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Increasing Salt Tolerance and Antioxidant Activity in *Artemisia aucheri* by H_2O_2-Priming

Forouzan Azimian and Parto Roshandel*

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Biology Department, Faculty of Sciences, Shahrekord University, Shahrekord, Iran
*Corresponding author; Email: roshandelparto@gmail.com

Abstract
Recent studies have introduced seed priming with H_2O_2 as an effective technique to alleviate abiotic stresses in plants. In the current study, accomplished at Faculty of Sciences, Shahrekord University, seeds of medicinal plant *Artemisia aucheri* were primed with H_2O_2 (0, 10, 50, 90 and 140 µM) and grown under salt stress (0 and 150 mM NaCl) for one month. Results showed a decrease in H_2O_2 and malonyldialdehyde concentrations by H_2O_2 priming leading to diminish lipid peroxidation at the cellular level. Moreover, seed priming with H_2O_2 (particularly at 90 µM) increased biomass, total water content, chlorophyll (a+b) and carotenoids concentrations, total phenolic content and antioxidant capacity in the plants from primed seeds under both normal and saline conditions. Higher activities of superoxide dismutase and catalase were observed in the primed *A. aucheri* with 90 µM H_2O_2, while the activity of ascorbate peroxidase was at the maximum level at 140 µM H_2O_2 priming condition. Additionally, hydroxyl and super oxide radicals scavenging activities were at the maximum level in the plants from primed seeds with 90 µM H_2O_2. Data revealed that H_2O_2 priming can induce salt tolerance in *A. aucheri* plants by adjusting physiological and metabolic processes such as photosynthesis, ROS scavenging and detoxification and brings about an improved growth and development in this species. Furthermore, H_2O_2 priming at 90 µM augmented antioxidant activity and reducing power in *A. aucheri* suggesting an increase in its medicinal properties.

Keywords: Antioxidant capacity; *Artemisia aucheri;* Radical scavenging; Salinity; Seed priming

Introduction

Artemisia plants, from Asteraceae, are perennial species distributed in arid and semi-arid lands of Iran. *Artemisia aucheri* Boiss. -locally named 'Dermaneh-Koohi'- is an aromatic species that is used in traditional medicine for treatment of various diseases such as astringent, antipoisoning, antiseptic, antiparasitic, stimulants and reduces the rheumatic pains (Pellicer *et al*. 2011; Asghari *et al*. 2012). This species is relatively resistant to severe environmental conditions and also has ecological benefits in soil stabilization and great forage value. Babaahmadi *et al*. (2013) reported that the phosphate-buffered saline extract of *A. aucheri* flowering tops have an allergenic effect. Moreover, it contains santonian, coumarin and flavonoids that have antioxidant activity (Bahrami Karkondi *et al*. 2010; Dinani *et al*. 2010) and is recommended for human health (Siahpoosh *et al*. 2011). Khajehzadeh *et al*. (2014) reported that alternation in antioxidant enzymes activity and polyphenols production was resulted by the adaptation of *A. aucheri* to the related factors of altitude such as soil moisture percentage, temperature and radiation. In another study, Mousaei Sanjerehei *et al*. (2013) stated ecological characters of *A. aucheri* such as tolerance to increasing elevation.

Salinity increasingly limits plant growth and development. Under stressful conditions such as salinity, reactive oxygen species (ROS) consisting of superoxide radicals, hydroxyl radicals and hydrogen peroxide are commonly generated and accumulated, resulting in oxidative stress (Ozgur *et al*. 2013). The ROS are strong oxidizing agents that

cause oxidative damage to biomolecules such as lipids, proteins, DNA and eventually lead to cell death. On the contrary, plants produce antioxidants (Gill and Tuteja 2010) including enzymatic (such as superoxide dismutase, catalase and peroxidases) and non-enzymatic (e.g. phenolic compounds and flavonoids) antioxidants to alleviate oxidative stress (Gill and Tuteja 2010; Khajehzadeh *et al.* 2014). On the other hand, it is well-documented that plants with strong antioxidant properties (containing high levels of polyphenols and radical scavenging activity) are valuable for human health (Krishnaiah *et al.* 2010). Thus, introducing plant materials rich in phenolics or applying treatments to elevate antioxidant capacity in plants could be of importance to the food industry. These treatments would be more appropriate as enable plants to increase their tolerance to environmental stresses such as salinity.

Seed priming is an easy, low cost and effective approach to augment plant tolerance to stressful environments (Tanou *et al.* 2012). In priming techniques, prior exposure of seeds to a natural or synthetic compound, as an eliciting factor, can hinder the deleterious effects of abiotic stress and make plants more tolerant to future stressful conditions.

Hydrogen peroxide (H_2O_2), as a final product of ROS (Quan *et al.* 2008; Karuppanapandian *et al.* 2011), is considered as a toxic chemical at high levels resulting in oxidative damage, but it could also act as a signal molecule at low concentrations leading to develop adaptive mechanisms in plants under stress (Hossein *et al.* 2015). It is claimed that the effect of H_2O_2 priming on plants is not stress-specific since its ameliorative effects was observed in rice seedlings stressed by NaCl or by high temperatures (Uchida *et al.* 2002). Also, the benefit of H_2O_2 priming to alleviate other abiotic stresses such as drought, chilling and heat stress is already reported in wheat (He *et al.* 2009), *Brassica juncea* (Kumar *et al.* 2010) and cucumber (Gao *et al.* 2010). Wahid *et al.* (2007) indicated that seed pretreatment of wheat with H_2O_2 caused an improvement in salt tolerance via mitigation of oxidative damage and expression of stress proteins. Azevedo-Neto *et al.* (2005) illustrated the involvement of H_2O_2 metabolism in increasing of salt acclimation in maize. In addition, a number of studies pointed out that H_2O_2 priming could also be useful in Al and Cd stress alleviation (Chao and Kao 2010; Xu *et al.* 2011). Apart from priming technique, the role of H_2O_2 treatment to reduce harmful effects of abiotic stresses has been also reported. For instance, Gondim *et al.* (2012) stated that H_2O_2 foliar spraying improved maize seedling growth under normal and saline conditions. Tanou *et al.* (2009) indicated that pre-exposure to H_2O_2 resulted in long-term antioxidant activity in citrus plants at presence or absence of NaCl. 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 current study we investigated the effect of H_2O_2 priming of seeds of *A. aucheri* on the physiological and biochemical changes in the seedlings arising from H_2O_2 primed seeds under saline condition. Here, we hypothesize that seed priming with H_2O_2 might enhance antioxidant capacity and salt tolerance in *A. aucheri.*

Materials and Methods

Seed priming

Seeds of *Artemisia aucheri* Boiss. were purchased from Pakan-Bazr (Isfahan, Iran) and sterilized with 70% ethanol for 2 min. Then, seeds were washed with sterilized distilled water for several times. Seed priming with H_2O_2 was accomplished according to Wahid *et al*. (2007) with some modifications. Seeds were soaked in 0, 10, 50, 90 and 140μM H_2O_2 solution 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

Our preliminary experiments showed that among the 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 the designed exposure times (2, 3, 4, 7 and 9h) for seed priming 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 seed tissues. Accordingly, treatments for the main experiment were: 1) control (with no H_2O_2 or NaCl treatment), 2) seed priming with in 0, 10, 50, 90 and 140μM H_2O_2 for 7h, 3) irrigation of 14-day-old seedlings with Hoagland solution (pH 6.8) containing 150 mM NaCl (no priming 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 or untreated seeds were sown in polystyrene boxes, filled with a potting mixture composed of 50% perlite and 50% fine sand. The plants were raised in a greenhouse under controlled conditions (16/8 h light/dark period, 32/25°C temperature, 60-70% RH and 1000-1200 μmol m^{-2} s^{-1}PAR). The experiment lasted for one month, and at the end, 45-day-old plants were sampled to determine dry weight production. Samples were weighed and dried by analytical balance (±0.0001) (model: JB1603-C/FACT) and oven (model: SHIMAZCO). Spectrophotometric analysis was accomplished by UV-VIS spectrophotometer (model: 2500, ShimadzuCorp).

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 extract 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 sulfuric acid. The absorbance of orange-yellow H_2O_2-Ti complex was recorded at 410 nm against blank. Concentration of H_2O_2 was determined using standard curve plotted with known concentration of H_2O_2.

Measurement of total water content

The water content was determined by drying the leaves at 80˚C for 48 h and calculated as:

Water content (%) = ((fresh weight - dry weight)/ fresh weight) ×100.

Estimation of photosynthetic pigment content

The content of total chlorophyll (Ch-a + Ch-b) and carotenoids were determined according to the method of Strain and Svec (1966) with 80% acetone as the solvent.

Chl a ($mgmL^{-1}$)= $12.25A_{663.2} - 2.79A_{646.8}$

Chl b ($mgmL^{-1}$)= $21.5A_{646.8} - 5.1A_{663.2}$

Chl Total ($mgmL^{-1}$)= Chl a + Chl b

Car= ($1000A_{470}$ – 1.82 Chl a – 85.02Chl b)/198

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

Estimation of 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 a 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 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 Chen *et al*. (2000) with some modification. 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 in the -20˚C freezer. Superoxide dismutase (SOD) activity was assayed in 100 mM potassium phosphate buffer, pH 7.5, 150 mM methionine L-methionine, 840 mM Nitroblue tetrazolium (NBT) and 24 µM riboflavin by using the photochemical NBT method in terms of superoxide dismutase'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 (CAT) activity was evaluated 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 mM H_2O_2 (Nemat-Ala and Hassan 2006). Total ascorbate peroxidase (APX) activity was evaluated spectrophotometrically according to Kato & Shimizu (1985) at 280 nm in 0.2 mM potassium phosphate buffer, pH 7.5, 15mM ascorbic acid and 50 mM H_2O_2, as ascorbate (ε= 2.8 mM^{-1} cm^{-1}) was oxidized. Guaiacol peroxidase activity was assayed in 44 mM H_2O_2 and 45 mM guaiacol. 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 Bradford (1976).

Polyphenol extraction and estimation

Fresh shoots of *A. aucheri* plants were shade dried for one week and ground to fine powder. A sample (1 g) of this dry powder was extracted with 80%

methanol with stirring for 30 min. The extracts were then kept for 24h at 4°C, filtered through a Whatman No. 4 filter paper and evaporated under vacuum. Phenolic compounds were assayed using the Folin-Ciocalteu reagent, following Singleton's method with slight modification (Ksouri *et al*. 2007). A sample of extract (0.125 mL, diluted 10-fold) was added to 0.5 mL of deionized water and 0.125 mL of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before adding 1.25 mL of 7% Na_2CO_3 solution. The solution was then diluted with deionized water to a final volume of 3 mL and mixed thoroughly. After incubation for 80 min at 23°C, the absorbance of sample reaction was read at 760 nm. Total phenolic concentration of plants (three replicates per treatment) was expressed as mg gallic acid equivalents (GAE) g^{-1}dry weight through a calibration curve with gallic acid.

Assay of 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 mM methanolic 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 ability to scavenge the DPPH radical was calculated as:

% Inhibition= $[(A_0-A_1)/A_0] \times 100$

where A_0 was the absorbance of the control and A_1 absorbance of extract or standard.

Assay of superoxide anion radical ($^{\cdot}O_2^-$)-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 wt.). The superoxide anion 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/standard.

Assay of hydroxyl-radical ($^{\cdot}OH$) scavenging activity

Hydroxyl radical scavenging activity of methanolic extracts was measured according to 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. aucheri*, 1mM $FeCl_3$, 1mM Na_2EDTA, 10 mM H_2O_2, 1 mM L-ascorbic acid, 36 mM 2-deoxy-D-ribose in 25 mM phosphate buffer (pH7.4). 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 523 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 ability to scavenge the hydroxyl radical was calculated using the following formula:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0] \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance of extract/standard.

Reducing power determination

The reducing power of methanolic extracts of shoots of *A. aucheri* 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 measured at 700 nm. Increase in absorbance of the reaction mixture indicated increased reducing power.

Statistical analysis

The experiment was laid as factorial based on completely randomized design. The data was analyzed using the SAS (V. 9.0) software and the least significant difference (LSD) among treatment means for each trait was calculated. All the measurements were carried out in triplicate and were expressed as means of three analyses ± standard error. P values less than 0.05 were considered to be statistically significant. The relationships between dependent and independent variables were carried out using the polynomial regression function in the Excel program 2007.

Results

Results showed that salinity (150 mM) significantly decreased the biomass of *A. aucheri* by 54% as compared to control ($p \leq 0.05$) (Figure 1A). H_2O_2 priming, at all applied levels, brought about a large increase in the biomass of salinized plants of *A. aucheri* (between 1.7 to 2.2 folds compared to the saline condition alone). H_2O_2 priming also caused a significant increase in the biomass of non-salinized plants (+1.7 folds) compared to control. H_2O_2 priming at 90 μM was the most effective level to increase the biomass under both control and saline conditions.

Total water content significantly decreased by salt stress ($p \leq 0.05$) (Figure 1B). H_2O_2 priming at 90 μM, increased this variable significantly ($p \leq 0.05$). Under non-saline condition, total water content increased by H_2O_2 priming, but it didn't significantly differ from control (Figure 1B).

Data analysis revealed that salt stress (150mM) decreased total chlorophyll content by 58% as compared to control (Figure 2A). H_2O_2 priming significantly increased chlorophyll (a+b) concentration under salinity ($p \leq 0.05$). H_2O_2 priming, particularly at 50 and 90 μM, augmented total chlorophyll content in the aerial parts of *A. aucheri* under both normal and saline conditions (Figure 2A). The percentage increase ranged from 9 to 16% and 38 to 41% under normal and saline conditions, respectively.

Carotenoids content decreased (75%) by 150 mM of NaCl (Figure 2B). Priming with H_2O_2

significantly increased carotenoids level in *A. aucheri*, but this positive effect was dominant under normal condition. At the absence of NaCl, applied concentrations of H_2O_2 increased carotenoids content from 1.4 to 2.2 folds, as 10 μM H_2O_2 was the best priming level. Under salt stress, carotenoids concentration augmented up to 47% by H_2O_2 priming, but there was no significant difference between different levels of H_2O_2 priming (Figure 2B).

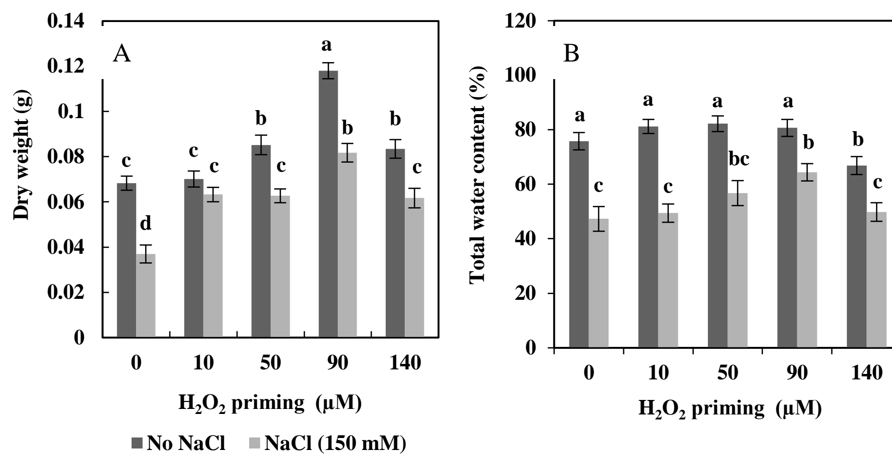

Figure 1. Plants of 45-day-old *Artemisia aucheri* primed with H_2O_2 and/or irrigated for 31 days with NaCl (150 mM). A) Dry weight, B) Total water content. Means (three replicates) with the same letter are not significantly different at $p \leq 0.05$. Bars represent standard errors.

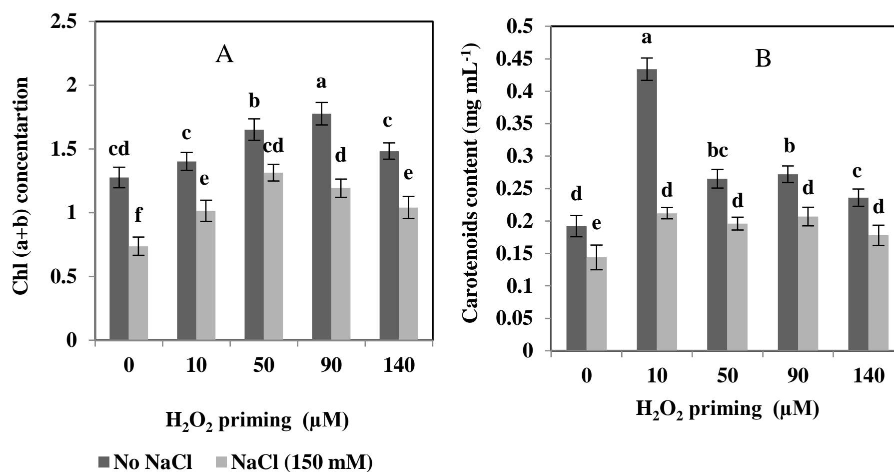

Figure 2. Plants of 45-day-old *Artemisia aucheri* primed with H_2O_2 and/or irrigated for 31 days with NaCl (150 mM). A) Chlorophyll (a+b) concentration, B) Carotenoids content. Means (three replicates) with the same letter are not significantly different at $p \leq 0.05$. Bars represent standard errors.

Results revealed that salinity (150 mM) increased the concentration of H_2O_2 (over 2 folds) in the aerial parts of *A. aucheri* compared to control (Figure 3A). H_2O_2 priming significantly reduced the level of H_2O_2 (from 20 to 43%) in the shoots of salinized plants ($p \leq 0.05$). H_2O_2 priming at 90µM showed the highest effect to diminish H_2O_2 level in the shoot. At the absence of NaCl, H_2O_2 priming resulted in reducing of H_2O_2 level in the tissues by 35%, particularly at 50 and 90µM.

As is shown in Figure 3B, NaCl at 150 mM significantly increased the MDA concentration by 2 folds as compared to control, indicating an increase of lipid peroxidation in the salinized shoots of *A. aucheri.* H_2O_2 priming, however, decreased MDA concentration in the plants grown in the saline culture solution ($p \leq 0.05$). This decrease ranged from 20 to 42% by different levels of H_2O_2 priming. At the absence of NaCl, the lowest amount of MDA was recorded in plants of pretreated seeds with 90 µM H_2O_2 (29% compared to control). Also, the most effective level of H_2O_2 priming to reduce MDA (by 19% compared to control) in the salinized plants was 90 µM (Figure. 3B).

Salt stress alone, significantly decreased the level of polyphenols by 25% as compared to control ($p \leq 0.05$) (Figure 4A). H_2O_2 priming significantly increased the total phenolic concentration in both normal and saline conditions ($p \leq 0.05$). At the absence of NaCl, H_2O_2 priming augmented the level of polyphenols from 1.2 to 2 folds. In this case, H_2O_2 priming at 50 and 90 µM displayed the best outcome, with no significant difference between them. At the presence of NaCl (150 mM), H_2O_2 priming brought about an increase in phenolic concentration from 1.6 to 2.7 folds. The highest value was obtained from the H_2O_2 priming at 90 µM.

Results showed that DPPH-radical scavenging activity of the methanolic extract of *A. aucheri* (IC_{50}= 245.1 µg g^{-1}dry wt.) was significantly affected by H_2O_2 priming and salinity ($p \leq 0.05$) (Figure 4B). Salt stress reduced DPPH-radical scavenging activity (IC_{50}= 301.7 µg g^{-1}dry wt.), while priming with H_2O_2 increased antioxidant properties in *A. aucheri* under both saline and non-saline conditions. The most efficient H_2O_2 priming was observed at 90 µM which caused a decrease in the IC_{50} values by 55 and 60% in the saline and control samples, respectively. Apart from the effect of H_2O_2 at 90µM, hydrogen peroxide at 50 and 140µM were more effective to increase this variable compared to the primed plants with 10 µM.

IC_{50} for superoxide anion radical scavenging activity in the aerial parts of *A. aucheri* was 116.7 µg g^{-1}dry wt. in the non-saline condition, which increased to 175.3 µg g^{-1}dry wt. under salt stress (near to +1.5 folds) (Figure 5A). Exposure of seeds to different concentrations of H_2O_2 significantly increased superoxide anion radical scavenging activity and the IC_{50} values ranged from 53.3 to 86.5 µg g^{-1}dry wt. under control and saline conditions. H_2O_2 priming at 90µM was the most effective level in decreasing IC_{50} for superoxide radical scavenging activity under saline condition (40.5% as compared with the treated plants only by NaCl).

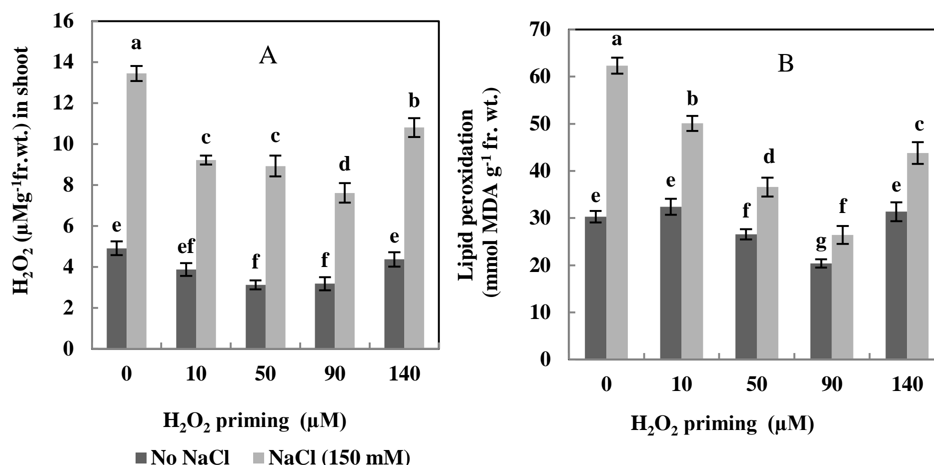

Figure 3. Plants of 45-day-old *Artemisia aucheri* primed with H_2O_2 and/or irrigated for 31 days with NaCl (150 mM). A) H_2O_2 concentration, (B) MDA concentration in the shoots. Means (three replicates) with the same letter are not significantly different at $p \leq 0.05$. Bars represent standard errors.

The IC_{50}value for hydroxyl radical scavenging activity in the aerial parts of *A. aucheri* was 183.6 μg g^{-1}dry wt .in normal condition, which increased to 247.5 μg g^{-1}dry wt. under salinity (near to +1.4 folds) (Figure 5B). Introduction of seeds to the utilized levels of H_2O_2 significantly augmented $^{\bullet}OH$-scavenging activity; the IC_{50} values ranged from 103.7 to 202.7 μg g^{-1}dry wt .in both control and saline conditions. Under saline condition, H_2O_2 priming at 90μM was most effective to decrease the IC_{50} value for $^{\bullet}OH$-scavenging activity (42% decrease compared to the primed plants only by NaCl). Furthermore, H_2O_2 priming at 50 and 140 μM showed the second ranking with no statistical

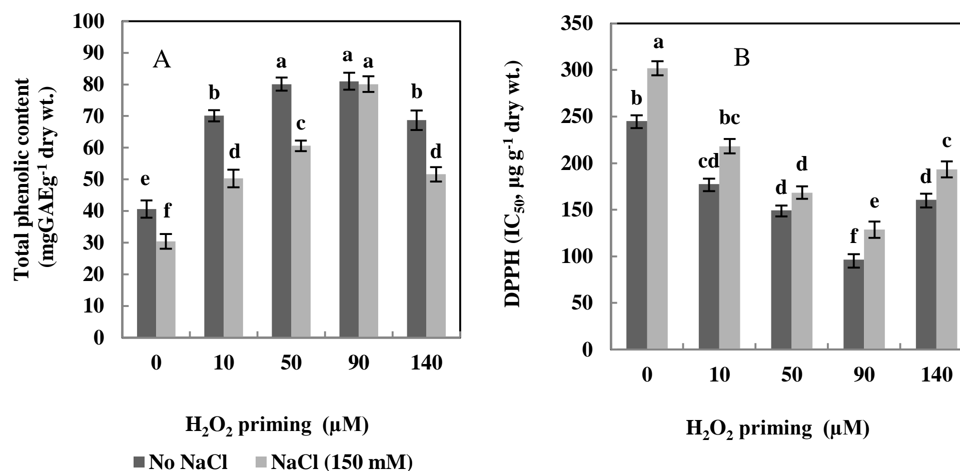

Figure 4. Plants of 45-day-old *Artemisia aucheri* primed with H_2O_2 and/or irrigated for 31 days with NaCl (150 mM). A) Total phenolic content, (B) DPPH scavenging activity of the shoots. Means (three replicates) with the same letter are not significantly different at $p \leq 0.05$. Bars represent standard errors.

difference. Under non-saline condition OH-scavenging activity of the extracts of primed *A. aucheri* was the highest at 50 and 90µM H_2O_2.

Data analysis indicated that salt stress (150 mM) resulted in a significant increase in IC_{50} (264.6 µg g^{-1}dry wt) for reducing power of *A. aucheri* (9% increase compared to control) ($p \leq 0.05$) (Figure 5C). H_2O_2 priming significantly increased the reducing power in both non-salinized and salinized plants (ranged from 113.3 to 206.8 µg g^{-1}dry wt). Increasing the level of H_2O_2 from 10 to 90 µM enhanced the effects of H_2O_2, but plants from H_2O_2-primed seeds at 90 µM exhibited the highest level of reducing ability in both normal and saline conditions compared to the control values. After 90 µM level and at the second rank, H_2O_2 at 50 µM was more efficient to enhance reducing power of *A. aucheri* compared to other H_2O_2 priming.

The lowest activity of superoxide dismutase was recorded in the control plants (Figure 6A). Salt stress and H_2O_2 priming significantly increased the activity of superoxide dismutase compared to control ($p \leq 0.05$). Almost in all H_2O_2-primed salinized plants, however, the activity of this enzyme was significantly higher than that in the plants from H_2O_2-primed seeds grown in normal condition. Under saline condition, the highest value of superoxide dismutase activity was found in the plants from primed seeds with 90 µM (+ 1.2 folds more than that in the exclusively salinized plants). At the absence of NaCl, primed *A. aucheri* with 90 µM H_2O_2 exhibited the highest value for the superoxide dismutase activity (+1.5 folds compared to control).

Catalase activity significantly increased by NaCl and H_2O_2 ($p \leq 0.05$) (Figure 6B). At the absence of salt, all plants from H_2O_2-primed seeds showed higher activity of catalase compared to control (ranged from +39% to 1.9 folds associated with 140 and 90 µM of H_2O_2, respectively). Under salt stress, catalase activity increased from 43 to 81% in all primed *A. aucheri* compared to the exclusively salinized plants. Plants from primed seeds with 10, 50 and 90 µM of H_2O_2 were at the same position, but higher than the salinized plants from primed seeds with 140 µM H_2O_2.

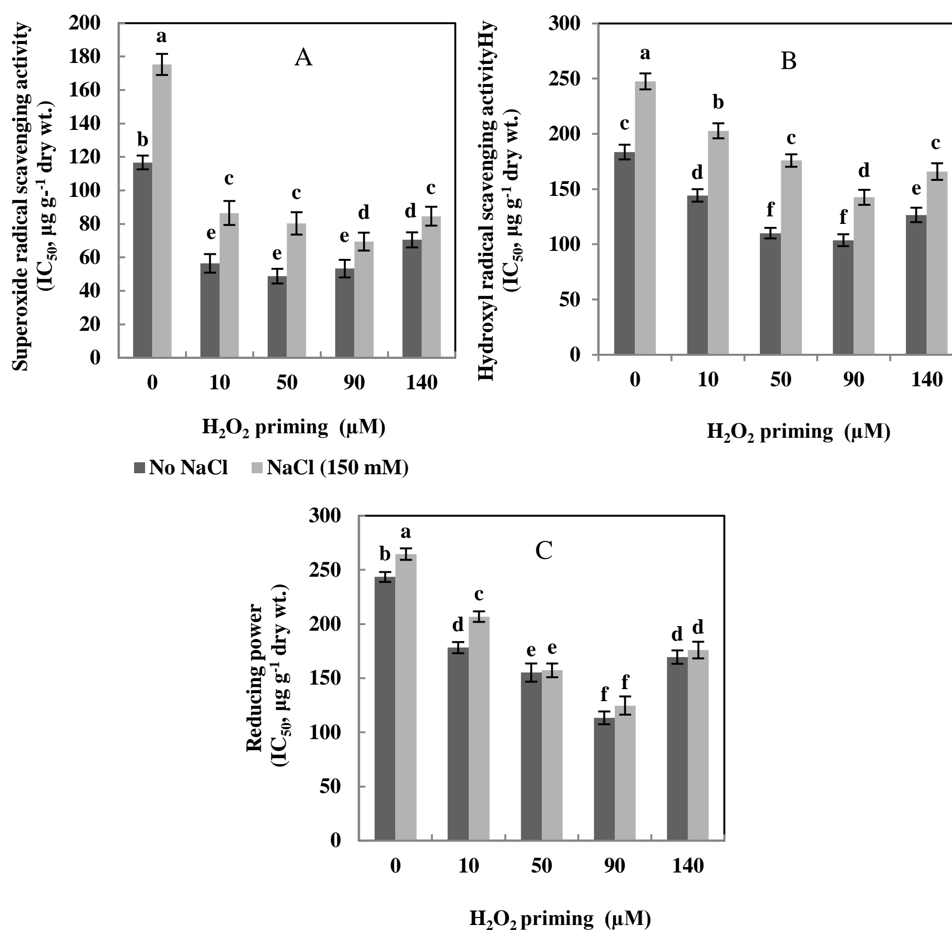

Figure 5. Plants of 45-day-old *Artemisia aucheri* primed with $H_2O_2$and/or irrigated for 31 days with NaCl (150 mM). A) Superoxide anion radical scavenging activity, B) Hydroxyl radical scavenging activity, C) Reducing power of the shoots. Means (three replicates) with the same letter are not significantly different at $p \leq 0.05$. Bars represent standard errors.

Data analysis showed that salt stress alone at 150 mM caused an increase in ascorbate peroxidase activity by 1.2 folds compared to control ($p \leq 0.05$) (Figure 6C). Under non-saline condition, H_2O_2 priming at 10, 50 and 90 µM decreased ascorbate peroxidase activity from 32% (at 10 µM H_2O_2) to 50% (at 50 and 90 µM H_2O_2). Ascorbate peroxidase activity, however, in the plants from primed seeds with 140 µM H_2O_2 increased by 1.6 folds compared to control ($p \leq 0.05$). Under saline condition, H_2O_2 priming significantly decreased ascorbate peroxidase activity from 18 to 39% ($p \leq 0.05$), excluding H_2O_2 priming at 140 µM. No significant difference was obtained for ascorbate peroxidase activity in the salinized plants from primed seeds with 140 µM H_2O_2 and control.

Results illustrated that guaiacol peroxidase activity had the highest values in the exclusively salinized and control *A. aucheri* plants (Figure 6D). Under salt stress, the activity of this enzyme was 1.1-fold more than that in the control ($p \leq 0.05$). Reduction of guaiacol peroxidase activity (ranged from -26 to -36%), however, occurred in the shoots

of primed *A. aucheri* under salinity. Under salinity (150 mM NaCl), the higher activity of guaiacol peroxidase was observed in the plants from primed at 90µM H_2O_2.

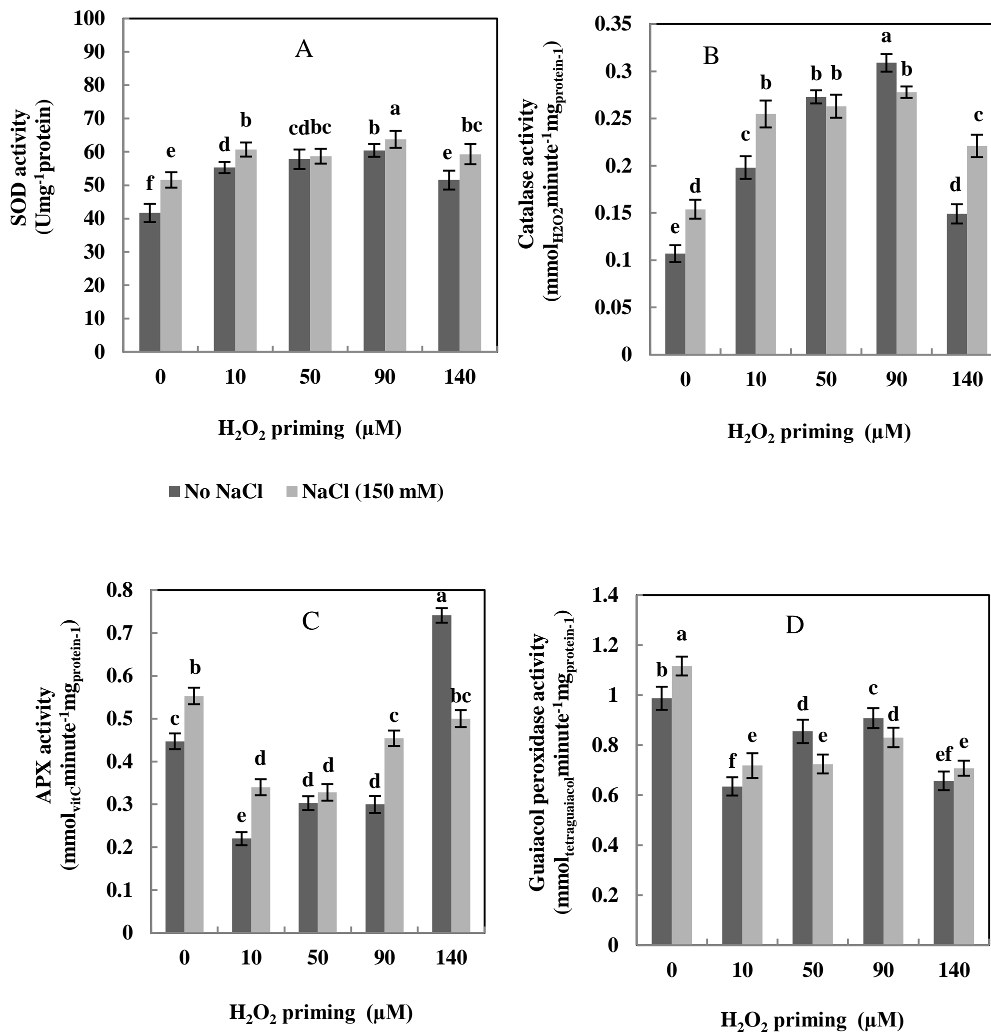

Figure 6. Antioxidant enzyme activity in the shoots of 45-day-old *Artemisia aucheri* plants primed with H_2O_2 and/or irrigated for 31 days with NaCl (150 mM). A) Superoxide dismutase activity, B) Catalase activity, C) Ascorbate peroxidase activity, D) Guaiacol peroxidase activity. Means (three replicates) with the same letter are not significantly different at $p \leq 0.05$. Bars represent standard errors.

Discussion

In the current study, we investigated the effect of H_2O_2 priming to reduce oxidative stress due to NaCl in *A. aucheri.* Numerous studies demonstrated that salinity provokes oxidative stress in plants, driving to cell damage or death, and the ability of plants to ROS detoxification through enhancing endogenous antioxidant system, leading to decrease 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. It includes enzymatic and non-enzymatic antioxidant components (Apel and Hirt 2004). 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 $^{\bullet}O_2^-$ to 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 mitochondrion and microbody (Shigeoka *et al*. 2002). Also, non-enzymatic antioxidants associate with radical scavenging activity and the maintenance of photosynthetic membranes integrity over oxidative stress. Thus, all these enzymes help in alleviation of the injurious effects of oxidative stress. Results obtained from evaluation of growth characters including dry weight, total water content and photosynthetic pigments concentration (Figures 1 & 2) suggested that H_2O_2 priming could increase salt tolerance in *A. aucheri* plants. This result was 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 several variables related to oxidative stress i.e. MDA and H_2O_2 concentrations, activities of antioxidant enzymes (particularly superoxide dismutase, catalase and ascorbate peroxidase) and radical scavenging activity (Figures 3-6) revealed that positive effects of H_2O_2 priming to enhance salt tolerance was associated with the reduction of oxidative damages in *A. aucheri* plants. This finding was in agreement with previous reports about other priming in *Artemisia* species (Azimian & Roshandel 2015; Roshandel & Azimian 2015).

Although salinity significantly increased H_2O_2 and MDA concentrations, but H_2O_2 priming (particularly at 90 μM) decreased the value of these variables at both normal and saline conditions. Similar results have already expressed by other researches (Lin *et al*. 2010; 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 systems (Wahid *et al*. 2007) and at the first stage a decrease in H_2O_2 concentration would appear in the tissues of salinized plant. Current data showed that H_2O_2 priming was also effective in increasing membrane integrity (subsequently a decrease in MDA concentration) (Figure 3), chlorophylls and carotenoids content in *A. aucheri* plants which were coincided with decreasing 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, MDA concentration 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 wheat seeds with H_2O_2. This occurrence was also reported by Azevedo-Neto *et al*. (2005), Gao *et al*. (2010) and Gondim *et al*. (2012). Protection of membrane integrity by H_2O_2 priming could be the main reason to protect chloroplasts ultrastructure, leading to an increase in total chlorophylls and carotenoids content in the salinized plants.

Data analysis showed that the activity of all evaluated antioxidant enzymes augmented in response to salinity (Figure 6). However, the effect of H_2O_2 priming on the activity of superoxide

dismutase, catalase, ascorbate peroxidase and guaiacol peroxidase appeared in different patterns. Superoxide dismutase and catalse showed maximum responses to H_2O_2 priming, particularly at 90 µM. It seems that the higher activity of these two enzymes would be the main causes for decreasing H_2O_2 and subsequently enhancing salt tolerance in H_2O_2 primed *A. aucheri*. Positive effects of H_2O_2 priming in triggering adaptive responses have been already reported (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 injurious effects of NaCl is highly associated with the increased activity of antioxidant enzymes. Furthermore, Gondim *et al*. (2012) showed that H_2O_2 treatment highly increased catalase activity which was mostly responsible for alleviation of oxidative damages due to salt stress. Under other abiotic stresses such as Al and Cd stresses, the benefit of H_2O_2 priming was also correlated with the induction of antioxidant defense capacity to scavenge generated ROS during stressful conditions (Chao and Kao 2010; Xu *et al*. 2011). Previous studies have indicated the improvement of relative water content 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 water content of primed *A. aucheri* at both normal and saline conditions. 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, present data revealed highly induced protein level in the aerial parts of primed *A. aucheri*, regardless of salt stress (data not shown).

Total phenolic content increased by different levels of H_2O_2 priming in all primed *A. aucheri*, regardless of NaCl presence (Figure 4). Seed priming with 90 µM H_2O_2 increased the values of polyphenols in both salinized and non-salinized plants. Polyphenols which are categorized in secondary metabolites have wide distribution and function in different manners such as electron donors, enzyme co-factors and reducing compounds (Arbona *et al*. 2003). Evidence strongly supports that plants with higher total phenolic content have higher antioxidant activities (Maisuthisakul *et al*. 2005). A higher radical scavenging activity which was observed in all primed *A. aucheri* plants (particularly at 90 µM) indicated the positive effect of H_2O_2 priming on increasing antioxidant activity. Our data indicated that antioxidant activities of superoxide dismutase and catalase were in coordinate with non-enzymatic antioxidant activity which measured as hydroxyl, superoxide anion and DPPH radical scavenging activities and reducing power of the methanolic extracts of primed salinized *A. aucheri* (particularly at 90 µM). 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

Overall, it could be concluded that H_2O_2 priming (especially at 90 µM) can increase salt tolerance in *A. aucheri* plants via mitigation of oxidative stress. Apparently, H_2O_2 acts as a signal molecule to enhance activation of enzymatic and non-enzymatic antioxidant systems in the seeds, which preserves in the seedlings to counteract the ion-induced oxidative damage. Furthermore, H_2O_2 priming can increase medicinal properties of *A. aucheri* through augmentation of total phenolic content and radical scavenging activity in this species under both normal and saline conditions. The current results suggest that enhancement of salt tolerance in *A. aucheri* by H_2O_2 priming is substantially due to decreasing 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, increase in the activity of antioxidant enzymes (particularly superoxide dismutase and catalase) could also increase salt tolerance in H_2O_2 primed *A. aucheri.* Among applied H_2O_2 concentrations in this study, 90 µM appeared as the most efficient level to obtain *A. aucheri* plants with enhanced salt tolerance as well as elevated medicinal properties.

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