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Induced changes by NaCl-Seed priming in *Dracocephalum moldavica* plants upon salinity

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ABSTRACT

Purpose: To evaluate the effects of NaCl priming to increase salt tolerance in *Dracocephalum moldavica* L. an experiment was conducted as factorial based on completely randomized design at Shahrekord University. **Research method: S**eeds of medicinal plant *D. moldavica* were primed with NaCl (1 M/ for 24 h/ at darkness/ under 25˚C) and then 30-day-old plants were introduced to salinity (0, 100 and 150 mM NaCl) for one month. The analyzed parameters consisted of dry weight, leaf area, total water content, leaf relative water content, electrolyte leakage, lipid peroxidation, photosynthetic pigments concentrations, total phenolic content and the activity of antioxidant enzymes. **Main findings:** NaCl-priming alleviated the injurious effects of salinity in the salinized plants. The biomass increased up to 64.5% and 3-fold at 100 and 150 mM NaCl, compared to exclusively salinity. Ion leakage and lipid peroxidation decreased as well. Moreover, NaCl-priming led to increase leaf area, improve water status, photosynthetic pigments content and antioxidant enzymes activities in favor of improving the biomass of salinized *D. moldavica.* Total phenolic content increased by salinity alone, but NaCl-priming markedly decreased it at normal condition. The pattern of polyphenols concentration and accumulation was different under NaCl-priming + salinity treatment. **Limitations:** No special limitations were founded. **Originality/Value:** Seed priming with NaCl enhanced salt tolerance in *D. moldavica* through improving water status and photosynthesis, protection of cellular membrane integrity and changes in antioxidant enzyme activity. Alternation in polyphenols concentration might be a signature of changes in the medicinal properties of different parts of this medicinal plant.

INTRODUCTION

Dracocephalum moldavica L. (belonging to Lamiaceae) is an endemic medicinal plant which grows in west Asia (such as Iran). This plant is used in traditional medicine as a painkiller to treat kidney complaints, toothache, colds, stomach-ache, rheumatism and bloat (Racz et al., 1978). The essential oil of *D. moldavica* has antiseptic and antibacterial properties (Omidbaigi, 2000). It is also helpful in regulating platelet aggregation of rate (Khapkin, 1994), stimulating ovulation in female rats and rabbits (Boikova & Akulova, 1995). In addition, it is applied as antioxidant, stimulant (Kakasy et al., 2002), and antitumor (Chachoyan & Oganesyan, 1996).

The experiments of Alaei et al. (2015) showed that *D. moldavica* was a moderately sensitive plant to NaCl and high levels of salinity caused a significant decrease in the percentage of germination and rate, seed vigor index and seedling length.

Salinity is one of the most common problems in arid and semiarid areas which negatively affects growth and development of crop plants and results in reducing agricultural production. During the last decades, along with climatic changes due to global warming, salinity problems have also increased in agriculture. Additionally, secondary salinization which is a result of direct human activities and inappropriate agricultural practices makes the problem worst. Hence, developing crops with higher salt tolerance is demanded more than ever. As declared, salt tolerance is established in plants by hardening which means exposing plants to saline conditions before beginning of stress (Farhoudi & Sharifzadeh, 2006). Therefore, seed priming with appropriate solutions could be an approach to increase salt tolerance in crop plants.

Seed priming is introduced as a practical, uncomplicated and reliable technique which enhances germination and early growth of seedling in comparison to plants raised from unprimed seeds. In seed priming process partial hydration occurs but not germination, and then the seeds were dried up to original moisture content. As follows, the germination is not completed, but particular metabolic activities initiate to organize seeds for radicle protrusion. Yet, different solutions and chemicals are used to prime seeds. Amongst and in the light of literature, the beneficial effects of NaCl priming to increase salt tolerance have been well-documented. Sedghi et al. (2010) believed the result of NaCl priming on seed performance is more efficient compared to that at seedling or maturity stage.

The positive effects of NaCl priming are reported, for instance, in watermelon (Armin et al., 2010), canola (Farhoudi & Harifzadeh, 2006), wheat (Fuller et al., 2012), mazie (Tian et al., 2014), sunflower (Afkari Bajehbaj, 2010), calendula and sweet fennel (Sedghi et al., 2010), muskmelon (Farhoudi et al., 2011), safflower (Elouaer & Hannachi, 2012), fenugreek (Soughir et al., 2013; Elouaer et al., 2013). Nevertheless, NaCl priming studies have generally carried out at seed germination and early growth of seedlings, but its advantages for later growth and development stages of plants remains to be investigated (Bakht et al., 2011).

The present study inspects the effect of seed priming with NaCl on the growth, physiological and biochemical characters of grown-up *D. moldavica* plants when subsequently exposed to long-term salinity.

MATERIALS AND METHODS

Plant material and preparation

Seeds of *Dracocephalum moldavica* were divided in two groups, those to be primed with NaCl and untreated. The seeds were primed with 1M NaCl for 24 h at 25˚C and darkness. The

main experiment started with 30-day-old plantlets (when they had fully developed leaves) and the treatments included 1: control (with no priming or salinity treatment), 2: seed priming with 1 M NaCl (no salinity treatment), 3: irrigation of the plantlets with Hoagland's solution (pH 6.8) containing 100 mM NaCl (no priming), 4: irrigation of the plantlets with Hoagland's solution (pH 6.8) containing 150 mM NaCl (no priming), 5: seed priming with NaCl (as in 2) along with irrigation of the plantlets with 100 mM NaCl, and 6: seed priming with NaCl (as in 2) along with irrigation of the plantlets with 150 mM NaCl. Treated and untreated seeds (15 seeds in each box, from which seedlings were reduced to 10 plantlets at the start of salinization) were sown in polystyrene boxes (32 cm diameter), filled with a potting mixture composed of 50% perlite and 50% fine sand. The plants were raised in the central green house of Shahrekord University under controlled conditions (16/8 h light/dark period, 32/25°C temperature, 60-70% humidity and 1000-1200 µM m^{-2} s^{-1} PAR).The experiments lasted for one month, and at the end of the experiments, 60-day-old plants were sampled to find out the changes in dry weight, total water content, relative water content of leaves, electrolyte leakage, lipid peroxidation, photosynthetic pigments content, total phenolic content and antioxidant enzymes in *D. moldavica* under salt stress. Dry weights were measured after drying plant for 5 days at 70 ºC until the materials reached a constant weight.

Measurement of total water content

The water content of whole plant (Gong et al., 2005) was calculated as follows (1):

$$\text{Water content (\%)} = ((\text{fresh weight} - \text{dry weight})/\text{fresh weight}) \times 100 \quad (1)$$

Assessment of leaf relative water content (RWC)

The leaf discs (2 cm^2) were weighed instantly to record fresh weight (FW), followed by floating on distilled water for 4 h. The turgid leaf discs were then quickly blotted to remove surface water and weighed to attain turgid weight (TW). The leaf discs were subsequently oven-dried at about 80˚C to obtain a constant weight as dry weight (DW). The RWC was calculated as (Lugojan & Ciulca, 2011) (2):

$$\text{RWC (\%)} = [\text{FW-DW})/(\text{TW-DW}] \times 100 \quad (2)$$

Photosynthetic pigments measurement

The content of total chlorophyll (a+b) and carotenoids were determined according to the method of Lichtenthaler and Buschmann (2001) with 80% acetone as the solvent (3-6):

$$\text{Chlorophyll a } (\mu g\ ml^{-1}) = 12.25A_{663} - 2.79A_{646} \quad (3)$$

$$\text{Chlorophyll b } (\mu g\ ml^{-1}) = 21.5A_{646} - 5.1A_{663} \quad (4)$$

$$\text{Total Chlorophyll } (\mu g\ ml^{-1}) = \text{Chlorophyll (a+b)} \quad (5)$$

$$\text{Car} = [1000A_{470} - (1.82)\ (\text{Chlorophyll a}) - (85.02)\ (\text{Chlorophyll b})/198 \quad (6)$$

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

Evaluation of electrolyte leakage

Leaves were sampled to measure membrane electrolyte leakage according to the method of Campos et al. (2003). Results were expressed as percentage of total conductivity.

Assessment of lipid peroxidation

Lipid peroxidation was evaluated by measuring of malondialdehyde (MDA) concentration in the aerial parts of *D. moldavica* according to the method of Ksouri et al. (2007). MDA concentration was determined using the extinction coefficient 155 mM^{-1} cm^{-1}.

Polyphenol extraction and estimation

Flowers, shoots and roots of *D. moldavica* plants were shade dried for one week and ground to fine powder. Total phenolic content was estimated using the Folin-Ciocalteu reagent, following Singleton's method with some modifications (Ksouri et al., 2007). Polyphenols 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.

Enzyme extraction and assay

Enzyme extraction procedure was accomplished according to the method of Chen et al. (2000) with some modification. All of the following operations were performed at 4 ˚C. The extract was transferred to Eppendorf tubes and kept in the -20˚C freezer. Catalase activity was assessed by means of spectrophotometer via 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 & 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, 15 mM ascorbic acid and 50 mM H_2O_2,, as ascorbate (ε = 2.8 mM^{-1} cm^{-1}) was oxidized. Guaiacol peroxidase activity was measured 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 & Balm, 2005).

Statistical analysis

The experiments were laid as factorial based on completely randomized design. The data was analyzed using the software SAS (V. 9.0) and the least significant difference (LSD) among treatments for each trait was calculated. All the measurements were carried out in triplicate. *P* values less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Salinity decreased the biomass of *D. moldavica* plants significantly, ($P < 0.05$) (Fig. 1A). At 100 and 150 mM NaCl, the biomass reduced by 36.7% and 46.9% compared to the control. By application of seed priming with NaCl (1 M), the biomass of *D. moldavica* plants increased under both normal and saline conditions (Fig. 1A). At the absence of NaCl in the medium, the biomass of *D. moldavica* plants raised from primed seeds augmented by 82.4% compared to the control. Under salinity, the biomass of primed plants increased by 64.5% and 3-fold -in turn- at 100 and 150 mM NaCl compared to the exclusively salt stressed-plants ($P < 0.05$).

Results showed that the leaf area of *D. moldavica* plants significantly reduced by salt stress ($P < 0.05$) (Fig. 1B). Salinity at 100 and 150 mM lessened this parameter by 4.6% and 7.3%, respectively, compared to the control. The leaf area rose up to 28.6% in the plants grown from NaCl-primed seeds at normal solution ($P < 0.05$). Under salt stress, the leaf area of NaCl-pretreated plants increased by 61.9% at 100 mM and 82.4% at 150 mM NaCl (Fig. 1 B).

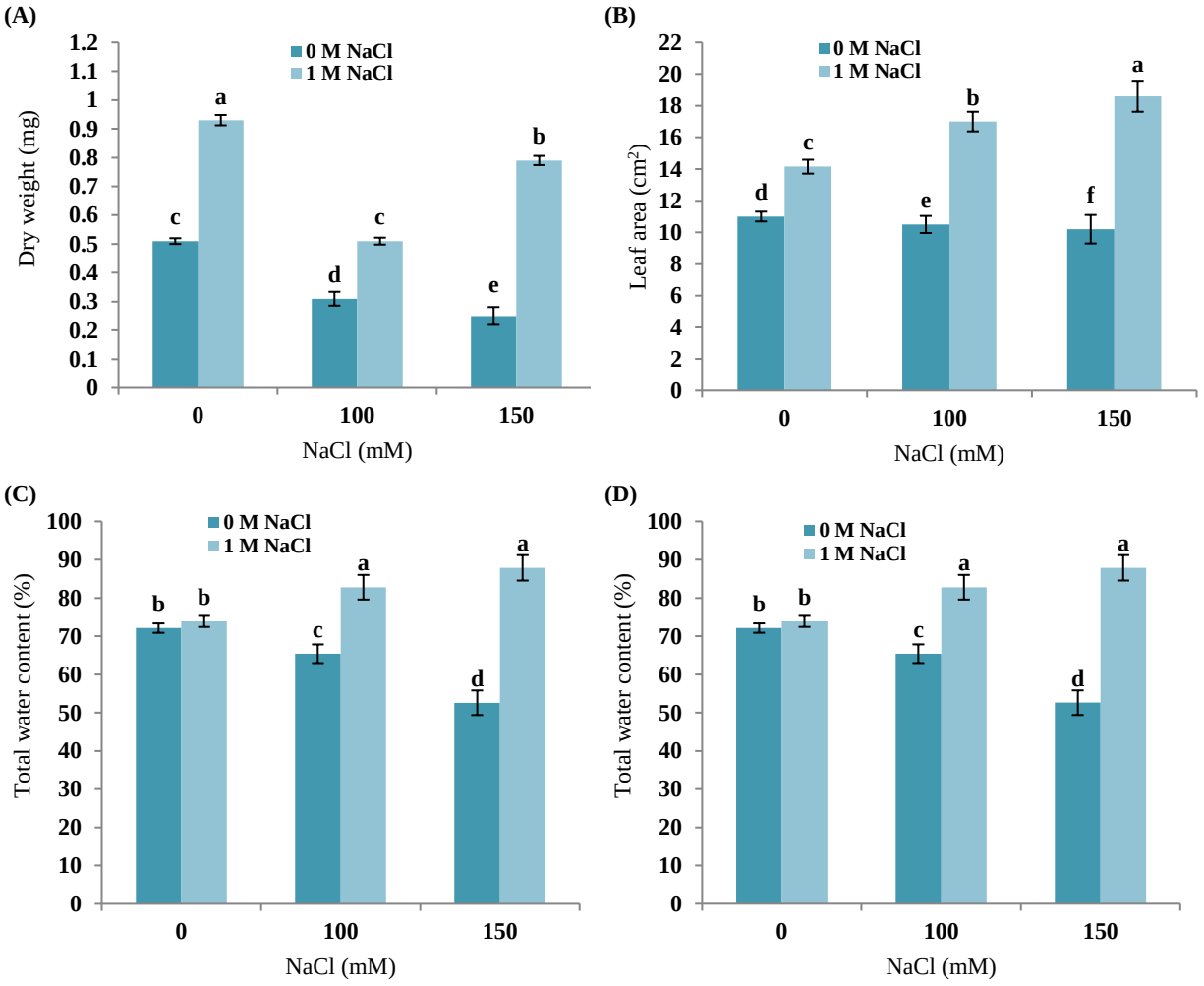

Fig. 1. 60-day-old *Dracocephalum moldavica* plants raised from non-primed or primed seed with NaCl (1 M) and grown under normal or saline conditions (100 and 150 mM NaCl). (A) Dry weight; (B) leaf area; (C) total water content; (D) leaf relative water content (RWC). Means (three replicates) with the same letter are not significantly different at $P < 0.05$ in LSD test. Bars indicate ± standard error.

Results confirmed that total water content in *D. moldavica* decreased with increasing NaCl degrees ($P < 0.05$) (Fig. 1C). At 100 and 150 mM NaCl, total water content reduced up to -in turn- 9.3% and 27.1% in comparison to salinity alone. Seed priming with NaCl (1 M) supported grown *D. moldavica* plants to preserve total water content from 26.6% (at 100 mM NaCl) to 67.1% (at 150 mM NaCl) compared to salinity alone. Also, at normal condition, seed priming with NaCl caused to increase total water content of the plants by 2.4% in comparison to the control (Fig. 1C).

Salinity decreased leaf relative water content (RWC) in *D. moldavica* with increasing NaCl concentration in the medium (Fig. 1D). This parameter reduced by 9.3% at 100 mM NaCl and 27.1% at 150 mM NaCl. However, seed priming with NaCl (1 M) caused to increase/maintain water content of leaves in grown *D. moldavica* plants at both normal and saline conditions. At normal condition, this priming drove *D. moldavica* plants to have a 6.4% increment in water content of leaves compared to the control. In addition, this priming directed mature *D. moldavica* plants to conserve their relative water content of leaves up to 13.6% at 100 mM NaCl and to 20% at 150 mM NaCl.

The level of total chlorophyll extremely affected by increasing salt tolerance (Fig. 2A). As, it decreased by 80.3% at 100 mM NaCl and 96.7% at 150 mM NaCl compared to the

control ($P < 0.05$). Nevertheless, seed priming with NaCl (1 M) caused to preserve total chlorophyll content in grown *D. moldavica* plant upon salinity. This parameter augmented up to 5-fold and 60% -in turn- at 100 and 150 mM NaCl in the pretreated plants under salt stress. At the absence of salinity, this priming increased total chlorophyll content by 73.7% compared to the control (Fig. 2A).

Data analysis showed that the concentration of carotenoids negatively influenced by salt stress ($P < 0.05$) (Fig. 2B). At 100 mM and 150 mM NaCl, it lessened by 32.2% and 79.2%, respectively, compared to the control. However, seed priming with NaCl (1 M) caused to sustain the level of carotenoids by 3.2-folds at 100 mM NaCl and by 93.3% at 150 mM NaCl compared to exclusively salinity. Besides, the concentration of carotenoids increased by 2-fold in the primed plants at non-saline condition (Fig. 2B).

Conductivity measurement showed the electrolyte leakage significantly increased (+ 4.2% at 100 mM NaCl and + 10.7% at 150 mM NaCl) in the leaves of salinized *D. moldavica* ($P < 0.05$) (Fig. 2C). NaCl-priming caused to reduce the electrolyte leakage in the leaves of *D. moldavica* up to 9% (in the non-stressed plants), 2.7% (at 100 mM NaCl) and 2.3% (at 150 mM NaCl).

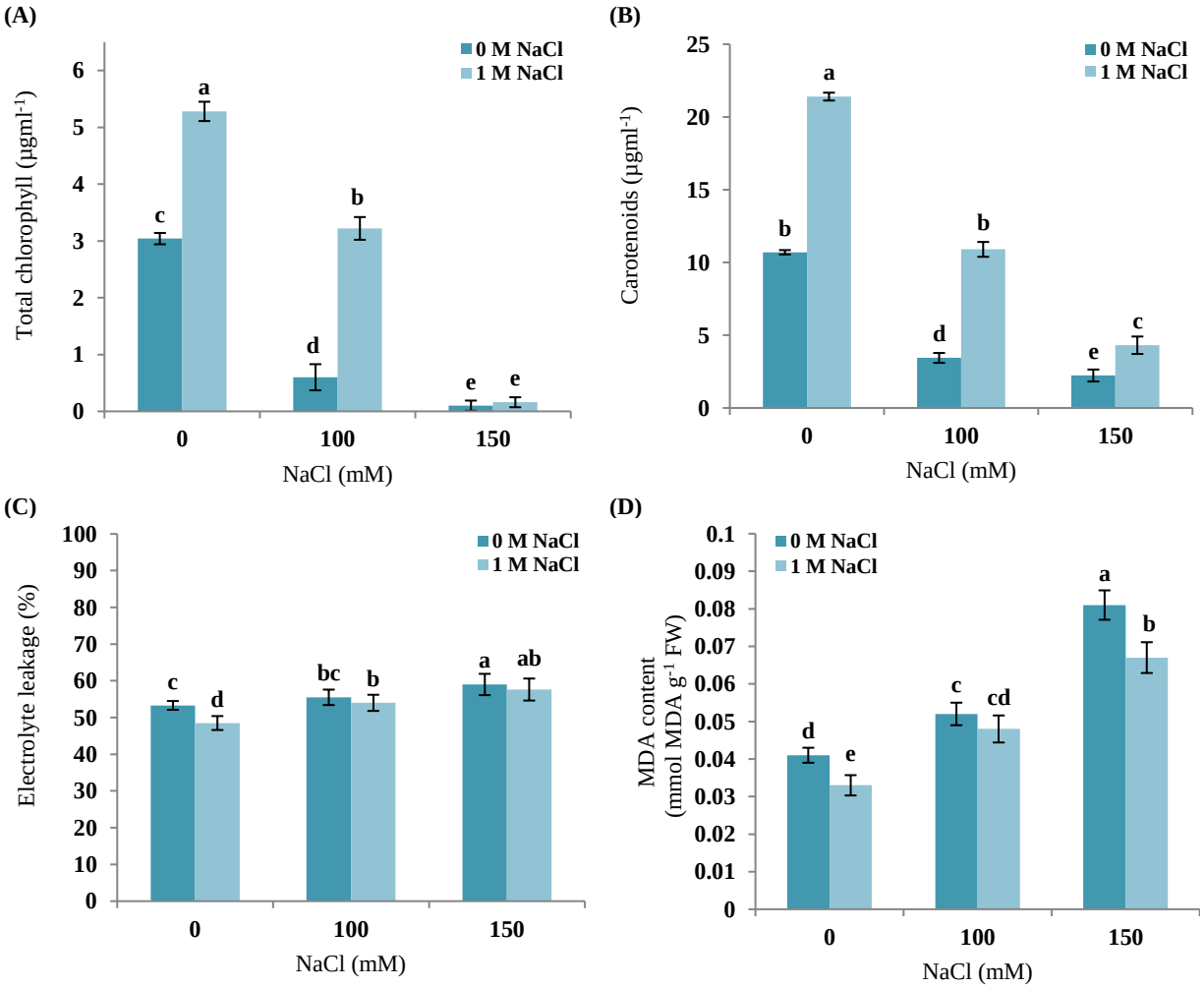

Fig. 2. 60-day-old *Dracocephalum moldavica* plants raised from non-primed or primed seed with NaCl (1 M) and grown under normal or saline conditions (100 and 150 mM NaCl). (A) Total chlorophyll; (B) carotenoids; (C) electrolyte leakage; (D) MDA concentration. Means (three replicates) with the same letter are not significantly different at $P < 0.05$ in LSD test. Bars indicate ± standard error.

Also, salinity significantly increased lipid peroxidation in the leaves of *D. moldavica* ($P < 0.05$) (Fig. 2D). At 100 and 150 mM NaCl, it increased –in turn- by 26.8% and 97.6%, compared to the control. NaCl priming significantly caused to decrease MDA concentration in the salt-stressed plants by 19.5% (at normal condition), 7.7% (at 100 mM NaCl) and 17.3% (at 150 mM NaCl) (Fig. 2D).

Results revealed the activities of the tested antioxidant enzymes were diverse at normal condition, salinity alone and NaCl-priming + salinity (Fig. 3). While the activities of ascorbate peroxidase and guaiacol peroxidase increased with increasing salinity (from 100 mM to 150 mM NaCl), but the activity of catalase reduced ($P < 0.05$). Instead, the activity of catalase increased in the NaCl-primed plants with increasing salinity. In contrast, ascorbate peroxidase and guaiacol peroxidase showed less activity at the same condition ($P < 0.05$). At normal condition, NaCl priming caused to diminish the activity of catalase and ascorbate peroxidase in the grown-up plants, but the activity of guaiacol peroxidase increased (Fig. 3). Data analysis detected that the highest level of polyphenols were found in the shoots of *D. moldavica* at normal condition. Though, dissimilar pattern of polyphenols distribution was evident at different conditions (Fig. 4). At salinity alone, the level of polyphenols increased with increasing salinity in all tested organs of *D. moldavica* (flowers, shoots and roots) ($P < 0.05$). At priming condition alone, the concentration of polyphenols significantly declined in all tested parts of *D. moldavica* (flowers, shoots and roots) ($P < 0.05$). At NaCl priming+100 mM NaCl, the level of polyphenols increased in the flowers but decreased in the shoots and roots compared to salinity alone. At NaCl priming + 150 mM NaCl, total phenolic content of the flowers significantly reduced while it increased in the shoots and roots ($P < 0.05$).

Principally, priming is considered as a method to enhance seed physiology and recover seed performance under stressful conditions. Seed priming leads to establish diverse defense mechanisms (such as osmotic adjustment and antioxidant defense system) in seeds to combat environmental stresses. These mechanisms construct a 'priming memory' in seeds which can be employed upon a later salinity stress-exposure and trigger greater stress tolerance in the plants grown-up from primed seeds (Chen & Arora, 2013).

In the current work the effect of seed priming with NaCl was studied on the growth and some physiological and biochemical characters in grown-up *D. moldavica* at normal and saline conditions.

The present study showed saline conditions inhibited plant growth to variable extent either by reducing plant osmotic potential or because of specific ion toxicity. Also, increasing salt tolerance from 100 to 150 mM NaCl exhibited stronger aspects of injurious effects of salt stress in *D. moldavica* plants. This result was in agreement with the reports (such as Alaie et al., 2015; Azimian & Roshandel, 2015; 2016). Seed priming with NaCl significantly caused to alleviate detrimental effects of salt stress in *D. moldavica* plants, confirming the previous results in other plants (e.g., Afkari Bajehbaj, 2010; Sedghi et al., 2010; Farhoudi et al., 2011; Fuller et al., 2012; Elouaer et al., 2013; Tian et al., 2014).

Photosynthetic pigments (total chlorophyll and carotenoids) of *D. maldavica* plants were markedly decreased by applied salinity levels. But, NaCl-priming mitigated the adverse effects of salinity and preserves the concentrations of these pigments. At salinity, Na^+ buildup in photosynthetic tissues causes to degrade photosynthetic pigments as well as reduction in their biosynthesis. Moreover, chloroplasts are membrane bound organelles and their stability is reliant to the membrane integrity which under high saline condition rarely remains intact. Subsequently, chloroplasts damages and the result would be a reduction in the content of photosynthetic pigments (Ali et al., 2004).

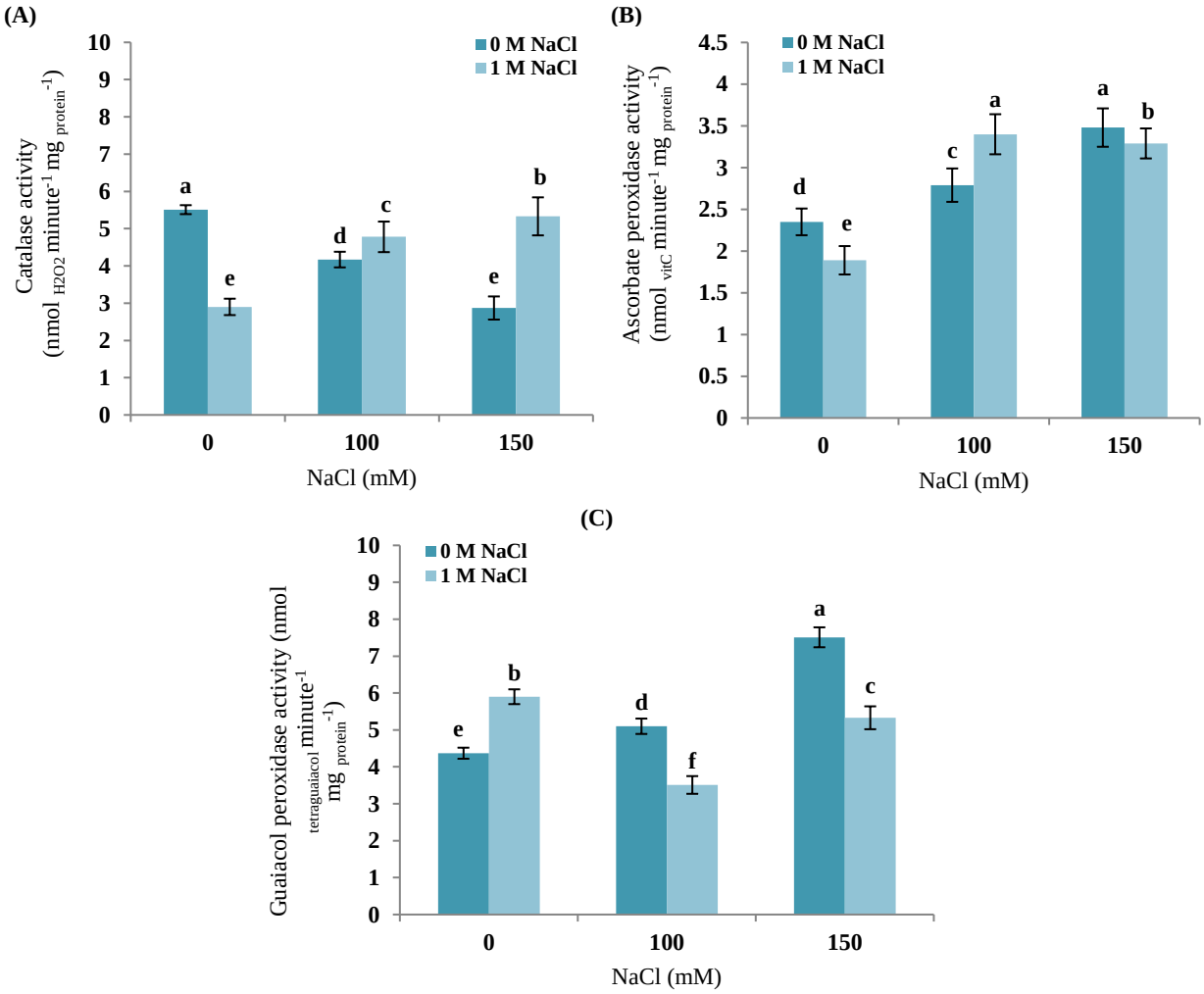

Fig. 3. 60-day-old *Dracocephalum moldavica* plants raised from non-primed or primed seed with NaCl (1 M) and grown under normal or saline conditions (100 and 150 mM NaCl). (A) Catalase activity; (B) ascorbate peroxidase activity; (C) guaiacole peroxidase activity. Means (three replicates) with the same letter are not significantly different at $P < 0.05$ in LSD test. Bars indicate ± standard error.

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Current data showed salinity caused to increase electrolyte leakage and lipid peroxidation in *D. moldavica* plants, but NaCl-priming led to protect cellular membrane stability and integrity in the derived plants. Protection of membrane integrity by NaCl-priming could be the main reason to preserve chloroplasts ultrastructure, also leading to an increase in total chlorophylls and carotenoids content in the salinized plants. The consequence result would be an improved CO_2 fixation and increased biomass under saline condition. Apart from chloroplasts, other membranous organelles in the cell would acquire the advantages of maintenance of cellular membrane integrity. Wahid et al. (2007) believed that improved membrane integrity was a consequence of low level of H_2O_2 due to seed pretreatment.

Antioxidant enzymes have a fundamental role to scavenge reactive oxygen species (such as H_2O_2) generated by salinity. Reactive oxygen species are toxic molecules degrading vital macromolecules like nucleic acids, proteins and lipids leading to cell damage and death. Peroxidases have an essential role in scavenging H_2O_2 which is produced through dismutation of superoxide anions catalyzed by superoxide dismutase. Catalase, as a main enzyme, removes or reduces H_2O_2 in the mitochondrion and microbodies. Data analysis revealed that the activity of all evaluated antioxidant enzymes (CAT, APX and GPX), augmented in response to salinity. However, the effect of NaCl-priming on the activity of tested enzymes appeared in different patterns. In merely salinized *D. maldavia*, the activity of ascorbate peroxidase and guaiacol peroxidase increased with increasing salinity degree, whilst the activity of catalase decreased. In the salt-stressed plants raised from primed seeds the incident was in contrast. It means, in this case, peroxidases and catalase worked consecutively. It is stated that seed priming can alter the activity of antioxidant enzymes somehow to gain improved plant vigor (Wahid et al., 2007; Kumar et al., 2010; Azimian & Roshandel, 2015; Azimian & Roshandel, 2016; Hossain et al., 2015). The results were in agreement with this implication.

The present results indicated NaCl-priming caused to increase total water content and RWC in the salinized plants rose from primed seeds. This finding was in the line of the previous reports, but at germination stage (Sedghi et al., 2010; Elouaer & Hannachi, 2012; Shehzad et al., 2012; Abraha & Yohannes, 2013). RWC commonly demonstrates the balance between water supply to the leaf tissue and transpiration rate and is considered as an important indicator of water status in plants (Lugojan & Ciulca, 2011). In other word, RWC has been applied to evaluate water deficit in the leaf which occurs upon salinity. Increase in RWC under salinity affects on the capability of the plant to recover from stress and therefore improves yield and yield stability. Additionally, total water content reflects the level of water use efficiency which drives plant to an improved growth and development under salt stress. Increase in total water content and maintenance of turgor under salinity can represent a suitable and efficient osmosis regulation of seed-primed derived plants compared to unprimed ones. Thus, the positive effect of NaCl-priming on water status of *D. moldavica* plants leads to increased salinity tolerance in this species in terms of agronomic and physiological traits (Jamal et al., 2011).

The content and composition of secondary metabolites (such as polyphenols) in medicinal and aromatic plants are affected by environmental stresses. Phenolic acids and flavonoids are

known as typical phenolics that possess antioxidant activity. Phenolic compounds act as antioxidants because their chemical structures allow them to donor electrons or hydrogen atoms to free radicals (Rice-Evans et al., 1996). Many researchers have verified a great positive relationship between total phenols and antioxidant activity in many plant species (Rainha et al., 2011). As reported, abiotic stresses (such as salinity) cause to increase total phenolic content in a number of species (Ksouri et al., 2007; Gill & Tuteja, 2010). Our results confirmed the earlier reports, as, polyphenols concentration increased in flower, shoot and root of *D. moldavica* under increasing salinity. However, NaCl-priming caused to reduce the level of polyphenols in *D. moldavica* at normal condition compared to the control. On the other hand, the effect of NaCl-priming on the pattern of polyphenols accumulation was different in the tested organs of *D. moldavica* compared to the control or salinity alone. The response of plants to salt stress (e.g., alternation in the total phenolic content) is very complex. Probably, the degree of salinity, the level of plant salt tolerance and plant species determine the capacity of plant tissues to accumulate polyphenols (Ksouri et al., 2007).

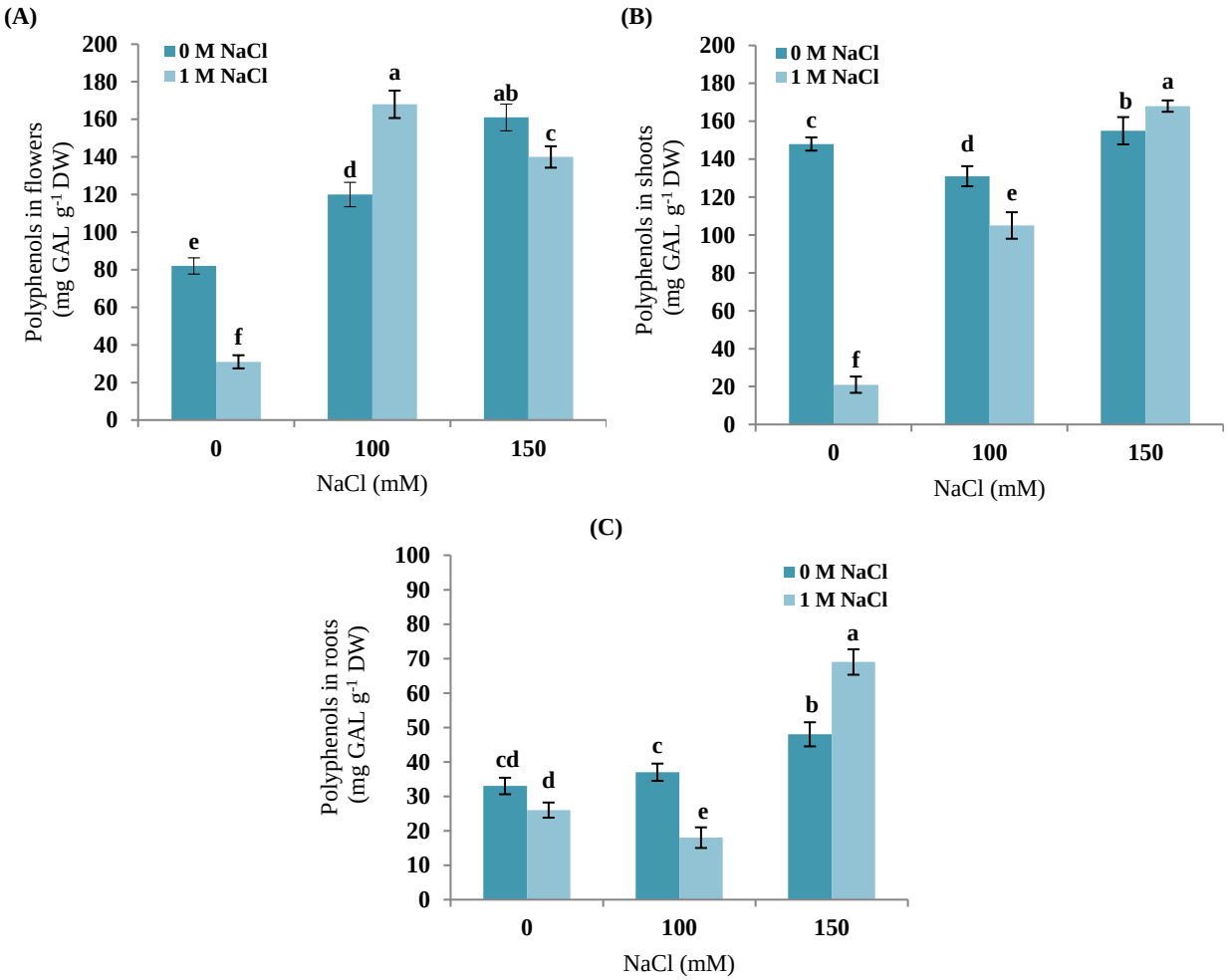

Fig. 4. 60-day-old *Dracocephalum moldavica* plants raised from non-primed or primed seed with NaCl (1 M) and grown under normal or saline conditions (100 and 150 mM NaCl). (A) Polyphenols in flower; (B) Polyphenols in shoots; (C) Polyphenols in roots. Means (three replicates) with the same letter are not significantly different at $P < 0.05$ in LSD test. Bars indicate ± standard error.

CONCLUSION

Overall, the present results suggest seed priming with NaCl could lessen the detrimental effects of salt stress in *D. moldavica* by employing antioxidant enzymes to scavenge ROS and protect cellular membrane stability. Furthermore, increase in the level of total water content and RWC, photosynthetic pigments and leaf area would enhance the growth of *D. moldavica* under salinity.

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Conflict of interest
The authors have no conflict of interest to report.

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