty acid oxidation, has been considered as an indicator of oxidative damages (Radic *et al.*, 2006; Sudhakar *et al.*, 2001; De Azevedo-Neto *et al.*, 2006). *Acanthophyllum* C.A. Meyer is a genus belonging to Caryophyllaceae family with a total of 61 species in the world, 33 species of them are distributed in Iran and some of them are found in salt affected areas (Ghaffari, 2004). *Acanthophyllum* species are known as important pharmaceutical plants in Iran, Turkey, Afghanistan, Pakistan and central Asia. So far, in Caryophyllaceae family more than 7 species with B-chromosome have been reported (Jones and Rees, 1982). *Acanthophyllum laxiusculum* is a diploid species with and without B-chromosome populations (Ghaffari and Bidmeshkipoor, 2002). B-chromosomes are additional chromosomes found in some, but not all, individuals of a species. Now-a-days, the existence of B-chromosomes in more than 1372 flowering plants has been proved; among them 12 species belong to coniferals and 1360 species to angiosperm. Approximately in 500 animal species B-chromosomes have been reported (Trivers *et al.*, 2003). The evidences for the effects of B-chromosomes on cellular and physiological processes in both plants and animals are vast (Camacho *et al.*, 2000). B-chromosomes enable some plants to be adaptive to special ecological conditions (Jones and Rees, 1982). In B-chromosome population of *A. laxiusculum* 1 to 3 B-chromosomes exists. Studies on these plants suggest an additional role for B-chromosomes: increasing seed production and pollen fertility. However, B-chromosomes in rye species decrease the rate of the above-mentioned traits (Ghaffari and Bidmeshkipoor, 2002). Therefore, it seems that B-chromosomes in different taxa have different roles which depends on environmental conditions acting upon population (Camacho *et al.*, 2000). The main objective of the present study is to compare the antioxidative responses, malondialdehyde (MDA), H<sub>2</sub>O<sub>2</sub> and proline accumulations between two *A. laxiusculum* variants under NaCl stress, with the aim of determining salt tolerance capacity in the two mentioned variants.

## MATERIALS AND METHODS

**Mitotic studies:** Seeds of *A. laxiusculum* without B-chromosomes (variant A) and with B-chromosomes (variant B) were collected from Iran, Tehran (north) and Semnan (Garmsar, Behbar), respectively. For the study of mitotic chromosomes, seeds were germinated in Petri dishes with moist paper filter in room temperature.

Appropriate size of root tips were pretreated in either 0.002 M 8-hydroxyquinoline or 0.1% colchicines for 3 h at 20°C and then fixed in 6:3:2 ethyl alcohol (96%): chloroform: propionic acid respectably at 4°C for 48 h. After rinsing in distilled water, root tips were hydrolyzed in the hydrochloric acid 1 N for 10 min at 60°C. Staining was carried out with the Feulgen reaction enhanced by squashing in 2% acetocarmine. Suitable slides were studied by Olympus microscope and were permanent by Venetian turpentine method (Wilson, 1945).

**Plant materials and salinity treatments:** For callus generation, approximately 0.5 cm sections of 30 days seedling were cultured in murashige and skoog (MS) medium (Murashige and Skoog, 1962) without NaCl, transferred to MS medium without NaCl and treated with 2,4-dichlorophenoxy acetic acid (2,4-D) and Kinetin (Kin) (4.52:4.65 µM) hormones. Petri dishes were maintained at 25°C, relative humidity of 60% and photoperiod of 16 h for 30 days. The produced calli were transferred to MS medium including 2, 4-D and Kin (4.52:4.65 µM) hormones and were treated with 50, 100, 125 and 150 mM NaCl for 40 days. The MS medium without NaCl concentration was used as control. After 40 days calli were harvested and used for further analysis.

**Relative growth rate measurement:** For growth estimating, plants were randomly selected and Relative Growth Rate (RGR) was calculated according to Beadle (1993).

**Proline determination:** Free proline was extracted from 1 g of calli in 3% (w/v) aqueous sulfosalicylic acid and estimated by using ninhydrin reagent according to the method of Bates *et al.* (1973). The absorbance of fraction with toluene aspired from liquid phase was read at 520 nm. Proline concentration was determined using calibration curve and expressed as µmol proline g<sup>-1</sup> FW.

**Lipid peroxidation determination:** The amount of MDA, a product of lipid peroxidation, was determined for lipid peroxidation as described by Heath and Packer (1968). The concentration of MDA was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) by using extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup>. The MDA content was expressed as µmol g<sup>-1</sup> FW.

**Estimation of H<sub>2</sub>O<sub>2</sub> content:** H<sub>2</sub>O<sub>2</sub> content was determined according to Velikova *et al.* (2000). One gram of calli was homogenized with 5 mL 0.1% (w/v) tri carboxylic acid

(TCA) in an ice bath. The homogenate was centrifuged at 12000 xg for 15 min and 0.5 mL of the extract was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI. Absorbance of the supernatant was measured at 390 nm and H<sub>2</sub>O<sub>2</sub> content was calculated using a standard curve.

**Enzyme extraction and assays:** For enzyme extraction, the calli of two populations of *A. laxiusculum* were homogenized separately in a pre-cooled mortar and pestle over ice-cold buffer (62.5 mM Tris-HCl, pH 6.7) containing 0.3 M sucrose. The supernatant was centrifuged at 20,000 xg for 30 min at 4°C. The extract obtained after centrifugation was used for the different assays. Protein concentrations were estimated according to Bradford (1976), using bovine serum albumin as standard.

Total SOD activity was assayed as described by Beauchamp and Fridovich (1971), by measuring its ability for inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8) with 0.1 mM ethylenediaminetetraacetic acid (EDTA), 75 µM NBT, 13 mM methionine, 2 µM riboflavin and 20 µL of enzyme extract. Reactions were carried out under two 30 W fluorescent lamps. The non-irradiated reaction mixture served as control and was deducted from absorption at 560 nm. One unit of SOD was defined as the amount of enzyme which caused 50% inhibition in the NBT reduction rate under the assay condition and the results were expressed as U mg<sup>-1</sup> protein.

CAT activity assay was performed according to the method of Aebi and Bergmeyer (1983). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 15 mM H<sub>2</sub>O<sub>2</sub> and 20 µL enzyme extract. The decrease of H<sub>2</sub>O<sub>2</sub> was monitored and quantified by its molar extinction coefficient (36 M<sup>-1</sup> cm<sup>-1</sup>) and the results were expressed as µmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> protein.

GPX and APX activities were determined following the method of Jebara *et al.* (2005). For GPX activity assay, the reaction mixture (1.0 mL) contained 50 mM sodium phosphate buffer (pH 7.0), 9 mM guaiacol, 19 mM H<sub>2</sub>O<sub>2</sub> and 10 µL enzyme extract. The reaction was started by addition of enzyme extract and the increase in absorbance was recorded at 470 nm for 1 min. Enzyme activity was quantified by the amount of tetraguaiacol formed using its molar extinction coefficient (26.6 mM<sup>-1</sup> cm<sup>-1</sup>).

For APX activity assay, the reaction mixture (1.0 mL) contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H<sub>2</sub>O<sub>2</sub>, 0.5 mM ascorbate and 15 µL enzyme extract. The reaction was started by the addition of H<sub>2</sub>O<sub>2</sub> and ascorbate oxidation was measured at 290 nm for 1 min. Enzyme activity was quantified using the molar extinction

coefficient for ascorbate (2.8 mM<sup>-1</sup> cm<sup>-1</sup>) and the results were expressed as µmol of ascorbate oxidized min<sup>-1</sup> per mg protein.

GR activity was assayed by the oxidation of NADPH using its extinction coefficient (6.2 mM<sup>-1</sup> cm<sup>-1</sup>) at 340 nm as described by Lee and Lee (2000). The reaction mixture (1.0 mL) consisted of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and the appropriate volume of enzyme extract. The reaction was initiated by the addition of NADPH and the results were expressed as µmol of NADPH oxidized min<sup>-1</sup> per mg protein.

**Native gel electrophoresis and isozyme staining:** Native polyacrylamide gel electrophoresis (PAGE) was performed at 4°C, 120 v (for SOD and GPX) and 140 v (for CAT) as described by Laemmli (1970). For SOD and GPX the electrophoretic separation was performed on native 10% polyacrylamide gels and for CAT the enzyme solution was subjected to native gels with 7% polyacrylamide. Staining for SOD was achieved using the procedure described by Beauchamp and Fridovich (1971). Identification of SOD isoforms was achieved by incubation of incubating gels in 50 mM potassium phosphate buffer (pH 7.0) containing 3 mM KCN or 5 mM H<sub>2</sub>O<sub>2</sub> for 30 min before staining for SOD activity (Lee and Lee, 2000).

Electrophoretic patterns of CAT and GPX were determined as described by Dewir *et al.* (2006). For CAT staining, the gel was soaked in 5 mM H<sub>2</sub>O<sub>2</sub> for 10 min. After a brief rinse, CAT isoforms were detected by incubating the gels in 2% (w/v) FeCl<sub>3</sub> and 2% (w/v) K<sub>3</sub>[Fe(CN)<sub>6</sub>] until achromatic bands appeared.

For GPX isoforms analysis, the gels were washed in distilled water, soaked in 25 mM potassium buffer (pH 7.0) for 15 min and then gels were submerged again in a freshly prepared 25 mM potassium buffer (pH 7.0) containing 18 mM guaiacol and 25 mM H<sub>2</sub>O<sub>2</sub> at room temperature until the GPX activity containing bands appeared.

**Statistics analysis:** Each experiment was repeated three times and data were analyzed using ANOVA and subsequent comparison of means was performed by Duncan test at 5% probability.

## RESULTS

**Mitotic studies result:** Two karyotypic variants were recognized in populations of *A. laxiusculum* (Ghaffari and Bidmeshkipoor, 2002). Variant A showed 2n = 30 chromosomes and variant B showed the presence of 1 to 3 B-chromosomes in addition to 2n = 30 (Fig. 1a, b).

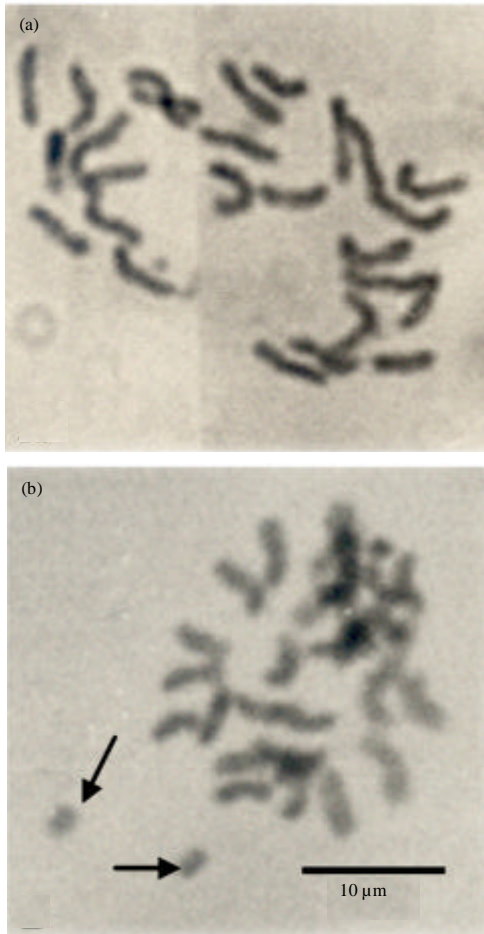


Fig. 1(a-b): Somatic metaphase in variant A with (a)  $2n = 30$  and in variant B with and (b)  $2n = 30 + 2B$  (arrows) Bar = 10  $\mu\text{m}$

**Effects of salinity stress on RGR, proline accumulation, lipid peroxidation and  $\text{H}_2\text{O}_2$  content:** Based on callus dry weight, the RGR of the two *A. laxiusculum* variants subjected to salinity treatment are shown in Fig. 2. In spite of the reduction in RGR which was observed in variant A and B, the reduction in growth rate varied significantly among two variants, so that reduction level was more pronounced in variant B than variant A.

The effects of salt stress on proline accumulation in two *A. laxiusculum* variants are shown in Fig. 3. Under salinity stress condition, there was a gradual increase in proline concentration in both variants, but significantly higher accumulation of proline was observed with 150 mM NaCl in variant A compared to in variant B.

Lipid peroxidation levels in calli of the two *A. laxiusculum* variants, measured as the MDA content,

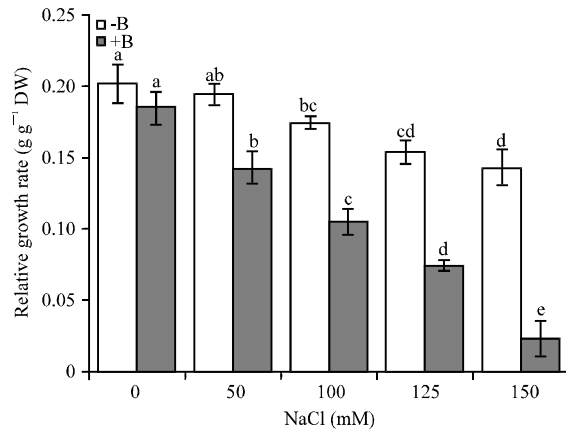


Fig. 2: Changes in the levels of RGR in the calli of variants A and B of *A. laxiusculum* cultured in medium with various salt concentration. Vertical bars indicate Mean  $\pm$  SE of three replicates. Means followed by the same letter were not significantly different at  $p < 0.05$

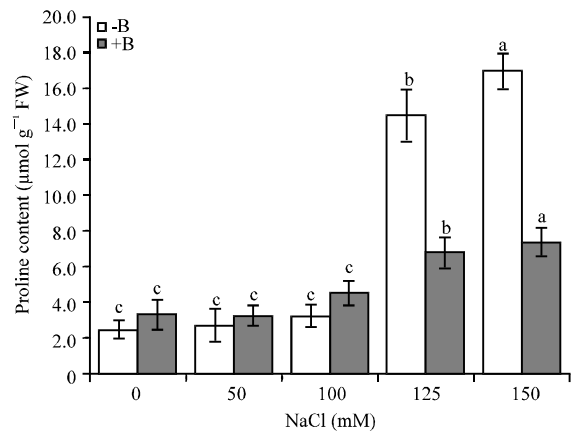


Fig. 3: Changes in the level of proline content in the calli of variants A and B of *A. laxiusculum* cultured in medium with various salt concentration. Vertical bars indicate Mean  $\pm$  SE of three replicates. Bars followed by the same letter were not significantly different at  $p < 0.05$

are given in Fig. 4a. Under salinity stress, variant A showed a gradual decrease in lipid peroxidation level, while calli of variant B showed an increase in lipid peroxidation concomitant with increasing salinity.

The inherent  $\text{H}_2\text{O}_2$  content was higher in variant A than variant B. However, under salt treatment it decreased in calli of variant A and increased in calli of variant B (Fig. 4b).

**Changes of antioxidative enzymes activity under salinity stress:** There were striking differences in antioxidant

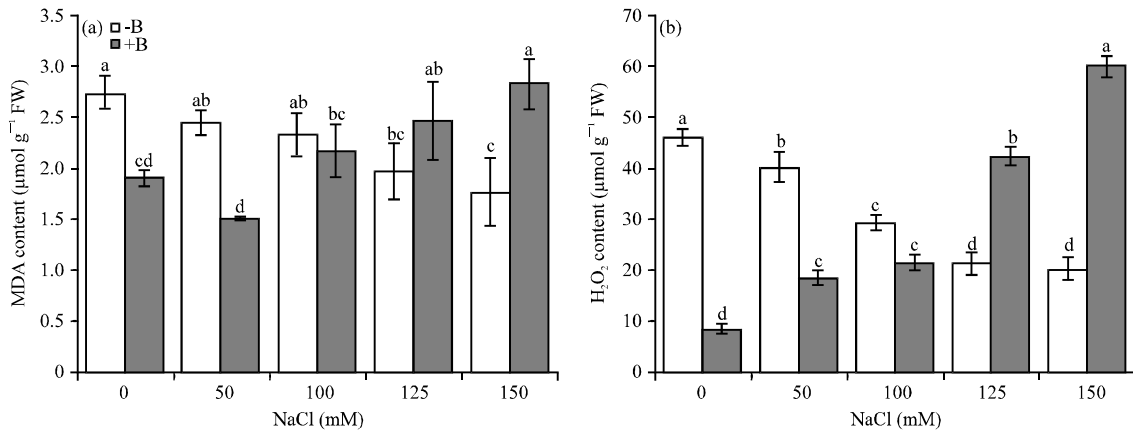


Fig. 4(a-b): Changes in the level of (a) MAD and (b) H<sub>2</sub>O<sub>2</sub> contents in the calli of variants A and B of *A. laxiusculum* cultured in medium with various salt concentrations. Vertical bars indicate Mean±SE of three replicates. Bars followed by the same letter were not significantly different at p<0.05

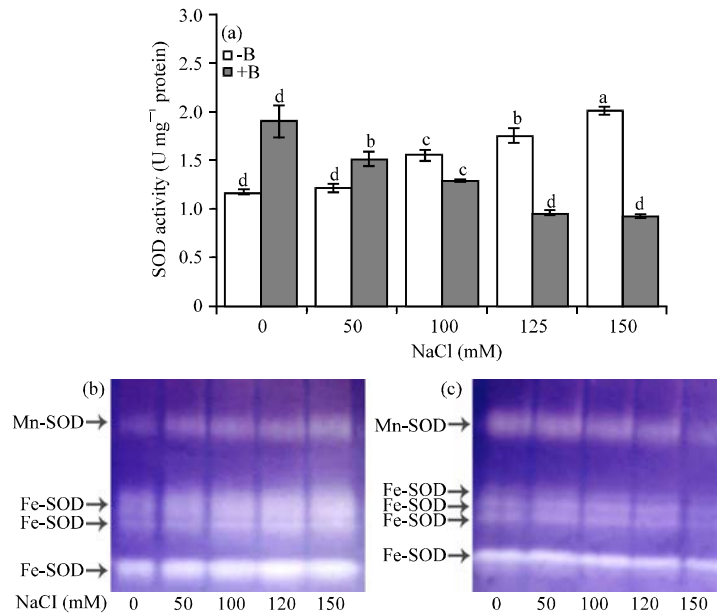


Fig. 5(a-c): Changes in the activity (a) and isoform patterns of SOD enzymes in the calli of variants A (b) and B (c) of *A. laxiusculum* cultured in medium with various salt concentrations. Vertical bars indicate Mean±SE of three replicates. Means followed by the same letter were not significantly different at p<0.05

enzyme activities between the two variants with the increasing of NaCl concentration. SOD activity in the calli of both variants showed remarkable changes with increased salinity. SOD activity enhanced in variant A; while decreased with increased NaCl concentration in variant B. However, the basal level of SOD activity was higher in variant B than variant A. The calli of variant A displayed the highest SOD activity at 150 mM NaCl as compared to control, whereas in variant B salinity caused a significant reduction at 150 mM NaCl (Fig. 5a). The separation of SOD isoforms confirmed the evolution of

the spectrophotometric activities. Total SOD activity represents the combined action of Mn-SOD and Fe-SOD isoforms (Fig. 5b, c). One isoform of SOD in the calli of variant A was identified as Mn-SOD and the other three isoforms were identified as Fe-SOD (Fig. 5b). In variant B, the SOD isoforms could be characterized as follows: one isoform was identified as Mn-SOD and the other four isoforms were identified as Fe-SOD (Fig. 5c). Cu/Zn-SOD isoform was not observed in native gels of any of both variants.

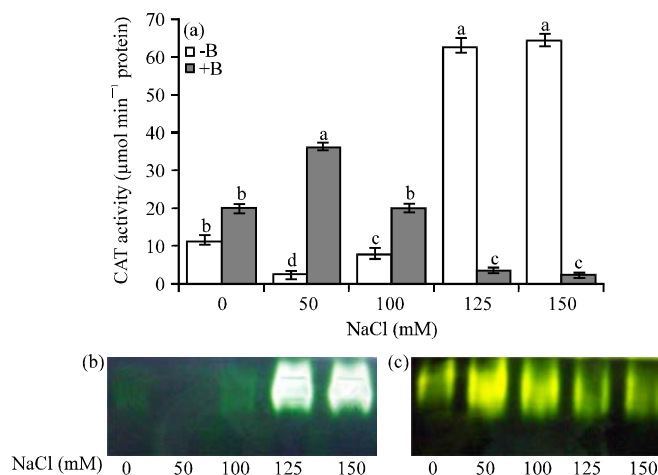


Fig. 6(a-c): Changes in the activity (a) and isoform patterns of CAT enzymes in the calli of variants A (b) and B (c) of *A. laxiusculum* cultured in medium with various salt concentrations. Vertical bars indicate Mean±SE of three replicates. Means followed by the same letter were not significantly different at  $p < 0.05$

CAT is an important antioxidant enzyme in scavenging of  $H_2O_2$  formed by the dismutation of superoxide anions catalyzed by SOD. Salt treatment led to a significant increase of CAT activity in variant A with a maximum at 150 mM NaCl compared to control. In variant B our results showed a gradual reduction in CAT activity with increasing NaCl concentration (Fig. 6a). These data were supported by native PAGE gels which revealed more intense CAT isoforms at high NaCl concentrations (125 and 150 mM) in variant A; while in the case of variant B, more intense CAT isoforms was observed in 50 mM NaCl and then declined with increasing salinity level (Fig. 6b, c).

Peroxidases are known to utilize different substrates to metabolize  $H_2O_2$ . When guaiacol was used as a substrate, although the results obtained showed higher inherent levels of GPX activity in variant A than variant B, but salinity treatment induced significant decrease in GPX activity of variant A. Unlike variant A, GPX activity increased with increasing NaCl concentration in variant B (Fig. 7a). GPX pattern showed only one isoform in both variants. As for SOD and CAT, obtained results corroborated the spectrophotometrically measured activities (Fig. 7b, c).

Salt-induced changes in the activities of APX and GR in both *A. laxiusculum* variants were depicted in Fig. 8a, b, respectively. There was a linear increase in APX activity with increasing concentrations of NaCl in variant A; while it gradually decreased with enhancing salinity levels in variant B (Fig. 8a). The activity of GR enzyme increased in the calli of variant A under stress condition, whereas it showed a significant reduction at high concentrations of NaCl (150 mM) in variant B (Fig. 8b).

## DISCUSSION

In the present study, callus culture was used to demonstrate different induced-salinity responses of two *A. laxiusculum* variants subjected to salinity stress. As shown in Fig. 2, under salinity stress, the calli of A variant revealed relatively lower RGR reduction compared with variant B; indicating greater tolerance of variant A to salt stress than variant B. Decrease in growth due to salinity has also been reported in some species of salt-sensitive plants (Abdel-Rahman *et al.*, 2005; Meratan *et al.*, 2008; Niknam *et al.*, 2006). Since, proline can contribute to regulation of osmotic balance in stressed plants (Ueda *et al.*, 2007), higher accumulation of proline in variant A than variant B suggests that variant A possesses a better potential to maintain osmotic balance than variant B under salinity stress (Fig. 3). Many workers found more proline accumulation levels in the salinity-tolerant plants than salinity-sensitive ones (Niknam *et al.*, 2011; Sumithra *et al.*, 2006; Koca *et al.*, 2007). Peroxidation of membrane lipids, by measuring the amount of MDA produced has often been used as an indicator of membrane damage and a tool to assess the degree of plant sensitivity to oxidative damage (Radic *et al.*, 2006). In the case of variant A, that showed a decrease in MDA content in stress condition, salinity treatment did not induce membrane lipid peroxidation. On the other hand in variant B, with increasing level of salinity stress, MDA content showed marked increase; indicating an increase in lipid peroxidation (Fig. 4a). Salt tolerance tomato cultivar (Juan *et al.*, 2005) and salt tolerance maize genotype (De Azevedo-Neto *et al.*, 2006) had lower levels of lipid peroxidation; while higher lipid

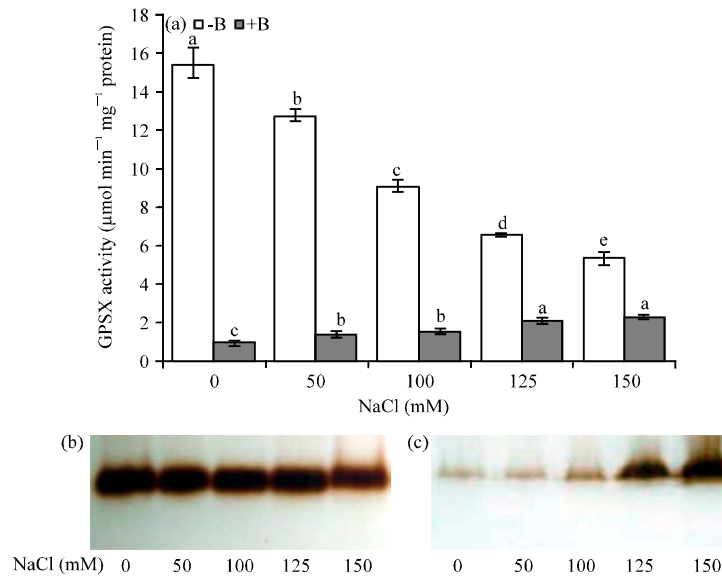


Fig. 7(a-c): Changes in the activity (a) and isoform patterns of GPX enzymes in the calli of variants A (b) and B (c) of *A. laxiusculum* cultured in medium with various salt concentrations. Vertical bars indicate Mean±SE of three replicates. Means followed by the same letter were not significantly different at  $p < 0.05$

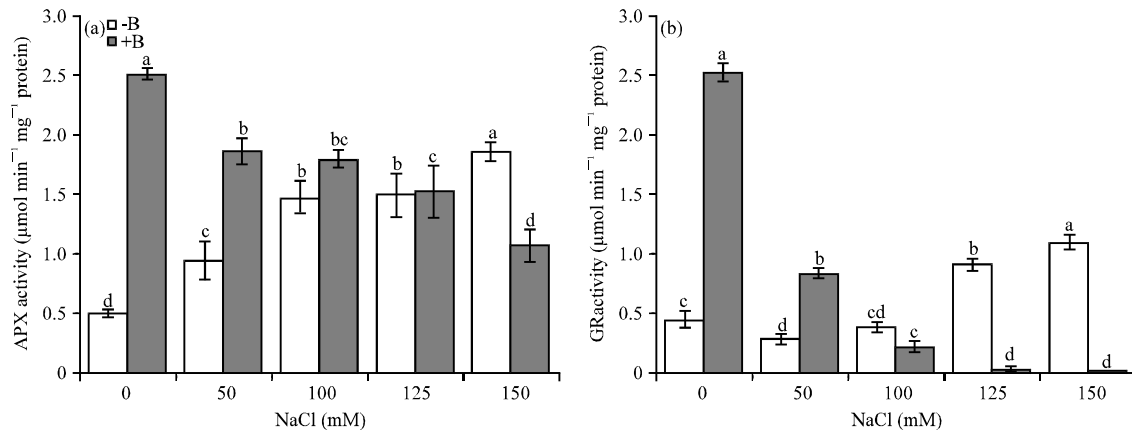


Fig. 8(a-b): Changes in the activities of APX (a) and GR (b) in the calli of variants A and B of *A. laxiusculum* cultured in medium with various salt concentrations. Vertical bars indicate Mean±SE of three replicates. Means followed by the same letter were not significantly different at  $p < 0.05$

peroxidation levels in sensitive cv. of pea (Sairam and Srivastava, 2002) and rice (Dionisio-Sese and Tobita, 1998) have been reported earlier. It has been proven that stress condition enhances H<sub>2</sub>O<sub>2</sub> production in different compartment of plant cells by enzymatic and non-enzymatic process (Chaparzadeh *et al.*, 2004). Since, H<sub>2</sub>O<sub>2</sub> is able to induce °OH formation, lipid peroxidation rate, to some extent, coincides with the amount of H<sub>2</sub>O<sub>2</sub> (Lin and Kao, 2000). In the case of variant A, H<sub>2</sub>O<sub>2</sub> content was declined with increasing salinity concentration. When NaCl concentration increased unlike variant A, a marked

increase in H<sub>2</sub>O<sub>2</sub> content was observed in variant B (Fig. 4b). Therefore, the maintenance of a low level of H<sub>2</sub>O<sub>2</sub> and lipid peroxidation may be one of the reasons for the observed tolerance of variant A calli when exposed to high levels of salinity. The role of antioxidant enzymes in protecting the cell structure against the ROS generated by stress condition has been proven (Ashraf, 2009; Turkan and Demiral, 2009; Niknam *et al.*, 2011; Reddy *et al.*, 2004). According to present works, it is clear that salinity tolerance is closely related to the efficiency of antioxidant enzymes (Turkan and Demiral, 2009;



Ashraf and Harris, 2004; Amor *et al.*, 2005). Major antioxidant enzymes involving in scavenging ROS include SOD, CAT, APX (Mittler, 2002) and GR which is last and rate limiting step for the removal of  $H_2O_2$  through the Halliwell-Asada pathway (Demiral and Turkan, 2005). As shown in Fig. 5a, salt stress causes a significant increase of SOD activity in the variant A; while in variant B SOD activity is markedly reduced in response to salt treatment, indicating the better  $O_2^{\bullet-}$  radical scavenging capacity of variant A than variant B. Similar results have been reported that show salt stress enhances SOD activity in salt-tolerant species and decreases it in salt-sensitive ones (Niknam *et al.*, 2011; Kholova *et al.*, 2010). Moreover, previous works on transgenic tobacco plants demonstrated that overexpression of SOD protects plants from oxidative stress (Fadzilla *et al.*, 1997). SOD isoforms according to their metal co-factor binding at the active site; can be distinguished into three classes: Cu-Zn, Mn and Fe-SOD (Laemmli, 1970). Since Mn-SOD is reported to be located in mitochondria, Fe-SOD in chloroplast and Cu/Zn-SOD in chloroplast and/or in cytosol, compartment-specific responses of the antioxidant enzymes can be distinguished (Amor *et al.*, 2005). As the Cu/Zn-SOD isoform did not detect in any of variants, our data (Fig. 5b, c) suggest that mitochondrial and chloroplastic compartments are essential in protection of calli against superoxide radical formation when exposed to salinity stress. Exposure to salt stress increased both Mn-SOD and Fe-SOD expressions in variant A; while it reduced the expression of them in variant B. Therefore, having more Mn-SOD and Fe-SOD activity in variant A than variant B, indicates that capacity for scavenging superoxide radicals is more efficient in the calli of variant A than variant B. Similarly, NaCl-tolerant pea cultivars showed an increase of Mn-SOD activity; while in sensitive cultivars the activity of this isoenzyme decreased (Olmos *et al.*, 1994). Moreover, salinity-induced increase in chloroplastic Fe-SOD has been reported in pea (Gomez *et al.*, 1999; Hernandez *et al.*, 2000). Sairam and Srivastava (2002) reported higher chloroplastic Fe-SOD and mitochondrial Mn-SOD activities in tolerant wheat genotype under salinity stress. Elevated SOD activity in variant A was accompanied with a significant increase in the activity of major antioxidant enzymes involving in removing  $H_2O_2$  including CAT, APX and GR which is a fine indication of this variant's salt tolerance as compared to the other one. In contrast, accumulation of  $H_2O_2$  that was correlated with more lipid peroxidation in the variant B can be attributed to a decline in activity of SOD, CAT, APX and GR enzymes under salinity treatment. CAT, the main scavenger of  $H_2O_2$  in peroxisomes (Koca *et al.*, 2007),

converts  $H_2O_2$  to water and molecular oxygen. A significant enhancement in CAT activity was also observed in variant A which showed a marked negative relation with  $H_2O_2$  content (Fig. 6a), that suggest a significant role for CAT in  $H_2O_2$  detoxification under salt treatment. Therefore, the combined activities of SOD and CAT play a key role in the scavenging of ROS in variant A, thus reduces the oxidative damages caused by ROS under salinity stress condition. These results are in good agreement with those of Shalata *et al.* (2001), who found that SOD and CAT activities increased in salt-tolerance tomato cultivar and decreased in salt-sensitive ones under salt stress. On the other hand,  $H_2O_2$  accumulation in the calli of variant B under salinity stress was related to a decline in CAT activity (Fig. 6a). Beside the involvement of POXs enzymes in scavenging of  $H_2O_2$  produced under salinity stress, the role of them in growth and development process, including lignification and suberization, have been proven (Dionisio-Sese and Tobita, 1998). In agreement with results presented here, Mittal and Dubey (1991) found a negative correlation between POX activity and salt tolerance of rice cvs. Moreover, there was an increase in POX activity in roots of salt-sensitive IR-28; while it decreased in roots of salt-tolerance ones (Demiral and Turkan, 2005). Thus, the reduction in growth of variant B induced by salinity, at least in part, might have been involved in the increasing of the non-specific peroxidase (GPX) activity (Fig. 7a). APX that uses ascorbate as the electron donor is one of the key enzymes which plays an important role in  $H_2O_2$  alleviation under stress condition (Demiral and Turkan, 2005). Activity of APX was also higher in variant A than in variant B (Fig. 8a). Hence, it suggested that ascorbate-glutathione cycle, in which APX acts as a strong catalyst together with GR, is very efficient in eliminating  $H_2O_2$  in the calli of variant (Sharata and Tal, 1998; Lopez *et al.*, 1996), reported an inherently higher and induced level of APX in wild salt-tolerant tomato and radish plants, respectively. GR which is known to maintain higher ratios of GSH/GSSG which are necessary for regeneration of ascorbate, plays a key role in ascorbate-glutathione pathway (Sumithra *et al.*, 2006). In variant A calli, GR activity was enhanced by NaCl stress as compared to control; while salinity treatment caused a significant reduction in GR activity of variant B when exposed to salt stress, therefore in high concentrations of NaCl we did not observed any level of GR activity (Fig. 8b). Similarly, GR activity increased in salt-tolerant pea variety as compared to salt-sensitive ones under salt stress condition (Hernandez *et al.*, 2000). Moreover, there are many reports indicating high levels of GR in

salt-tolerant plants under stress condition (Kholova *et al.*, 2010; Sudhakar *et al.*, 2001; Sairam and Srivastava, 2002; Sumithra *et al.*, 2006; Koca *et al.*, 2007; Niknam *et al.*, 2011). B-chromosomes are extra chromosomes over the standard diploid or polyploid chromosome complement and are usually smaller in size than normal A-chromosomes (Jones and Rees, 1982). Because many of B-chromosomes have been found to carry ribosomal genes; some of B-chromosomes can be attributed to the production of genes. In spite the role of B-chromosomes in the processes or characters related with vigor, fertility and fecundity (Camacho *et al.*, 2000), Jones and Rees (1982) summarized a board range of deleterious effects from B-chromosomes in many species of plants and animals. In the present study, possible effects of occurrence and lack of B-chromosomes in salt-induced responses in two variants of *A. laxiusculum* have been investigated. According to our results, higher SOD, CAT, APX and GR activities, decreased the lipid peroxidation level and H<sub>2</sub>O<sub>2</sub> content and finally higher proline accumulation in variant A than variant B under salinity stress can be attribute to the increased capacity for ROS scavenging and preserving membrane structures in variant A than variant B, indicating the relationship between salt tolerance and antioxidant defensive systems. Considerably, both populations of *A. laxiusculum* were collected from different areas with same salinity and drought parameters. However, it should be emphasized that because B-chromosomes effect depends on the environmental condition acting upon population, it will be risky for the effects detected in one population to be extrapolated over the entire distribution range of that species (Camacho *et al.*, 2000). Therefore, further investigations are necessary in order to state the probable effects of B-chromosomes in antioxidant responses against stress conditions in plants.

#### ABBREVIATIONS

MDA = Malondialdehyde  
SOD = Superoxide dismutase  
CAT = Catalase  
APX = Ascorbate peroxidase  
GR = Glutathione reductase  
GPX = Guaiacol peroxidase  
2,4-D = 2,4-dichlorophenoxy acetic acid  
Kin = Kinetin  
RGR = Relative growth rate  
ROS = Reactive oxygen species  
MS = Murashige and skoog  
NBT = Nitroblue tetrazolium

TCA = Tri carboxylic acid  
EDTA = Ethylenediaminetetraacetic acid  
PAGE = Polyacrylamide gel electrophoresis

#### REFERENCES

- Abdel-Rahman, N.M., R.A. Shibli, K.I. Ereifej and M.Y. Hindiyeh, 2005. Influence of salinity on growth and physiology of *In vitro* grown cucumber (*Cucumis sativus* L.). Jordan J. Agric. Sci., 1: 93-106.
- Aebi, H.E. and H.U. Bergmeyer, 1983. Methods of Enzymatic Analysis. Vol. 3, Verlag Chemic, Deerfield Beach, FL, pp: 273-286.
- Amor, N.B., K.B. Hamed, A. Debez, C. Grignon and C. Abdely, 2005. Physiological and antioxidant responses of the perennial halophyte *Crithmum maritimum* to salinity. Plant Sci., 168: 889-899.
- Ashraf, M. and P.J.C. Harris, 2004. Potential biochemical indicators of salinity tolerance in plants. Plant Sci., 166: 3-16.
- Ashraf, M., 2009. Biotechnological approach of improving plant salt tolerance using antioxidants as markers. Biotechnol. Adv., 27: 84-93.
- Bates, L.S., R.P. Waldren and I.D. Teare, 1973. Rapid determination of free proline for water-stress studies. Plant Soil, 39: 205-207.
- Beadle, C.L., 1993. Growth Analysis. In: Photosynthesis and Production in a Changing Environment: A Field and Laboratory Manual, Hall, D.O., J.M.O. Scurlock, H.R. Boolhar-Nordenkampf, R.C. Leegood and S.P. Long (Eds.). Chapman and Hall, London, pp: 22-46.
- Beauchamp, C. and I. Fridovich, 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal. Biochem., 44: 276-287.
- Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein using the principle of protein dye binding. Anal. Biochem., 72: 248-254.
- Camacho, J.P.M., T.F. Sharbel and L.W. Beukeboom, 2000. B-chromosome evolution. Phil. Trans. R. Soc. Lond. B, 355: 163-178.
- Chaparzadeh, N., M.L. D'Amico, R.A. Khavari-Nejad, R. Izzo and F. Navari-Izzo, 2004. Antioxidative responses of *Calendula officinalis* under salinity conditions. Plant Physiol. Biochem., 42: 695-701.
- De Azevedo-Neto, A.D., J.T. Pitsco, J. Eneas-Filho, C.E.B. de Abreu and E. Gomes-Filho, 2006. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. Environ. Exp. Bot., 56: 87-94.

- Demiral, T. and I. Turkan, 2005. Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. *Environ. Exp. Bot.*, 53: 247-257.
- Dewir, Y.H., D. Chakrabarty, M.B. Ali, E.J. Hahn and K.Y. Paek, 2006. Lipid peroxidation and antioxidant enzyme activities of *Euphorbia millii* hyperhydric shoots. *Environ. Exp. Bot.*, 58: 93-99.
- Dionisio-Sese, M.L. and S. Tobita, 1998. Antioxidant responses of rice seedlings to salinity stress. *Plant Sci.*, 135: 1-9.
- Fadzilla, N.M., R.P. Finch and R.H. Burdon, 1997. Salinity, oxidative stress and antioxidant responses in shoot cultures of rice. *J. Exp. Bot.*, 48: 325-331.
- Ghaffari, S.M. and A. Bidmeshkipoor, 2002. Presence and behaviour of B-chromosomes in *Acanthophyllum laxiusculum* (Caryophyllaceae). *Genetica*, 115: 319-323.
- Ghaffari, S.M., 2004. Cytotaxonomy of some species of *Acanthophyllum* (Caryophyllaceae) from Iran. *Biologia Bratislava*, 59: 53-60.
- Gomez, J.M., J.A. Hernandez, A. Jimenez, L.A. del Rio and F. Sevilla, 1999. Differential responses of antioxidative enzymes of chloroplasts and mitochondria to long-term NaCl stress of pea plants. *Free Radic. Res.*, 31: S11-S18.
- Heath, R.L. and L. Packer, 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.*, 125: 189-198.
- Hernandez, J.A., A. Jimenez, P. Mullineaux and F. Sevilla, 2000. Tolerance of pea (*Pisum sativum* L.) to long term salt stress is associated with induction of antioxidant defences. *Plant Cell Environ.*, 23: 853-862.
- Jebara, C., M. Jebara, F. Limam and M.E. Aouani, 2005. Changes in ascorbate peroxidase, catalase, guaiacol peroxidase and superoxide dismutase activities in common bean (*Phaseolus vulgaris*) nodules under salt stress. *J. Plant Physiol.*, 162: 929-936.
- Jones, R.N. and H. Rees, 1982. B Chromosomes. Academic Press, New York, USA.
- Juan, M., R.M. Rivero, L. Romero and J.M. Ruiz, 2005. Evaluation of some nutritional and biochemical indicators in selecting salt-resistant tomato cultivars. *Environ. Exp. Bot.*, 54: 193-201.
- Kholova, J., R.K. Sairam and R.C. Meena, 2010. Osmolytes and metal ions accumulation, oxidative stress and antioxidant enzymes activity as determinants of salinity stress tolerance in maize genotypes. *Acta Physiologiae Plantarum*, 32: 477-486.
- Koca, H., M. Bor, F. Ozdemir and I. Turkan, 2007. The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars. *Environ. Exp. Bot.*, 60: 344-351.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature*, 227: 680-685.
- Lee, D.H. and C.B. Lee, 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: In gel enzyme activity assays. *Plant Sci.*, 159: 75-85.
- Lin, C.C. and C.H. Kao, 2000. Effect of NaCl stress on H<sub>2</sub>O<sub>2</sub> metabolism in rice leaves. *Plant Growth Regul.*, 30: 151-155.
- Lopez, F., G. Vansuyt, F. Casse-Delbart and P. Fourcroy, 1996. Ascorbate peroxidase activity not the mRNA level is enhanced in salt stressed *Raphanus sativus* plants. *Physiologia Plantarum*, 97: 13-20.
- Meratan, A.A., S.M. Ghaffari and V. Niknam, 2008. Effects of salinity on growth, proteins and antioxidant enzymes in three *Acanthophyllum* species of different ploidy levels. *JSUT*, 33: 1-8.
- Mittal, R. and R.S. Dubey, 1991. Behaviour of peroxidases in rice: Changes in enzyme activity and isoforms in relation to salt tolerance. *Plant Physiol. Biochem.*, 29: 31-40.
- Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.*, 7: 405-410.
- Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15: 473-497.
- Niknam, V., A.A. Meratan and S.M. Ghaffari, 2011. The effect of salt stress on lipid peroxidation and antioxidative enzymes in callus of two *Acanthophyllum* species. *In vitro Cell. Dev. Biol. Plant*, 47: 297-308.
- Niknam, V., N. Razavi, H. Ebrahimzadeh and B. Sharifzadeh, 2006. Effect of NaCl on biomass, protein and proline contents and antioxidant enzymes in seedlings and calli of two *Trigonella* species. *Biologia Plantarum*, 50: 591-596.
- Olmos, E., J.A. Hernandez, F. Sevilla and E. Hellin, 1994. Induction of several antioxidant enzymes in the selection of a salt-tolerant cell line of *Pisum sativum*. *J. Plant Physiol.*, 144: 594-598.
- Radic, S., M. Radic-Stojkovic and B. Pevalak-Kozlina, 2006. Influence of NaCl and mannitol on peroxidase activity and lipid peroxidation in *Centaurea ragusina* L. roots and shoots. *J. Plant Physiol.*, 163: 1284-1292.
- Reddy, A.R., K.V. Chaitanya, P.P. Jutur and K. Sumithra, 2004. Differential responses to water stress among five mulberry (*Morus alba* L.) cultivars. *Environ. Exptl. Bot.*, 52: 33-42.

- Sairam, R.K. and G.C. Srivastava, 2002. Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. *Plant Sci.*, 162: 897-904.
- Shalata, A., V. Mittova, M. Volokita, M. Guy and M. Tal, 2001. Response of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii* to salt-dependent oxidative stress: The root antioxidative system. *Physiol. Plant.*, 112: 487-494.
- Sharata, A. and M. Tal, 1998. The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. *Physiol. Plant.*, 104: 169-174.
- Sudhakar, C., A. Lakshmi and S. Giridarakumar, 2001. Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. *Plant Sci.*, 161: 613-619.
- Sumithra, K., P.P. Jutur, B.D. Carmel and A.R. Reddy, 2006. Salinity-induced changes in two cultivars of *Vigna radiate*: Responses of antioxidative and proline metabolism. *Plant Growth Regul.*, 50: 11-22.
- Trivers, R., A. Burt and B.G. Palestis, 2003. B chromosomes and genome size in flowering plants. *Genome*, 47: 1-8.
- Turkan, I. and T. Demiral, 2009. Recent developments in understanding salinity tolerance. *Environ. Exp. Bot.*, 67: 2-9.
- Ueda, A., Y. Yamamoto-Yamane and T. Takabe, 2007. Salt stress enhances proline utilization in the apical region of barley roots. *Biochem. Biophys. Res. Commun.*, 335: 61-66.
- Velikova, V., I. Yordavov and A. Edreva, 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. *Plant Sci.*, 151: 59-66.
- Wilson, G.B., 1945. The Venetian turpentine mounting medium. *Stain Technol.*, 20: 133-135.