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SALINITY TOLERANCE THRESHOLD OF BERSEEM CLOVER (*TRIFOLIUM ALEXANDRINUM*) AT DIFFERENT GROWTH STAGES

SEYED ABDOLREZA KAZEMEINI[1*], HADI PIRASTEH-ANOSHEH[2], ABDOLRASOL BASIRAT[1], AND NUDRAT AISHA AKRAM[2]

[1]*Department of Crop Production and Plant Breeding, College of agriculture, Shiraz University, Shiraz, 7196484334, Iran*
[2]*National Salinity Research Center, Agricultural Research, Education and Extension Organization, Yazd, 8917357676, Iran*
[3]*Department of Botany, Government College University, Faisalabad, 38000, Pakistan*
[*]*Corresponding author's email: kazemin@shirazu.ac.ir*

Abstract

The most crops are sensitive to salt stress, however, this sensitivity is varied among different growth stages. Therefore, in this study salt stress tolerance threshold at different growth stages was examined in berseem clover (*Trifolium alexandrinum* L.). The treatments included five saline irrigation levels: 0.62 (tap water as control), 3, 6, 9 and 12 dS m^{-1} and three growth stages at which salinity was applied: 2-leaf, 6-leaf and flowering. Growth parameters, ions accumulation and oxidative damage of berseem clover plants were evaluated. The results showed that salinity stress caused a significant reduction in plant shoot and root dry weights, root length, height, and potassium (shoot and root) concentration, but the impact of salinity varied from stage to stage. On the other hand, tissue sodium concentration and antioxidant enzymes activities were found to be enhanced due to salt stress. Higher levels of salinity had more negative effects on growth parameters and biochemical attributes. Salt stress imposed at the early growth stage had more severe effect on plant growth than that applied at the later growth stages. In view of salt tolerance threshold analysis worked out as 50% reduction based on Van-Genuchtan and Hoffman equation of berseem clover were 6.54, 7.99 and 10.00 dS m^{-1} at 2-leaf, 6-leaf and flowering, respectively. However, when salt stress was imposed at the 2-leaf stage, plants were able to recover in terms of ion accumulation and antioxidant enzymes, and they were as good as the non-stressed plants. Overall, it was revealed that berseem clover is a sensitive plant at early growth stage, but is relatively tolerant at the later growth stages.

Key words: Catalase, Dry weight, Oxidative damage, Potassium and sodium.

Introduction

Legume crops are exceptionally important in agriculture due to root nodulation and symbiotic relation with nitrogen fixing bacteria. Recently, cultivation of berseem clover is increases due to number of harvests, fast growth, freshness, and quality of forage production (Rouhi *et al*., 2012). Clover species are widely cultivated in Iran, where the soil salt concentration is one of the important adversaries for crop growth and yield (Pirasteh-Anosheh *et al*., 2014a). Berseem clover (*Trifolium alexandrinum* L.), like other plants in the legume family, is placed among the most sensitive plants, because its tolerance threshold was estimated to be close to 1.5 dS m^{-1} (Maas & Hoffman, 1977). High salinity can damage the plant at cellular or/and whole-plant level resulting in severe reduction in plant growth and yield production. However, salt tolerant plants can maintain growth by excluding salts or causing osmotic adjustment within the cells. Under saline conditions, all major processes within a plant are usually affected such as energy and lipid metabolism, protein synthesis, photosynthesis, and ion accumulation (Parida & Das, 2005). Salt stress causes osmotic effects which leads to over-generation of reactive oxygen species (ROS) including singlet oxygen, hydrogen peroxide, superoxide and hydroxyl radical (Pakar *et al*., 2016). In varying degrees, plants overexpress antioxidant enzymes such as catalase, peroxidase, superoxide dismutase and ascorbic peroxidase which protect the cells/tissues against cytotoxic species of reactive oxygen (Ashraf & Harris, 2004).

Plant sensitivity depends on soil salinity conditions as well as age of plants. The seedling stage of plant growth is more sensitive to salt stress relative to later plant growth stages including the vegetative and reproductive stages (Mass & Hoffman, 1977; Siddiqi *et al*., 2007). In contrast, Maas *et al*., (1983) reported that sixteen cultivars of maize were more tolerant to salinity at germination stage, although plant seedlings were susceptible to soil salinity. So, the salinity threshold level at the reproductive (ear and grain yields) and vegetative stages were relatively higher than that at the seedling stage. In *Sorghum* it was observed some selected cultivars were sensitive to salt stress at the vegetative stage as compared to that at reproduction (Maas *et al*., 1986). Maas & Poss (1989a) reported that cowpea was less sensitive to salinity at the later plants growth stages than that the initial growth stages. Similar results were observed by Maas & Poss (1989) in wheat exhibiting that plants were more tolerant to saline stress at later growth stages. They recommended that wheat plants (durum and bread) were comparatively tolerant to salinity during the latter 90 days of plant growth. According to what has been stated above, salt sensitivity of some plants such as maize, sorghum, cowpea and wheat at various growth stages has been studied, however, there is no/little information about salinity tolerance threshold at different growth stage for berseem clover. Therefore, this study was conducted to examine the effect of salt stress imposed at different growth stages on growth, ion accumulation and distribution, and antioxidant enzymes in berseem clover.

Material and Methods

A completely randomized design (CRD) with four replicates was carried-out at the Research Greenhouse of Shiraz University, Iran. The pots of uniform size were filled with field soil (Fine, mixed, mesic, Cacixerollic Xerochrepts), sand and humus as 2:1:1 ratio. The used soil texture was sandy clay with pH = 7.05 and EC = 0.52 dS m^{-1}. The seeds of berseem clover were sterilized in sodium hypochlorite solution (5%) and then in 96% ethanol for 30 sec. Ten seeds of berseem clover were sown in 2-3 cm depth in each pot and after seedling emergence they were thinned to five. The illumination, 14 h daily; relative humidity, 60-65% and temperature, 15 (minimum) and 26°C (maximum) in the greenhouse were recorded during the entire period of the experiment. Treatments were combination of two factors: salinity levels and growth stage at which salinity was applied, i.e. 2-leaf, 6 leaf and flowering. Salinity levels included 3, 6, 9 and 12 dS m^{-1}, which were achieved using 2:1 weight ratio of NaCl: $CaCl_2$. The combination of these two salts were used for avoiding the toxic impact of sodium and approaching to natural salinity. Furthermore, the berseem clover plants were irrigated with tap water (EC = 0.62 dS m^{-1}) throughout the experiment as control. Electrical conductivity of irrigation water and drainage were controlled by a portable EC meter (Model 2052 digital USA) in each irrigation. The pots were irrigated to attain field capacity every week.

The characters measured included shoot height (Ht), shoot dry matter (SDM), root length (RL), root dry matter (RDM) as well as activities of peroxidase (POD), ascorbic peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) enzymes. Furthermore, shoot sodium (Na) and potassium (K) concentration of root and shoot were measured. One plant in each plot was considered for antioxidant enzymes assessment, and remaining four plants in each pot were harvested for other measurements. The samples were oven-dried at 70°C for 48 h, and weighed after separation of shoot and root. To assay concentrations of different ions in the shoots and roots, they were digested in 40 mL of 4% HNO_3 at 95°C for 6 hours in a 54-well Hot Block (Environmental Express, Mt Pleasant, South Carolina, USA). The concentrations of Na^+ and K^+ in the digested samples were determined using a flame photometer (SL-CC-102 India). The activities of SOD and CAT enzymes were estimated following Dhindsa *et al.*, (1981), the POD enzyme activity following Chance & Maehly (1995) and that of APX following Nakano & Asada (1981). The data so collected were subjected to analysis of variance (ANOVA) and multivariate regression using SAS *v*. 9.1 software. Threshold values (50% reduction) were estimated based on the Van-Genuchtan & Hoffman (1984) method using SAS *v*. 9.1 software.

Results

Salinity imposed at different stages significantly reduced plant height (Fig. 1a) and shoot dry weight (Fig. 1b) of berseem clover, however, the extent of reduction was found to be dependent on the severity of the stress. So, 3 dS m^{-1} salinity level imposed at flowering and 6-leaf stages, and 6 dS m^{-1} salinity level applied at flowering had no significant effect on plant height. The higher reduction in plant height was recorded when salinity was imposed at the 2-leaf stage followed by at 6-leaf, and higher salinity level had more negative effect (Fig. 1a). Indeed, salinity stress as 3, 6, 9 and 12 dS m^{-1}imposed at 2-leaf stage led to 36.0%, 38.7%, 46.6% and 59.4% reduction in plant height, respectively. These values were 10.6%, 20.0%, 27.7% and 39.4% for 6-leaf stage and were 3.8%, 8.5%, 14.4% and 28.3% for flowering, respectively. Although salt stress of any levels imposed at any stage had a significant negative effect on shoot dry weight, the negative effect was more at 2-leaf and due to higher salt stress levels. On average, salinity stress imposed at 2-leaf, 6-leaf and flowering reduced shoot dry weight by 58.4%, 48.6% and 37.1%, respectively.

Salt stress significantly decreased root length (Fig. 1c) as well as root dry weight (Fig. 1d) of berseem clover, however, there were significant differences among the treatments. Root length decreased due to salinity of 6, 9 and 12 dS m^{-1} imposed at 6-leaf and flowering stages, and due to all salt levels when imposed at the 2-leaf stage. Salinity treatment as 3, 6, 9 and 12 dS m^{-1} reduced root length by 31.7%, 35.8%, 47.5% and by 60.8% at 2-leaf, 3.9%, 15.3%, 30.4% and 40.6% at 6-leaf and by 2.6%, 16.5%, 22.9% and 25.4% at the flowering stage, respectively. The negative effect of salinity was more when applied at the 2-leaf stage and at higher salt stress levels (Fig. 1c). The reductions in root dry weight were greater when salinity was applied at the 2-leaf (63.2%) and 6-leaf stages (45.8%) than that at the flowering stage (28.1%) as shown in Fig. 3d. On the other hand, higher salinity levels imposed at all different growth stages considerably reduced root dry weight. For example, 3, 6, 9 and 12 dS m^{-1} salt levels imposed at the 2-leaf stage reduced root dry weight by 37.1%, 60.9%, 67.5% and 87.2%, respectively. These reductions were 12.0%, 38.4%, 54.2% and 78.6% when salt stress was imposed at the 6-leaf stage and were 4.5%, 21.0%, 31.7% and 55.2% when salt stress imposed at flowering, respectively.

Tolerance threshold analysis also showed that threshold levels of berseem clover were 6.54, 7.99 and 10.00 dS m^{-1} when salt stress was imposed at the 2-, 6-leaf and flowering stages, respectively. The difference in sensitivity of berseem clover also could be observed in growth parameters (Fig. 1), or in the slope of reduction in plant dry matter (Fig. 3). The slope of reduction due to salt stress in plant dry matter was more when it was imposed at the 2-leaf (-0.26) followed by at the 6-leaf (-0.25) compared to that at flowering (-0.23).

Except in 12 dS m^{-1} salt level, the lowest shoot and root Na^+ concentrations were found in plants subjected to salt stress at 2-leaf, the treatment applied at flowering showed the highest Na^+ concentrations. Reduced shoot and root growth due to salt stress in the current study might be attributed to the negative effect of this ion. Salinity stress increased Na^+ accumulation, but decreased that of potassium (K^+) in the shoot and root of berseem clover plants (Fig. 2c and 2d).

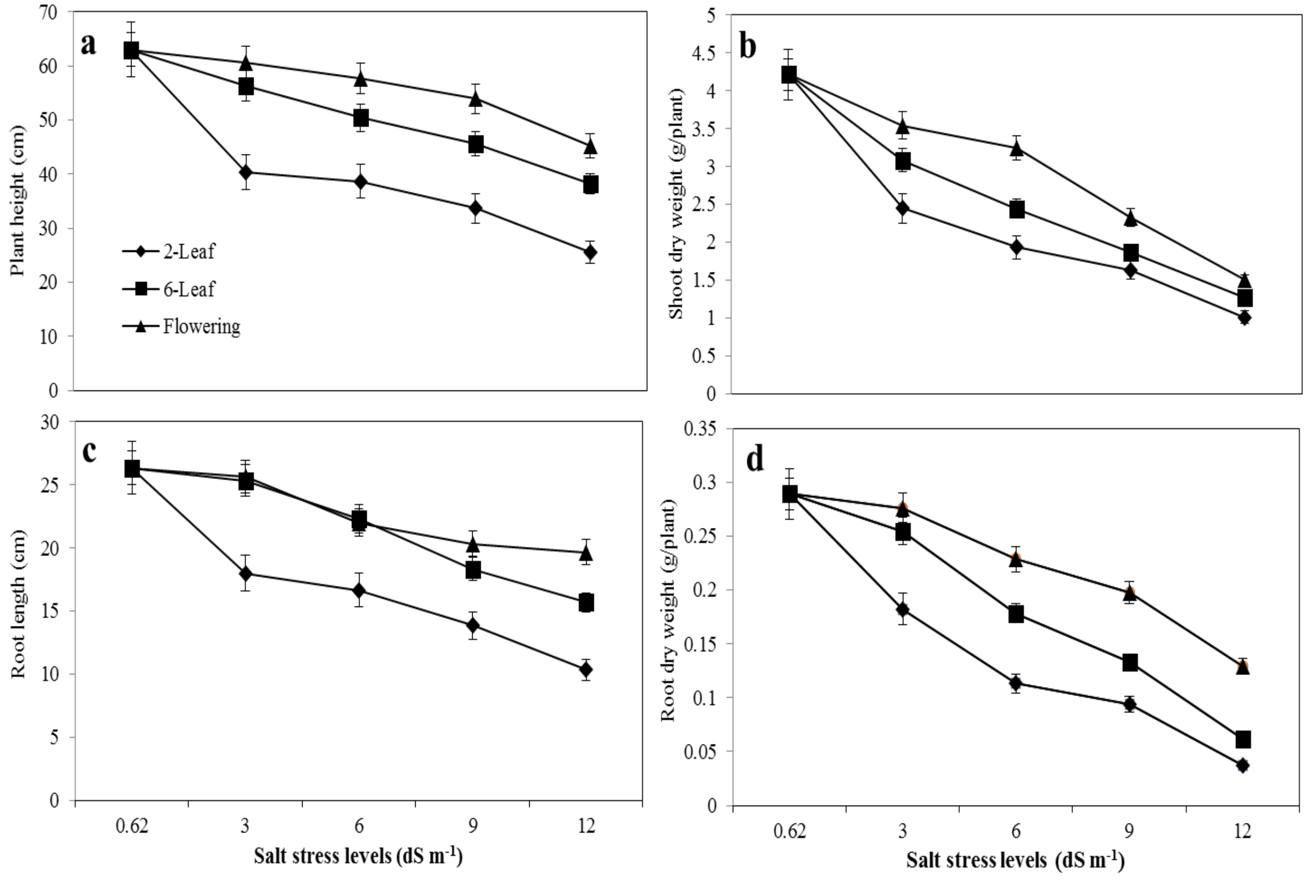

Fig. 1. Effect of different salt stress levels imposed at three growth stages on growth parameters. Bars represent standard errors (±SE).

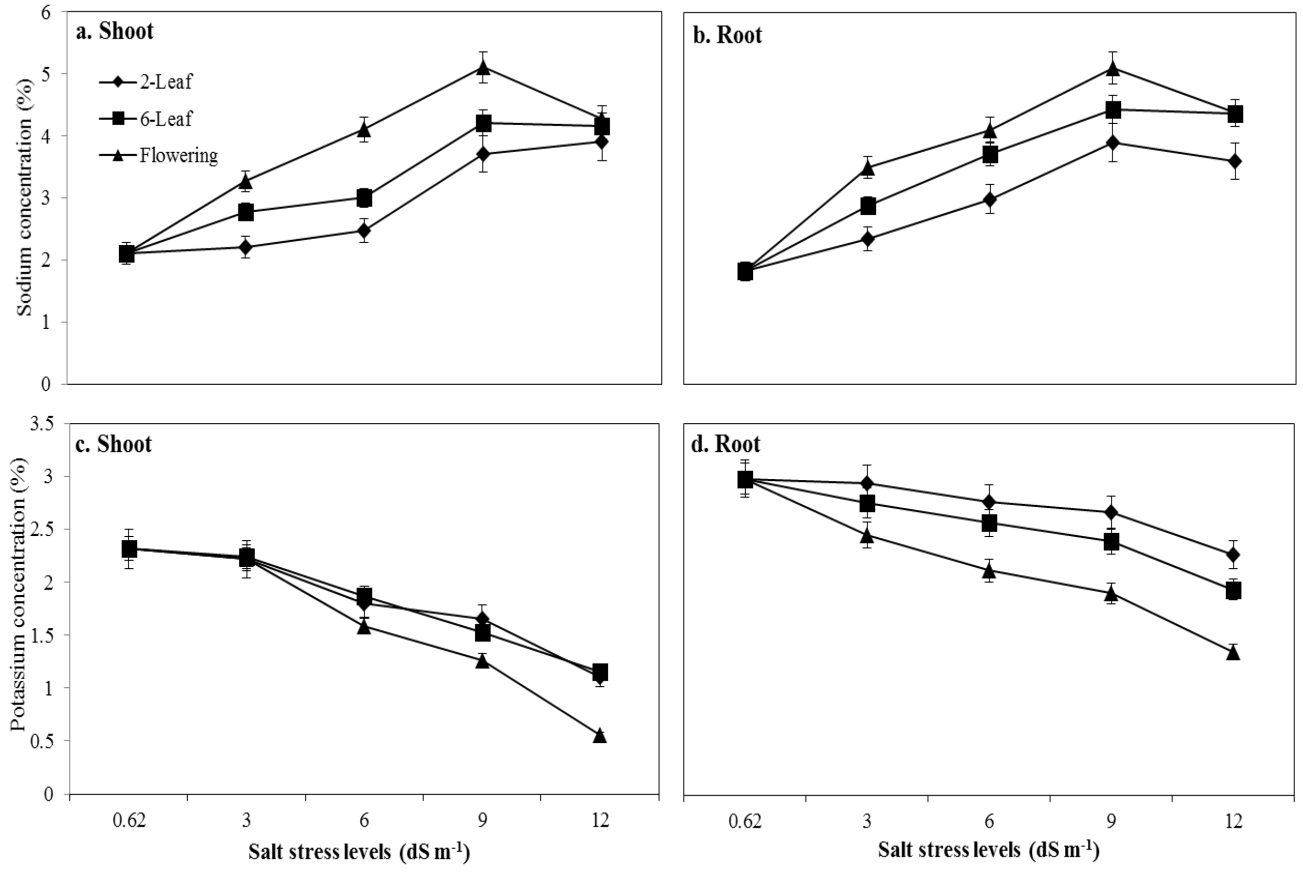

Fig. 2. Effect of different salt stress levels imposed at three growth stages on ion accumulation in shoot and root. Bars represent standard errors (±SE).

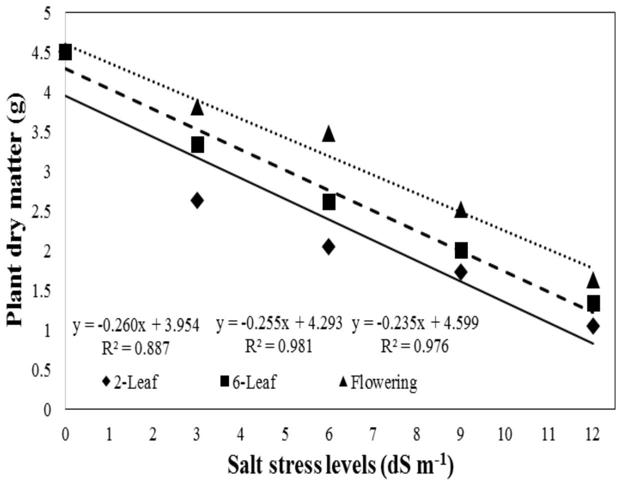

Fig. 3. Relationship between different salt stress levels imposed at three growth stages with plant dry matter (shoot + root).

There was no significant difference among the three stages at which salinity stress was imposed particularly 3 dS m^{-1} level; however, in other salt levels (e.g. 6, 9 and 12 dS m^{-1}), shoot K^+ concentration was greater in plants which were subjected to salt stress at the flowering stage (Fig. 2c). The highest and the lowest root K^+ concentrations were observed in stressed plants when salt stress was imposed at flowering and 2-leaf stages, respectively (Fig. 2d).

Salt stress over 3 dS m^{-1} imposed at flowering stage and over 6 dS m^{-1} imposed at 2- and 6-leaf stages significantly enhanced the activity of catalase (CAT) as shown in Fig. 4a. There was no significant difference between the stressed and non-stressed plants in terms of CAT activities particularly when salinity was imposed at 2- and 6-leaf stages, however when salt stress was imposed at flowering, the CAT activity was much higher. So that on average, salt stress imposed at 2-leaf, 6-leaf and flowering caused 28.3%, 31.3% and 45.3% increase in CAT activity, respectively.

Although salt stress imposed at any stage enhanced superoxide dismutase (SOD) activity, however, there was no significant difference among the three stages (Fig. 4b). The effect of all salt levels (3, 6, 9 and 12 dS m^{-1}) averaged over three growth stages showed 128.7%, 151.2%, 175.8 and 201.0% increasing in SOD activity, respectively.

All levels of salt stress at flowering and more than 3 dS m^{-1} at 2- and 6-leaf stages caused enhanced peroxidase (POD) activity (Fig. 4c). At all salinity levels, stressed plants at flowering had greater POD activity than that at the two other stages, so that at 3, 6, 9 and 12 dS m^{-1} salinity treatments the POD activity was higher by 23.0%, 44.6%, 56.3% and 44.8% in stressed plants at flowering.

Salt stress significantly increased shoot and root sodium (Na^+) concentration, however, these increasing trends were obtained up to 9 dS m^{-1}, but higher salinity level, i.e. 12 dS m^{-1} did not significantly change or decrease Na^+ concentrations (Fig. 2a and 2b).

Salt stress at 6, 9 and 12 dS m^{-1} enhanced ascorbic peroxidase (ASP) activity only when it was imposed at flowering, however salinity stress imposed at 2- or 6-leaf stage had no significant effect on ASP activity (Fig. 4d). Salt stress at 6, 9 and 12 dS m^{-1} imposed at flowering caused 20.9%, 28.3% and 31.5% increase in ASP activity, respectively.

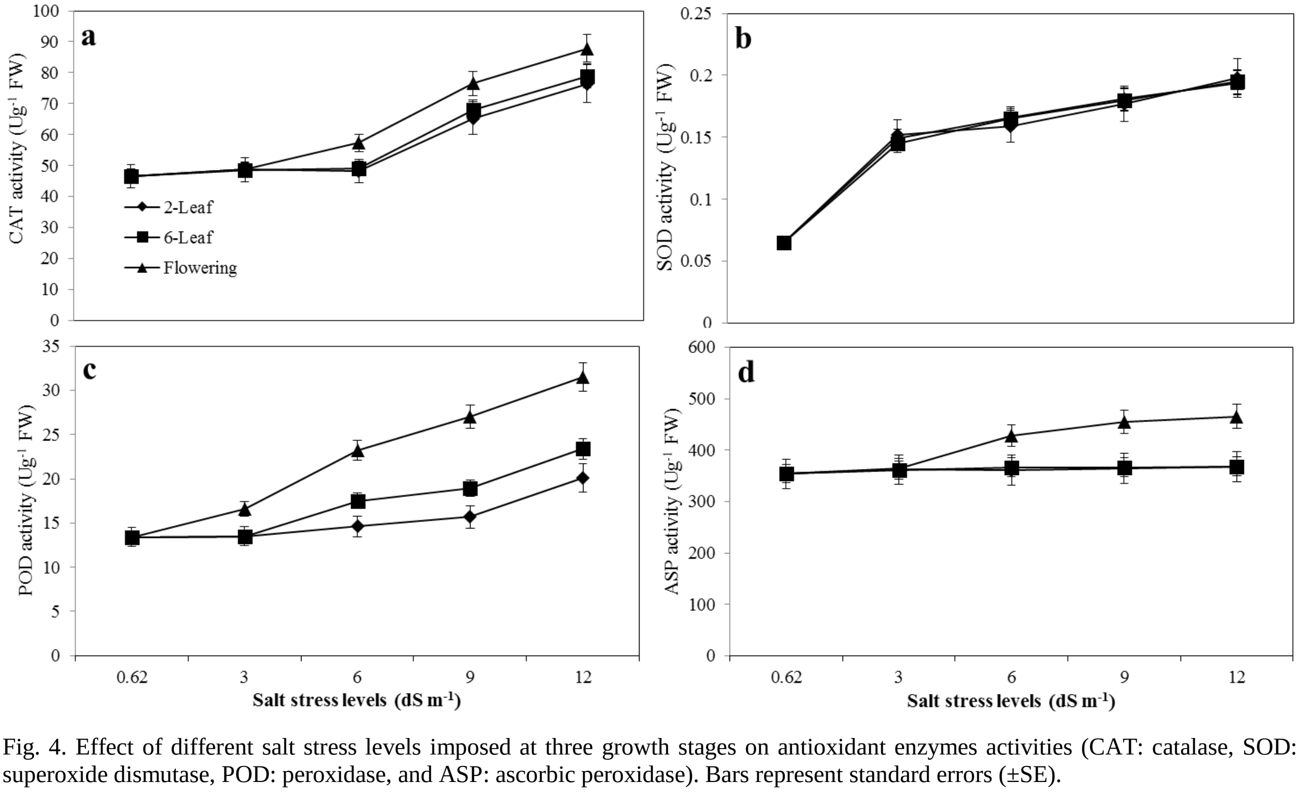

Fig. 4. Effect of different salt stress levels imposed at three growth stages on antioxidant enzymes activities (CAT: catalase, SOD: superoxide dismutase, POD: peroxidase, and ASP: ascorbic peroxidase). Bars represent standard errors (±SE).

Discussion

Our results of reduction in growth of berseem are in agreement with those of Tavakkoli *et al*., (2010), Pirasteh-Anosheh *et al*., (2014a,b, 2015), who reported reduction in plant height due to salinity in different crops. They also reported that high salinity levels were very effective in decreasing plant height in different crops. The reductions in growth parameters such as plant height and shoot dry matter might be due to the effect of salinity on resource use efficiency, such as water and nutrition. Salt stress reduces the ability of plants to utilize such resources as water and results in a reduction in growth (Munns, 2002, Pirasteh-Anosheh *et al*., 2015). At different stages, salinity has been reported to reduce the water imbibition by roots that results in reducing osmotic potentials in the root zone. This alteration may lead to alterations in metabolic activities and reduction in crop growth (Parida & Das, 2005; Ashraf & Orooj, 2006; Aftab *et al*., 2011). Higher sodium and lower potassium concentrations in stressed plants shown in the current study may also be responsible for reduced shoot growth. Pakar *et al*., (2016) showed that the activity of SOD and K+/Na+ ratio were found to be useful in salt tolerance manipulation in barley plants.

Salt stress is reported to be responsible for both inhibition and delay in germination and emergence (Aftab *et al*., 2011), decrease in water uptake (Ashraf & Harris, 2004), and enhanced leaf senescence (Munns, 2002), that might be involved for reduced root growth. Some researchers attributed reductions in root growth to reduced photosynthetic rate and changes in plant metabolic processes, which led to lower transported assimilates (Tavakkoli *et al*., 2010).

The response of growth parameters to salt stress treatments showed that berseem clover was most sensitive at early growth and at later stage it was relatively tolerant. It is argued that salt stress at early growth leads to develop weak seedlings, which may subsequently yield weak adult plants. For example, earlier Maas & Poss (1989) for bread and durum wheat, Maas *et al*., (1983) for maize, Maas *et al*., (1986) for sorghum, Maas & Poss (1989a) for cowpea showed that all these plants were more sensitive to a stress at the seedling stage.

Monovalent ions such as Na^+ may have adverse effects on plant biochemical processes (Ashraf *et al*., 2010). High concentrations of Na^+ in soil can cause deterioration in the soil structure and may exacerbate the effects of salinity by impeding drainage as well as affecting the availability of water to the plant as the soil dries (Ashraf & Harris, 2004). Salt stress is known to alter the ion equilibrium in plant tissues and resultantly, some important ions could be effectively used as important selection criteria for salt tolerance (Ashraf *et al*., 2010). Indeed, K^+ concentration is believed to be an index of salinity tolerance in most crop species (Ashraf & Harris, 2004). In the present study, the lowest root K^+ concentrations were observed in stressed plants when salt stress was imposed at flowering and 2-leaf stages, respectively. Mittova *et al*., (2002) reported that salt stress tolerance was correlated with increased activities of antioxidant enzymes such as SOD, ASP, and POD. The role of SOD in scavenging of active oxygen species has been well focused in the literature. It was indicated that plants generally are able to eliminate superoxide (O_2^-) using SOD, which catalyzes the dismutation of superoxide into hydrogen peroxide and oxygen, and is important in preventing the reduction of metal ions and hence the synthesis of hydroxyl radicals (Ashraf & Harris, 2004; Ashraf & Ali, 2008). Ashraf and Harris (2004) believed that POD and some other antioxidant enzymes played a crucial role in salt stress tolerance. They reported that salt stress caused a considerable increase in the activities of POD and glutathione reductase in the salt tolerant plants, whereas the activities of these enzymes remained unchanged or decreased in salt sensitive plants. Furthermore, it has been noted that SOD could catalyze O_2^- to hydrogen peroxide and oxygen. Hydrogen peroxide can be eliminated by ASP located in the thylakoid membrane (Chen & Asada, 1989). Our results about the ability of berseem clover to get recovered form saline stress could also be observed in terms of ion accumulation and antioxidant enzymes activities. Ashraf & Harris (2004) in a peer review noted considerable variations in the protective mechanisms of antioxidant enzymes such as CAT, SOD, POD and ASP against activated oxygen species in different plant species. Thus, further work is required to establish the general validity of this phenomenon in salinity tolerance in different plants.

Conclusion

Salt stress reduced growth and K^+ concentration, and enhanced Na^+ concentration and antioxidant enzymes activities. Both shoot and root tissues had relatively similar responses. Higher salt stress levels, especially at early growth had more negative effect on the attributes measured. It also was shown that when salt stress was imposed at early growth stage, plants were able to recover in terms of ion accumulation and antioxidant enzymes. Response of growth parameters to salt stress at different stages and threshold analysis revealed that berseem clover was a most sensitive plant at early growth, but less sensitive at later growth stages.

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