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The effects of salt stress on the germination and antioxidative enzyme activity of Hungarian vetch (*Vicia pannonica* Crantz.) varieties

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ABSTRACT

Among abiotic stresses, salinity is one of the most important affecting agricultural production world-wide. In this study, 5 different salt (NaCl) concentrations (0, 50, 100, 150 and, 200 mM) were investigated on the germination and antioxidative enzyme activity of Hungarian vetch seeds. The germination rates, shoot and root lengths, shoot and root fresh weights, shoot/root ratios, lipid peroxidation (MDA), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activities were evaluated and analyzed at 15 days after placing the seeds in petri dishes. The results indicated a significant variation in the germination and early seedling growth characteristic responses of the varieties to NaCl salinity. It was seen that increasing the NaCl concentration resulted in a decrease in the early seedling growth characteristic germination rates and values of all of varieties used in experiment. However, these decreased less in the tolerant varieties than in the sensitive ones. Under stress conditions, the tolerant varieties showed less of an increase in MDA and more of an increase in SOD, CAT, APX, and GR than the intolerant varieties, indicating that saline stress induces the production of reactive oxygen radicals, resulting in an increase MDA and oxidative stress in the varieties. In Hungarian vetch, this increase in the antioxidant enzyme activities is associated with salt stress tolerance. These results might be beneficial in breeding programs relevant to salinity stress resistance.

Key words: Ascorbate peroxidase, catalase, glutathione reductase, malondialdehit, superoxide dismutase, salinity.

INTRODUCTION

Salinity is the major environmental factor that limits plant growth, productivity, and distribution. Therefore, understanding the biochemical and physiological mechanisms of salt stress is essential for breeding tolerant cultivars and improving environmental conditions to increase crop productivity (Wang *et al.*, 2009). A water deficit is also induced by salinity, caused by a decrease in the osmotic potential of the soil solutes, making it difficult for roots to absorb water from their surrounding media, even in well watered soils. A reduction in the growth of plants is mainly due to the severe effects of salinity on various biochemical and physiological processes (Khatoon *et al.*, 2010). Salinity is an important abiotic stress that significantly affects seedling growth and seed quality (Amar *et al.*, 2014). Some studies in the literature have aimed at determining the effects of salinity on the germination of various plant species. It has been reported that a correlation exists between the activities of antioxidative enzymes and the salt tolerance of plants. In their study, Qian *et al.* (2007) reported the effects of varying salt concentrations (from 2 to 48 dSm^{-1}) on turfgrass germination. Dai *et al.* (2009) reported that an increase in the salt concentration greatly decreases the percentage and rate of germination, and increases the germination time. In another study, the effect of NaCl concentrations of 0%, 0.5%, and 1.5% on the germination and growth of wheat genotypes was studied (Zabihi-e-Mahmoodabad *et al.*, 2011). Coelho *et al.* (2014) reported that salinity levels did not affect the seed germination of varieties of forage sorghum until reaching a conductivity level of 10 dSm^{-1}.

Salt stress causes a reduction in seed germination by limiting water absorption, affecting the mobilization of stored reserves, and directly affecting the protein synthesis or structural organization in embryos as they germinate (Almansouri *et al.*, 2001). In plants, exposure to environmental stresses like drought and salinity causes a disruption in the balance between reactive oxygen species (ROS) production and antioxidant quenching activity, often causing oxidative damage (Gossett *et al.*, 1994). ROS overproduction results in oxidative damage of cell

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biomolecules (lipids, proteins, and DNA) or possibly even cell death. The presence of lipid soluble antioxidants, water-soluble reductants, and antioxidant enzymes like catalase (CAT) (EC 1.11.1.6), ascorbate peroxidase (APX) (EC 1.11.1.11), and superoxide dismutase (SOD) (EC 1.15.1.1) prevents the harmful effects caused by ROS (Patade et al., 2012; Kusvuran et al., 2013). H_2O_2 is a ROS produced in response to environmental stresses that acts as a major signaling molecule and serves as an effective mode of defense. A further reduction of H_2O_2 to H_2O occurs in the peroxisomes by CAT, in the chloroplasts and cytosol by APX, and in the cell wall by glutathione reductase (GR) (Kachout et al., 2012). It has been reported that plants with constitutive or induced high levels of antioxidants have higher resistance to oxidative damage, and that in plants exposed to abiotic stress, the extent of oxidative cellular damage is controlled by their antioxidant system capacity.

Ashraf *et al.* (1987) and Almansouri *et al.* (2001) showed that salinity resistance is a heritable trait that could be used as efficient criteria in the selection of salt-resistant populations. To better understand plant adaptability to salt stress in their germination phase, the present study exposed Hungarian vetch varieties to different concentrations of NaCl with the aim of evaluating their stress tolerance by assessing the seed, MDA content, and antioxidant enzyme activity conditions in the developing seedlings.

MATERIALS AND METHODS

In this study, 6 different Hungarian vetch varieties that are commonly used under Mediterranean climate conditions were used as the plant materials. Prior to seeding, the seed surface was sterilized with 2% sodium hypochlorite for 10 min. After sterilization, the seeds were washed with distilled water three times (Bilgili *et al.* 2011). As the next step, 25 representative seeds of each cultivar were placed in 10-cm petri dishes containing 2 sheets of Watman No. 1 filter paper, which were initially moistened with either 5 mL of distilled water (control) or the different saline treatment solutions: 50, 100, 150, and 200 mM NaCl. The germination chamber was set at 25 °C under darkness and seed germination was determined when the radicle protruded from the seed coat (Wang *et al.* 2009).

Germination percentage (GPs) (%): From each of the Hungarian vetch varieties, 25 seeds were placed in petri dishes for germination. The germinated seeds were counted after 15 days in the petri dishes and the GPs was calculated. Seedlings were discarded from the petri dishes until only 20 remained for further study of their characteristics.

Shoot and root length (cm): The 20 remaining seedlings in each petri dish were used for measuring the shoot and root characteristics. After 15 days in the petri dishes, the roots and shoots of the seedlings were separated. Measurements were taken of the distances from the crown to the leaf and root tips as the shoot and root lengths, respectively.

Shoot and root fresh weight: The root and shoot fresh weights of each seedling were measured. The average fresh weights of the root and shoot of each plant seedling were measured by dividing the total weight by the total number of seedlings.

Shoot/root ratio: This calculation was made for the length and weight via the division of the shoot values by the root values.

Enzyme extraction and assay: Liquid nitrogen was used to rinse the seedling samples for 5 min. The frozen cotyledons were then stored at –80 °C until further analyses. Using a mortar and pestle, extraction of the enzymes was performed using 0.5 g of cotyledon tissue, with 5 mL extraction buffer that contained 50 mM potassium-phosphate buffer (pH 7.6) and 0.1 mM Na-EDTA. Centrifugation of the homogenate took place for 15 min at 15.000 × g and the supernatant fraction was then used for the enzyme assays. All of the enzyme extraction preparation procedures were performed at 4 °C (Kusvuran *et al.* 2007). The method of Karanlik (2001) was used for the SOD was assays, which included monitoring of the superoxide radical-induced nitro blue tetrazolium (NBT) reduction at 560 nm (Karanlik, 2001). One unit of SOD activity was defined as the amount of enzyme resulting in 50% inhibition of the photochemical reduction of the NBT. Determination of the CAT activity was done according to the method of Karanlik (2001), by monitoring the disappearance of HO. Determination of the APX activity was done by measuring the ascorbate consumption by following the absorbance at 290 nm. One unit of APX activity was defined as the amount of enzyme needed to consume (Cakmak and Marschner, 1992) 1 ìmole of ascorbate min^{-1}. Determination of the GR activity was done by measuring the enzyme-dependent oxidation of nicotinamide adenine di nucleotide phosphate-oxidase (NADPH) by following the absorbance at 340 nm. One unit of GR activity was defined as the amount of enzyme that oxidized 1 ìmole of NADPH min^{-1} (Karanlik, 2001).

Lipid peroxide content (MDA): Measurement of the MDA was taken as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). Homogenization of the frozen samples was done in a prechilled mortar using 2 volumes of ice-cold 0.1% (w/v) trichloroacetic acid (TCA). Centrifugation was then performed at 15.000 × g for 15 min. An assay mixture

consisting of a 1-mL aliquot of the supernatant and 2 mL of 0.5% (w/v) TBA in 20% (w/v) TCA was heated to 95 °C for 30 min, and then cooled rapidly in an ice bath. The supernatant absorbance (532 nm) was read after centrifugation (10.000 × g at 4 °C for 10 min) and values that corresponded to nonspecific absorption (600 nm) were subtracted. Calculation of the MDA content was done according to the molar extinction coefficient of the MDA (155 mM^{-1} cm^{-1}).

Experimental design and statistical analysis: The experiment was designed as a completely randomized plot with 4 replicates. Data were analyzed statistically and the means of each treatment were analyzed using Duncan's multiple range test ($P < 0.05$) and the SAS (9.0) packet program.

RESULTS AND DISCUSSION

In general, salt stress significantly decreased the GP of the Hungarian vetch varieties in comparison to the controls (Figure 1). The highest GP was observed in the control, and the lowest value was determined with the 150 and 200 mM NaCl applications. The Anadolu Pembesi and Altinova-2002 varieties had germination rates (GRs) higher than 80%, even with 150 mM NaCl, while the Tarim Beyazi-98 (24.16% decreasing) and Oguz-2002 (30.71% decreasing) varieties had lower GRs with the same NaCl treatment. Salt stress caused 17% reductions in the Hungarian vetch varieties with 200 mM NaCl. The highest GRs were observed in the Anadolu Pembesi and Altinova-2002 (84.25% and 84.75%, respectively) varieties. However, the Ege Beyazi-79 (56.25%) and Oguz-2002 (61.25%) cultivars showed the lowest GP with 200 mM NaCl. It was suggested by Cokkizgin (2012) that an increase in salinity brings about a reduction in the percentage of seed germination and the delayed onset of germination. The results of our study are in agreement with those of Kokten *et al.* (2010), who reported a significant difference in the salt tolerance of lentil genotypes and their responses to increasing salt concentrations. The GPs decreased when the NaCl levels increased. Furthermore, NaCl stress had a negative impact on the GPs. The present study identified and compared the salinity tolerance levels of 6 varieties of Hungarian vetch during germination, where the Anadolu Pembesi and Altinova-2002 varieties showed more performance than the others. In addition, because of the depressing effect of NaCl, seeds saturated with 150 and 200 mM NaCl had a lower GP. Increasing salt concentrations cause a reduction of the soil water potential. The osmotic potential of media solutions are decreased by salts, reducing the availability of water to the plant. Similarly, a decrease in the water potential of the solutions following NaCl treatments, resulting in decreased water imbibitions, was also reported by Katembe *et al.* (1998) and Cokkizgin (2012). The cause for an increase in the NaCl concentration is most likely a decrease in the gradient of the water potential between the seeds and their surrounding media. Salinity stress negatively affects seed germination through reduced osmotic water absorption or the accumulation of Na^+ and Cl^-, causing an imbalance in the nutrient uptake and toxicity effects (Shokohifard *et al.*, 1998; Cokkizgin, 2012).

Among the varieties, significant differences were observed in the shoot and root lengths (Figures 2 and 3). Increasing the NaCl treatment resulted in a significant decrease in the shoot elongation. The longest shoot lengths were obtained in the Ege Beyazi-79 and Tarm Beyazi-98 varieties (11.31 and 11.69 cm, respectively). The Anadolu Pembesi and Tarm Beyazi-98 varieties showed the longest

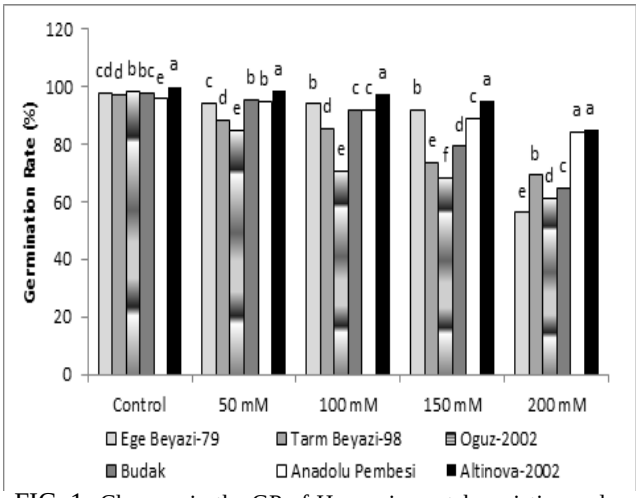

FIG. 1: Changes in the GP of Hungarian vetch varieties under salt stress.

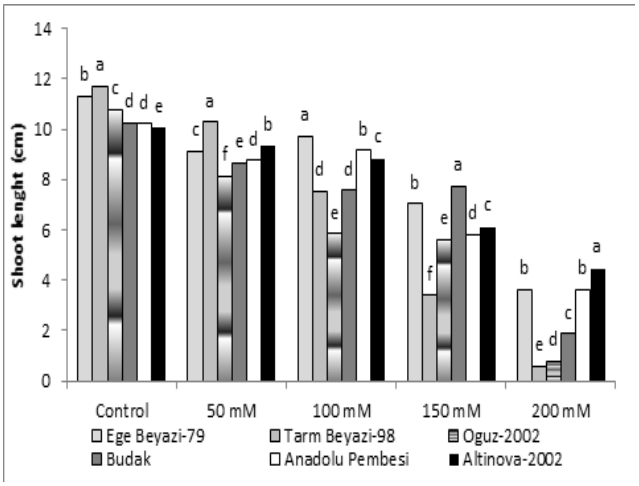

FIG. 2: Changes in the shoot length of Hungarian vetch varieties under salt stress.

root lengths under the control treatment, with 6.87 and 5.59 cm, respectively. On the other hand, the shortest root length was determined in the Oguz-2002 and Budak varieties (0.28 and 0.30 cm, respectively), with 200 mM NaCl. Compared to the control plants, the shoot and root length decrease averaged 32% and 54% with 200 mM NaCl, respectively. The least reductions were in the Anadolu Pembesi and Altinova-2002 varieties, with decreases of 55% and 64%, respectively. However, the Tarm Beyazi-98, Oguz-2002, and Budak varieties showed important shoot and root reductions, with decreases of 92%–94%. Furthermore, the shoot/root ratio of the salt-tolerant varieties was 3.26–4.09 with 200 mM NaCl (Figure 4). Zabihi-e-Mahmoodabad (2011) reported that salt stress during germination is a reliable test for the tolerance evaluation of many species, due to a reduction in the root and shoot growth caused by salinity. Some varieties are affected less and grow equally with the control plants and no inhibition effects are caused under saline growth. This is in accordance with the previous reports in melon, eggplant, bean, and tomato (Kusvuran *et al.*, 2007; Yasar *et al.*, 2006; Kaya *et al.*, 2007; Dasgan and Koc, 2009). The general effects of salinity on plant growth reported in the literature were a growth reduction with shorter stature and occasionally, fewer leaves, and reduced root length and mass (Shannon and Grieve, 1999).

The salinity treatments had a significant effect on the fresh weights of the shoots and roots (Figures 5 and 6.). Increasing the salinity reduced the shoot and root fresh weights by approximately 53% and 92% compared to those of the control group. When the salinity was increased from 50 mM to 200 mM, the shoot and root fresh weights were markedly decreased in the varieties by 79% and 74%, respectively. Salt-tolerant and salt-sensitive varieties showed

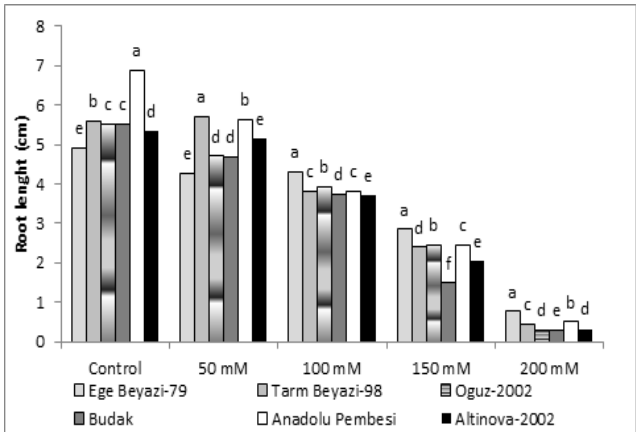

FIG. 3: Changes in the root length of Hungarian vetch varieties under salt stress.

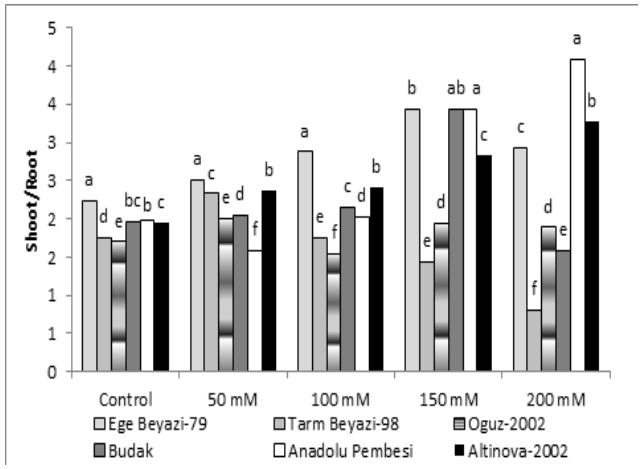

FIG. 4: Changes in the shoot/root ratios of Hungarian vetch varieties under salt stress.

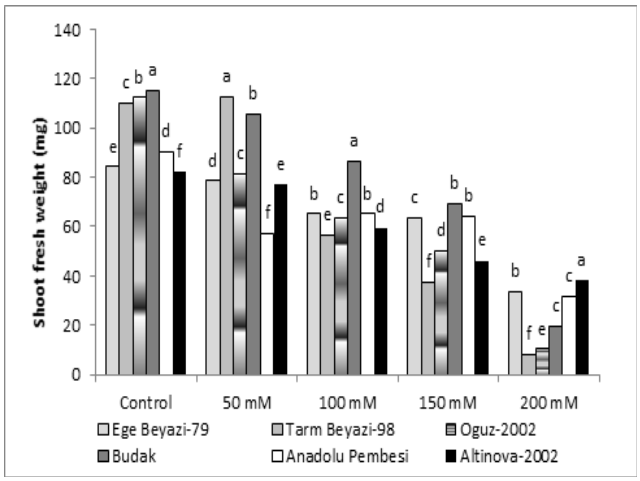

FIG. 5: Changes in the shoot fresh weight of Hungarian vetch varieties under salt stress.

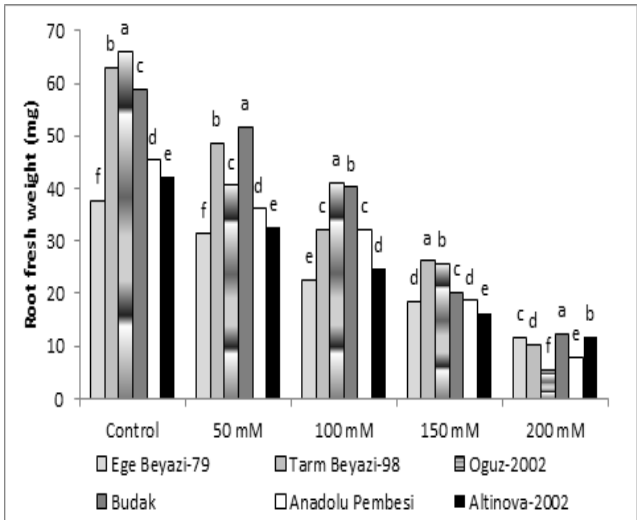

FIG. 6: Changes in the root fresh weight of Hungarian vetch varieties under salt stress.

very different development patterns. While the sensitive varieties Ege Beyazi-79, Tarm Beyazi-98, and Oguz-2002 had high reductions in the shoot and root fresh weights (83% and 91% decrease, respectively), in the tolerant varieties, Anadolu Pembesi and Altinova-2002, the shoot and root fresh weights decreased less in comparison to the control (53% and 72%, respectively).

The shoot and root fresh weight activities of all 6 varieties also decreased significantly with increasing NaCl concentrations. The average shoot and root fresh weights of the varieties were 99.13 g plant^{-1} and 52.18 g plant^{-1} under control conditions. However, these values decreased to 23.64 g plant^{-1} and 9.84 g plant^{-1} with 200 mM NaCl, respectively (75.85% and 81.14% reduction). Salt stress includes osmotic and ionic stresses, and the suppression of growth is directly contingent on the total soluble salt concentration and soil osmotic potential. At a whole-plant level, the detrimental effect is easily observed as decreased productivity or plant death (Kusvuran, 2012). Plant growth in the Hungarian vetch varieties was significantly reduced by 200 mM NaCl. Salt stress inhibits the efficiency of translocation and assimilation of photosynthetic products (Xiong and Zhu 2002), which may have caused this reduction in the shoot growth and seedling fresh weight. Zabihi-e-Mahmoodabad *et al.* (2011) reported a decrease with increasing salinity in the shoot and root fresh and dry weights, and many other studies also reported this trait as the main indicator of salinity tolerance. Moreover, Hussein *et al.* (2007) and Carpici *et al.* (2009) reported that a negative relationship was detected between the vegetative growth parameters and increasing salinity.

Lipid peroxidation levels in the leaves of the Hungarian vetch varieties, as measured by the MDA content and a product of lipid peroxidation, are shown in Figure 7. Salt stresses significantly affected the accumulation of the MDA content in the stressed seedlings of the Hungarian vetch varieties when compared to the controls. The concentration of MDA under salt stress, was seen to increase in the Ege Beyazi-79 and Oguz-2002 varieties and reached 8.95 and 10.26 μmol g^{-1} FW with 200 mM NaCl (410% increase). The increase was more significant in the Ege Beyazi-79 and Oguz-2002 varieties when compared with those of the other 4 cultivars, indicating a higher degree of lipid peroxidation in response to salt stress in this cultivar. On the other hand, the increase in the MDA content was much less in the Anadolu Pembesi and Altinova-2002 varieties (6.75 and 5.82 μmol g^{-1} FW, respectively) with 200 mM NaCl (Figure 7). In terms of the MDA, the lipid peroxidation rate can be used to evaluate a plant's tolerance to oxidative stress and oxidative stress sensitivity (Wang *et al.*, 2009). Navari-Izzo *et al.* (1996) and Wang and Hun (2009) reported that at the cellular level, ROS formation increases peroxidation, and the rate of increase depends on the species of the plant and the stress severity. We observed significant increases in the MDA contents of all of the Hungarian vetch cultivars; however, the magnitude of lipid damage was lower in the Anadolu Pembesi and Altinova-2002 varieties than in the sensitive ones. This indicates that under salt stress, the Anadolu Pembesi and Altinova-2002 varieties might have a higher capability of adopting improved antioxidant mechanisms than the other varieties.

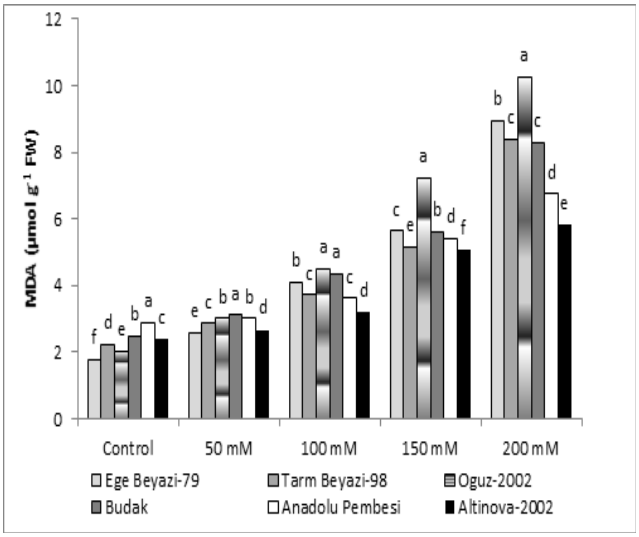

FIG. 7: Changes in the MDA of Hungarian vetch varieties under salt stress.

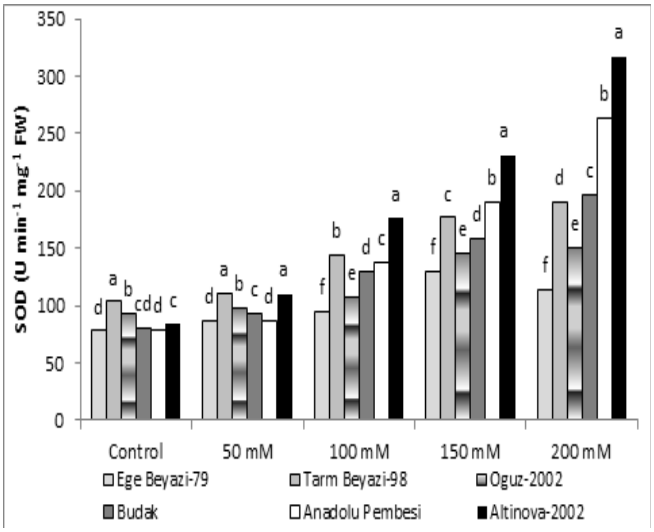

FIG. 8: Changes in the SOD of Hungarian vetch varieties under salt stress.

The antioxidant enzyme activities were also affected differently in the Hungarian vetch varieties by the NaCl treatments. The increasing salt levels increased the SOD activities of all of the varieties. Nevertheless, in the Anadolu Pembesi and Altinova-2002 varieties, the SOD activity increased more than in the others (Figure 8). With 200 mM NaCl, the SOD activity in the Ege Beyazi-79 and Oguz-2002 varieties increased by 45.1% and 62.0%, and in the Anadolu Pembesi and Altinova-2002 varieties, it increased by 236.2% and 277.5%, above the level of the control groups.

The CAT enzyme activity in the Hungarian vetch varieties increased under stress conditions. With 50 mM NaCl, the CAT activity began to increase in the all of the varieties. With this treatment, the CAT activity of the Anadolu Pembesi and Altinova-2002 varieties was 66.4 and 53.2 μmol min^{-1} mg^{-1} FW. While under the same saline conditions, the CAT activity of the sensitive Ege Beyazi-79, Tarm Beyazi-98, and Oguz-2002 varieties was 55.2, 47.1, and 46.2 μmol min^{-1} mg^{-1} FW, respectively (Figure 9). After exposure to 200 mM NaCl, the CAT activity of the Anadolu Pembesi and Altinova-2002 varieties was 205.9 and 155.0 μmol min^{-1} mg^{-1} FW, respectively. While under the same saline conditions, the CAT activity of the Ege Beyazi-79, Tarm Beyazi-98, and Oguz-2002 varieties was 90.3, 81.8, and 71.5 μmol min^{-1} mg^{-1} FW, respectively.

Compared to the control plants, when we observed the GR activity under salt stress, all of the varieties showed an increase. The GR activity reached 552.7 and 443.5 μmol min^{-1} mg^{-1} FW (a 355.1% and 337.4% increase) with 200 mM NaCl, respectively, in the Anadolu Pembesi and Altinova-2002 varieties under salt stress. However, under the same saline conditions, the GR activity of the Ege Beyazi-79 and Oguz-2002 varieties was 170.1 and 196.3 μmol min^{-1} mg^{-1} FW (a 50% and 46% increase), respectively (Figure 10). In a comparison against the control plants, the GR activity was generally increased in the Hungarian vetch varieties under salt stress. Nonetheless, in the sensitive varieties of Hungarian vetch, the increase was significantly less when compared to the tolerant varieties.

The APX activities of the Hungarian vetch varieties under the effect of salt stress increased. With 50 mM NaCl, the APX activity was seen to increase in the tolerant varieties. With 200 mM NaCl, we observed increased APX activity in the Anadolu Pembesi and Altinova-2002 varieties, at 156.1 and 168.4 μmol min^{-1} mg^{-1} FW (a 294.5% and 299.1% increase) under salt stress, respectively. On the other hand, in the Ege Beyazi-79, Oguz-2002, and Budak varieties, the APX activity showed similarities with the control plants with 200 mM NaCl, and the increasing rates with stress were only 89.5%, 77.9%, and 99.1% (Figure 11). Under such adverse conditions and to survive oxidative damage, plants produce antioxidants like ascorbic acid, glutathione, tocopherol, carotenoid, and flavonoids, as well as enzymes like SOD, POX, APOX, CAT, and GR. Conversion of the superoxide anion to H_2O_2 is catalyzed by SOD (Turkan *et al.*, 2005). According to Kachout *et al.* (2012), SOD is a significant superoxide (O_2^-) scavenger in the formation of H_2O_2 and O_2, and it plays a key role in the defense against cellular damage resulting from environmental stress. The different salt concentrations increased the SOD activity in all of the pumpkin genotype calli. The increasing in SOD activity in the Hungarian vetch was more remarkable in the Anadolu Pembesi and Altinova-2002 varieties than in the sensitive ones (Ege Beyazi-79 and Oguz-2002). Similar results were

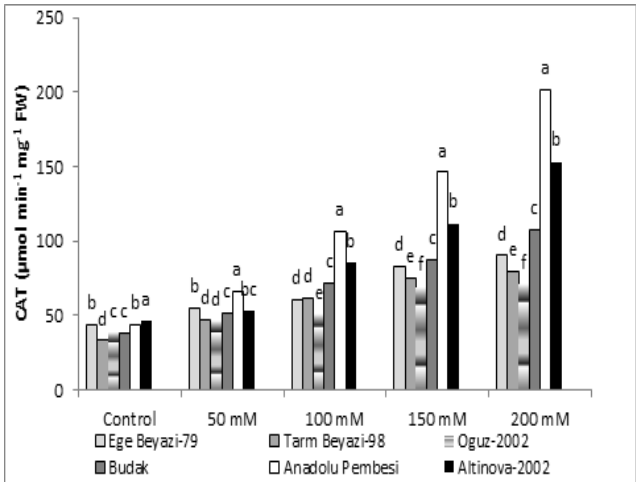

FIG. 9: Changes in the CAT of Hungarian vetch varieties under salt stress.

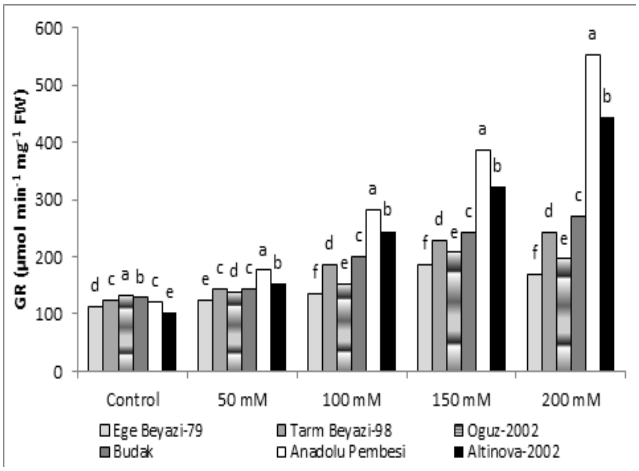

FIG. 10: Changes in the GR of Hungarian vetch varieties under salt stress.

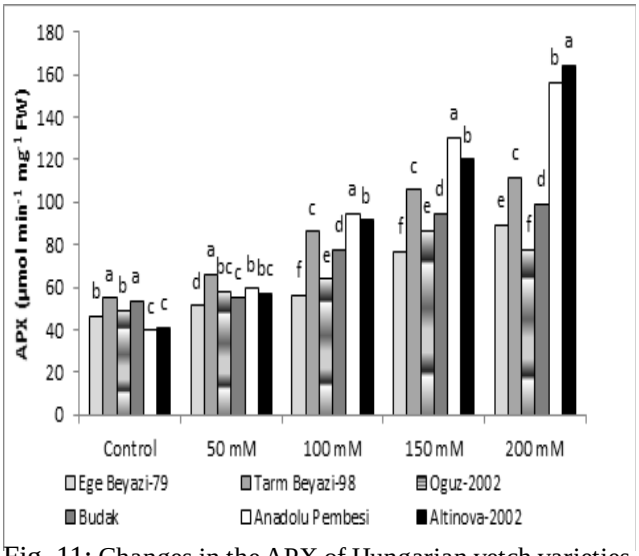

Fig. 11: Changes in the APX of Hungarian vetch varieties under salt stress

reported by Yasar (2003) and Sevengor *et al.* (2011). CAT and SOD activities are considered as the most effective antioxidant enzymes in the prevention of cellular damage (Scandalios, 1993; He *et al.,* 2009). In this study, the increasing salt concentrations were seen to cause an increase in the CAT activity. Moreover, when compared to the sensitive Ege Beyazi-79 and Oguz-2002 varieties, the tolerant ones (Anadolu Pembesi and Altinova-2002) showed higher constitutive levels of CAT. These results indicated that the increased CAT activities observed in the Ege Beyazi-79 and Oguz-2002 varieties might be of benefit in scavenging ROS. Similar results were indicated by Gossett et al. (1994) in cotton under salt stress. APX seemed to be the enzyme that removes the excess of H_2O_2 that is formed (Davenport et al., 2003). In the ascorbate-glutathione cycle, monodehydroascorbate radicals are produced by APX enzymatic activity, and this can spontaneously dismutate or be reduced enzymatically to dehydroascorbate by NADPH-dependent monodehydroascorbate radical reductase. The reduced glutathione then nonenzymatically reduces the dehydroascorbate back to ascorbate or enzymatically reduces it in a dehydroascorbate reductase-mediated reaction. The NADPH-dependent GR then converts the resulting oxidized glutathione back into its reduced form (Hossain *et al.,* 1984; Gosset *et al.,* 1994; Sevengor *et al.,* 2011; Foyer *et al.,* 1991; Polle *et al.,* 1992). In Hungarian vetch, the APX activity increased with 50 mM NaCl and continued to increase with 200 mM NaCl. However, this increase was remarkable in the tolerant varieties. An increase of GR activity enhances the tolerance to oxidative stress (Nunez *et al.,* 2003). The GR activity was more increased in the Anadolu Pembesi and Altinova-2002 varieties under salt stress compared to the sensitive Hungarian vetch varieties (Ege Beyazi-79 and Oguz-2002). This result, supporting the tolerant varieties, may be an effective protection against salt stress. Molina et al., (2002), when comparing salt-adapted cells to unadapted cells, indicated that a higher APX might be related to the adapted cells having a greater ability to decompose H_2O and control the reduced state of the glutathione pool; thus, making it the main adaptation of cell growth in tomatoes under saline conditions.

CONCLUSIONS

The present study on the salt tolerance of Hungarian vetch varieties showed a marked variation in their sensitivity to salt tolerance. The increasing NaCl concentrations caused harmful effects on seed germination in the evaluated properties, such as the shoot and root lengths. The results of this study suggest effective antioxidative defense mechanisms in Hungarian vetch under salt stress. Thus, the increased antioxidative enzyme activity together with the low increase in the MDA content may contribute to salt tolerance. Antioxidative enzyme activities play a protective role against salt-stress, and that antioxidative defense mechanism was effective in providing a tolerance to salt stress in Hungarian vetch varieties. These results suggest that the salt-tolerant varieties exhibit a better protection mechanism against salt stress by maintaining a higher inherited and induced activity of antioxidant enzymes than the sensitive varieties. Hence, the MDA content and antioxidative enzymes activities of the 6 Hungarian vetch varieties investigated in this study were also found as a significance indicator of salt tolerance.

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