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Morphological and biochemical responses of sainfoin (*Onobrychis viciifolia* Scop.) ecotypes to salinity

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ABSTRACT
This study was conducted to evaluate the effect of different NaCl concentrations (0, 100 and 150 mM) on the morphological and biochemical parameters of different sainfoin ecotypes 'Koçaş', 'Malya,' 'Altınova' and 'Ulaş' under controlled conditions. The morphological parameters included seed germination, seedling and root lengths, and seedling fresh and dry weights while the biochemical parameters included chlorophyll and proline contents, lipid peroxidation (MDA), and the activities of antioxidant enzymes (SOD, CAT, APX, and GR). The results demonstrated that increase in NaCl concentration caused an overall decrease in morphological parameters and an increase in the biochemical parameters in all ecotypes. The findings showed that among all ecotypes, 'Koçaş' had higher chlorophyll and proline content, increased activity of CAT, GR, and APX, and increased fresh and dry weight in response to salt stress. To the best of our knowledge, this is the first report on the evaluation of enzymatic and non-enzymatic defense systems in sainfoin plants under salt stress.

Key words: Antioxidant enzymes, Chlorophyll contents, Malondialdehyde (MDA), Proline content.

INTRODUCTION

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial forage legume that is beneficial in animal husbandry due to its high protein content and the presence of secondary metabolites (Kempf *et al.*, 2016). *Onobrychis*, commonly known as sainfoin, is an important plant that can thrive and proliferate throughout the world especially in arid and semiarid regions (Erkovan *et al.*, 2016). In arid and semiarid areas, soil salinity is recognized as a major threat to agriculture and food production (Chaitanya *et al.*, 2014). Although sainfoin is considered as a relatively salt tolerant species compared with other legume forages, it is sensitive to salt stress in the seedling stage (Temel *et al.*, 2016; Wu *et al.,*2017). Harmful stress conditions such as salinity can cause morphological and biochemical changes in plants. There are limited reports on the effects of salt stress in sainfoin plants. Moreover, to the best of our knowledge, so far, there have been no studies on the response of the enzymatic and non-enzymatic defense system of sainfoin under salt stress conditions. Therefore, the purpose of this study was to assess the morphological and biochemical responses of sainfoin (*O. viciifolia* Scop.) ecotypes and the ecotype that is least affected by salt stress.

MATERIALS AND METHODS

Plant materials: In the present study, sainfoin (*O. viciifolia* Scop.) ecotypes 'Koçaş,' 'Malya,' 'Altınova,' and 'Ulaş,' which are widely cultivated in Central Anatolia in Turkey, were used as plant materials for the experiments.

Salt treatments: For pretreating, seeds of various ecotypes were placed into a solution containing different NaCl concentrations (0, 100 and 150) for 10 minutes. The pretreated seeds were germinated in the magenta caps (14x14) filled with commercial peat (250 g) in a growth chamber with a 16 photoperiod at 25 C^0 and 70% humidity. In the salt treatment, the plants were exposed to salt stress by adding 25 ml solution (containing 0, 100 and 150 mM NaCl) every other day for 30 days. The treatments were replicated four times, and each replication consisted of 25 plants.

Morphological observation: For morphological analysis, leaf samples were randomly taken from 30-day-old plants in each replication. The seed germination rate, seedling shoots and root lengths, and the seedling fresh and dry weights were recorded as morphological parameters. The germination percentages were recorded on the 30^{th} day. The shoot and root lengths and fresh and dry weights of the seedlings were measured on the 30^{th} day. The dry weight was measured after drying the samples at 70 C° for 48 hours in an oven.

Biochemical observation

Chlorophyll contents: Chlorophyll a, chlorophyll b and total chlorophyll content of the leaves from plants treated with

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different doses of boron were determined as described by Curtis and Shetty (1996).

SOD, CAT, SOD and APX activity

Superoxide dismutase (SOD) activity was determined using the method proposed by Çakmak and Marschner (1992), and Çakmak *et al.* (1994), based on the reduction of NBT (nitro blue tetrazolium chloride) by O_2- under light.

Catalase activity (CAT) was measured based on the decomposition rate of H_2O_2 at 240 nm (E = 39.4 mM cm^{-1}) (Çakmak and Marschner, 1992; Çakmak *et al.*, 1994).

Ascorbate peroxidase (APX) activity was measured using the method proposed by Cakmak and Marschner (1992), and Cakmak *et al.* (1994), based on the oxidation of ascorbate at 290 nm (E =2.8 mM cm^{-1}).

Glutathione reductase (GR) activity was measured using the method proposed by Cakmak and Marschner (1992), and Cakmak *et al.* (1994), based on the oxidation of NADPH at 340 nm (E =6.2 mM cm^{-1}).

Malondialdehyde (MDA) and Proline content

The malondialdehyde (MDA) was determined according to Lutts *et al.* (1996). The proline assay was based on the method of Bates *et al.* (1973), which uses 3% sulfosalicylic acid for grinding the fresh plant samples.

Statistical analysis: Data were performed by Duncan's multiple range test using 'IBM SPSS Statistics 22' software. Data given in percentages were subjected to arcsine (√X) transformation before statistical analysis(Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

To evaluate the morphological parameters in sainfoin ecotypes under salinity stress, the ecotypes were exposed to different concentrations (50, 100, and 150 mM) of NaCl. Fig. 1 shows that salt stress significantly ($p<0.05$) reduced seed germination and morphological parameters including seedling and root length and plant fresh and dry weight in all ecotypes ('Koçaş,' 'Malya,' 'Altınova' and 'Ulaş'). However, the responses of the ecotypes to the salt levels varied. 'Ulaş' ecotypes showed the highest seed germination percentages and root lenght at 150 mM NaCl (Figure 1). In term of growth parameters, specifically fresh and dry weights, 'Koçaş' performed better than all other ecotypes under salt stress. Therefore, it could be concluded that salt stress less affects the morphological traits of 'Koçaş' less compared with other ecotypes. The findings also show that although 'Koçaş' has an underdeveloped root system at the highest NaCl (150 mM) concentration compared with other ecotypes, it has remarkably higher dry matter content (Fig. 1). This could be attributed to high chlorophyll and proline contents, high antioxidant enzymes activity, and secondary low MDA content of 'Koçaş' at 150 mM NaCl (Fig. 2). The reduction in growth and productivity when plants are grown under conditions of salt stress is accompanied by a strong reduction in the rate of photosynthesis (Zhang *et al.,* 2014). Between the increase in total chlorophyll and the increase in dry matter was reported by Bokari (1983). On the other hand, a close correlation between the activities of antioxidant enzymes and proline accumulation and dry have been showed in many studies (Lum *et al.,* 2014; Balal *et al.,* 2012). In the present study, reduction in the percentage of seed germination, seedling and root length, and plant fresh and dry weight are in agreement with those of Cokkızgın (2012) and Kusvuran (2015).

Chlorophyll a, b and total chlorophyll contents in the leaf tissues were determined in normal plants and those subjected to stress (Fig. 1). Regarding chlorophyll contents, the responses of ecotypes to salt concentration were different. As shown in Fig. 1, the contents of Chl a, Chl b, and total chlorophyll in plants significantly ($P<0.05$) decreased in 'Altınova' and 'Ulaş' ecotypes, and increased in 'Koçaş' and 'Malya' in response to increase in salinity. Compared with control, chlorophyll a, b and total chlorophyll contents respectively increased by 4.5% and 8.4% and 4.9% in 'Koçaş' and 7%, 47% and 44% in 'Malya' in response to 150 mM NaCl application. For 'Altınova,' the chlorophyll a, b and total chlorophyll contents were a reduction of 47, 48 and 47 %, respectively. The plants of the 'Ulaş' ecotype treated with 150 mM NaCl showed chlorophyll a, b and total chlorophyll contents representing 32, 28 and 22% reduction respectively compared with control plants. Our results regarding the decrease in chlorophyll a and b contents corroborate those of Taibi *et al.,* (2016) and Tayyab *et al.,* (2016). Conversely, the results regarding the increase in chlorophyll content with the increase in salt concentrations concur with the results reported by Amira and Abdul (2011).

Salt stress affects not only the morphological parameters but also the biochemical parameters of plants (Fang *et al.*, 2017). The results obtained for the current investigation indicate that salinity stress significantly ($p<0.05$) affects all the biochemical parameters of ecotypes (Fig. 2). A significant change was observed in all ecotypes regarding the activity of antioxidant enzymes (CAT, SOD, GR, and APX). These antioxidative enzymes may play important roles in the rapid defense responses of plant cells against oxidative stress (Kusvuran and Dasgan, 2017). Catalases are responsible for the removal of H_2O_2 by reducing H_2O_2 to $2H_2O$ (Caverzan *et al.*, 2016). Our findings show that CAT increased with increased concentration of NaCl in all ecotypes. The 261% increased rate was observed in the 'Koçaş' ecotype at 150 mM NaCl. However, CAT activities in 'Ulaş,' 'Altinova' and 'Malya' ecotypes were showed an increase of 94%, 15%, and 17%, respectively compared with the control. Kusvuran (2015) reported that salt stress

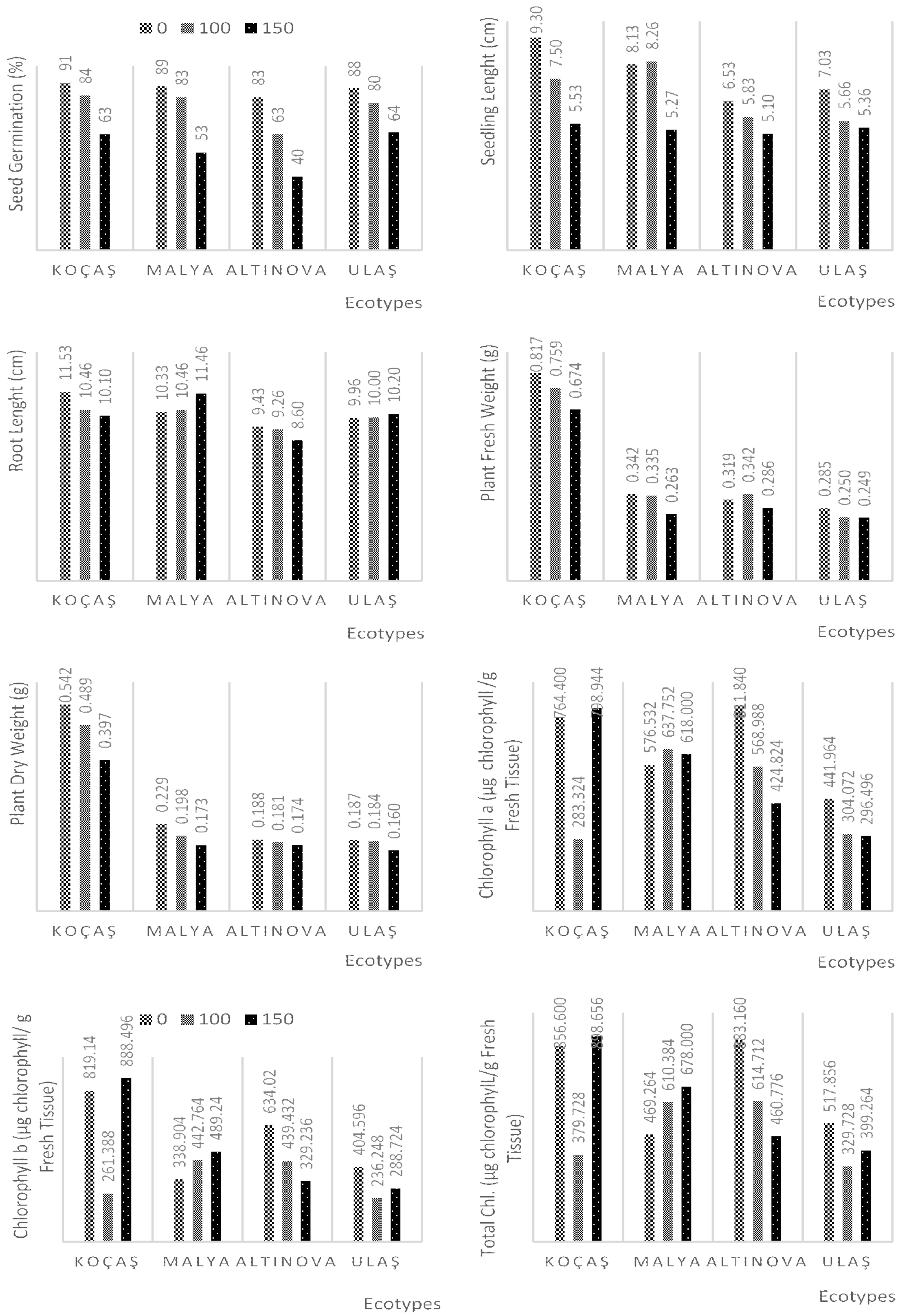

Fig 1: Seed germination, seedling and root length, and fresh, dry weight and cholorophyll a, b and total content of ecotypes under different salt concentrations (0, 100 and 150 mM NaCl). The values represent the mean of 4 replications.

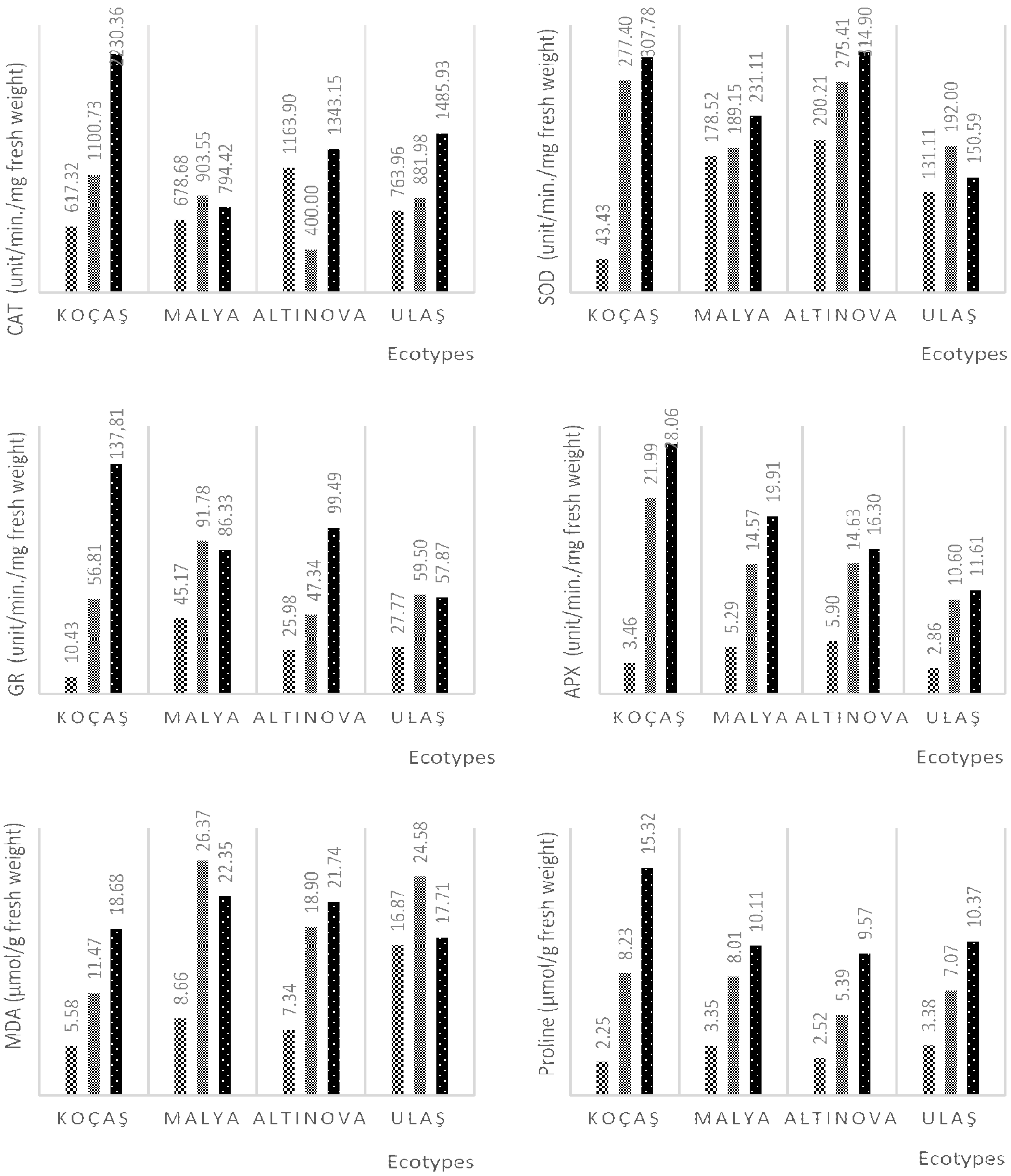

Fig 2: Antioxidant enzyme activities, MDA and proline contents of different ecotypes under different salt concentrations (0, 100 and 150 mM NaCl). The values represent the mean of 4 replications.

increased the CAT activity of the Hungarian vetch (*Vicia pannonica* Crantz.) varieties.

In an oxidative defense system, SOD has an important role in plants and the first dismutation ROS. Our results showed that the activity of SOD significantly increased with increasing NaCl concentration in all ecotypes. The maximum increased activity rate (58%) of SOD was noted in the 'Altınova' ecotype at 150 mM of NaCl. The SOD activity at 150 mM NaCl showed a 608%, 29%, and 14% increase in 'Koçaş', 'Malya,' and 'Ulaş,' respectively compared with the control.

Glutathione Reductase (GR) is a potential enzyme of the ASH-GSH cycle and plays an essential role in the defense system against ROS by sustaining the reduced status of GSH (Gill and Tuteja, 2010). Our findings showed that salt treatment increased the GR activities in all ecotypes compared with the control plants (Fig. 3). The highest GR activity (1221% increase) was measured in 'Koçaş'. The increase in NaCl increased the GR activity by 282, 91, and 108% in 'Altınova,' 'Malya,' and 'Ulaş,' respectively.

APX is regarded as one of the most widely distributed antioxidant enzymes in plant cells. Moreover, the isoforms of APX have a much higher affinity for H_2O_2 than CAT, making them efficient scavengers of H_2O_2 under stress conditions (Sharma *et al.*, 2012). Our results indicate that the APX activity increased significantly due to salt stress in all ecotypes (Fig. 3). The highest increases (710%) was recorded in 'Koçaş' at 150 mM of NaCl. Hovewer, 305, 276, and 176% increase in APX activity were observed for 'Ulaş,' 'Malya,' and 'Altınova,' respectively.

'Koçaş' showed the highest CAT, APX, and GR enzyme activities (Fig. 2). Thus, the antioxidant enzymes of 'Koçaş' show a more effective response to salt stress than the other ecotypes. Moreover, our findings indicate that CAT played a more active role than the other enzymes in all ecotypes under salt stress conditions suggesting that it plays a key role in the defense against ROS in sainfoin leaves. The increased antioxidant enzymes activity was reported in other legumes under salt stress condititons (Rasool *et al.,* 2013; Praxedes *et al.,* 2014; Yasar *et al.,* 2016). Contrary, Ozturk *et al.* (2012) indicated that salt treatments decreased the CAT and APX activities in *Pisum sativum* L. seedlings.

Lipid molecules, specifically unsaturated lipids, are sensitive to oxidation by ROS (reactive oxygen species) (Rasool *et al.*, 2013). Membrane lipid peroxidation is often used as a marker of an adverse effect of oxidative stress (Ozturk *et al.*, 2012). In the present study, the lipid peroxidation was measured as the amount of MDA. The results showed that MDA contents significantly increased under stress conditions compared with the control in all ecotypes (Fig. 3). The ecotype 'Ulaş' recorded the lowest increase rate of (5%) MDA content at 150 mM NaCl. The Hovewer, the MDA contents increased by 234, 158, and 196% in 'Koçaş,' 'Malya,' and 'Altınova,' respectively. The increase in MDA contents concurs with the results of Yasar *et al.,* (2016). Our results show that 'Koçaş' has the second lowest MDA content at 150 mM NaCl (Fig. 2). These results can be attributed to the high antioxidant enzyme activities (except SOD) and proline content. High antioxidant enzyme activities decrease ROS induced lipid peroxidation (Balal *et al.,* 2012; Dhanyalakshmi *et al.,* 2013). Hovewer, it was reported that the highest increase in lipid peroxidation and low antioxidant enzyme activities by Rasool *et al.,* (2013).

Proline is a widely accumulated osmolyte in a wide range of plant species (Ramana *et al.*, 2012). However, proline is a good indicator for determining the salt tolerant species of leguminous plants (Cha-um *et al.*, 2013). In the present study, we observed a positive correlation between the proline accumulation and salinity stress in the ecotypes. The higher increased rate was (580%) observed in 'Koçaş' at 150 mM NaCl (Fig. 2). Hoverwer, proline accumulation increased by 279, 206 and 201% in 'Altınova,' 'Ulaş,' and 'Malya,' respectively, compared with the control. These results are in accordance with the findings of El-Sabagh *et al.* (2015).

CONCLUSION

In the present study, all sainfoin ecotypes responded differently to different concentrations of NaCl. Overall, our results show that in term of some morphological (seedling length, plant fresh and dry weight) parameters, highest scores obtained from 'Koçaş' ecotypes under salt stress (150 mM). However, considering biochemical (chlorophyll a, b and total contents, CAT, GR and APX, proline and MDA (as secondly) contents) parameters, highest scores also obtained from 'Koçaş' ecotypes under salt stress (150 mM). Thus, the changes in these parameters related to salt tolerance, in 'Koçaş' ecotypes, are more active and/or sensitive to salt stress. Conversely, further investigations are needed such as Na^+ uptake, N fixation, tannin expression or quality under salt stress to say something more about salt tolerance of sainfoin. Among the antioxidant enzymes, CAT seems to be more active in scavenging ROS in sainfoin. To the best of our best knowledge, this is the first report on changes in the antioxidant defense systems of sainfoin under salt stress.

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