Biochemical and Antioxidant Responses of Borage Seedlings in Saline Environments

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Abstract

Background: Although borage (*Borago officinalis* L.) is a valuable medicinal plant, no information is available on the responses of this plant to salinity. For this reason, it is necessary to determine responses of this plant to salinity.

Objective: Since germination and early growth stage is one of the most critical phases of plant life under salinity condition; this experiment was conducted to determine some responses of borage to salinity at the seedling stage.

Methods: This experiment was laid out in a completely randomized design with three replications and four salinity treatments, including distilled water (EC=0.0dS m^{-1}) and three saline water conditions with EC of 5.0, 10.0 and 15.0 dSm^{-1}.

Results: With increasing EC, the content of free proline, soluble carbohydrates and proteins were increased. Moreover, the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and polyphenol oxidase (PPO) enzymes were significantly increased. Although seedlings dry weight and emergence percentage were declined with increasing EC, the seedlings had markedly growth/survival under salinity conditions.

Conclusion: The survival and little reduction in emergence under salinity conditions (12.5%) indicated that borage was a salt tolerant species at the early growth stage. This tolerant is certainly due to the enhancement of antioxidant enzymes activities and compatible solutes content.

Keywords: Antioxidant enzymes, *Borago officinalis* L., Osmotic adjustment, Salinity, Seedling stage.

Abbreviates: Catalase (CAT), Dry Weight (DW), Fresh Weight (FW), Peroxidase (POD), Polyphenol Oxidase (PPO), Reactive Oxygen Species (ROS), Superoxide Dismutase (SOD), Water content (WC)

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Introduction

Salinity is considered as a major environmental stress that can limit plant growth. It is expected that salinization of arable lands will result in 30 percent land loss in next 25 years and 50 percent by the middle of the 21 st century [1]. On the other hand, rapid growth of human population has led to rising demand for food and usage of saline soils or water for crop production. Successful plant production under salt stress conditions requires an adequate understanding of how salts affect soil characteristics and plant performance [2].

The ability of plants to grow in high salt concentrations is known as salt tolerance [3]. Evidence collected from various species suggests that plant salinity tolerance varies depending on many factors such as environmental conditions, agronomical practices, irrigation management, soil fertility, cultivars, and growth stage. The early growth stages is one of the most critical phases of plant life which is greatly influenced by salinity [4, 5]. For this reason, many plants are extremely sensitive to salinity during the early growth stages [6].

It has been reported that the content of reactive oxygen species (ROS) was elevated with increasing salinity, due to the imbalance in the production and destruction of ROS [7]. ROS attack the most sensitive biological macromolecules and membranes to impair their functions [8]. Plants employ biochemical and molecular mechanisms to cope with salt stress such as induction of antioxidative enzymes and synthesis of compatible solutes [9].

One mechanism of ROS detoxification is activation of enzymatic antioxidants (SOD, CAT, POD, etc.) which exist in all the plants [8, 10]. Hence, a strong correlation between the antioxidant defense system and salt tolerance in many plants are reported [7].

Furthermore, a major effect of salinity stress is the loss of intracellular water. Plants accumulate many metabolites as “compatible solutes” which prevent the water loss from the cell and protect the cellular proteins [11]. The accumulation of these osmolytes, facilitate the osmotic adjustment [12]. This phenomenon is an effective mechanism for salinity tolerance in many plants (Ashraf and Orooj, 2006). Carbohydrates and proline are two major compatible solutes [9].

Borage (*Borago officinalis* L.) is an annual herbaceous plant which is well suited for cultivation in certain countries of the world including Iran [14]. Recently, borage has been the subject of increasing agricultural interest because researches showed that seeds and other parts of this plant have valuable fatty acids, particularly gamma linolenic acids. This compound has potential to prevent cardiovascular disease, cancer and infectious diseases. So it was subjected that borage could be a ‘power food’ of the future [15, 16].

Although borage is one of valuable medicinal plants and its cultivation is continuously being extended in the world, no information is available on the responses of this plant to salinity. Since early growth stages is one of the most critical phases of plant life [4, 5], the present study was carried out to explore the effects of salinity on borage at seedling stage.

Materials and Methods

This study was conducted in the Plant Science Laboratory at Tarbiat Modares University, Tehran, Iran from September to November 2006. Seeds of *Borago officinalis* L. were obtained from the Institute of

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The experiment was laid out in a completely randomized design with four salt treatments and three replicates. The treatments were distilled water (control) and three saline water treatments with electrical conductivities of 5.0, 10.0 and 15.0 dS m^{-1}. The treatment solutions were made with saline water and distilled water depending on target salinity. Natural saline water was obtained from Hoz-e-Soltan Lake in Qom, Iran. The major ions of saline water were:128 g/l Na^+, 218.7 g/l Cl^-, 1.23 g/l K^+, 19.5 g/l Mg^{2+}, 0.086 g/l Ca^{2+} and 48.8 g/l SO_4^{2-}.

The seeds were selected for uniformity in size, shape and color. The seeds surfaces were sterilized with 0.5% sodium hypochlorite for 1 minute and rinsed thoroughly with distilled water. Each replicate contained 1.0 L plastic container in which 50 seeds were sown with dry washed sand. Then, one concentration of the treatment solutions was added to the medium up to the soil field capacity (sufficient water to initiate drainage).

The salinity levels were kept constant throughout the experiment period by using containers sealed with plastic bags to avoid evapotranspiration. The growth room conditions were maintained at 24.5 ± 0.5 ^{0}C, relative humidity of 35 ± 5% and photoperiod of 12 h.

Fifteen -day-old seedlings were used for evaluation fresh and dry weight, soluble protein, free proline, soluble carbohydrates and antioxidant enzyme activities. The emergence of seedlings was recorded at the end of experiment and the emerged percentage was calculated. Then, the seedlings were harvested/washed with distilled water and the surface moistures were wiped out. Seedling growth (fresh and dry weight) was determined using ten seedlings from each salinity levels in triplicate. The seedlings were oven dried at 70 ˚C for 72h (until there was no decrease in weight). Water content (WC), as the percentage of fresh weight, was calculated using the following formula (Misra and Dwivedi, 2004):

$$WC (\%) = [(FW - DW) / FW] \times 100$$

Free proline content was extracted from the leaves and roots using 3% sulphosalicylic acid and L-proline (Sigma) as a standard [17]. Water-soluble carbohydrates concentration in the leaves and roots was measured by phenol-sulfuric acid [18]. Protein content was determined with method of Bradford using bovine serum albumin as standard for the calibration curve [19].

A sample of seedling tissue (0.5 g) was homogenized in 5ml of 50mM Tris-HCl buffer (pH 7.5). The homogenate was filtered and then centrifuged at 4 °C for 20 min at 15000×g [7]. The obtained supernatant was used for the measurement of enzyme activity.

Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT), as described by Beauchamp and Fridovich (1971).

Catalase (CAT, EC 1.11.1.6) activity was assayed in the extract by measuring the level of decrease in absorption of 240nm (Kar and Mishra, 1976) in the reaction mixture containing 30µml of the enzyme extract, 50mM of Tris-buffer (pH 7.0) and 5 mM of H_2O_2.

Peroxidase (POX, EC 1.11.1.7) activity was determined as described by Kar and Mishra (1976). The reaction mixture contained 100mM of Tris-buffer (pH 7.0), 5mM of H_2O_2, and 10mM of pyrogallol. Some 12 µml of the extract enzyme was added to the mixture to initiate the reaction, measured

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spectrophotometrically at 425 nm.

Polyphenol oxidase (PPO, EC1.14.18.1) activity was assayed using the method of Kar and Mishra (1976). The reaction mixture contained 100mM of Tris–buffer (pH 7.6); and 10 mM of pyrogallol and the enzyme solution was incubated at 25 °C for 5 minutes. The absorbance of the purpurogallin form was taken at 420 nm.

One-way analysis of variance was applied to evaluate the salt-effect. The mean differences were compared by "Duncan's multiple range test" at $p< 0.05$.

Results and Discussion

Seedlings emergence percentage of borage was significantly different ($p<0.05$) among the various treatments (Table 1). Although, the salinity reduced seedlings emergence percentages, the reduction in seedling emergence was quite small (12.5%). In addition, with increasing EC from 5 to 15 dS m^{-1}, no more reduction in seedling emergence was observed (Fig. 1A).

At seedling emergence stage, salt tolerance is usually determined based on the survival rates [3]. The results indicated that seedlings emergence was about 80 percent in non-saline treatment, declined to about 69% when EC increased up to 15 dS m^{-1} (Fig.1A). Also, ECt (the solution electrical conductivity at which germination starts to decrease) for borage was 5 dS m^{-1} which amount of this reduction (12.5%) was little. Several studies have shown different results from other plants. Martín Alemán et al. (1999) reported EC_t value of 4.9 dS m^{-1} for *Phoenix canarien* and 1.6 dS m^{-1} for *Sabal palmetto* [22].

Additionally, the electrical conductivity at which germination declines by 50 percent (EC_{50}) was reported to be 21.2 dS m^{-1} for sorghum [23], 4.3 for salt-tolerant barley [24] and 10.4 dS m^{-1} for *Salvadora persica* [25]. In this study, the decline in the seed germination was 12.5% at EC of 15 dS m^{-1} which could be suggested that borage seed had high potential of germination under saline conditions. Also, according to salt tolerance classification system of the Mass and Hoffman (1977), it can be suggest that borage had a salt tolerance potential at the seedlings emergence stage.

The fresh weight (FW) was significantly ($p<0.01$) improved at EC of 5 and 10 dS m^{-1}, but drastically reduced with increasing salinity i.e EC of 15 dS m^{-1}. However, the difference in terms of the seedlings FW was not significant at EC of 0 and 15 dS m^{-1} (Fig. 1B). Therefore, after an initial increase in the FW with increasing salinity, it declined at EC of 15 dS m^{-1}. With increasing salinity, dry weight (DW) of seedlings was significantly reduced. Of course, the growth performance of the seedlings was satisfactory (Fig. 1C).

Water content (WC) of the seedlings increased up to EC of 10 dS m^{-1} and then declined with further increment of salinity. The highest percentage of WC was observed at EC of 10 dS m^{-1}, whereas the lowest value was observed at EC of 0 dS m^{-1} (Fig. 1D).

Trends in WC of the seedling were similar to those of their FW (Fig. 1B and D). In other words, WC and FW increased with increasing salinity up to EC of 10 dS m^{-1} and then decreased at EC of 15 dS m^{-1}. Our findings showed that increasing salinity caused an increment in the soluble osmolytes (Fig. 1E and F) and consequently caused a significant increase in FW and WC of the seedlings at EC of 5 and 10 dS m^{-1} (Fig. 1D). This phenomenon is an effective mechanism for salinity tolerance in many plants to prevent the water loss from the cell and protect the cellular proteins [11, 12, 13].

However, in excess of salinity (EC of 15 dS m^{-1}), FW and WC of the seedlings were

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declined probably due to a reduction of ability to adjust osmotically, or the high demand of energy requirements for such adjustment [26].

Although, the seedlings DW significantly decreased ($p<0.01$) with increasing salinity (Fig.1 C), it was adequate well up to EC of 15 dS m^{-1}. This reduction in DW is probably due to the usage of carbohydrate compounds to synthesize specific osmolytes such as proline [27]. In addition, other factors such as the toxic effect of salt, unbalanced nutrient uptake and nutrient deficiencies may also play a role in reduction of dry weight [28]. It was previously reported that salt stress also results in a significant decrease in DW of leaves, stem, and roots in plants [29, 30].

Increasing salinity of the growth medium had a significant increasing effect ($p<0.01$) on free proline content of shoots and roots. It was maximal at the highest salinity level, i.e. EC of 15 dS m^{-1}. The free proline content was higher in the roots than the shoots at different salinity levels (Fig. 1E).

The soluble carbohydrates content of the roots and shoots were significantly increased ($p<0.01$) with increasing salinity. Unlike to proline, the content of soluble carbohydrates in the shoots was higher than the roots at different salinity treatments (Fig. 1F).

The soluble protein content of the seedlings was significantly ($p<0.01$) increased with the enhancement of salinity levels (Table 1 and Fig. 2A). The protein content was 31.32 mg g^{-1} (FW) at salinity level of 0 dS m^{-1} of but it increased significantly up to 53.53 mg g^{-1} (FW) at the EC of 15 dS m^{-1}.

To cope with salt stress, plants employ different biochemical processes. One of the vital processes is production of some metabolites which called compatible solutes or osmolytes. The accumulation of these osmolytes facilitates osmotic adjustment [11, 12, 13].

Proline is a main osmolyte which accumulate under saline conditions in many plants including some medicinal plants such as ajwain [13], anise and coriander [31]. Proline can be utilized both as a carbon and/or nitrogen source for rapid recovery from the salinity stress [32]. Also, proline have a dual

Table 1: Analysis of variance for different parameters

Source of variation	df	Mean squares				
		Seedling Emergence	FW	DW	WC	Shoot proline
Treatments	3	72.89*	3087.15***	12.37***	13.281***	0.323***
Error	8	16.00	67.37	0.136	0.227	0.002
		Root proline	Shoot carbohydrates	Root carbohydrates	Soluble protein	SOD activity
Treatments	3	3.90 ***	24599.82***	3525.43***	374.04***	2139.44***
Error	8	0.011	84.88	65.20	8.04	51.04
		POX activity	CAT activity	PPO activity		
Treatments	3	33789.42***	731187.97***	27.354***		
Error	8	47.18	2477.65	0.468		

***: significant at 0.001.
*: significant at 0.05

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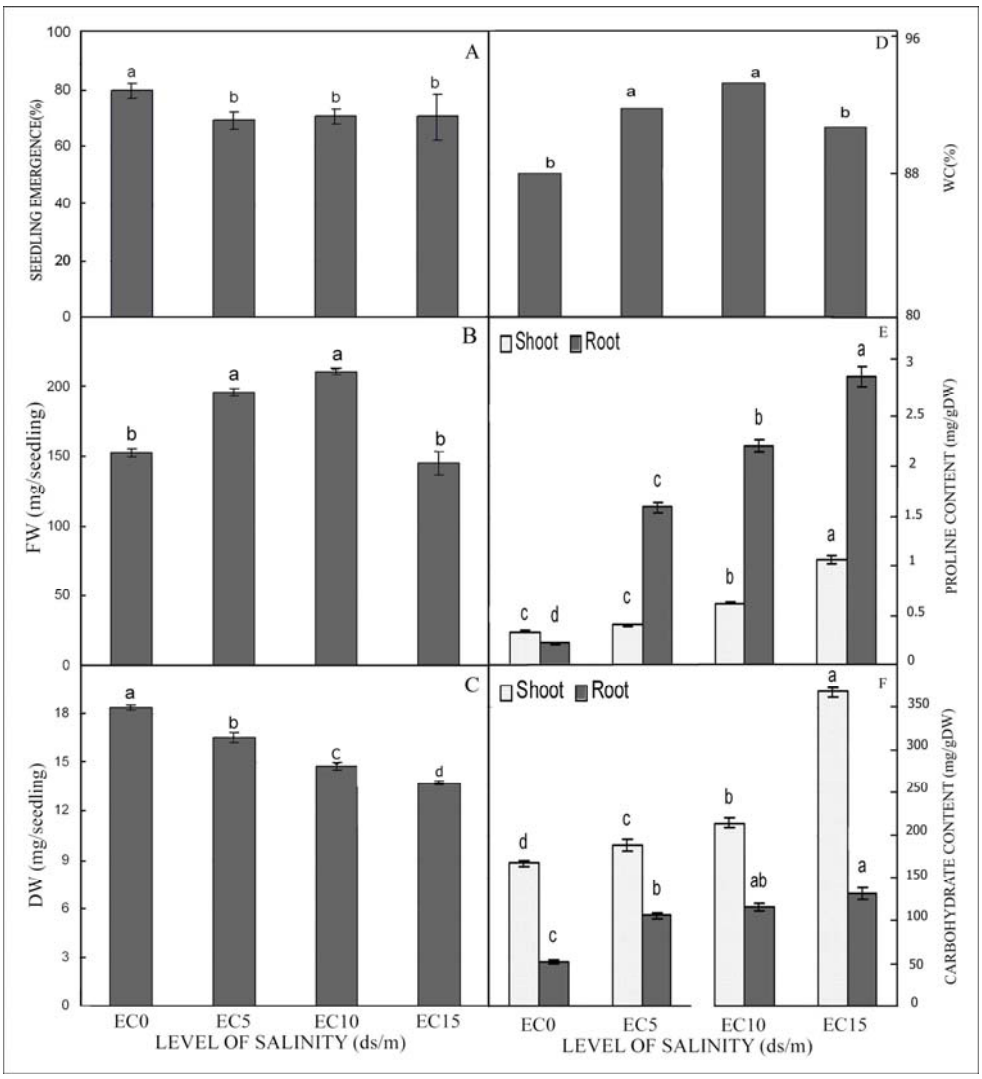

Fig. 1- Effect of salinity (EC) on seedling emergence (A), fresh weight production (B), dry weight production (C), water content (D), free proline content (E) and soluble carbohydrate content (F) in *B. offıcinalis* seedlings. Bars with different letters are significantly different at P<0.05. Each Bar represents a mean of three replicates ± standard error (SE).

role in improving salt stress tolerance as they are able to act in a similar way to the peroxidase enzymes and scavenge reactive oxygen species [33].

The results indicated that the proline concentration was much greater in the root than in the shoot tissues which cause to decrease in water potential in the root and increase in water uptake. Therefore, the enhancement of proline content in root is a valuable strategy which can be a reason of the seedlings survival at saline condition. In other words, this mechanism could cause to increasing salt tolerant potential of borage at early growth stage.

In saline conditions, carbohydrates are other major compatible solutes [9] which prevent the water loss from the cell and protect the cellular proteins [11]. The carbohydrates accumulation facilitates the osmotic adjustment [12] and has an effective role in salinity tolerance in borage seedlings under

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saline conditions. Also, the previous study indicated that accumulation of carbohydrates provides the carbon skeleton to synthesize proline that is used for adaptive and/or defensive responses against stress including salinity [27].

The protein accumulation in borage seedlings may be part of the protection mechanism against salinity (Fig. 2A). In addition, proteins accumulation under saline conditions may provide a storage form of nitrogen that is re-utilized when the stress is over and may play a role in osmotic adjustment [34]. It was previously reported that a higher soluble proteins content has been observed in salt tolerant than in salt sensitive cultivars of barley [35].

The salinity caused significant ($p<0.01$) increase in the antioxidant enzymes activities (Table 1 and Fig.2). Activity of SOD was significantly increased with increasing salinity up to EC of 5 dS m^{-1}, but it remained constant with further increasing salinity (Fig. 2B). The activities of POD, CAT and PPO in the seedlings were significantly ($p<0.01$) enhanced with increasing salinity. The highest activity of these enzymes activities were observed at the maximum level of salinity i.e. EC of 15dS m^{-1} (Fig. 2C, 2D and E).

An excessive ROS production occurs in response to stress conditions such as salt stress [11]. Under such condition, ROS production overcomes the antioxidant system capacity, and thus oxidative stress occurs. Afterwards, the lipid metabolism of plant is interrupted as a result of oxidative damage to membrane lipids by ROS [7]. This could lead to the damage of the components of electron transfer chain in mitochondrial [36].

SOD converts superoxide radicals into hydrogen peroxide and oxygen. Hydrogen peroxide is eliminated by action of CAT and different classes of peroxidases [37]. Therefore, higher SOD, POD, CAT and PPO activities in borage seedling under salinity stress (Fig. 2) probably results from an increased capacity for oxygen radical scavenging and maintenance of cellular membrane. These enzymes are considered to be the main protective enzymes that play a key role in the removal of ROS [8, 10].

Also, increase in SOD, CAT, POD and PPO activities in different tissues under increasing salinity have also been reported in a number of plants [38]. In addition, many researchers have reported that the antioxidant enzymes have relatively higher activities in tolerant cultivars than in the sensitive ones, suggesting that higher antioxidant enzymes activities have a role in imparting the tolerance to these cultivars against environmental hazards such as salinity [39].

On the other hand, a strong correlation between the antioxidant defense system and salt tolerance in many plants is reported [7]. Thus, increasing the antioxidant enzymes activities had an important role in salt tolerance potential of borage seedlings.

Although antioxidant activity has been reported in the extract of borage seeds (Wettasinghe and Shahidi, 1999), however, the present research is the first report on antioxidant activity of SOD, CAT, POD and PPO enzymes in borage seedling.

Conclusion

This study indicated that borage had a salt tolerance potential up to EC of 15 dS m^{-1} at the early growth stage. Also, this salt tolerant potential was due to increase in the antioxidant enzymes activities and the compatible solutes content. However, this study is only a step towards evaluation of salt tolerance and further investigations are needed to determining of phytochemical and production potential of borage at saline conditions.

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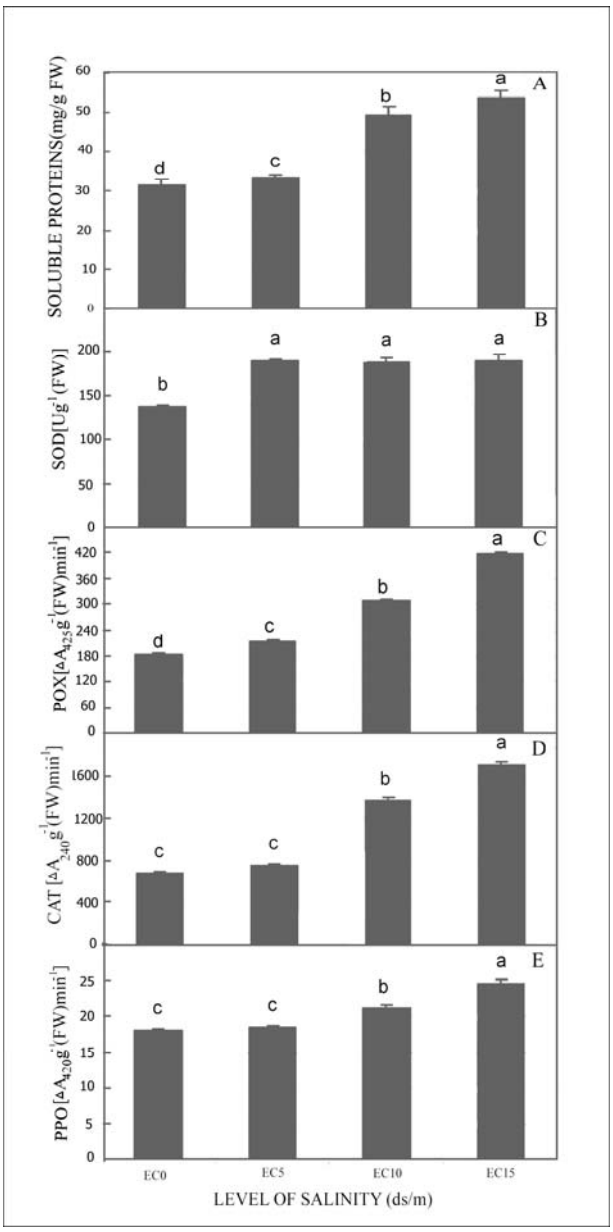

Fig. 2- Effect of salinity (EC) on soluble proteins (A), and activities of SOD (B), POX (C), CAT (D) and PPO (E) in *B. officinalis* seedlings. Bars with different letters are significantly different at $p<0.05$. Each Bar represents a mean of three replicates ± standard error (SE).

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