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Effect of Salt Stress on Growth, Membrane Damage, Antioxidant Metabolism and Artemisinin Accumulation in *Artemisia annua* L.

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ABSTRACT

Salinity in soil and irrigation water is one of the major factors that limit crop productivity. The effects of salinity on growth, lipid peroxidation, the antioxidant defence system and changes in artemisinin content were studied in *Artemisia annua* L. Salinity treatments were established by adding 0, 50, 100, 150 and 200 mM of sodium chloride (NaCl) to the soil. Salt stress negatively affected the growth of plants, measured in terms of shoot and root length and dry weight. Photosynthetic attributes and total chlorophyll content were also reduced by salinity stress. Salinity treatments inhibited the activity of carbonic anhydrase and significantly increased electrolyte leakage and proline content. Moreover, salt stress induced oxidative stress, as indicated by the level of lipid peroxidation. The activities of antioxidant enzymes viz. catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) were upregulated by salt stress. Most importantly, a synergistic relationship was noted between endogenous H_2O_2 and artemisinin content i.e. both contents increased under low levels of salinity (50 and 100 mM) and thereafter decreased. Thus, it can be concluded that *A. annua* species is very sensitive to soil salinity; however, moderate saline conditions can be utilized to obtain more artemisinin.

Keywords: electrolyte leakage, lipid peroxidation, photosynthesis, salinity stress

INTRODUCTION

Agricultural productivity is severely affected by soil salinity and the damaging effect of salt accumulation in agricultural soils has become an important environmental concern. Every year more and more land becomes non-productive due to salt accumulation. In India, of the 9.38 million ha of salt-affected soil, 3.88 million ha are alkali soil and 5.5 million ha (including coastal lands) are saline soil (Jaleel *et al.* 2007). Plants grown in agricultural systems are exposed to many environmental stresses limiting their yield potential. Salinity is one of the limiting environmental factors for soil fertility and plant production. Excess salt in soil may adversely affect plant growth either through osmotic inhibition of water uptake by roots or specific ion effects. In many crop plants, seed germination and early seedling growth are the most sensitive stages to environmental stresses such as salinity (Yildirim *et al.* 2006; Khan and Panda 2008). Salinity has been shown to increase the uptake of Na or decrease the uptake of Ca and K (Yildirim *et al.* 2006). Specific ion effects may cause direct toxicity or, alternatively, the insolubility or competitive absorption of ions may affect plant nutritional balances (Greenway and Munns 1980). Plants exposed to salt stress adapt their metabolism in order to cope with the changing environment. Survival under these stressful conditions depends on the plant's ability to perceive the stimulus, generate and transmit signals and instigate biochemical changes that adjust metabolism accordingly (Hasegawa *et al.* 2000).

Salinity stress is known to trigger oxidative stress in plant tissues through the increase in reactive oxygen species (ROS). Chloroplasts are the major organelles producing ROS such as superoxide radicals, hydrogen peroxide (H_2O_2) and singlet oxygen during photosynthesis (Apel and Hirt 2004). Salt stress induces a significant reduction in photosynthesis, which depends on photosynthesizing tissue (leaf area) and photosynthetic pigments (Dubey 2005). ROS cause chlorophyll (Chl) degradation and membrane lipid peroxidation. So, malondialdehyde (MDA) accumulation as a product of lipid peroxidation and Chl retention are two oxidative stress indicators that are tools for determining salt tolerance in plants (Yildirim *et al.* 2008). To scavenge ROS, plants possess specific mechanisms, which include activation of antioxidant enzymes and non enzymatic antioxidants (Mittler 2002; Jaleel *et al*. 2006). ROS deteriorate membrane function (Mishra and Choudhuri 1999); NO_3^- uptake and its reduction were limited to nitrogen assimilation and plant growth under saline conditions (Silveira *et al.* 2001). To scavenge ROS, plants synthesize different types of antioxidant compounds or activate key antioxidant enzymes (Mittler 2002). The alleviation of oxidative damage and increased resistance to salinity and other environment stresses are often correlated with an efficient antioxidative system (Jaleel *et al*. 2007).

Being the world's most severe parasitic infection, malaria causes more than a million deaths and 500 million cases annually. Artemisinin, a sesquiterpene lactone containing an endoperoxide bridge and its derivatives are effective against multi-drug resistant, *Plasmodium falciparum* strains mainly in Southeast Asia and more recently in Africa, without any reputed cases of resistance (Kremsner and Krishna 2004). It is a promising drug, as it has a lack of cross resistance with other anti-malarial drugs, no known adverse effects to humans, and the ability to clear the blood of parasites more rapidly than other available drugs (Meshnick *et al.* 1996). Since 2001 artemisinin-based combination therapies (ACTs) are recommended by the World Health Organization (WHO 2006). This recommendation increased the demand for artemisinin, leading to supply shortages, although the situation has been recently reported as being under control (Roll Back Malaria 2004). It is important to explore new methods to enhance productivity of this immensely important drug plant and also to cut short its cost. Its chemical synthesis is possible but is complicated and uneconomically viable due to poor yields (Abdin *et al.* 2003), therefore the intact plant remains the only viable source of the artemisinin production,

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and the enhanced production of the artemisinin content in the whole plant is highly desirable (Abdin *et al.* 2003; Aftab *et al.* 2010a, 2010c, 2011).

Although there are some reports regarding the responses of this immensely important antimalarial plant to salinity, studies on the damaging effects of salt stress on growth and productivity in terms of oxidative stress and antioxidant enzymes are, however limited. Prasad *et al.* (1998) found that the yield increased significantly with increasing salinity stress, but further increases in salinity decreased the yield. The artemisinin content in the vegetative tissue was not influenced with a salinity stress. Qureshi *et al.* (2005) and Qian *et al.* (2007) reported that the yield was negatively influenced by salt stress, however, artemisinin content increased. The current study was therefore undertaken to determine the growth, photosynthetic measurements, lipid peroxidation, antioxidant enzymes and changes in artemisinin content associated with salinity stress.

MATERIALS AND METHODS

A greenhouse pot experiment was conducted to analyze the effects of different concentrations of soil salinity *viz.* 0, 50, 100, 150 and 200 mM at the flowering stage (150 days after sowing) of *A. annua* plants. Salinity was applied when the plants were at the stage of 3-4 true leaves. To avoid osmotic shock, NaCl concentration was increased gradually by 25 mM every day, from zero, until the final concentration of 200 mM was reached. All the chemicals used were of analytical grade and purchased from Sigma-Aldrich, USA.

Analyses of growth and yield parameters

The plants from each treatment were uprooted carefully and shoot height was recorded. Plants were washed with tap water to remove adhering foreign particles. Roots of the plant were removed and fresh mass of the shoots was recorded individually. The shoots were dried at 80°C for 48 h, and dry mass was then recorded. Total leaves of the plants were weighed to determine leaf yield.

Determination of photosynthetic parameters and pigments

Net photosynthetic rate (P_N) was measured on sunny days at 11:00 am using fully expanded leaves of *A. annua* with the help of an IRGA (Infra Red Gas Analyzer, LI-COR 6400 Portable Photosynthesis System, Lincoln, Nebraska, USA). Before recording the measurement, the IRGA was calibrated and zero was adjusted approximately every 30 min during the measurement period. Each leaf was enclosed in a gas exchange chamber for 60 s. All the attributes measured by IRGA were recorded three times for each treatment. Total Chl content in fresh leaves was estimated by the method of Lichtenthaler and Buschmann (2001). The fresh tissue from young leaves was ground using a mortar and pestle containing 80% acetone. The absorbance of the solution was recorded at 662 and 645 nm for Chl estimation using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan).

Assay of carbonic anhydrase activity

Carbonic anhydrase (CA; E.C. 4.2.1.1) activity was measured in fresh leaves using the method as described by Dwivedi and Randhawa (1974). 200 mg of fresh leaf pieces were weighed and transferred to Petri dishes. The leaf pieces were dipped in 10 mL of 0.2 M cystein hydrochloride solution for 20 min at 4°C. To each test tube, 4 mL of 0.2 M sodium bicarbonate solution and 0.2 mL of 0.022% bromothymol blue were added. The reaction mixture was titrated against 0.05 N HCl using methyl red as indicator. The enzyme was expressed as μM CO_2 kg^{-1} leaf FW s^{-1}.

Determination of electrolyte leakage and proline (Pro) content

Electrolyte leakage was used to assess membrane permeability as described by Lutts *et al.* (1995). Samples were washed three times with double distilled water (DDW) to remove surface contamination. Young leaf discs from each sample were taken. Leaf discs were placed in a closed vial containing 10 mL of DDW and incubated on a rotatory shaker for 24 h. Subsequently the electrical conductivity of solution (EC_1) was determined. Samples were then autoclaved at 120°C for 20 min and last electrical conductivity (EC_2) was noted after cooling the solution at room temperature. Electrolyte leakage was calculated as:

$$\text{Electrolyte leakage (\%)} = (EC_1/EC_2) \times 100$$

The Pro content was estimated by the method of Bates *et al.* (1973). The leaves were homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 10,000 rpm. The supernatant was used for the estimation of Pro content. The reaction mixture consisted of 2 mL acid ninhydrin and 2 mL of glacial acetic acid, was boiled at 100°C for 1 h. After termination of the reaction in an ice bath, the reaction mixture was extracted with 4 mL of toluene and absorbance was read at 520 nm.

Lipid peroxidation rate (TBARS content)

Oxidative damage to leaf lipids was estimated by the content of total 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA). TBARS content was estimated by the method of Cakmak and Horst (1991). TBARS were extracted from 0.5 g chopped fresh leaves, ground in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA). Following the centrifugation at 12000 × *g* for 5 min, a 1-mL aliquot from the supernatant was added to 4 mL of 0.5% (w/v) TBA in 20% (w/v) TCA. Samples were incubated at 90°C for 30 min. Thereafter, the reaction was stopped in an ice bath. Centrifugation was performed at 10000 × *g* for 5 min, and absorbance of the supernatant was read at 532 nm on a spectrophotometer and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. TBARS content was expressed as nmol g^{-1} FW.

Antioxidant enzymes assay

Catalase (CAT; E.C. 1.11.1.6) activity was measured according the method given by Chandlee and Scandalios (1984) with a small modification. The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer (pH 7.0), 0.4 mL of 15 mM H_2O_2 and 0.04 mL of enzyme extract. The decomposition of H_2O_2 was followed by a decline in absorbance at 240 nm. Enzyme activity was expressed in U mg^{-1} protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein).

Peroxidase (POX; E.C. 1.11.1.6) activity was assayed by the method of Kumar and Khan (1982). The assay mixture of POX contained 2 mL of 0.1 M phosphate buffer (pH 6.8), 1 mL of 0.01 M pyrogallol, 1 mL of 0.005 M H_2O_2 and 0.5 mL of enzyme extract. The solution was incubated for 5 min at 25°C after which the reaction was terminated by adding 1 mL of 2.5 N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a reagent blank prepared by adding the extract after the addition of 2.5 N H_2SO_4 at zero time. The activity was expressed in U mg^{-1} protein. 1 U of the enzyme activity corresponded to an amount of enzyme that caused an increase in the absorbance of 0.1 min^{-1} mg^{-1} protein.

Superoxide dismutase (SOD; E.C. 1.15.1.1) activity was assayed as described by Beauchamp and Fridovich (1971). The reaction mixture contained 1.17×10^{-6} M riboflavin, 0.1 M methionine, 2×10^{-5} M KCN and 5.6×10^{-5} M nitroblue tetrazolium salt (NBT) dissolved in 3 mL of 0.05 M sodium phosphate buffer (pH 7.8) and 3 mL of the reaction medium was added to 1 mL of enzyme extract. The mixtures were illuminated in glass test tubes by two sets of Philips 40W fluorescent tubes in a single row. Illumination was started to initiate the reaction at 30°C for 1 h. Identical solutions that were kept under dark served as blanks. The absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity was expressed as U mg^{-1} protein. 1 U was defined as the amount of change in the absorbance by 0.1 h^{-1} mg^{-1} protein.

Determination of endogenous H_2O_2 production

The content of H_2O_2 in the leaves was determined according to the method of Mukherjee and Choudhuri (1983). Fresh leaves of *A. annua* (0.5 g) were homogenized using a cold mortar and pestle in pre-cooled acetone (5 mL) and the homogenate was centrifuged at 12000 × *g* for 5 min. One mL of the supernatant was mixed with 0.1 mL of 5% $Ti(SO_4)_2$ and 0.2 mL 19% ammonia. After a precipitate was formed, the reaction mixture was centrifuged at 12000 × *g* for 5 min. The resulting pellet was dissolved in 3 mL of 2 M H_2SO_4 and the absorbance was read at 415 nm using a spectrophotometer. The H_2O_2 concentration was calculated according to a standard curve of H_2O_2 ranging from 0 to 10 µM.

Artemisinin extraction and estimation

Dry leaf material (1 g) was used to estimate artemisinin modified to a compound Q_{260} (artemisinin was converted to compound Q_{260} as this is a UV absorbing compound to detect on UV-detector) and quantified using HPLC method (Zhao and Zeng 1986). A standard curve was prepared using 1 mg of standard artemisinin dissolved in 1 mL of HPLC-grade methanol to make the stock solution. It was extracted with 20 mL petroleum ether in shaker at 70 rpm for 24 h. After 24 h, the solvent was decanted and pooled and 20 mL of petroleum ether added again and this step was repeated three times. Petroleum ether fractions were pooled and concentrated under reduced pressure and residues de-fatted with CH_3CN (10 mL × 3). Precipitated fat was filtered out and filtrate concentrated under reduced pressure. Residues were dissolved in 1 mL of methanol. 100 µL aliquot of each sample of each treatment was taken and to this 4 mL of 0.3% NaOH was added. The samples were incubated in shaking water bath at 50°C for 30 min, thereafter cooled and neutralized with glacial acetic acid (0.1 M in 20% MeOH). The pH of the solution was maintained at 6.8. Derivatized artemisinin was analyzed and quantified through reverse phase column (C18; 5 µm; 4.6 mm; 250 mm) using premix methanol: 10 mM K-Phosphate buffer (pH, 6.5) in the ratio of 60:40 as mobile phase at constant flow rate of 1 mL/min, with the detector set at 260 nm. Artemisinin was quantified against the standard curve of artemisinin, obtained from Sigma-Aldrich, USA.

Statistical analysis

Each pot was treated as one replicate and all the treatments were replicated five times. The data were analyzed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Mean values were statistically compared by Duncan's multiple range test (DMRT) at $P < 0.05\%$.

RESULTS

The presence of salinity in the soil medium significantly lowered the values for growth attributes (Shoot and root lengths, shoot and root dry weights). Shoot length in 200 mM NaCl-treated plants was reduced by 60.5% compared to control plants (**Table 1**). Root length was more severely affected by NaCl toxicity compared to shoots. In 200 mM NaCl-treated plants, root length was reduced 75.4% com-

Table 1 Effect of different salinity treatments on growth attributes of *Artemisia annua* L. Means within a column followed by the same letter are not significantly different compared by Duncan's multiple range test ($P \leq 0.05$). The data shown are means of five replicates ± SE.

Treatments	Shoot length (cm)	Root length (cm)	Shoot dry mass (g)	Root dry mass (g)
Control	89.4 ± 3.21 a	30.7 ± 0.97 a	102.5 ± 3.74 a	26.3 ± 0.83 a
50 mM NaCl	84.1 ± 3.23 b	28.7 ± 0.81 b	93.8 ± 3.17 b	24.7 ± 0.81 b
100 mM NaCl	77.9 ± 2.21 c	24.8 ± 0.91 c	82.4 ± 3.08 c	21.6 ± 0.72 c
150 mM NaCl	67.3 ± 2.36 d	21.4 ± 0.84 d	71.2 ± 2.53 d	17.6 ± 0.67 d
200 mM NaCl	55.7 ± 1.14 e	17.5 ± 0.79 e	56.3 ± 2.31 e	14.8 ± 0.54 e

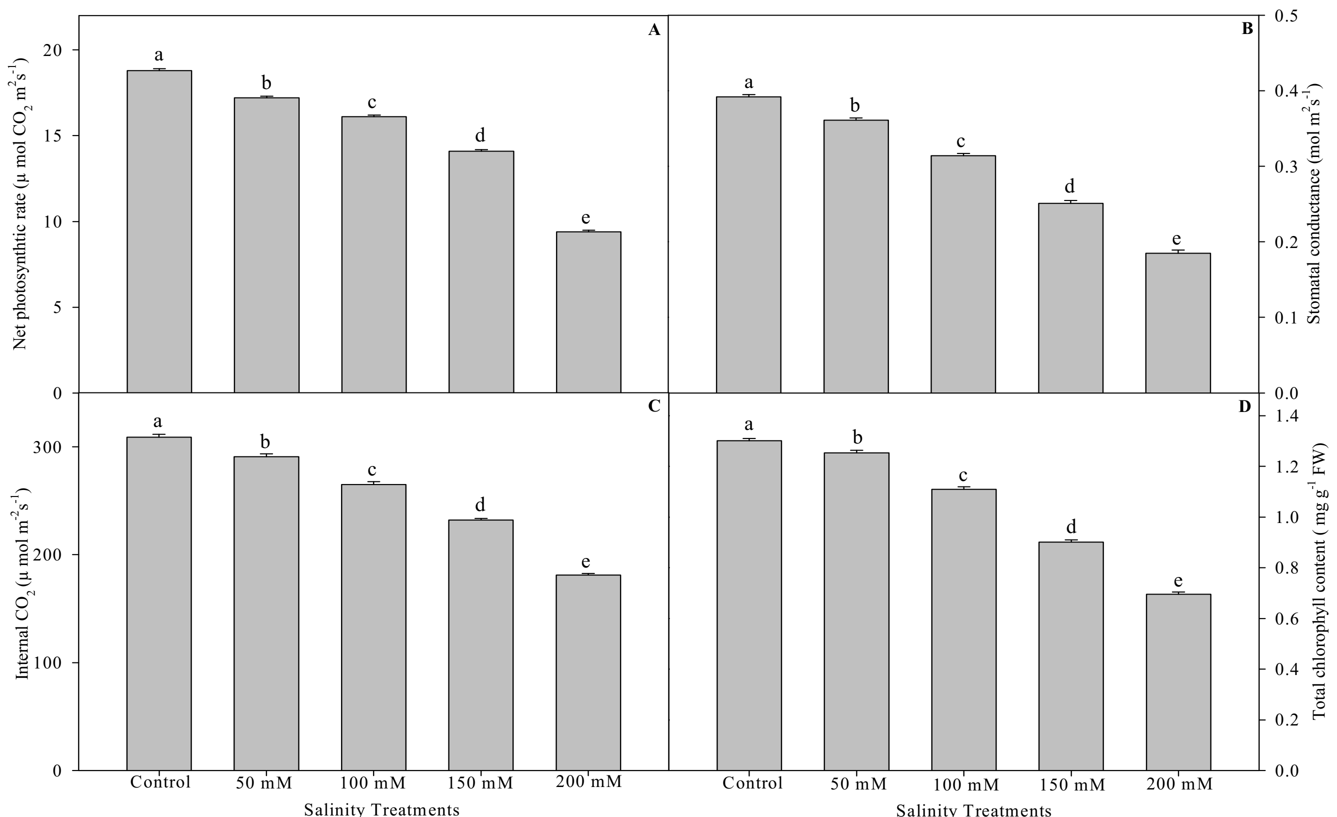

Fig 1 Effect of different salinity treatments on net photosynthetic rate (**A**), stomatal conductance (**B**), internal CO_2 (**C**) and total chlorophyll content (**D**) of *Artemisia annua* L. Bars showing the same letter are not significantly different at $P \leq 0.05$ as determined by Duncan's multiple range test. The data shown are means of five replicates and error bars show SE.

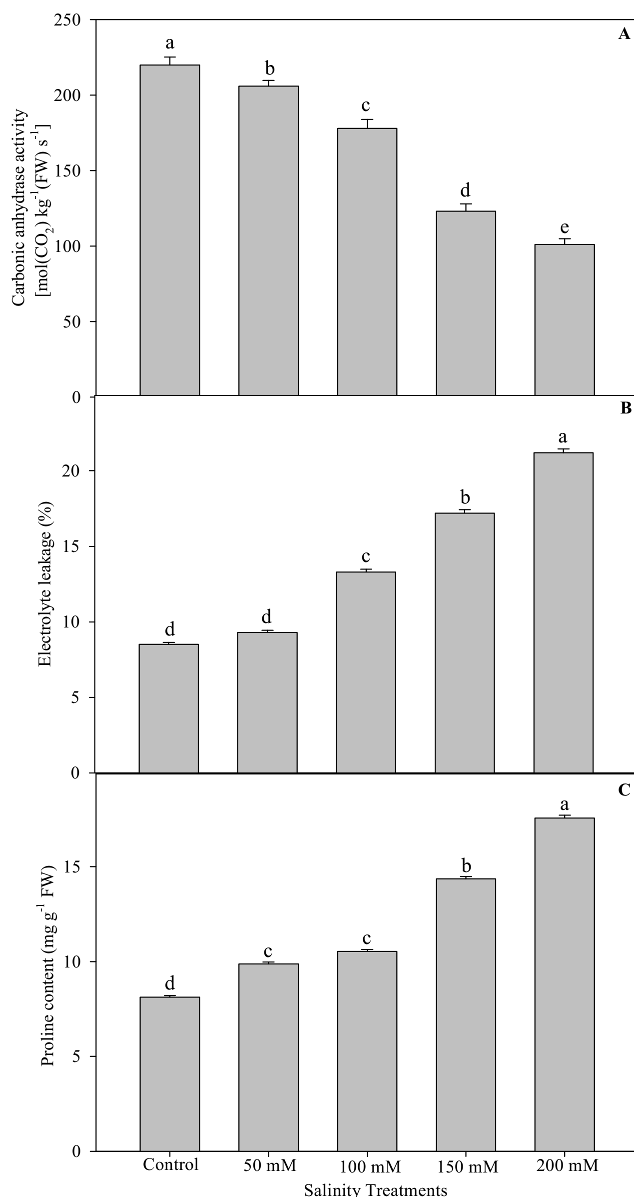

Fig. 2 Effect of different salinity treatments on CA activity (**A**), electrolyte leakage (**B**) and proline content (**C**) of *Artemisia annua* L. Bars showing the same letter are not significantly different at $P \leq 0.05$ as determined by Duncan's multiple range test. The data shown are means of five replicates and error bars show SE.

pared to untreated plants (**Table 1**). Shoot and root dry weights were also significantly reduced by different salinity treatments. At 200 mM NaCl (the highest applied concentration), the shoot and root dry weights of the plant were reduced by 82.1 and 77.8%, respectively, compared to the control (**Table 1**).

A reduced rate of photosynthetic activity was observed in the salinity treated plants. In comparison to control, at 200 mM NaCl concentration, the net photosynthetic rate was 98.4% lower showing the degree of damage to the leaf tissues (**Fig. 1A**). Both, stomatal conductance and internal CO_2 was observed the lowest (111.8 and 70.7% less than the control, respectively) at 200 mM NaCl (**Fig. 1B, 1C**). Chl content was also reduced in salt-stressed plants and the most toxic effect was noted at 200 mM NaCl at which the content was 87.9% less than that of untreated plants (**Fig. 1D**).

Salinity stress decreased the activity of CA measured in the leaves of treated plants; at 200 mM NaCl, the activity of CA was reduced by 116% compared to the control (**Fig. 2A**). The toxic effect of salinity on electrolyte leakage and Pro was proportionate to the concentration applied and the highest applied concentration (200 mM NaCl) decreased both by 143 and 115%, respectively compared to the control (**Fig. 2B, 2C**).

The values of TBARS content and antioxidant enzymes significantly enhanced in the plants subjected to salt stress. The highest TBARS content was noted when the plants were supplied with 200 mM NaCl through the soil (**Fig. 3A**). CAT activity was high in the plants receiving different NaCl treatments, the most toxic effect being at 200 mM NaCl (**Fig. 3B**). Compared to the control, the activity of POX and SOD also significantly increased in salt-treated plants and the application of 200 mM NaCl to *A. annua* plants showed highest activity of POX while SOD activity was maximum at 150 mM of NaCl (**Fig. 3B, 3C**).

The content of endogenous H_2O_2 was measured in order to determine the internal ROS status by salt stress in *A. annua* plants. H_2O_2 content was higher in plants treated with 50 and 100 mM NaCl, compared to the control, suggesting an increment in the level of ROS in cells. At 100 mM of salt, the increase in internal H_2O_2 was 44.9% compared to the control (**Fig. 4A**). The artemisinin content was highest when 100 mM NaCl was applied through soil (47.8% more) compared to untreated plants, although a decrease in artemisinin content was noted when higher doses of salt were applied (**Fig. 4B**).

DISCUSSION

Growth parameters

From the results of the present study, it is clear that salinity caused an overall reduction in growth of *A. annua* plants when compared with the control (**Table 1**), which is in agreement with what has been earlier reported in different crops (Athar *et al.* 2008; Siddiqui *et al.* 2008; Khan *et al.* 2010). Prasad *et al.* (1998) found that the vegetative yield increased significantly with increasing salinity stress to 6.0 dS/m, but further increases in salinity decreased the yield. Qureshi *et al.* (2005) and Qian *et al.* (2007) reported that the yield was negatively influenced by salt stress. Salinity reduces the imbibition of water because of lowered osmotic potentials of the medium and causes changes in metabolic activities, thereby leading to a reduction in growth (Meneguzzo *et al.* 1999; Jaleel *et al.* 2007). Also, salt stress limits plant growth by adversely affecting various physiological and biochemical processes, including antioxidant capacity and homeostasis (Ashraf 2004). Reduction in growth of *A. annua* in response to increasing salt stress might have been due to variation in a number of biochemical or physiological traits that are associated with mechanisms of salt tolerance such as photosynthesis, nutrient homoeostasis, accumulation of compatible solutes and activities of antioxidant enzymes, etc.

Photosynthetic parameters

Extensive literature is available which shows that plant growth is dependent on plant photosynthetic capacity. Therefore, a decline in productivity because of salinity stress is often associated with a reduction in photosynthesis capacity (Ashraf 2004; Dubey 2005). In the present study, a reduced net photosynthetic rate, stomatal conductance and internal CO_2 concentration was noticed when plants were exposed to salinity. Salt stress damages the photosynthetic machinery at multiple levels, such as pigments, stomatal functioning and gaseous exchange, structure and function of thylakoid membrane, electron transport and enzymes (Sudhir and Murthy 2004). Excess salt causes stomata to close, thereby decreasing the partial CO_2 pressure (Bethkey and Drew 1992) as well as internal CO_2 concentration and consequently a decrease in the net photosynthetic rate. In a number of studies with different crop species, salt stress reduced photosynthetic rate (Ashraf 2004; Khan *et al.* 2010). As observed in the present study, oxidative stress might be induced due to decreased stomatal conductance in response to abiotic stress. Consequently, due to limitation of CO_2,

Fig. 3 Effect of different salinity treatments on TBRAS content (**A**), CAT (**B**), POX (**C**) and SOD (**D**) activities in leaves of *Artemisia annua* L. Bars showing the same letter are not significantly different at $P \leq 0.05$ as determined by Duncan's multiple range test. The data shown are means of five replicates and error bars show SE.

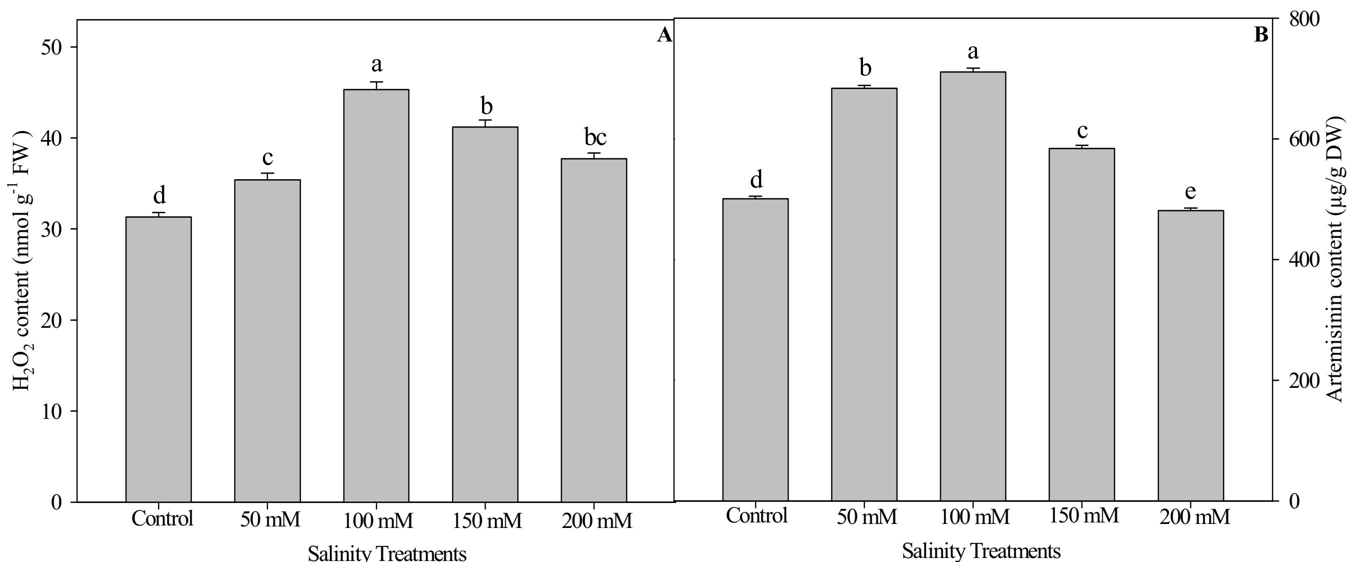

Fig. 4 Effect of different salinity treatments on H_2O_2 (**A**) and artemisinin (**B**) content of *Artemisia annua* L. Bars showing the same letter are not significantly different at $P \leq 0.05$ as determined by Duncan's multiple range test. The data shown are means of five replicates and error bars show SE.

electrons from PSII are available to form fatty acids of thylakoids and stroma (Qureshi *et al.* 2005). The possible disruption of thylakoid and stomatal membranes results in chlorosis and necrosis leading ultimately to decrease in photosynthesis and photosynthetic pigments, hence reduced availability of photosynthates for biomass accumulation. The decrease in Chl content can be attributed to oxidation of Chl and chloroplastic membranes; instability of the pigment protein complex, which might be exacerbated by excess salt, as reported by Stepien and Klobus (2006) in *Cucumis sativus* on applying 100 mM of salt. These observations of lower Chl content are consistent with those of Shim *et al.* (2003) and Stepien and Klobus (2006) who indicated that Chl content considerably decreased in the leaves of spinach and cucumber plants, respectively with increasing NaCl concentrations from 0 to 100 mM. Parida and Das (2005) reported that salt stress inhibited the Chl content in the leaves of many crops.

Physiological and biochemical parameters

CA is the enzyme that plays many diverse roles in physiological processes such as ion exchange, acid–base balance, carboxylation/decarboxylation reactions and inorganic carbon diffusion between the cell and its environment as well as within the cell (Georgios *et al.* 2004). Also, the activity of CA, which catalyzes the interconversion of CO_2 and HCO_3, is regulated by photon flux density, CO_2 concentration, availability of Zn (Tiwari *et al.* 2005) and expression of genes encoding CA protein (Kim *et al.* 1994). NaCl-fed plants showed the lowest CA activity, which may be due to inactivation of Rubisco, which sequentially reduces the net photosynthetic rate, carbon metabolism, leaf Chl content and photosynthetic efficiency (Seeman and Critchley 1985).

Electrolyte leakage enables cell membrane injury to be assessed when plants are subject to salinity stress. Maintaining integrity of cellular membranes under salt stress is considered an integral part of salinity tolerance mechanism (Stevens *et al.* 2006). The plants treated with NaCl exhibited a significant increase in electrolyte leakage compared to the control plants. It has been reported that salt stress led to a significant increase in the level of electrolyte leakage in many crop (Parida and Das 2005; Khan *et al.* 2007; Siddiqui *et al.* 2008). A compatible solute which accumulates under salt stress in plants is Pro. In the present study, an increase in Pro content in the NaCl-treated plants was noted, being highest at 200 mM (**Fig. 2C**). Although the precise role of Pro accumulation is still debated, it is often considered as a compatible solute involved in osmotic adjustment (Azooz *et al.* 2004). The accumulation of Pro may be through an increase in its synthesis constantly with inhibition of its catabolism (Yoshiba *et al.* 1997) and may be a mechanism for stress tolerance. However, its role in imparting stress resistance under saline conditions is controversial. Anyway, understanding the biosynthesis, degradation, transport and role of Pro during stress and the signalling events that regulate stress-induced accumulation is vital in developing plants for stress tolerance (Jaleel *et al.* 2007).

The lipid peroxidation rate (TBARS content) was measured in leaves as an indicator of oxidative stress. Increment in oxidative stress was noted when the plants were exposed to different concentrations of salinity (**Fig. 3A**). Mittler (2002) proposed that membrane damage might be caused by high H_2O_2 levels, which could accelerate the formation of hydroxyl radical and thus lipid peroxidation. In NaCl-treated plants, oxidative stress might be induced due to the decreased stomatal conductance in response to the osmotic imbalance and reduced leaf water potential. Lipid peroxidation has been associated with damages provoked by a variety of environmental stresses (Hernàndez *et al.* 2003). The increase in lipid peroxidation can be correlated with the accumulation of ions and ROS production under salt stress (Hernàndez *et al.* 2003; Misra and Gupta 2006). In accordance with the present results, others have found that excess salt increased TBARS content in *A. annua* and other plants (Qureshi *et al.* 2005; Pan *et al.* 2006; Jaleel *et al.* 2007, 2008).

Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to oxidative damage (Sudhakar *et al.* 2001). Environmental stresses increase the formation of ROS that oxidize membrane lipids, protein and nucleic acids (Gong *et al.* 2005). Under salt stress, plants are overloaded with ROS, which inhibit several plant processes and cause damage to the plants in different ways. ROS generate hydroxyl radicals and other destructive species such as lipid peroxides (Vaidyanathan *et al.* 2003), which causes destruction of cell membrane as reflected by increased values for TBARS under salt stress. Thus, to maintain metabolic functions under stress, the scavenging of ROS is required. ROS scavenging depends on detoxification mechanism provided by antioxidant enzymes. CAT and POX activities increased significantly in NaCl-treated plants with respect to the control, while SOD activity increased up to 150 mM NaCl and thereafter a decrease in its activity was noted (**Fig. 3B-D**). An increase in CAT, POX and SOD, was documented by many others investigating the salinity stress in several plant species (Koca *et al.* 2007; Pan *et al.* 2006; Jaleel *et al.* 2007, 2008). CAT and POX appear to play an essential protective role in scavenging process of ROS when coordinated with SOD (Jaleel *et al.* 2009). SOD initiates detoxification of singlet oxygen by forming H_2O_2, which is also toxic and must be eliminated by conversion to H_2O in subsequent reactions. In plants, a number of enzymes regulate intracellular H_2O_2 levels, but CAT and POX are considered the most important (Noctor and Foyer 1988). Superoxide radicals are toxic by-products of oxidative metabolism and can interact with H_2O_2 to form highly reactive hydroxyl radicals, which are thought to be primarily responsible for oxygen toxicity in the cell (Azevedo *et al.* 2005). The dismutation of superoxide radicals into H_2O_2 and oxygen is an important step in protecting the cell and is catalyzed by SOD.

The H_2O_2 concentration was increased significantly by applying 50 and 100 mM NaCl and thereafter a decrease in the concentration of H_2O_2 was observed (**Fig. 4A**). A common denominator in all adverse conditions (abiotic stresses) is the over production of ROS within different cellular compartment of the plant cell (Pinheiro *et al.* 2004). Enhanced ROS production in *A. annua* was detected by others due to different environmental stresses (Qureshi *et al.* 2005; Ferreira 2007; Guo *et al.* 2010). In our previous study, we reported that mild boron stress (0.50 and 1.00 mM) increase the H_2O_2 production in *A. annua* plants (Aftab *et al.* 2010b). As CAT, POX and SOD are known to be scavengers of ROS, when the content of ROS reaches above the threshold, they scavenge them and the observed reduction in the content of H_2O_2 after a certain NaCl concentration is obvious.

Artemisinin content and yield

Prasad *et al.* (1998) found that the artemisinin content in the vegetative tissue was not influenced with a salinity stress while Qureshi *et al.* (2005) and Qian *et al.* (2007) reported that the artemisinin content increased on salinity stress. An association between H_2O_2 and artemisinin production was noted in the present study. Overproduction of artemisinin content was observed when 50 and 100 mM NaCl was applied and at further high concentrations it was decreased (**Fig. 4B**). Wallaart *et al.* (2000), Ferreira (2007), Pu *et al.* (2009) and Guo *et al.* (2010) showed a direct relationship between ROS and artemisinin content. In our lab study, we observed that artemisinin content enhanced significantly upon exposure to boron stress (0.50 and 1.00 mM) and decreased by applying higher concentrations (Aftab *et al.* 2010b). From the current research it is clear that low levels of salinity (50 and 100 mM) promoted the biosynthesis of artemisinin and also we have established the possible connection between artemisinin and H_2O_2, which is considered to play an important role of converting dihydroartemisinic acid to artemisinin during artemisinin synthesis. Wallaart *et al.* (1999) suggested that dihydroartemisinic acid might act as a scavenger of ROS that are released in plant cells when they are exposed to oxidative stress. Our results are in agreement with the observations recorded by Wallaart *et al.* (2000), Ferreira (2007), Pu *et al.* (2009) and Guo *et al.* (2010) who advocated that relatively high levels of ROS were produced on occurrence of various stresses that in turn, resulted in enhanced conversion of dihydroartemisinic acid to artemisinin.

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