Physiological and phytochemical changes induced by seed pretreatment with hydrogen peroxide in *Artemisia sieberi* under salt stress

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Abstract

Seeds of medicinal plant *Artemisia sieberi* were pretreated with H_2O_2 (0, 10, 50, 90, and 140 µM) and grown in saline condition (0 and 150 mM NaCl) for one month. Phytochemical properties such as antioxidant capacity and also salt tolerance in the plans arising from H_2O_2 pretreated seeds under salt stress were examined. Results showed a decrease in H_2O_2 and malondialdehyde concentrations in the shoots of pretreated *A. sieberi*. Furthermore, seed pretreatment with H_2O_2 (particularly at 50 µM) increased photosynthetic pigments content, antioxidant capacity and dry and fresh weights of shoots in the pretreated plants under both normal and saline conditions. The activity of the antioxidant enzymes increased in all pretreated *A. sieberi* plants particularly at 50 µM H_2O_2. Data indicated H_2O_2pretreatment can induce salt tolerance in *A. sieberi* by supporting physiological and phytochemical processes such as photosynthesis, reactive oxygen species scavenging and detoxification, and also membrane stability in this species. Additionally, hydrogen peroxide pretreatment enhanced reducing power and antioxidant activity in *A. sieberi* suggesting an increase in its medicinal properties.

Key words: Artemisia *sieberi;* antioxidant activity; H_2O_2-priming; salt tolerance; oxidative stress

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Introduction

Artemisia sieberi Bess., belonging to Asteraceae, is broadly distributed in the desert area of Iran and locally named 'Dermaneh-Dashti'. This species is resistant to drought and plays an important role in preserving the soil in dry regions (Ehsani, 2013). Furthermore, this plant has medicinal benefits for humans such as spasmolytic, vermicidal, insecticidal, and anticandidal properties (Negahban et al., 2007; Mahboubi et al., 2008; Sharafi et al., 2014).

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Baghery and Abbaszadeh (2014) reported that saline condition influenced phytochemical characters of *A. sieberi* as the essential oil of its flowering shoot increased under salinity. Salinity as an abiotic stress negatively affects plant growth and development. Under saline condition, reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, and hydrogen peroxide are generally generated and accumulated, resulting in oxidative stress (Ozgur et al., 2013). ROS are detrimental to biomolecules such as DNA, proteins, and also cellular membranes, which all lead to cell death. In contrast, plants ameliorate harmful effects of

oxidative stress due to ROS by producing enzymatic (such as superoxide dismutase, catalase, and peroxidase) and non-enzymatic antioxidant compounds (e.g. phenolic compounds and flavonoids) (Gill and Tuteja, 2010). In addition, it is well-documented that plants with strong antioxidant properties which have high levels of radical scavenging activity are valuable for human health (Krishnaiah et al., 2010). As such, introducing plant materials comprising this characteristic or applying treatments to elevate antioxidant capacity in plants could be of value. These treatments would be more appropriate as they enable plants to increase their tolerance to environmental stresses such as salinity.

It is confirmed that hydrogen peroxide (H_2O_2), is toxic at high levels and causes oxidative damage but could also act as a signal molecule at low concentrations leading to development of adaptive mechanisms in plants under stressful conditions (Hossein et al., 2015). Yet, various studies have detected the role of exogenous H_2O_2, either as addition of H_2O_2 to the nutrient solution or leaf spraying, to reduce injurious effects of abiotic stresses such as salinity (Azevedo-Neto et al., 2005; Tanou et al., 2009; Gondim et al., 2012), heavy metal stresses (Chao and Kao, 2010; Xu et al., 2011), chilling (Kumar et al. 2010) and heat stress (Gao et al., 2010). On the other hand, the benefit of seed pretreatment with H_2O_2 has also been reported to alleviate abiotic stresses. For instance, Wahid et al. (2007) indicated that pretreatment of seeds with H_2O_2 in *Triticum aestivum* caused an improvement in salt tolerance of the seedlings via mitigation of oxidative damage and expression of stress proteins. He et al. (2009) reported a higher germination rate in the H_2O_2-pretreated seeds of *Triticum aestivum* under drought condition compared to control. Besides, these seedlings exhibited increased growth characteristics such as dry weight. Likewise, Gondim et al. (2012) showed that seed pretreatment with H_2O_2 decreased the deleterious effects of salt stress on the growth of *Zea mays*. Also, Abass and Mohamed (2011) stated that H_2O_2pretreatment of seeds alleviated the adverse effects of drought stress in *Phaseolus vulgaris*. In another report, Çavusoglu and Kabar (2010) explained the affirmative effects of H_2O_2priming on the germination and early seedling growth of *Hordeum vulgare* under salt and temperature stress. Moreover, Kumar et al. (2010) declared that exogenous pretreatment of H_2O_2 to seeds of *Brassica juncea* acclimatized the seedlings to tolerate chilling stress. Alternatively, Lin and Block (2010) claimed that the positive effect of exogenous H_2O_2 utilization is not obvious under all experimental conditions tested.

In the present work we studied the effect of H_2O_2 pretreatment of seeds of *A. sieberi* on phytochemical properties such as antioxidant capacity and also salt tolerance in the plans arising from H_2O_2 pretreated seeds under salt stress.

Materials and Methods

Seed pretreatment

Seeds of *Artemisia sieberi* Boiss. were purchased from Pakan-Bazr (Isfahan, Iran) and sterilized with 70% ethanol for 2 min. Then, the seeds were washed with sterilized with distilled water for several times. Seed pretreatment with H_2O_2 was accomplished according to the protocol of Wahid et al. (2007) with some modifications. Seeds were soaked in 0, 10, 50, 90, and 140 µM H_2O_2solution for 2, 3, 5, 7, and 9 hours. Seeds were washed with distilled water and blot dried to assess the absorbed H_2O_2 as described below.

Treatments and plant growth conditions

The preliminary experiments showed that among 50, 100, 150, 200, and 250 mM NaCl treatments, application of 150 mM NaCl reduced germination percentage by 50%. Also, it was revealed that among designed exposure times (2, 3, 4, 7, and 9h) for seed pretreatment with H_2O_2, seed exposure for 7 hours was the most effective time to enhance ROS scavenging activity (Data not shown) as the concentration of H_2O_2 was at the minimum level in the tissues of seeds. Also, seeds pretreatment with 140 µM did not germinate at all. Accordingly, the main experiment treatments included 1: control (with no H_2O_2 or NaCl treatment), 2: seed pretreatment with 0, 10, 50, and 90 µM H_2O_2 for 7h, 3: irrigation of 14-day-old seedlings with Hoagland

solution (pH 6.8) containing 150 mM NaCl (no pretreatment with H_2O_2), and 4: seed pretreatment with H_2O_2 (as in 2) along with irrigation of 14-day-old seedlings with 150 mM NaCl. Treated and untreated seeds (15 seeds in each box, from which seedlings were reduced to 5 seedlings at the start of salinization) were sown in polystyrene boxes, filled with a potting mixture composed of 50% perlite and 50% fine sand. The plants were raised in a green house under controlled conditions (16/8 h light/dark period, 32/25° C temperature, 60-70% RH and 1000-1200 µM m^{-2} s^{-1}PAR).The experiments lasted for one month, and at the end of the experiments, 45-day-old plants were sampled to determine shoot fresh and dry weight.

Estimation of H_2O_2 content

Hydrogen peroxide content was evaluated by measuring the absorbance of titanium-hydroperoxide complex (Nag *et al*., 2000). Fresh leaf samples (1 g) were homogenized in 12 ml of cold acetone. Then, 4 ml of titanium reagent was added to the mixture followed by 5 ml of concentrated ammonium solution to precipitate hydroperoxide-titanium complex. The mixture was centrifuged in the refrigerated centrifuge for 5 min at 8500 g. The pellet was washed twice with 5 ml acetone followed by dissolving in 1 M sulphuric acid. The absorbance of orange-yellow H_2O_2-Ticomplex was recorded at 410 nm against blank. Concentration of H_2O_2 was determined using standard curve plotted with known concentrations of H_2O_2 (a range of 10-100 µM).

Total water content

The water content was determined based on Gong et al. (2005) by drying the leaves at 80˚C for 48 h and calculated as follows:
water content (%) = (fresh weight - dry weight)/fresh weight ×100.

Photosynthetic pigments

The contents of total chlorophyll (Ch-a + Ch-b) and carotenoids were determined according to the method of Lichtenthaler and Buschmann (2001) with 80% acetone as the solvent.

$$\text{Ch-a } (\mu g mL^{-1}) = 12.25A_{663} - 2.79A_{646}$$

$$\text{Ch-b } (\mu g mL^{-1}) = 21.5A_{646} - 5.1A_{663}$$

$$\text{Ch-Total } (\mu g mL^{-1}) = \text{Ch-a} + \text{Ch-b}$$

$$\text{Car} = (1000A_{470} - 1.82 \text{ Ch-a} - 85.02\text{Ch-b})/198$$

where, A_{663}, A_{645}, and A_{470} represent absorbance values read at 663, 645, and 470 nm wavelengths, respectively.

Lipid peroxidation

Lipid peroxidation was evaluated in terms of malonyldialdehyde (MDA) content (Ksouri et al., 2007). Fresh samples of shoots (250 mg fresh weight) were homogenized in 5 ml of 0.1% trichlroacetic acid (TCA). The homogenate was centrifuged at 10000g for 10 min at 4°C, and 1 ml supernatant was mixed with 5 ml of 0.5% thiobarbituric acid (TBA) prepared in TCA 20%, and incubated at 95°C for 30 min. Reaction was stopped by placing the tubes in an ice bath, and samples were centrifuged at 10000g for 5 min. The absorbance of the supernatant was measured at 532 nm and after subtracting the non-specific absorbance at 600 nm, MDA concentration was determined using the extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Enzyme extraction and assay

Enzyme extraction procedure was accomplished according to the method of Chen et al. (2000) with some modifications. All of the following operations were performed at 4˚C. Fresh leaf samples (1g) were ground in a mortar with liquid nitrogen and extracted in 100 mM Na-phosphate buffer (pH 6), containing 0.1 mM EDTA. The homogenate was centrifuged at 12000 g for 20 min. The supernatant was transferred to Eppendorf tubes and kept at -20˚C in a freezer.

Total SOD activity was assayed in 100 mM potassium phosphate buffer, pH 7.5, 150 mM methionine L-methionine, 840 mM Nitrobluetetrazolium (NBT), and 24 µM riboflavin by using the photochemical NBT method in terms of SOD's ability to inhibit reduction of NBT to

form formazan by superoxide (Sairam et al., 2002). The photoreduction of NBT was measured at 560 nm. Catalase activity was evaluated spectrophotometrically by determining the consumption of H_2O_2 (ε= 39.4 mM^{-1} cm^{-1}) at 240 nm in 50 mM phosphate buffer, pH 7.5 and 200 mMH_2O_2 (Nemat- Ala and Hassan, 2006). Total ascorbate peroxidase activity was evaluated spectrophotometrically according to the method of Kato and Shimizu (1985) at 280 nm in 0.2 mM potassium phosphate buffer, pH 7.5, 15mM ascorbic acid and 50 mMH_2O_2,, as ascorbate (ε= 2.8 mM^{-1} cm^{-1})was oxidized. Guaiacol peroxidase activity was assayed in 44 mMH_2O_2, and 45 mMguaiacol. The absorption at 470 nm was recorded and the activity was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} (Buchanan and Balm, 2005).

All enzyme activities were expressed as units per mg of protein. Protein content in all enzyme extracts was determined according to the method of Bradford(1976).

DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical-scavenging activity

The antioxidant activity of extracts was assayed based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrozyl (DPPH) free radical (Ksouri et al., 2007). Methanolic extracts of shoots (2 ml) were mixed with 0.5 ml of 0.2 mMmethanolic DPPH, the mixture was shaken vigorously and left standing at room temperature for 30 min. The absorbance of resulting solution was measured at 517 nm. The scavenging activity was expressed as IC_{50} (μgg^{-1}dry wt.). The percentage inhibition activity of DPPH radical was calculated as:

% Inhibition= $[(A_0-A_1)/A_0]\times100$, where A_0 was the absorbance of the control and A_1 was absorbance of the extract.

Superoxide anion radical ($^{\bullet}O_2^-$)- scavenging activity

Measurement of superoxide anion scavenging activity was based on the method of Kumaran and Joel karunakaran (2006). The reaction mixture consisted of 50 mM phosphate buffer, pH 7.6, 20 μg riboflavin, 12 mM EDTA and NBT 0.1 mg 3ml^{-1}, added in that sequence. Reaction was started by illuminating (fluorescent lamp) the reaction mixture with different concentrations of the extract for 80 seconds. Immediately after illumination, the absorbance was read at 580 nm. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes, with reaction mixture were kept in the dark and served as blanks. The antioxidant activity of the extracts was based on IC_{50} (μg g^{-1}dry weight). The percentage of inhibition of superoxide anion generation was calculated using the following formula:

% Inhibition= $[(A_0- A_1) /A_0]\times100$, where A_0 was the absorbance of the control and A_1 was the absorbance of the extract.

Hydroxyl-radical ($^{\bullet}OH$) scavenging activity

Hydroxyl radical scavenging activity of methanolic extracts was measured according to the method of Yuan et al. (2005). The final reaction solution (1 ml) consisted of aliquots (500 μl) of various concentrations of the methanolic extracts of shoots of *A. sieberi*, 1mM $FeCl_3$, 1mM Na_2EDTA, 10 mM H_2O_2, 1 mM L-ascorbic acid, and 36 mM 2-deoxy-D-ribose in 25 mM phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 h at 37°C, and further heated in a boiling water bath for 15 min after addition of 1 ml of 2.8% TCA and 1 ml of 1% TBA. The color development was measured at 532 nm. Evaluating the antioxidant activity of the extracts was based on IC_{50} (μg g^{-1}dry wt.). The antiradical activity was expressed as IC_{50} (μg g^{-1}dry wt.). The inhibition percentage of hydroxyl radical scavenging activity was calculated using the following formula:

% Inhibition= $[(A_0-A_1) /A_0] \times100$, where A_0 was the absorbance of the control and A_1 was the absorbance of the extract.

Reducing power determination

The reducing power of methanolic extracts of shoots of *A. sieberi* was determined according to the method of Kumaran and Joel karunakaran (2006). Different amounts of the extract (50- 1500 μgml^{-1}) were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide $[K_3Fe(CN)_6]$. Mixture was incubated at 50°C for 20 min, followed by

addition of 2.5 ml of 10%TCA, and then centrifuged for 10 min. The upper layer of solution (2.5 mL) was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated increased reducing power.

Statistical analysis

The experiment was arranged as factorial in a completely randomized design with three replications. The data was analyzed using the SAS software (V. 9.0) and the least significant difference (LSD) among treatments for each trait was calculated. P values less than 0.05 were considered to be statistically significant.

Results

Results showed salinity (150 mM) significantly decreased shoot dry weight of *A. sieberi* by 45% compared to control (p<0.05) (Fig. I-A). H_2O_2 pretreatment, at all applied levels,brought about a considerable increase in the shoot dry weight of salinized plants of *A. sieberi*(between 1.6 to 2 folds compared to saline condition alone). H_2O_2 pretreatment also caused a significant increment in the shoot dry weight of unsalinized plants (+ 1.2 folds) compared to control. H_2O_2 pretreatment at 50 μM was the most effective one to increase shoot dry weight under both control and saline conditions.

Shoot fresh weight significantly decreased by salt stress (-29%) (p<0.05) (Fig. I-B). Under non-saline condition, seed pretreatment with H_2O_2 (at 50 and 90 μM) increased this parameter significantly (p<0.05); the positive effect of H_2O_2 pretreatment was the best (+1.9 folds) at 50 μM. Under saline condition, seed pretreatment with H_2O_2 increased shoot fresh weight of *A. sieberi*; while it was merely significant at 50 μM (+1.6 folds) (p<0.05).

Data analysis revealed that salt stress (150 mM) significantly decreased chlorophyll a, b and total chlorophyll concentrations from 26 to 55% compared to control (Figs. II-A, B, C). Seed pretreatment with H_2O_2 significantly increased chlorophylls concentration under both normal and saline conditions (p<0.05). Under salt stress,

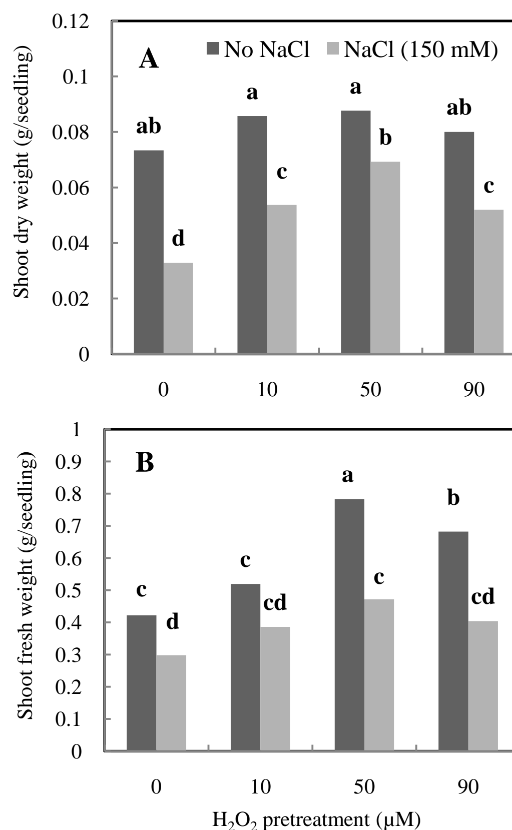

Fig. I. 45-day-old *Artemisia sieberi* plants pretreated with H_2O_2 and/or irrigated for 31 days with NaCl (150 mM). (A) Shoot dry weight, (B) Shoot fresh weight; Means (three replicates) with the same letter are not significantly different at p<0.05.

the most effective level of H_2O_2 to increase total chlorophyll content in *A. sieberi* was found at 50 μM (near to +2 folds) (Fig. II-C). Carotenoids content decreased (-70%) by 150 mM of NaCl (Fig. II-D). Seed pretreatment with H_2O_2 significantly increased carotenoids level in *A. sieberi*; this positive effect was dominant under normal condition (+32% compared to control), but there was no significant difference between different levels of H_2O_2. Under salinity, carotenoids concentration augmented up to 51% by H_2O_2 pretreatment; but there was no statistically significant difference between applied levels of H_2O_2(Fig. II-D).

Results revealed that salinity (150 mM) increased the concentration of H_2O_2 (over 2.4 folds) in the aerial parts of *A. sieberi* compared to control (Fig. III-A). Seed pretreatment with H_2O_2significantly reduced the level of H_2O_2 (from 31 to 39%) in the shoots of salinized plants (p<0.05). H_2O_2 pretreatment at 50μM showed the

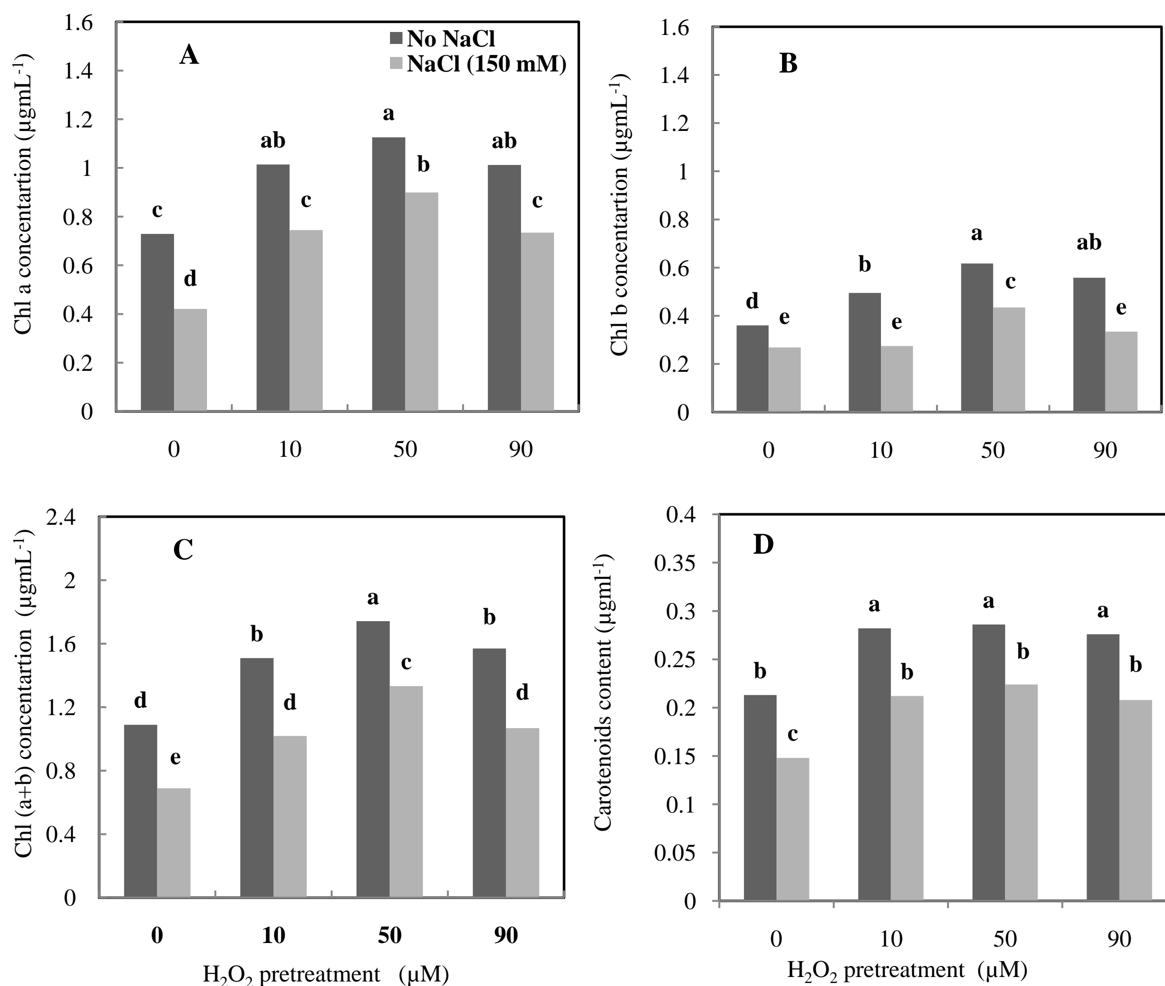

Fig. II. 45-day-old *Artemisia sieberi* plants pretreated with H_2O_2 and/or irrigated for 31 days with NaCl (150 mM); (A) Chlorophyll a, (B) Chlorophyll b, (C) Chlorophyll (a+b), and, (D) carotenoids concentrations; Means (three replicates) with the same letter are not significantly different at p<0.05.

most effects to diminish H_2O_2 level in the shoot. At the absence of NaCl, H_2O_2 pretreatment resulted in reducing of H_2O_2 level in the aerial tissues by 42% at 50 µM H_2O_2.

As is shown in (Fig. III-B), NaCl at 150 mM significantly increased the MDA concentration by 2.5 folds compared to control, indicating an increment of lipid peroxidation in the salinized shoots of *A. sieberi.* Seed pretreatment with H_2O_2, however, decreased MDA concentration in the plants grown in the saline culture solution (p<0.05). This decrease ranged from 46 to 55% by different levels of H_2O_2. At the absence of NaCl, the least amount of MDA was obtained from those plants pretreated with 90 µM H_2O_2 (-29% compared to control). Also, the most effective level of H_2O_2 to reduce MDA (by -19% compared to control) in the salinized plants was 90 µM (Fig. III-B).

Results showed DPPH-radical scavenging activity of the methanolic extract of *A. sieberi* (IC_{50}= 533.1 µg g^{-1}dry wt.) was significantly affected by H_2O_2 and salinity (p<0.05) (Fig. IV-A). Salt stress increased DPPH-radical scavenging activity (IC_{50}= 425.2 µg g^{-1}dry wt.). Seed pretreatment with H_2O_2 also increased antioxidant properties in *A. sieberi* under both saline and non-saline conditions. The most efficient H_2O_2 level was 90 µM which caused a decrease in the IC_{50} values by 50 and 73% in the saline and control samples, respectively. Apart from the effect of H_2O_2 at 90 µM, hydrogen peroxide at 50 µM was more effective to increase this parameter compared to the plants pretreated with 10 µM.

IC_{50} for superoxide anion radical scavenging activity in the aerial parts of *A. sieberi* was 181.6 (µg g^{-1}dry wt.) in non-saline condition, which decreased to 192.9 (µg g^{-1}dry wt.) under salt stress (+7%) (Fig. IV-B). Exposure of seeds to different concentrations of H_2O_2 significantly increased superoxide anion radical scavenging activity and the IC_{50} values ranged from 50.3 to 90.5 µg g^{-1}dry wt. under control and saline conditions. Seed pretreatment with H_2O_2 at 90 µM was most effective in decreasing IC_{50} for superoxide radical scavenging activity under saline condition (-67% compared to the treated plants with only NaCl).

The IC_{50} value for hydroxyl radical scavenging activity in the aerial parts of *A. sieberi* was 184.1 (µg g^{-1}dry wt.)in normal condition, which increased to 248.1 (µg g^{-1}dry wt.) under salinity (around +1.4 folds) (Fig. IV-C). Introduction of seeds to the utilized levels of H_2O_2 significantly augmented $^{\bullet}$OH-scavenging activity and the IC_{50} values ranged from 104.3 to 203.2 µg g^{-1}dry wt. in both control and saline conditions. Under saline condition, seed pretreatment with H_2O_2 at 90 µM was most effective to decrease the IC_{50} value for $^{\bullet}$OH-scavenging activity (-43% compared to the plants treated only by NaCl). Under non-saline condition, $^{\bullet}$OH-scavenging activity of the extracts of pretreated *A. sieberi* was the most at 50 and 90 µM H_2O_2.

Data analysis indicated that salt stress (150 mM) resulted in a significant increase in IC_{50} (changing from 135.6 to 147.3 µg g^{-1}dry wt) for reducing power of *A. sieberi* (+9% compared to control) ($p<0.05$) (Fig. IV-D). Seed pretreatment with H_2O_2 significantly increased the reducing power in both salinized and not salinized plants (ranging from 91.8 to 124.7 µg g^{-1}dry wt). The effect of H_2O_2 to decrease IC_{50} was much greater in the unstressed plant compared to salinized ones. H_2O_2-preteated plants at 90 µM exhibited the highest level of reducing ability in both normal and saline conditions compared to control.

The least activity of SOD enzyme was recorded in control plants (Fig. V-A). Combined treatment of salt stress and seed pretreatment with H_2O_2 significantly increased the activity of SOD enzyme compared to control ($p<0.05$). In all H_2O_2-pretreated salinized plants, however, the

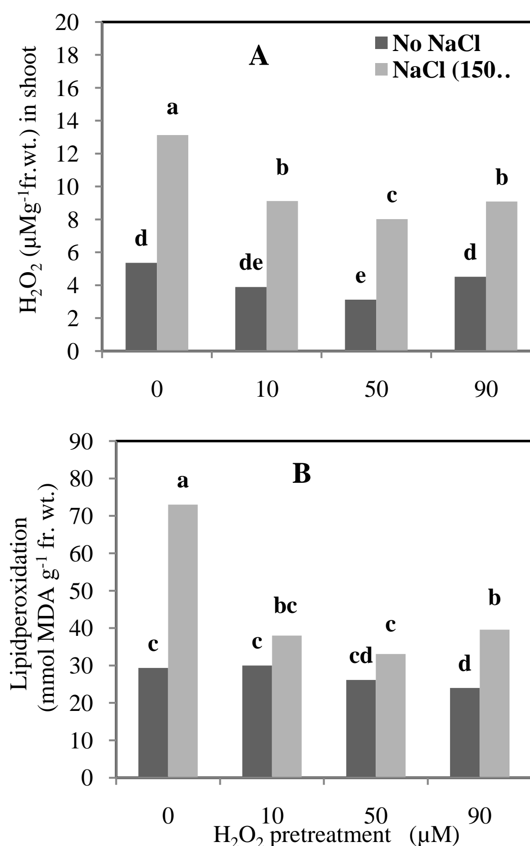

Fig. III. 45-day-old *Artemisia sieberi* plants pretreated with H_2O_2 and/or irrigated for 31 days with NaCl (150 mM). (A) H_2O_2 and, (B) MDA concentration in the shoots; Means (three replicates) with the same letter are not significantly different at $p<0.05$.

activity of this enzyme was significantly higher than that of the H_2O_2-pretreated plants grown in normal condition. There was no significant difference between salinized plants or between unsalinized ones regarding SOD enzyme activity.

Catalase activity significantly increased by NaCl and H_2O_2 ($p<0.05$) (Fig. V-B). At the absence of salt, all H_2O_2-preteated plants showed higher activity of catalase compared to control (ranging from +2 to 2.7 folds); no difference was found between H_2O_2-pretreated plants at 50 and 90 µM. Under salt stress, catalase activity increased by 22% in pretreated *A. sieberi* at 50 and 90 µM compared to the exclusively salinized ones.

Data analysis showed that salt stress (alone) at 150 mM caused an increment in APX activity by 37% compared to control ($p<0.05$) (Fig. V-C). Under non-saline condition, APX activity also significantly increased in all pretreated *A. sieberi* from 32% (at 90 µM H_2O_2) to 2.3 folds (at 50 µM H_2O_2) compared to control ($p<0.05$).

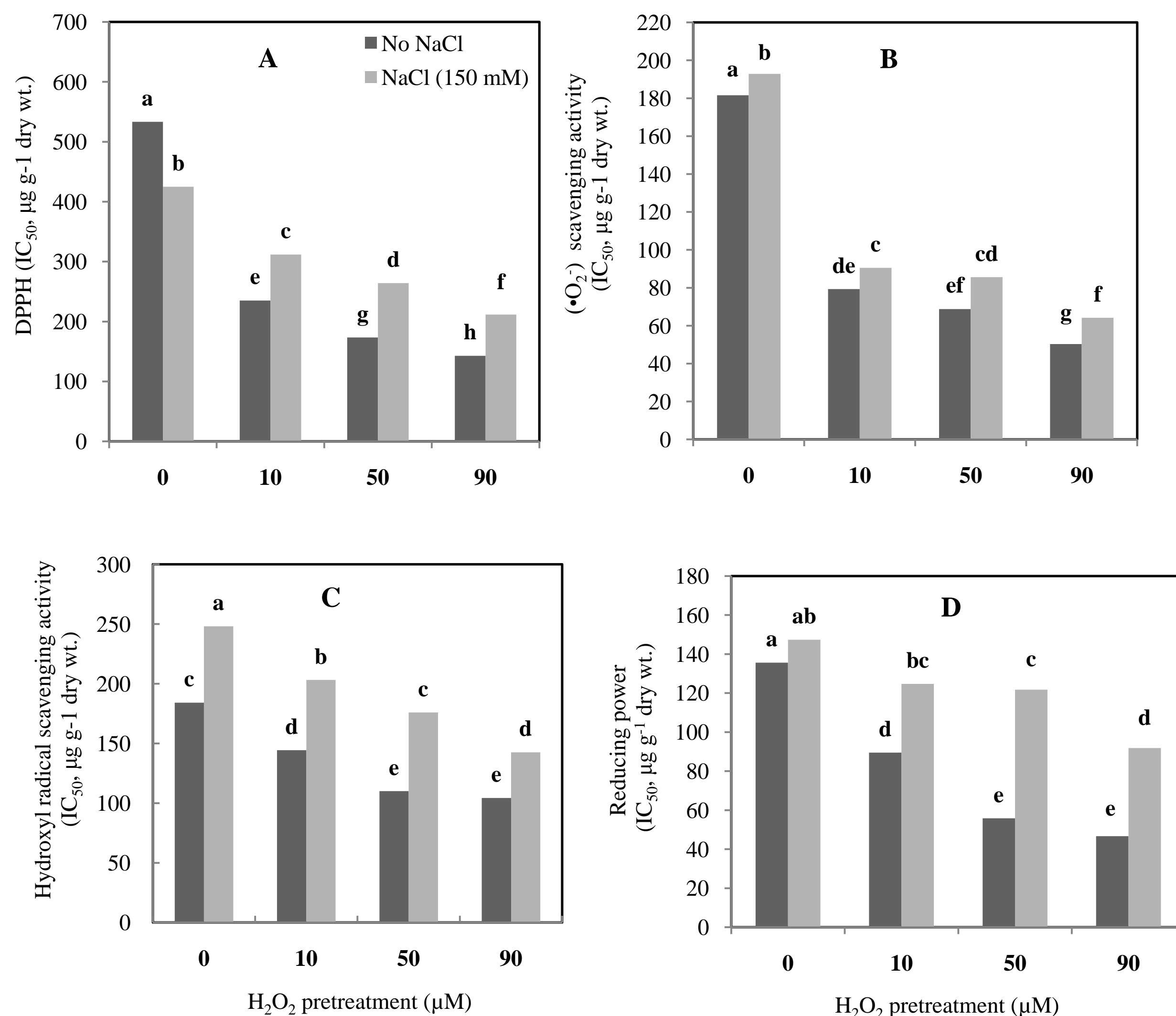

Fig. IV.45-day-old *Artemisia sieberi* plants pretreated with H_2O_2 and/or irrigated for 31 days with NaCl (150 mM); (A) DPPH scavenging activity, (B) superoxide anion radical scavenging activity, (C) hydroxyl radical scavenging activity and, (D) reducing power of the shoots; Means (three replicates) with the same letter are not significantly different at $p<0.05$.

Under saline condition, seed pretreatment with H_2O_2significantly increased APX activity by 21%, but it decreased up to 20% compared to the exclusively salinized plants($p<0.05$).

Results suggested that seed pretreatment with H_2O_2 increased guaiacol peroxidase activity in all pretreated *A. sieberi* plants, but this influence was much greater in the unstressed ones (Fig. V-D). Under normal condition, the increment of guaiacol peroxidase activity ranged from 2.3 (at 10 µM H_2O_2) to 3 folds (at 50 µM H_2O_2). Under salt stress, the activity of this enzyme ranged from +16 (at 10 and 90 µM H_2O_2) to 42% (at 50 µM H_2O_2) compared to only salt-treated *A. sieberi* ($p<0.05$).

Discussion

In the current study, we investigated the effect of seed pretreatment with H_2O_2 on oxidative stress due to NaCl and radical scavenging activity of the aerial parts of *A. sieberi* as a medicinal plant. It is believed that alternation of the latter characteristic would affect the medicinal properties.Numerous studies demonstrated that salinity provokes oxidative stress in plants, leading to cell damage or death,

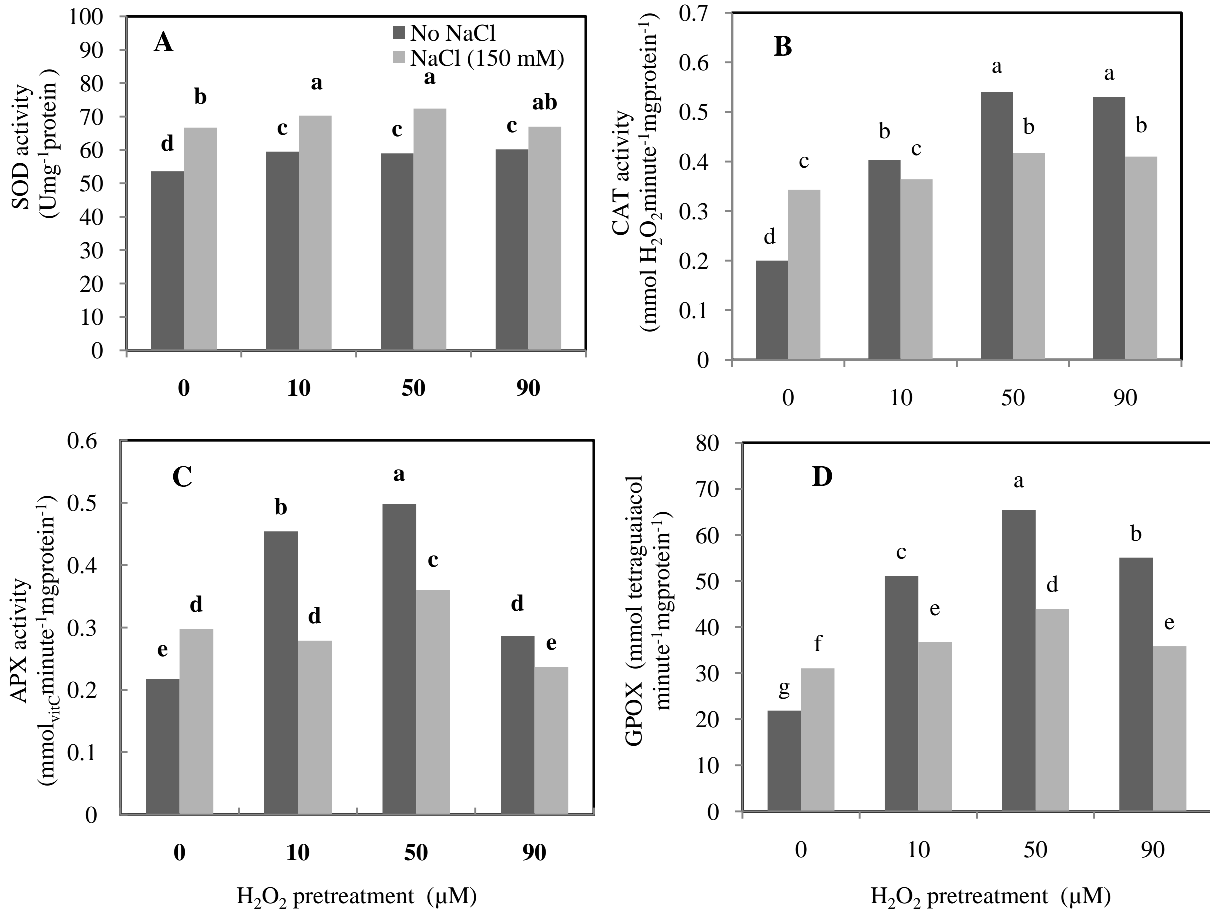

Fig. V. 45-day-old *Artemisia sieberi* plants pretreated with H_2O_2 and/or irrigated for 31 days with NaCl (150 mM); (A) Superoxide dismutase activity, (B) catalase activity, (C) ascorbate peroxidase activity, and (D) guaiacol peroxidase activity of the shoots; Means (three replicates) with the same letter are not significantly different at p<0.05.

and the ability of plans to ROS detoxification through enhancing endogenous antioxidant system leads to reduced deleterious effects of salt stress (Gill and Tuteja, 2010). Plants employ an internal complex defensive system to eliminate or reduce detrimental effects of oxidative stress. This includes enzymatic and non-enzymatic antioxidant components (Apel and Hirt, 2004). Non-enzymatic antioxidants associate with radical scavenging activity and the maintenance of photosynthetic membranes integrity over oxidative stress. Antioxidant enzymes can directly operate ROS detoxification or cooperate by producing non-enzymatic antioxidants. A concerted action of antioxidant components is required to detoxify ROS such as $^{\bullet}O_2^{-}$ and H_2O_2. Superoxide dismutase converts $^{\bullet}O_2^{-}$ into H_2O_2. Peroxidase has an essential role in scavenging H_2O_2 which is produced through dismutation of $^{\bullet}O_2^{-}$ catalyzed by superoxide dismutase. Catalase, as a main enzyme, removes or reduces H_2O_2 in the mitochondria and microbodies (Shigeoka et al., 2002).Thus, all mentioned enzymes help in alleviation of the injurious effects of oxidative stress.

Results obtained from evaluation of growth characteristics including dry and fresh weights and photosynthetic pigments concentration suggested that H_2O_2 pretreatment could increase salt tolerance in *A. sieberi* plants. This result is consistent with the previous studies on wheat and citrus under salt stress (Wahid et al., 2007; Tanou et al., 2009). On the other hand, measuring of parameters of oxidative stress i.e. MDA and H_2O_2 concentrations, activities of antioxidant enzymes (SOD, CAT, APX and GPOX) revealed that positive effects of H_2O_2 pretreatment to enhance salt tolerance was associated with the reduction of oxidative damages in *A. sieberi* plants.

Although, salinity significantly increased H_2O_2 and MDA concentrations, H_2O_2 pretreatment (particularly at 50 µM) decreased the value of these parameters at both normal and saline culture solutions. This finding is in agreement with the previous reports (Li et al., 2011; Gondim et al., 2012). It has been suggested that H_2O_2 pretreatment operates a H_2O_2 signaling process in seed and subsequently seedlings to activate antioxidant system (Wahid et al., 2007) and at the first stage a decrease in H_2O_2 concentration appears in the tissues of salinized plant. Current data showed that H_2O_2 pretreatment was also effective in increasing membrane integrity (subsequently a decrease in MDA concentration), chlorophylls, and carotenoids content in *A. sieberi* plants which were coincident with decreasing of H_2O_2 in the tissues. At the cellular level, the intensity of lipid peroxidation of the membranes (occurred by ROS) increases in salinized plants. As a result, malonyldialdehyde (MDA) content builds up, which is often used as an indicator of oxidative damage. Wahid et al. (2007) believed that improved membrane integrity and decreased ion leakage were a consequence of low level of H_2O_2 due to pretreatment of seeds with H_2O_2. This occurrence has also been reported by Azevedo-Neto et al. (2005), Gao et al. (2010) and Gondim et al. (2012). Protection of membrane integrity by H_2O_2 pretreatment could be the main reason to protect chloroplasts ultrastructure leading to an increase in total chlorophyll and carotenoids content in the primed salinized plants compared to the exclusively salt stressed ones.

Data analysis showed that the activity of all tested antioxidant enzymes augmented in response to salinity which are supposed to be important in salt tolerance in plants. These antioxidant enzymes exhibited their highest activity at 50 µM H_2O_2 pretreatment which was in coordinate with the best results obtained for dry and fresh weights of *A. sieberi*. It seems the higher activity of these enzymes would be of the main causes for decreasing H_2O_2 and subsequently enhancing salt tolerance in H_2O_2 primed *A. sieberi*. Positive effects of H_2O_2 pretreatment in triggering adaptive responses have already been reported (Among them: Wahid et al., 2007; Kumar et al., 2010; Hossain et al.,2015) and proposed that the ability of H_2O_2-treated plants to reduce detrimental effects of NaCl is highly associated with increased activity of antioxidant enzymes. In addition, Gondim et al. (2012) showed that H_2O_2 treatment caused a high increase in CAT activity which was mostly responsible for alleviation of oxidative damages due to salt stress. Under other abiotic stresses such as Al and Cd stress, the benefit of H_2O_2pretreatment was also correlated to the induction of antioxidant defense capacity to scavenge generated ROS during stressful condition (Chao and Kao, 2010; Xu et al., 2011).

Previous studies have shown the improvement of relative water content (RWC) in plants by H_2O_2 treatment (Kukerja et al., 2005; He et al., 2009). In line with these findings, our results showed an increment in fresh weight of primed *A. sieberi* at both normal and saline culture solutions. Kukerja et al. (2005) expressed that augmentation of ABA content and induction of antioxidant system were involved in this phenomenon. Tanou et al. (2009) revealed that treatments with low levels of H_2O_2, regardless of NaCl presence, altered specific proteins involved in photosynthesis, defense, and energy metabolism. Moreover, a stimulation of protein S-nitrosylation was observed in H_2O_2-primed citrus plants under salinity (Tanou et al., 2009). In this way, current data revealed highly induced protein level in the aerial parts of primed *A. sieberi*, regardless of salt stress (Data not shown).

In the current study, radical scavenging activity of the aerial parts of *A. sieberi,* as some signatures of medicinal characters, were also evaluated. Results showed along with an increase in the level of H_2O_2, so did the level of radical scavenging activity increase which indicates the positive effect of H_2O_2 pretreatment on increasing antioxidant activity. Some reports have suggested the central role of non-enzymatic antioxidants in H_2O_2-signaling to ameliorate oxidative damage due to NaCl. For instance, Yu et al. (2003) stated that H_2O_2 treatment increased chilling tolerance by enhancing the glutathione level in mung bean seedlings. In another study, Chao and Kao (2010) showed the up-regulation of ascorbate production in H_2O_2-treated rice seedlings under Cd stress.

Conclusion

Generally, it could be concluded that H_2O_2pretreatmentcan increase salt tolerance in *A. sieberi* plants via mitigation of oxidative stress. Apparently, H_2O_2acts as a signal molecule to enhance activation of enzymatic and non-enzymatic antioxidant systems in the seeds, which is preserved in the seedlings to counteract the ion-induced oxidative damage. Furthermore, H_2O_2pretreatment can increase medicinal properties of *A. sieberi* through augmentation of radical scavenging activity in this species under both normal and saline conditions. The current results suggest that enhancement of salt tolerance in *A. sieberi* by H_2O_2pretreatment substantially is due to the decrease in H_2O_2 concentration and lipid peroxidation. This reduction of lipid peroxidation results in improvement of cellular membrane integrity helping to maintain the ultrastructure of chloroplasts and vacuoles. Furthermore, an increase in the activity of antioxidant enzymes could be of other important reason to increase salt tolerance in H_2O_2-pretreated *A. sieberi.* Among applied H_2O_2 concentrations in this study, 50 µM appeared as the most efficient level to obtain *A. sieberi* plants with enhanced salt tolerance as well as elevated medicinal properties.

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