Effects of priming with salicylic acid on germination traits of *Dracocephalum moldavica* L. under salinity stress

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**Abstract**

In order to evaluate the effects of seed priming with salicylic acid (SA) (0, 2, 10, and 20 mM) to increase salt tolerance (0, 50, 100, 150, and 200 mM NaCl) in medicinal plant *Dracocephalum moldavica* at seed germination, an experiment was conducted as factorial in a completely randomized design with four replicates. The assessed parameters included germination percentage and rate, length, dry weight, lipid peroxidation, electrolyte leakage, and the activity of catalase, ascorbate peroxidase, and guaiacol peroxidase of the seedlings. Results revealed the values of germination indices significantly decreased with increasing salinity levels. Nevertheless, seed priming with SA (2 mM) significantly mitigated the adverse effects of salinity in *D. moldavica*. Under salinity and at this level of SA priming, seed germination percentage increased by 24% and 75% at 100 and 150 mM compared to exclusive salt stress. Also, germination rate augmented by SA priming up to 9.2% at 100 mM and 2 folds at 150 mM NaCl. The values of weight (+ 9.1% at 100 mM and +8 folds at 150 mM) and length (+12.5% at 100 mM and + 15.1% at 150 mM NaCl) of seedlings significantly increased by SA priming compared to the exclusively salt-stressed ones. SA priming increased antioxidant enzymes activities while it decreased the level of lipid peroxidation and ion leakage in the seedlings of *D. moldavica*. As a conclusion, SA improved seed performance in *D. moldavica* under salt stress by reduction of detrimental effects of oxidative stress.

**Keywords:** antioxidative system; hormonal priming; moldavian balm; oxidative stress; seed priming


**Introduction**

Moldavian balm (*Dracocephalum moldavica* L., Lamiaceae) is a perennial herb native to West Asia which grows naturally in eastern and central Europe. It is often consumed for its known medicinal characteristics.

Salinity, as one of the major abiotic stresses, limits crop growth and production. Additionally, secondary salinization caused by poor irrigation and/or drainage practices is a continuing natural process in agriculture. Approximately, about 2000 and 4000 ha of irrigated land in arid and semiarid areas around the world are corrupted by salinity and become inappropriate for crop production (Shabala, 2013; Qadir et al., 2014). Simultaneously, restoration of
salt-affected agricultural soils is a costly, time consuming, and hard process on a large scale. Thus, introducing easy, economical, and practical techniques to increase salt tolerance in crop plants would be of interest to attain sustainable agriculture (Ondrasek et al., 2011).

Seed priming is identified as a beneficial technique of seed enhancement that would induce germination percentage and rate, seedling growth and uniformity of the seedling establishment. Inducing rapid and uniform germination, seed priming results in normal and vigorous seedlings and motivates faster and better germination and emergence in different crops. Utilization of proper seed priming could assist seedlings to grow safer in stressed conditions (Jafar et al., 2012).

Various studies have revealed that exogenous application of salicylic acid (2-hydroxybenzoic acid) can alleviate toxicity symptoms induced by salt stress in several plant species (reviewed in Ashraf et al., 2010; Hayat et al., 2010; Jayakannan et al., 2015). Salicylic acid, as a simple natural phenolic molecule, is a plant growth regulator which plays an important role in numerous physiological processes in plants. The ameliorative effects of SA for salt tolerance (which is used in different methods on plants) have been reported in many crops such as barley (El-Tayeb, 2005), wheat (Tammam et al., 2008), sunflower (Noreen and Ashraf, 2008), bean (Azooz, 2009), mung bean (Khan et al., 2010), violet (Hussain et al., 2011), tomato (Javaheri et al., 2012), maize (Tufail et al., 2013; Bagheri, 2014), rice (Joseph and Jinin, 2017), and broad bean (Anaya et al., 2018).

The present investigation was carried out to evaluate whether the utilization of SA enhanced salt tolerance in D. moldavica at seed germination and to study the possible physiological mechanisms.

**Materials and Methods**

**Priming and germination conditions**

Seeds of D. moldavica were purchased from Pakan-Bazr (Esfahan, Iran) and surface-sterilized with 70% ethanol for 2 min followed by repeated washing with double-distilled water and dried on filter paper. The surface-sterilized seeds were primed in 0, 2, 10, and 20 mM of salicylic acid for 24 h at 23 ± 2 °C under dark conditions. Afterwards, seeds were washed with distilled water and then dried at room temperature on filter paper for 24 h. Our preliminary experiment was accomplished at seed germination with four levels of salt stress including 50, 100, 150, and 200 mM NaCl. Accordingly, the levels of 50 and 200 mM NaCl were omitted for the main experiments since the seeds of D. moldavica displayed full germination at 50 mM NaCl, but no germination was observed at 200 mM NaCl. Consequently, for the main experiment the treatments included, 1: control (no treatments), 2: seed priming with in 0, 2, 10, and 20 mM SA, 3: saline condition containing 100 or 150 mM NaCl (no priming), and, 4: seed priming with SA (as in 2) along with saline condition at 100 or 150 mM NaCl. Seeds were placed on a filter paper in 9 cm Petri dish containing 3 cm³ of distilled water or saline solution. A factorial experiment was arranged based on a completely randomized design with four replicates. Each replication consisted of one Petri dish with 25 seeds. The Petri dishes were sealed with Parafilm to prevent evaporation and then carefully kept in a germinator at a temperature of 25 ± 1 °C and 12 h day length. The experiments lasted for 10 days; germinated seeds were counted per day. Seeds with 2 mm emerged radical were considered as germinated ones. Different every parameters of germination were evaluated (Vashista and Nagarajan, 2010) including germination percentage as (GP% = Number of germinated seeds/number of total seeds × 100), germination rate (GR= Σ (Gn/Dn), Gn: germinated seeds on the day n after sowing and Dn: the day n after sowing), the length of seedlings, and the dry weight of seedlings.

**Determination of electrolyte leakage percentage**

Electrolyte leakage percentage (ELP) is used to assess membrane permeability and measured using an electrical conductivity meter. The procedure used was based on the method of Lutts et al. (1996). Fifteen seedlings were placed in individual stoppered vials containing 10 ml of distilled water after three washes with distilled water to remove surface contamination. These
samples were incubated at room temperature (25 °C) on a shaker (100 rpm) for 24 h. Electrical conductivity of bathing solution (EC1) was read after incubation. Samples were then placed in thermostatic water bath at 95 °C for 15 min and the second reading (EC2) was determined after cooling the bathing solutions to room temperature. ELP was calculated as EC1/EC2 and expressed as percent.

**Lipid peroxidation measurement**

Lipid peroxidation was evaluated in terms of malondialdehyde (MDA) content (Ksouri et al., 2007). Fresh samples of seedlings (250 mg fresh weight) were homogenized in 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 g for 10 min at 4°C and the 1 mL supernatant was mixed with 5 mL 0.5% thiobarbituric acid (TBA) prepared in TCA 20% and incubated at 95 °C for 30 min. The reaction was stopped by placing the tubes in an ice bath and samples were centrifuged at 10000 g 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⁻¹cm⁻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 in the -20 °C freezer. Total 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 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 photo-reduction of NBT was measured at 560 nm. Catalase activity was evaluated spectrophotometrically by determining the consumption of H₂O₂ (ε= 39.4 mM⁻¹ cm⁻¹) at 240 nm in 50 mM phosphate buffer, pH 7.5 and 200 mM H₂O₂ (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, 5 mM ascorbic acid and 50 mM H₂O₂, as ascorbate (ε= 2.8 mM⁻¹ cm⁻¹) was oxidized. Guaiacol peroxidase activity was assayed in 44 mM H₂O₂ 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 Balmer, 2005). All enzyme activities were expressed as units per mg protein. Protein contents in all enzyme extracts were determined according to the method of Bradford (1976).

**Statistical Analysis**

The experiments (in a factorial arrangement) were carried out according to a completely randomized design with four replicates. The data were analyzed using 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 of variance analysis of the studied germination traits of *D. moldavica* are shown in Tables 1 and 2. According to these tables, salicylic acid and salinity affected the germination traits significantly (p<0.01). In addition, a significant interaction was obtained between salicylic acid and salinity (p<0.01).

Results showed that salt stress at both levels (100, 150 mM) significantly (p<0.01) decreased the germination percentage of *D. moldavica* by 20.2 and 52.4% compared to control, respectively (Fig. I-A). Priming with salicylic acid at 2 mM, brought about a significant increase in the germination percentage of seeds of *D. moldavica* under normal condition (+10%) compared to the
control and saline condition (+24% and +75%) compared to exclusive salt stress at 100 and 150 mM. SA priming at 10 and 20 mM could not increase this trait under salinity.

Germination rate of the seeds significantly diminished by salt stress (p<0.01) (Fig. I-B). At 100 and 150 mM NaCl, germination rate reduced in turn, up to 31.6% and 73.7% compared to control. SA priming, at 2 mM, increased this parameter significantly at both concentrations of applied NaCl (p<0.01). Under non-saline condition, the rate of seed germination increased (+6.3%) by SA priming (Fig. I-B). Also, SA priming augmented the germination rate by +9.2% at 100 mM and by +2 folds at 150 mM NaCl in comparison to the exclusively salt-stressed group.

Data analysis revealed that salt stress (100 and 150 mM) decreased the length of *D. moldavica* seedlings by 16% and 64% compared to control, respectively (Fig. II-A). SA priming at 2 mM significantly increased this parameter under both levels of NaCl (p<0.01). At normal condition, the length of primed seedlings increased by 26.1% compared to the control and saline condition (+24% and +75%) compared to exclusive salt stress at 100 and 150 mM. SA priming at 10 and 20 mM could not increase this trait under salinity.

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completely compared to control. At 100 and 150 mM NaCl, primed seedlings of *D. moldavica* displayed an augmentation in the length of seedlings up to 12.5% and 15.1%, respectively. The levels of 10 and 20 mM SA significantly decreased the value of this parameter even compared to saline condition alone.

The weight of seedlings in *D. moldavica* significantly declined under salinity (p<0.01). At 100 and 150 mM NaCl, this parameter reduced by 21.4% and 93%, respectively, compared to control (Fig. II-B). Priming with SA at 2 mM significantly increased the weight of seedlings under saline condition. At 100 and 150 mM NaCl, the weight of *D. moldavica* seedlings rose to 9.1% and 8 folds compared to the exclusive salt stress treatment. Other applied levels of SA significantly decreased this parameter compared to the exclusive salt-stressed treatment (Fig. II-B).

Results disclosed that salinity (100 and 150 mM NaCl) increased the level of electrolyte leakage in the seedlings of *D. moldavica* by 10.7% and 22%, respectively, compared to the control (Fig. III-A). However, SA priming at 2 mM significantly mitigated the level of electrolyte leakage in the seedlings under salinity (p<0.01). At 100 and 150 mM NaCl, SA priming (2 mM) decreased the level of this parameter by -23.7 % and -16.8%, respectively compared to saline condition alone. Moreover, SA priming at 10 mM decreased the level of electrolyte leakage though it was at the second rank compared to the pretreatment with 2 mM SA priming (Fig. III-A). Nevertheless, the injurious effect of SA priming at 20 mM was much higher than that of sole salinity and the ion leakage significantly increased in the seedlings.

As shown in Fig. III-B, NaCl at both 100 and 150 mM significantly increased MDA concentration by 97.6% and 2.3 folds, respectively compared to the control, indicating an increment of lipid peroxidation in the salinized seedlings of *D. moldavica*. SA priming at 2 mM, however, decreased MDA concentration in the seedling grown in the saline culture solution (p<0.01). This decrease was -74.1% at 100 mM NaCl and -39% at 150 mM NaCl. SA priming at 10 and 20 mM did not exhibit any positive effect on decreasing MDA concentration (Fig. III-B).

Catalase activity significantly decreased by salinity at both applied concentrations (P<0.01) (Fig. IV-A). The negative impact of 150 mM NaCl on reducing the activity of catalase was greater than that of 100 mM NaCl. However, SA priming at 2 mM increased the activity of catalase under both normal and saline conditions. At 2 mM SA priming, the activity of catalase at 150 mM NaCl was much less than that at 100 mM NaCl (Fig. IV-A). SA priming at 10 and 20 mM highly reduced catalase activity, regardless of NaCl presence.

Data analysis showed that salt stress (alone) at 100 and 150 mM caused an augmentation in ascorbate peroxidase activity by 52% and 36.5%, respectively, compared to control (p<0.01) (Fig. IV-B). SA priming increased ascorbate peroxidase activity at all three levels (2, 10 and 20 mM). This increment was particularly marked at 100 mM NaCl compared to the exclusively salinity treatment, ranging from +3 to
+3.2 folds. The positive effect of SA priming at 2 mM for increasing ascorbate peroxidase activity not only was evident at 100 mM NaCl but also was obvious at 0 and 150 mM NaCl compared to other treatments (Fig. IV-B).

Results illustrated that guaiacol peroxidase activity increased in non-primed seedlings under salinity. This augmentation was much higher (+86.7%) compared to the control (Fig. IV-C). Among three levels of SA applied in seed priming, the concentration of 2 mM was the most effective on raising the activity of guaiacol peroxidase at 0, 100, and 150 mM NaCl compared to control and the exclusively salinized seedlings. In fact, SA priming at 10 and 20 mM decreased the activity of this enzyme compared to other treatments (Fig. IV-C).

Discussion

The present study showed the reduction of germination percentage and rate of salinized *D. moldavica*, was a consequence of salt osmotic effects due to the reduced water availability. As believed, aquaporins are responsible for water entry into cell, but salinity reduces their expression (Boursiac et al., 2005). Moreover, the specific ionic effect of NaCl caused more reduction in the germination percentage and rate through imposing negative effects on the activation of embryo growth using reserve metabolites (Nonogaki et al., 2010). On the other hand, the inhibition of seed germination caused by salinity has been associated with the suppression of ethylene production during imbibition (Chang et al., 2010).

SA priming (at an appropriate concentration) improved seed germination percentage and rate, seedling length, and dry weight of salt-stressed *D. moldavica*. These observations were in agreement with the previous reports (Dolatabadian et al., 2008; Dallali et al., 2012; Enteshari et al., 2012; Boukraâ et al., 2013; Jini and Joseph, 2017; Anaya et al., 2018). Escobar et al. (2010) indicated SA priming increased osmotic adjustment at the imbibition stage. Other suggestion declared SA-induced acidification of the cytosol resulted in aquaporin activation and then faster seed imbibition (Boursiac et al., 2005; Verdoucq et al., 2008). Also, it is reported that SA priming enhanced synthesis of proteins that are essential for embryo growth at the second stage of germination (Rajjou et al., 2006) and the activation of the mobilization of reserve metabolites with low molecular weights (Nonogaki et al., 2010). As suggested, earlier germination of primed seeds is because of much earlier commence of metabolic activities compared to non-primed ones.

Farooq et al. (2007) stated that improved fresh and dry weights of the SA-primed salinized seedlings might be due to an increased cell division within the apical meristem of seedling roots, which causes an increase in plant growth. It is proposed that the exogenous utilization of salicylic acid results in the betterment of cell division in apical meristems through preventing the reduction of IAA and cytokinin levels in salinity-stressed plants (Shakirova et al., 2003), priming enhanced synthesis of proteins that are essential for embryo growth at the second stage of germination (Rajjou et al., 2006) and the activation of the mobilization of reserve metabolites with low molecular weights (Nonogaki et al., 2010). As suggested, earlier germination of primed seeds is because of much earlier commence of metabolic activities compared to non-primed ones.

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thus leading to increased growth and productivity of plants. They also indicated the pre-treatment with SA brought about the buildup of ABA which might involve in the pre-adaptation of seedlings to salt stress, because ABA stimulates the synthesis of a broad range of anti-stress proteins which provide a better protection to plants (such as wheat). Another suggestion was declared by Szalai et al. (2005) who believe the interaction between salinity and SA probably triggers the genes encoding salt resistance and proceeds on germination by increasing the physiological activity and mobilization of the reserved material required for the growth.

Several studies have confirmed that salinity initiates oxidative stress in plants causing cell damage or death. Oxidative stress is generated through high production of reactive oxygen species (ROS). However, plants utilize an internal complex defensive system to remove or decrease injurious effects of oxidative stress. Antioxidant enzymes can directly manage ROS detoxification in plant cells (Gill and Tuteja, 2010). Nevertheless, review of the literature indicates that exogenous application of salicylic acid to the stressed plants can lower the level of ROS generated by salinity (Shakirova et al., 2003; Hayat et al., 2010). This conclusion points out that the activities of antioxidant enzymes are directly or indirectly regulated by salicylic acid and provide an efficient protection against salinity stress. In line with the previous studies, the current results showed an augmentation in the activity of all tested antioxidant enzymes (CAT, POX and GPX), compared to control and salinity alone. These antioxidant enzymes exhibited their highest activities at 2 mM SA priming which was in agreement with the best results obtained for dry weights of the seedlings. Peroxidases has a crucial function in scavenging H$_2$O$_2$ which is generated during dismutation of $\cdot$O$_2^-$ catalyzed by superoxide dismutase. Catalase, as a major enzyme, eliminates or diminishes H$_2$O$_2$ in the mitochondria and microbodies. Thereby, all these enzymes assist in mitigation of the adverse effects of oxidative stress. It seems the higher activity of these enzymes would be of the main causes for decreasing H$_2$O$_2$ and subsequently enhancing salt tolerance in D. moldavica. On the other hand, another report indicates that the higher activities of antioxidant enzymes under SA + NaCl treatment are associated with the increase in free proline content which consequently leads to salt tolerance (Yusuf et al., 2008). Furthermore, it is suggested that SA treatment could stimulate seed

![Fig. IV. 10-day-old Dracocephalum moldavica seedlings primed with salicylic acid and/or moistened for 10 days with NaCl; (A) Catalase activity, (B) ascorbate peroxidase activity, (C) guaiacol peroxidase activity of the seedling; means (four replicates) with the same letter are not significantly different at p<0.05.](image-url)
germination via bio-synthesis of gibberellin and operates as a thermogene inducer (Shah, 2003).

Current data analysis showed that SA priming decreased the level of lipid peroxidation and electrolyte leakage in the seedlings of *D. moldavica* under saline condition. At the cellular level, the intensity of lipid peroxidation and ion leakage of the membranes (caused by ROS) augments in salinized plants. Consequently, malondialdehyde (MDA) concentration builds up. MDA is routinely assessed as an indicator of oxidative damage. Apparently, seed priming with SA (at 2 mM) improved cellular membrane integration in *D. moldavica* seedlings upon salinity leading to the enhancement of plant growth. Moreover, the reduction of MDA content was in coordinate with the higher activities of the antioxidant enzymes assayed in the study confirming the positive effects of SA to reduce oxidative stress. This finding was in agreement with the previous reports (such as Gunes et al., 2007).

The current results revealed that with increasing SA concentration, not only the ameliorative effects of SA under salinity disappeared, but also the high levels of SA by itself displayed as a stress to reduce germination characteristics much more compared to exclusively salt stress. This result was consistent with the previous reports (Arteca, 1995; Hayat et al., 2010; Anaya et al., 2018). Wu et al. (1998) believed increasing SA concentration led to induce ABA synthesis, which consequently stopped the seed germination.

**Conclusion**

Generally, it is concluded that seed priming with salicylic acid can increase salt tolerance in *D. moldavica* seedlings via mitigation of oxidative stress. It seems that SA acts as a signal molecule to induce activation of enzymatic antioxidant systems in seeds, which is preserved in the seedlings to combat destructive effects of oxidative damage. As a result, improved membrane stability and integration helps to maintain the ultrastructure and function of cellular organelles and to establish vigorous seedlings under stressful conditions.

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