Peer-reviewed version available at Biomolecules 2020, 10, 189; doi:10.3390/biom10020189

Article

Sodium Chloride-Induced Salt Stress Responses of Antioxidative Activities in Leaves and Roots of Pistachio Rootstock

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Running Title: Antioxidative response to Salt Stress in Pistachio

Abstract: Salinity substantially affects plant growth and crop productivity worldwide. Plants adopt several biochemical mechanisms including regulation of antioxidant biosynthesis to protect themselves against the toxic effects induced by the stress. One-year-old Pistachio rootstock exhibiting different degrees of salinity tolerance were subjected to sodium chloride induced salt stress to identify genetic diversity among cultivated pistachio rootstock for their antioxidant responses, and to determine the correlation of these enzymes to salinity stress. Leaves and roots were harvested following NaCl-induced stress. Results show that a higher concentration of NaCl treatment induced oxidative stress in the leaf tissue and to a lesser extent in the roots. Both tissues showed an increase in ascorbate peroxidase, superoxide dismutase, catalase, glutathione reductase, peroxidase and malondialdehyde. Responses of antioxidant enzymes were cultivar dependent, as well as temporal and dependent on the salinity level. Linear and quadratic regression model analysis revealed significant correlation of enzyme activities to salinity treatment in both tissues. The variation in salinity tolerance reflected their capabilities in orchestrating antioxidant enzymes at the roots and harmonized across the cell membranes of the leaves. The study provides a better understanding of root and leaf coordination in regulating the antioxidant enzymes to NaCl induced oxidative stress.

Keywords: antioxidant enzymes; lipid peroxidation; NaCl; *Pistacia vera*; rootstock; salinity stress

1. Introduction

Pistachio (*Pistacia vera* L. Anacardiaceae) is a widely cultivated and important tree nut crop. These nuts provide rich sources of health promoting nutrients such as proteins, phenols, antioxidants, and minerals[1]. The crop is grown in semi-arid regions where high soil salinity has adversely affected the pistachio cultivation and production. Soil salinization is a serious menace for crop cultivation, productivity, and has devastating global effects, estimated at 50 % of land loss by 2050, affecting the crop sustainability and food security[2]. Salinity affects the crop productivity through various physiological changes resulting in osmotic stress by making it harder for roots to absorb water, causing internal dehydration, and ion toxicity caused by the direct accumulation of salts. The ionic imbalance and hyperosmotic stress resulting from salinity manifest itself as oxidative stress and salinity-induced accumulation of Na, which competes with K ions, causing inhibition of metabolic enzymes[3,4]. The cascade of oxidative reactions causes the inactivation of enzymes and protein

degradation. These reactions damage the cell membranes, photosynthetic pigments, protein, nucleic acids, and lipids. Thus, it is very critical for plants to regulate the ROS levels in the cells[5]

Several studies suggest that the activity of antioxidant enzymes is correlated with plant tolerance to salinity[6]. In general, plants are fortified with different classes of protective and restitution systems to minimize the adverse effects of oxidative damage. The first order class enzymes are the scavenging ROS enzymes such as ascorbate peroxidase (APX), peroxidases (POX), catalase (CAT), superoxide dismutase (SOD), and the lipid peroxidation detoxification products (MDA). Additionally, a class of low molecular mass antioxidants, which regenerate the oxidized antioxidants and turn them into their active forms, namely, ascorbate, glutathione and glutathione reductase (GR), are associated with the protective mechanism[7]. Briefly, SOD acts as the first line antioxidant systems of plants, catalyzing the dismutation of the O_2^- to O_2 and H_2O_2 thus causing cell damage[8]. POXs have been commonly used as a marker in physiological studies and their activity level is used as an index for evaluating oxidative stress. POX is well acknowledged as 'stress enzyme' in plants, oxidizing numerous substrates using H_2O_2 and hampering the excess agglomeration of H_2O_2, generated by normal metabolism or stress conditions[9].

POXs catalyze the oxido-reduction between H_2O_2 and various reductants and enhance the plant tolerance to salinity[10]. Catalases, commonly located in peroxisomes, catalyze H_2O_2 to water and oxygen and are involved in photorespiration[1] . The most important H_2O_2 detoxification pathway is the ascorbate-glutathione system involving APX and GR enzymes, occurring in chloroplasts, cytosol, mitochondria, and peroxisomes. APX uses two molecules of ascorbate as a specific electron donor for reducing H_2O_2 to water[12]. Subsequently the oxidized ascorbate is regenerated by mono-dehydro ascorbate reductase and/or dehydro ascorbate reductase using reduced glutathione (GSH), generating glutathione disulfide (GSSG), which in turn, is then reduced into two molecules of glutathione with the aid of NADPH as the electron donor catalyzed by the enzyme GR[13]. Furthermore, ROS are able to affect the antioxidative enzyme activities leading to lipid peroxidation and MDA formation. The content of MDA, the main byproduct of lipid peroxidation, has commonly been considered as a biomarker of cell membrane damage in plants[14]

Thus, the relatively quantitative changes of these enzymes and their correlation analyses play a major role in determining the plant tolerance to the salinity stress, suggesting they can be applied as biochemical markers to select a more tolerant plant or to investigate the level of salinity tolerance in plants[15]. Very few studies are available on differences in oxidative stress and antioxidative defenses in different organs and prolonged periods of stress. *Pistachio*s are relatively better adapted to salinity compared to most tree nut crops[16]. Therefore, evaluating genetic diversity among pistachio germplasm for their salt tolerance will bestow a better understanding on salt tolerance in pistachio genotypes especially rootstock for introducing potential traits in the breeding program[17]. Correlating studies of the changes in these enzymes in different cultivars are necessary to determine the biochemical pathways regulating under various degrees of salt stress in pistachio. Hence, the present study was aimed to: 1) investigate the tissue-specific responses of the antioxidant enzymes associated with salinity in the leaves and roots 2) elucidate biochemical modification mechanisms for salt tolerance, and 3) establish suitable correlation and regression models to explain variability in salt tolerance among various rootstock. To our knowledge, this is the first report of the genetic variation studies on temporal antioxidant responses in root and leaf pistachio rootstock in response to different NaCl induced saline treatment.

2. Results

2.1. The Effect of salinity on plant growth and morphology:

After 100 days of salt treatments at 8dS/m concentration, rootstock did not show any symptoms. Whereas at 12dS/m NaCl, leaf tip necrosis was observed in Akbari and KG rootstock, and up to 50% of leaves showed senescence symptoms and desiccation at 16 dS/m NaCl treatment. Rootstock Ghazvini, followed by Badami and UCB-1 developed leaf tip necrosis at 16dS/m. We have further investigated the enzyme activity (SOD, POD, CAT, APX, and GR) and MDA content of five pistachio

rootstock in leaf tissues at different salinity levels, and measured at different time courses (25-100 days), while for roots the activities were measured at 100-day treatment and compared with that in leaf tissues. Results of these enzyme activities and the MDA content are as shown below:

2.2. Antioxidant enzyme activity:

The SOD activity was higher in leaves than in the roots under both normal and saline conditions. Under control conditions, the activity of SOD in leaf remained constant over time under control conditions, with the highest being in Ghazvini rootstock followed by Badami, Akbari, KG and UCB-1 (Figure 1). Leaf tissue displayed significantly higher SOD activity relative to NaCl treatments in all rootstock. NaCl at 8dS/m induced SOD activity in all cultivars. At 12dS/m it had significantly elevated SOD levels in rootstock UCB-1 (2.8 fold) followed by Badami, Ghazvini, KG, and Akbari in decreasing order. At 16dS/m NaCl concentration, increased SOD activity was noticed until 50 days, thereafter gradually declined in all rootstock thereafter, with the highest activity being in UCB-1, followed by Badami, Ghazvini, Akbari, and KG. In leaf tissue, the highest SOD activity was 209 in 12 dS/m in UCB-1, which decreased to 135.8 in 16 dS/m. In root tissue, the enzyme activity increased in salt treated samples, and at day 100 the maximum amount of SOD was observed in UCB-1 at 12dS/m (91.1), then decreased at 16dS/m (59.07) (Figure 2).

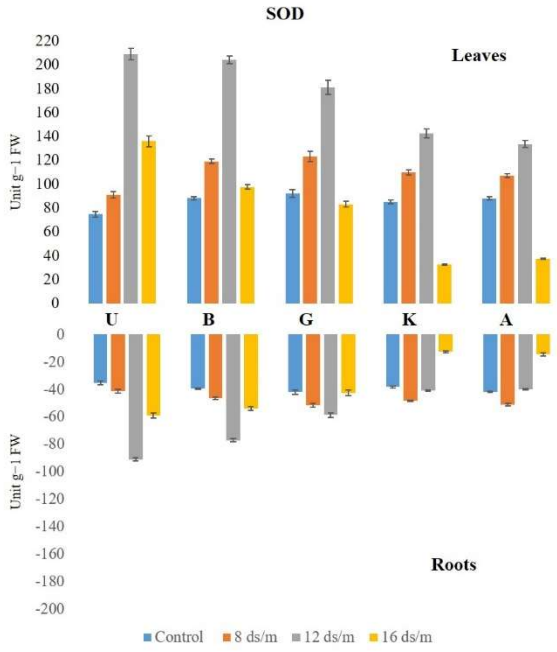

Figure 1. Effect NaCl-salinity on SOD activity in leaves of pistachio rootstock.

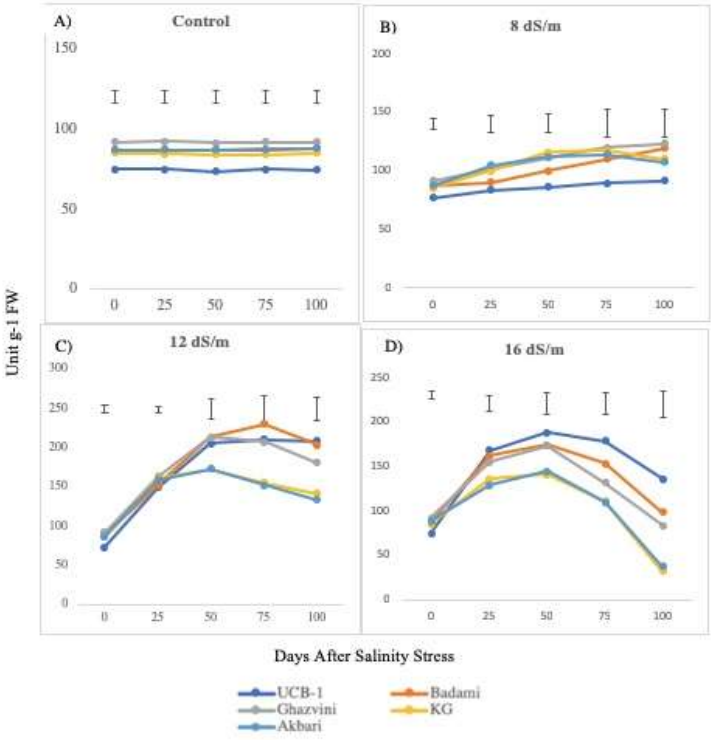

Figure 2. Effect NaCl-salinity at 100-day treatment on SOD activity in leaves and roots of pistachio rootstock.

The POD levels were higher in control leaf tissues than in root tissues and displayed significant elevations in both leaves and roots in response to NaCl treatments (Figure 3). Under control conditions, the POD activity levels remained unchanged in all rootstock under control conditions in both tissues in the descending order of UCB-1, followed by KG, Akbari, Badami, and Ghazvini. At 8dS/m NaCl concentration, all cultivars showed increased POD activity through 100 days in leaf tissue. At 12dS/m, the activity increased up to 75 days in all cultivars, except in Akbari and KG, where a decline occurred at day 50. Under 16dS/m NaCl treatment, the decline in enzyme activity started at day 50 in KG and Akbari, while in UCB-1, Ghazvini, and Badami, the activity declined at day 75. Among the rootstock, UCB-1 maintained higher enzymatic activity (1.4 fold) at 100 day. The maximum POD level in the root was observed in rootstock UCB-1 (1.5 fold), at 16dS/m salinity treatment, compared to the control (Figure 4). For POD, in both tissues, UCB-1 showed the highest activity and Akbari the lowest at 100 days. However, in the controls, UCB-1 showed the lowest values in both tissues, and during stress, they increased to the highest POD values in both tissues.

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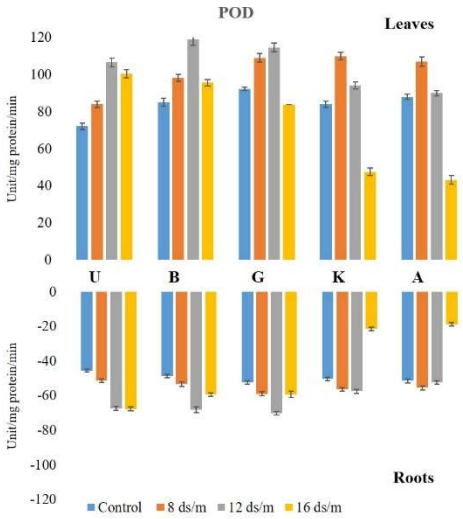

Figure 3. Effect NaCl-salinity on POD activity in leaves of pistachio rootstock.

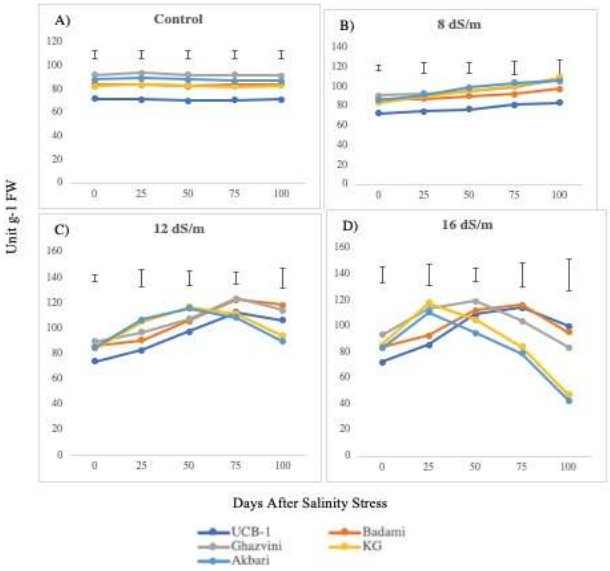

Figure 4. Effect NaCl-salinity at 100-day treatment on POD activity in leaves and roots of pistachio rootstock.

The activity of CAT was higher in leaf than in the root under control conditions in all rootstock (Figure 5). No significant change in CAT activity was observed in control leaf tissues over time, with Akbari being the highest, followed by KG, Badami, Ghazvini, and UCB-1. At both 8dS/m and 12dS/m, the activities increased at day 25 and relatively maintained same levels furthermore. However, at 16dS/m, the activity although increased at day 25, showed a gradual decline in all rootstock thereafter. The maximum level of CAT was observed in the leaves of rootstock UCB-1 at 25 days under 16dS/m saline treatment (5 fold), which had decreased further over the treatment time. In root tissue, maximum CAT enzyme amount was observed in Badami at NaCl 8dS/m (12.61), which further decreased at 12dS/m and 16dS/m. At 16dS/m, the maximum CAT level was observed in rootstock UCB-1 (9.29), followed by Badami, Ghazvini, KG, and Akbari (Figure 6).

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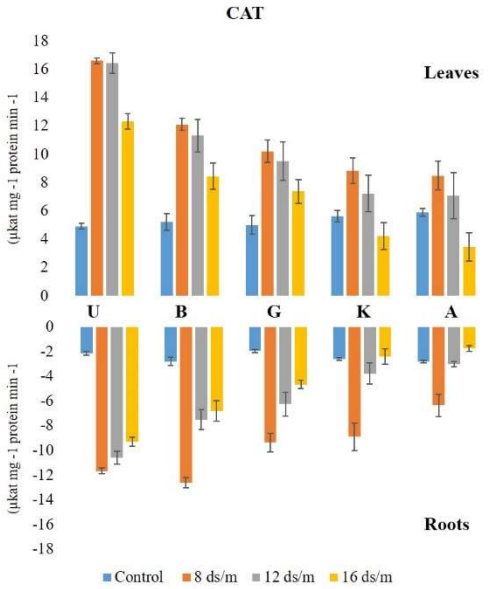

Figure 5. Effect NaCl-salinity on CAT activity in leaves of pistachio rootstock.

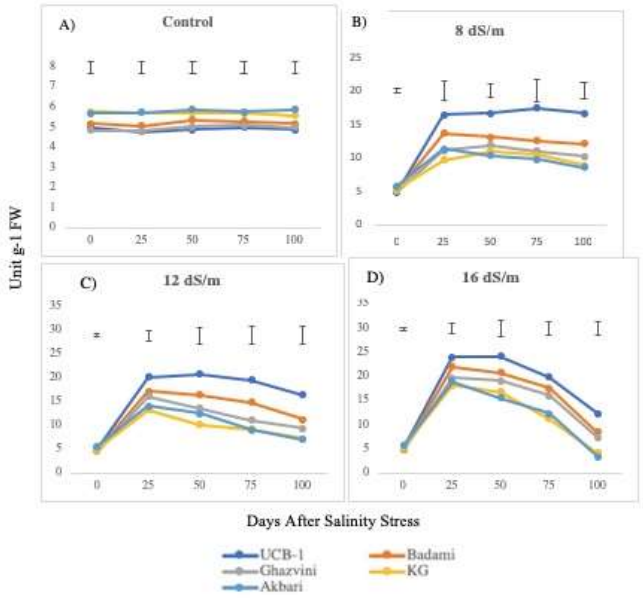

Figure 6. Effect NaCl-salinity at 100-day treatment on CAT activity in leaves and roots of pistachio rootstock.

The activity of APX was higher in leaves than in roots, both in controls and in saline conditions (Figure 7). The APX activity in the leaves under the control condition was in the following decreasing order; Ghazvini followed by UCB-1, Badami, Akbari, and KG, remaining relatively unchanged over 100 days, and then significantly increased due to salinity. At 8dS/m, 12dS/m, and 16dS/m NaCl treatments, all leaf tissues in cultivars showed a significant increase in APX activity up to 50 days and later declined gradually with UCB-1 being the highest, followed by Badami, Gahzvini, KG, and Akbari. In root tissues, APX exhibited a significant increase to salinity at 8dS/m, 12dS/m, and 16dS/m in all the cultivars except in KG and Akbari. At 16dS/m, UCB-1 was the highest, followed by Badami, Ghazvini, KG, and Akbari (Figure 8).

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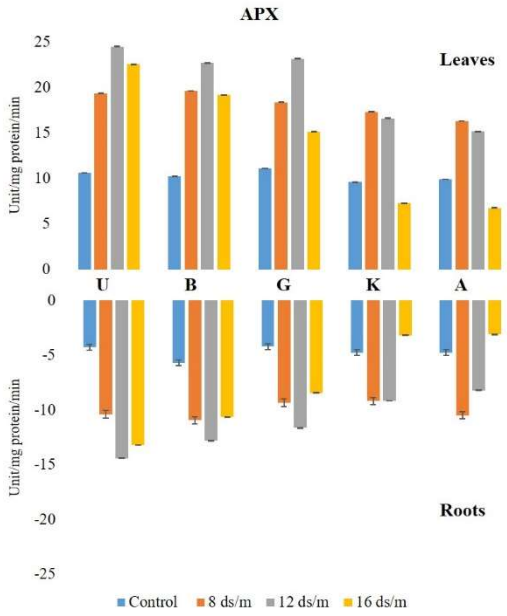

158

159 **Figure 7.** Effect of NaCl concentration on APX activity in pistachio rootstock.

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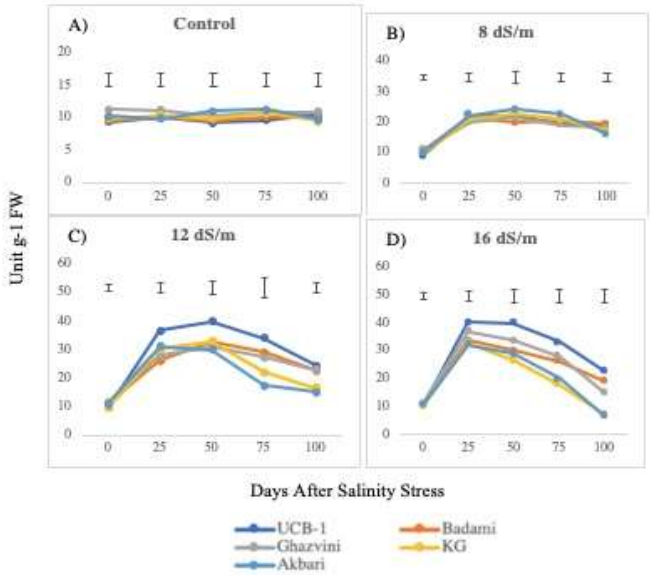

161

162 **Figure 8.** Effect NaCl-salinity at 100-day treatment on APX activity in leaves and roots of pistachio
163 rootstock.

164 Glutathione reductase (GR) activity exhibited significant changes in leaf and root tissues (Figure
165 9). GR activity was relatively low in leaves compared to roots, under control conditions in the
166 following decreasing order: Akbari followed by Ghazvini, UCB-1, KG, and Badami. At low
167 concentrations of salt (8dS/m) the GR levels elevated in all cultivars in both tissues. At moderate and
168 high salt stress (12dS/m and 16dS/m), the GR activity showed mixed responses among the cultivars.
169 For example, UCB-1 showed a linear increase in GR level at NaCl 12dS/m and reduced at 16dS/m in
170 leaf tissue, while all other rootstock showed a gradual decrease in GR activity in both tissues. Under
171 the 16dS/m saline treatment, the maximum increase in the amount of GR was observed in the UCB-
172 1 cultivar (1.9-fold in leaves; 2.2-fold in roots), followed by rootstock Badami, Ghazvini, KG, and
173 Akbari in decreasing order (Figure 10).

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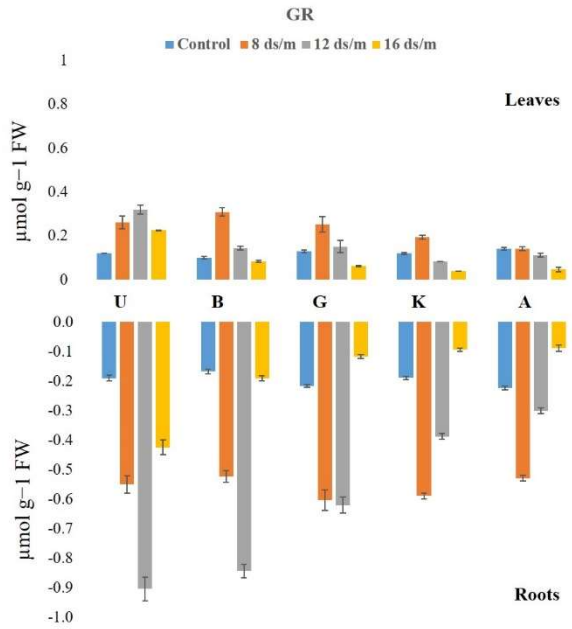

Figure 9. Effect of NaCl concentration on GR activity in pistachio rootstock.

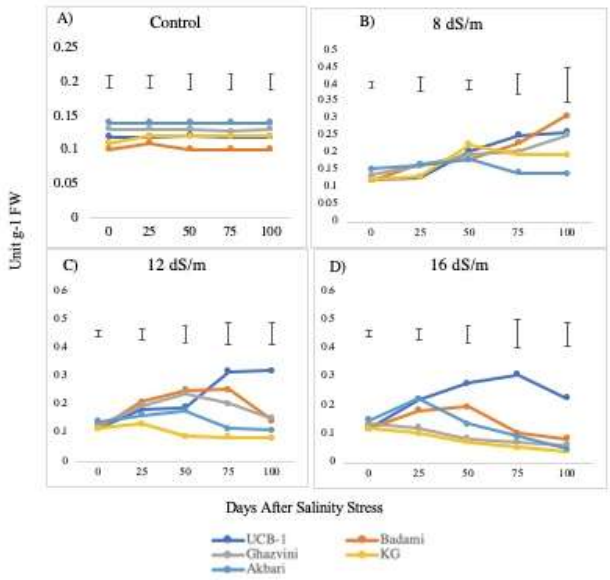

Figure 10. Effect NaCl-salinity at 100-day treatment on GR activity in leaves and roots of pistachio rootstock.

2.3. Lipid peroxidation:

Lipid peroxidation levels, measured from MDA concentration, were higher in leaves than in roots under control conditions (Figure 11). The MDA levels increased linearly from low to high levels of NaCl in all rootstock, generally being higher in leaf compared to root tissues. In leaf tissue, the level of MDA measured after 100-day treatment with NaCl 16dS/m was highest in Akbari in both the tissues, (leaf a 4.1-fold; roots a 4.5-fold) followed by KG, Ghazvini, Badami, and UCB-1 (Figure 12). A similar trend of MDA activity was observed in roots to NaCl treatments 8, 12, and 16 dS/m, with the maximum being in rootstock Akbari followed by KG, Ghazvini, Badami, and UCB-1.

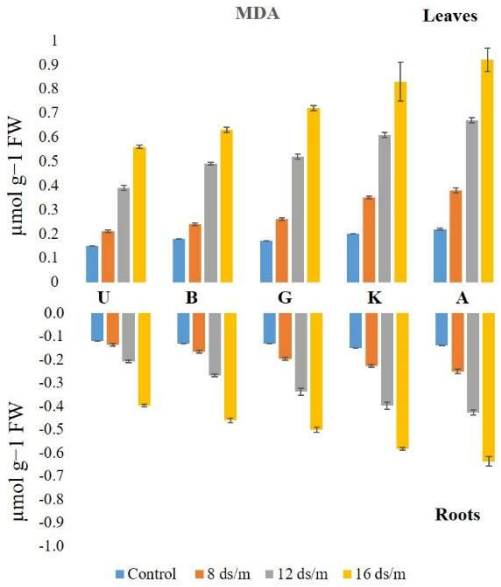

Figure 11. Effect of NaCl concentration on MDA activity in pistachio rootstock leaves at different time intervals.

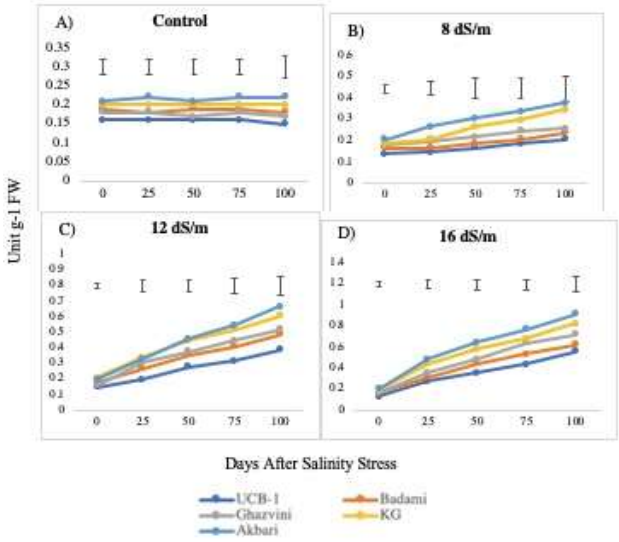

Figure 12. Effect NaCl-salinity at 100-day treatment on MDA activity in leaves and roots of pistachio rootstock.

2.4. Correlation analysis:

Four different correlation coefficients between the measured characters were investigated in leaves, roots, leaves with roots and roots with leaves were calculated (Table 1a).

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Table 1. Correlation analysis of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), malondialdehyde (MDA), ascorbate peroxidase (APX), glutathione reductase (GR) content in the leaves and roots of five pistachio rootstocks.

	Leaf						**Root**					
Leaf	APX	CAT	POD	SOD	MDA	GR	APX	CAT	POD	SOD	MDA	GR
APX	1.00						0.99	0.98	0.83	0.97	-0.99	0.99
CAT	0.56	1.00					0.51	0.55	0.35	0.45	-0.56	0.58
POD	0.96	0.34	1.00				0.97	0.94	0.86	0.98	-0.95	0.94
SOD	0.85	0.28	0.91	1.00			0.88	0.81	0.96	0.93	-0.84	0.81
MDA	-0.99	-0.55	-0.95	-0.85	1.00		-0.96	-0.96	-0.86	-0.95	1	-0.99
GR	0.95	0.65	0.88	0.70	-0.94	1.00	0.91	0.90	0.72	0.89	-0.94	0.96
Root												
APX							1.00					
CAT							0.97	1.00				
POD							0.83	0.79	1.00			
SOD							0.98	0.93	0.88	1.00		
MDA							-0.98	-0.97	-0.83	-0.95	1.00	
GR							0.96	0.96	0.80	0.94	-0.99	1.00

The values more than 0.44 and 0.59 are significant at 0.05 and 0.01, respectively.

All measured characters had a significant correlation with each other in leaves with roots and roots with leaves (except CAT in leaf with POD in root). The correlation coefficient of MDA content between leaves and roots was 1. The MDA content in leaves was negatively correlated with all the other enzymes in leaves, and its correlation coefficients reached up to -0.99 ($P<0.01$) with APX. A similar result was found in the root, as the MDA was negatively correlated with other studied characters in both roots and leaves. All correlation coefficients of the MDA contents in leaves were negatively correlated with the others in the roots. The maximum and minimum correlation coefficients between the enzymes in leaves were found between APX and MDA (-.99), and CAT and SOD (.29) respectively. The highest positive correlation coefficient in root enzymes was observed between APX and SOD (0.98), whereas the highest negative correlation coefficient was observed between MDA and GR (-0.99). According to the results, in both root and leaf the SOD, POD and APX were significant enzymes involved in salinity tolerance. Data was analyzed as an unbalanced complete randomized design (CRD) between the rootstock of two different groups of rootstock, tolerant: UCB-1, Badami, Ghazvini, and susceptible: Akbari and KG that showed SOD, POD and APX were the most effective indices, respectively (Table 1b).

Table 1. Unbalanced completely randomized design between tolerant (UCB-1, Badami, Ghazvini) and susceptible (Akbari and Kale-Ghouchi) rootstocks.

Source of variation	**df**	**Mean square (Leaf)**						**Mean square (Root)**					
		APX	CAT	POD	SOD	MDA	GR	APX	CAT	POD	SOD	MDA	GR
Group	1	64.7*	70	823.6*	1127**	0.02*	0.007	11.71*	12.19	205**	358.4*	0.01*	0.025
Error	3	5.49	60	36.8	9.69	0.001	0.002	0.84	1.54	2.08	11.69	0.001	0.003

* and ** are 0.05 and 0.01, respectively

2.5. Model analysis:

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The linear and quadratic regression models for the enzyme activities of *Pistachio* rootstock, in both leaves and roots, were evaluated. The enzymes, APX, CAT, POD, and MDA had a linear regression model in leaves and the enzymes; SOD and GR had a quadratic regression model with the salinity levels (Figure 13). In the present study, 9 of the 12 models had a high R-square. The three leaf enzymes, namely APX, CAT, and POD, had a linear regression model, whereas the root had a quadratic regression model in roots. Among these models, CAT in leaves had the highest R-square (97%). Therefore, by increasing the salinity level, the concentration of the APX, CAT and POD, enzymes in the leaves, as well as MDA in both leaves and roots increased. The SOD and GR enzymes had quadratic regression models in both roots and leaves. In all of the models, GR in leaves had the highest R-square. It could explain more than 99% of the variance of the data, whereas the model of SOD in root just explained about 55% of the variation. The MDA had a linear regression model with a high coefficient of determination in both leaves and roots.

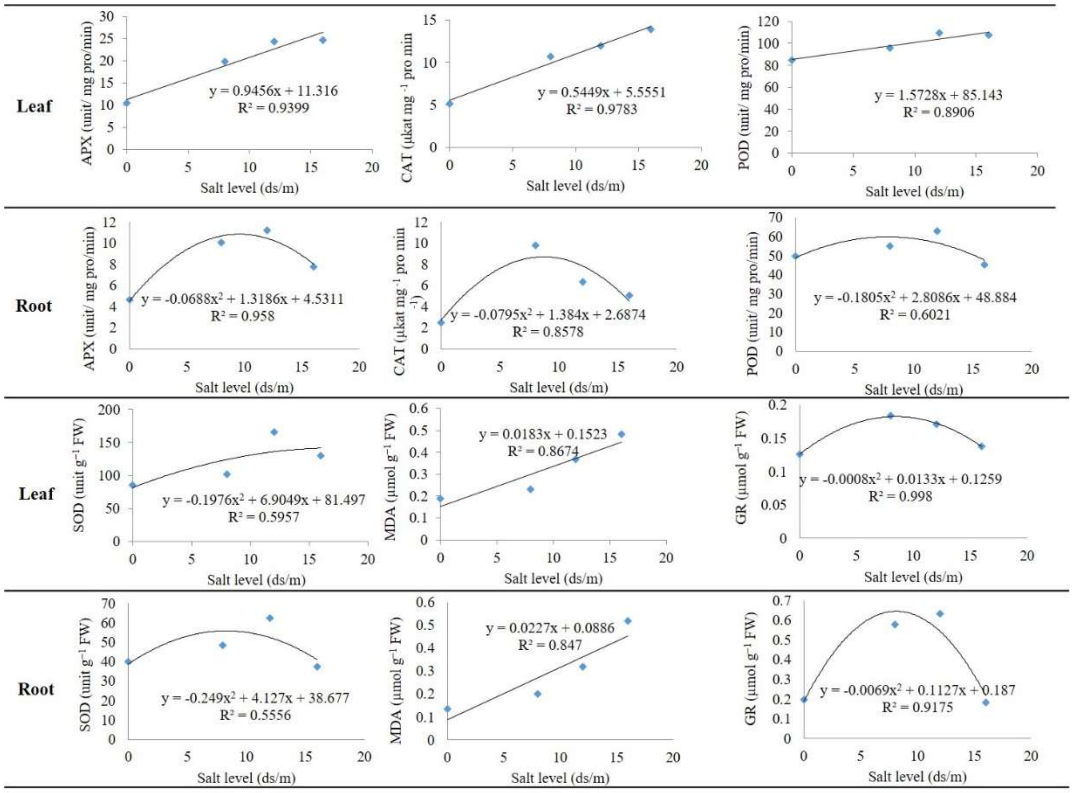

Figure 13. Linear and quadratic models of SOD, POD, CAT, APX, GRT and MDA indices in leaves and roots of pistachio rootstock.

3. Discussion

Salt stress results in the accumulation of ROS due to changes in the electron transport chain and induces protection mechanisms during salt stress[18]. Higher salinity soil conditions reportedly induce oxidative stress in plant organelles[19]. On the other hand, overexpression of antioxidant enzymes is associated with improvement in salt tolerance[20].

3.1. Quantitative variation among the antioxidative enzymes are tissue specific and depends on both duration and levels of NaCl-induced salt stress:

In our studies under normal irrigation conditions, antioxidative enzyme levels of SOD, POD, CAT and APX and lipid peroxidase activity were maintained higher in leaf tissues than in roots in all rootstock. On the contrary, GR showed lower levels in leaf compared to root tissues. Low (8dS/m) and moderate (12dS/m) salt stress elevated SOD, POD and CAT levels up to 100-day stress period in

leaf tissues, while, at higher salt levels (16dS/m), their activity was maintained for 75-days stress treatment, beyond that, it declined in all rootstock. However, APX activity was elevated only up to 50-days stress duration. Both in root and leaf tissues, these enzyme levels were elevated at 100-day stress duration low and moderate stress level, while the levels declined at higher salinity stress. Salt tolerant pistachio rootstock showed lower levels of enzyme activities compared to their levels in susceptible cultivars under normal growth conditions. However, upon the stress, tolerant rootstock exhibited higher levels over the susceptible tissues.

One of the enzymes, SOD, plays a major role in ROS scavenging in plants and is considered as the first line of defense against the toxic effects of elevated ROS levels[21,22]. SOD catalyzes the dismutation of superoxide radicals to H_2O_2 and O_2. The increase of SOD activity might be the reason for enhanced O_2 generation, as a result of electron leakage from the electron transport chains to molecular oxygen[23]. Similarly, POD activity was significantly altered in both tissues after salt treatments. POD levels were higher in control leaf tissues than in root tissues, produced by POD, CAT, APX, and other antioxidant enzymes. SOD levels were higher in control leaf tissues compared with root tissues, and its levels were significantly higher in both leaves and roots to salinity treatments. No significant difference was observed between control and $8dSm^{-1}$ in the leaves, but a significant difference was observed in the roots at a low salt concentration ($8dSm^{-1}$) and also at increasing salinity levels of $12dSm^{-1}$ and $16dSm^{-1}$.

The difference was significant in the roots compared to the increased SOD in leaves, suggesting SOD activity in roots was insensitive to low salinity and seems to be organ specific. By increasing salinity to $12dsm^{-1}$ and $16dsm^{-1}$, the rootstock of UCB-1, Badami, and Ghazvini exhibited higher SOD activity to overcome the oxidative stress compared to KG and Akbari rootstock in both tissues. Our previous studies on osmoregulation showed that the rootstock UCB-1, Badami, and Ghazvini are relatively salt tolerant compared to KG and Akbari[24]. Although SOD levels were low in tolerant rootstock, their increase in enzymatic activities was higher in response to salinity stress in relatively tolerant rootstock, suggesting their O_2- scavenging ability. A similar increase in SOD activity in tolerant genotypes has been observed in cotton in response to saline stress[25].

POD is the primary enzyme and the increase in its activity detoxifies H_2O_2 in chloroplast and cytosol during oxidative stress[26,27]. POD levels have been increased in tolerant rootstock (UCB-1 and Ghazvani) to high saline stress in both tissues, while the activity decreased in less tolerant rootstock (Badami, KG, and Akbari). POD activity has been reported to be high in salt tolerant cotton cultivar under salt stress, while the enzyme activity remained constant compared to the control[28]. Low levels of salinity (8dS/m, and 12dS/m) for prolonged periods induced higher activities of SOD and POD in the leaf tissue, while the higher salt concentration increased the enzyme activity for 25-50 days, reducing significantly thereafter. Rootstock UCB-1 maintained a higher enzymatic activity in both tissues (1.4-fold in leaf and 1.5-fold in root) during stress over 100 days of treatment compared to other cultivars in both tissues at 16dS/m salinity treatment (see Supplementary Figure 2 and 4). It is noteworthy that in both tissues, the POD activities were similar to the SOD in the rootstock in the following order: UCB-1 followed by Badami, Ghazvini, KG, and Akbari. POD activity in both tissues was the highest in UCB-1, and the lowest in Akbari, while the opposite results were seen in their controls.

CAT plays an important role in the antioxidant system. Both POD and CAT constitute a main H_2O_2 scavenging system in the cells[29]. The present data showed that CAT activity was higher in the leaves than that in the roots under both saline and control conditions, however, the rate of increase in enzyme activity to salt stress is relatively high in roots (3-4 times) compared to that in leaf tissues among all the rootstock. Compared to the controls, the activity of CAT in the roots had increased more than that in the leaves. Although the control CAT activity in leaf and root was relatively higher in Akbari compared to UCB-1, at 16dS/m, UCB-1 showed highest CAT activity while the Akbari performed the lowest among all the rootstock. CAT is involved in salinity stress and increases the resistance of plants to saline conditions[19,30]. The CAT activity was significantly increased in response to salinity.

Furthermore, the CAT activity against salt-induced oxidative stress appears to be organ specific, as well as cultivar and the duration of stress duration dependent. Increased CAT activity was recorded in leaf tissues of tolerant rootstock (UCB-1, Badami, and Ghazvini) up to 100 days at high NaCl stress. However, in susceptible rootstock (Akbari and KG), the amount of CAT decreased, when compared to the control. Similar findings have been reported that CAT activity has been increased gradually by increasing the salt treatments in the salt tolerant maize cultivars and reduced significantly in the salt-sensitive ones[31]. Concomitant with the results obtained here, salt-tolerant rice genotypes had shown significantly higher CAT activity compared with that of susceptible genotypes [19]. At high salinity, the APX activity was elevated in the tolerant rootstock of UCB-1, Ghazvini, and Badami in leaves, while it was decreased in less tolerant rootstock, Akbari and KG, suggesting a stronger correlation of APX activity to salt tolerance. Higher APX activity has been observed in salt tolerant genotypes compared to their salt sensitive counterparts, in different tissues[12,32]. Genetic engineering of plum plants by cytosolic APX gene enhanced the tolerance to salt stress in *in vitro* plum plants[33]. Accordingly, overexpression of cytosolic APX in tomato has been reported to confer tolerance to salt stress[34].

GR, belonging to the family of NADPH-dependent oxidoreductase, plays a key role in cell defense against ROS through maintaining the reduced GSH pool at a cellular level by catalyzing the reduction of GSSG (oxidized glutathione) to GSH. In this study, the GR activity was higher in the roots than that in the leaves under both saline and normal growth conditions. Salinity stress significantly enhanced GR activity in both leaves and roots, indicating its role in scavenging H_2O_2 formed as a result of increasing SOD activity under salinity stress. After 100 days of salinity stress, the salt tolerant rootstock, UCB-1, showed a remarkable increase in GR enzyme in root tissue. It has been reported that the salt-induced increase in GR activity is higher in salt-tolerant rice genotypes compared to salt-sensitive ones[35].

3.2:. Lipid peroxidation activity in relation to antioxidant enzymes among rootstock:

MDA is the final product of lipid peroxidation that has attracted widespread interest as an indicator of damage to the cell membrane system. High levels of ROS in the cells cause membrane lipid peroxidation. MDA content was increased due to salinity stress (at 16dSm^{-1}) in leaf and root tissues of the rootstock. In comparison with the control group, the MDA increments in the leaves of pistachio rootstock were higher than those in the roots, indicating that the leaves were more affected by the salinity induced oxidative damage, most likely due to the increased activity of enzymatic and non-enzymatic pathways detoxifying ROS. Similarly, in lentil, the shoot and root responses to salinity stress indicated that roots are less affected by the salinity-induced oxidative stress [36]. In another report, lipid peroxidation has been only observed in the roots of *Crithmum maritimum* L. under high salinity, while the leaves were not seriously influenced by salinity stress[37]. In the present study, susceptible rootstock Akbari had the maximum MDA content in the leaves and roots and the minimum MDA content in leaves and roots was observed in tolerant rootstock UCB-1 exposed to high stress. Unaffected MDA content suggests reduced membrane damage, which indicates the superior ability of UCB-1 over the other studied rootstock to cope with the saline conditions. The relationship between MDA content and enzyme activity shows that at 75-day higher NaCl treatment (16dSm^{-1}), the activity would increase in all rootstock. This is likely due to H_2O_2 accumulation, which causes lipid peroxidation and it could be seen an increase in MDA concentration. This increase in MDA damaged the membrane of cells causing up to a 50 percent leaf loss in Akbari and KG.

3.3:. Correlation between H_2O_2 activity and antioxidant responses:

A positive correlation was observed between SOD and CAT in the roots but the SOD and CAT activity was not correlated in the leaves. This is while the SOD was positively correlated with APX activity in both leaves and roots (Table 1a). It has been suggested that both CAT and APX, which are responsible for detoxification of H_2O_2, are equally important[38] herein, although the CAT activity is significantly increased, APX is more activated than CAT in detoxification of H_2O_2 in our study. It has been reported that in the tomato, CAT activity is not associated with significant changes in the leaves,

while significant changes have been observed in the roots[39]. Similar results have been observed in CAT with decreased activity under stress situations as compared with the control in the leaf tissue of cowpea[40]. The activities of CAT and APX enzymes were changed depending on the time of salt treatment and salt concentration and was highly correlated in the leaves (0.97) and roots (0.99), suggesting that both of these enzymes are essential for detoxification of H_2O_2 produced by SOD activity and photorespiration.

Parallel enhancements of CAT and APX activities also have been expressed in *Centauries tuzgoluensis* under 150 mM NaCl stress treatments but at 300 mM NaCl it was reported that CAT activity was increased, while APX activity remained unchanged. This suggests that CAT might be more important than APX in scavenging H_2O_2 during salinity stress[23]. The enzymes, SOD, POD, CAT, and APX are important factors and suggest that they are correspondingly organized in relation to each other under saline environments (Table 1b). The results showed that the models were significant at a 95% confidence limit and can be used to closely predict the values of the indices in the roots, as well as in the leaves of pistachio. Further studies on the localization of the models may require more validation nevertheless but they were statistically significant in this study. The activity of enzymes and MDA content in the leaves of pistachio rootstock were positively correlated with those in the roots except between CAT and POD. Also, all the measured values in both leaves and roots were significantly correlated to the saline water treatment regimes. Thus, it shows that the antioxidative responses of pistachio rootstock to salinity in both leaves and roots are cultivar dependent and have a relative adjustment mechanism.

3.4:. Genetic variation in antioxidant activity among rootstock to salt stress:

In this study, a significant correlation between the APX and GR activity in both leaves (0.95) and roots (0.96) were observed among the rootstock. Since the APX and GR activities have been increased under salt stress, it is likely that the oxidative stress was efficiently reduced by the activity of the ascorbate–glutathione cycle spatially in the tolerant rootstock (UCB-1) followed by Badami and Ghazvini. In addition, the activity of GR in the roots, which is positively correlated with the other enzymes, suggests that non-enzymatic routes are as important as enzymatic routes for controlling the oxidative stress caused by salinity in pistachio rootstock. H_2O_2 is a toxic compound, which is produced due to significant changes in SOD activity[37]. Thus, GR content was significantly higher after NaCl treatment and was negatively correlated with SOD activity in roots (Figure 1 & Figure 5.

4. Materials and methods

4.1. Plant material, growth conditions:

Four Iranian pistachio (*Pistacia vera* L.) rootstock (namely: Badami, Ghazvini, Akbari, and Kale-Ghouchi) and UCB-1, a hybrid (*P. atlantica* x *P. integerrima*) which has been extensively cultivated, were used in our studies (from this point on, Kale-Ghouchi will be abbreviated as KG). One-year-old uniform rootstock were prepared and transplanted to the 8L greenhouse pots filled with sieved 2-mm sandy-loam soil (pH 7.86, EC 0.71 dSm^{-1}, 0.84 % organic and15 % field capacity, available N, P, and K of 99.3, 29.6, and 289.4 mg kg^{-1} respectively).

4.2. Salinity treatment and sample Collection:

After eight weeks of pre-culture, irrigation with four different concentrations of saline water (0.5L per pot once in three days), with electrical conductivities (EC) including a control (0.42dSm^{-1}), 8dSm^{-1}, 12 dSm^{-1}, and 16dSm^{-1} were applied for 100 days. Salinity treatments were applied gradually to avoid osmotic shock. At three irrigations intervals, 200 mL of deionized water was applied to prevent salt accumulation. Greenhouse conditions were: temperature regime of 30/17°C (day/night), with an average relative humidity of 75% and 14/10h light/dark period at a photosynthetic photon flux density of about 400-500 µmol m^{-2} s^{-1}. On days 0, 25, 50, 75 and 100 after the beginning of the

salinity treatments, leaf samples were collected from each rootstock. Root samples were collected at day 100 after harvest and stored at −80°C for further biochemical analysis. The MDA and antioxidant enzyme activity of SOD, POD, CAT, APX, and GR of both leaf and root samples were measured as described below.

4.3. Determination of enzyme activity:

To determine the enzyme activity, both leaf and root samples were entirely milled using a cold mortar and pestle. One-gram sample was homogenized in 5mL of 50mM sodium phosphate buffer (pH 7.8) and centrifuged at 13,000g for 20 min at 4°C. The supernatant was used to measure the enzyme activities.

4.3.1. SOD activity:

To determine the SOD activity, 3 mL reaction solution containing 13mM methionine, 63mM r-nitro blue tetrazolium chloride, 1.3 mM riboflavin, 50mM phosphate buffer, and 50 mL of the enzyme extract[41] . The reaction mixture was incubated for 10 min and the absorbance was recorded at 560 nm. One unit of SOD activity corresponds to the amount of enzyme required for the inhibition of photochemical reduction of *p*-nitro blue tetrazolium chloride reduction by 50%.

4.3.2. POD activity:

One gram of each leaf and root sample was separately milled in 5 mL of assay buffer. The homogenates were centrifuged at 12,000 g for 30 min at 4 °C [42]. Five mL of the assay buffer for the peroxidase activity contained: 125 µM of phosphate buffer, 50 µM of pyrogallol, 50 mM of H_2O_2, pH 6.8, and one mL of the 20 times-diluted enzyme extract. This was incubated for five min at 25 °C and subsequently, the reaction was stopped by adding 0.5 mL of 5% (v/v) H_2SO_4. The amount of purpurogallin was determined by measuring the absorbance at 420 nm.

4.3.3. CAT activity

To determine the CAT activity, the 3 mL reaction solution contained 15 mM H_2O_2, 50 mM phosphate buffer (pH 7.0), and 50 mL of the enzyme extract[43]. The reaction was initiated by the addition of the 100 µL enzyme extract, and the decrease in absorbance of H_2O_2 at 240 nm for 30 s was recorded.

4.3.4. APX activity:

One gram of samples was milled in 3 mL of extraction solution including 50 mM phosphate buffer (pH 7.0), 2 mM ascorbate, and 5 mM EDTA at 4 °C [44]. The suspension was centrifuged for 20 min at 13,000 g. The supernatant was used for analyzing the enzyme activity. The 3 mL reaction solution of APX contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2, and 0.1 mL of enzyme extracts. The reduction in the absorbance of ascorbate indicates the APX activity within 1 min at 290 nm. One unit of APX activity was defined as the amount of enzyme required for catalyzing the oxidation of 1 mmol ascorbate per minute.

4.3.5. GR activity:

Extraction of GR was determined by measuring the reduction of GSSG by NADPH at 30°C through the decrease in absorbance at 340 nm and via the extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ [45]. The assay mixture contained 0.2 M potassium phosphate, 0.2 mM Na_2EDTA, 1.5 mM $MgCl_2$, 25 µM NADPH, 0.25 mM GSSH, pH 7.5, and 50 µL of enzyme extract in a 1-mL final volume. The reaction was initiated by the addition of NADPH.

4.4. Lipid peroxidation:

Lipid peroxidation was determined by measuring the amount of MDA produced by the thiobarbituric acid reactive substances assay. One gram of frozen leaf and root samples were milled separately in 5 mL of 1% trichloroacetic acid and centrifuged at 15000 rpm for 10 min at 4°C [46]. One mL of the supernatant was added to 4 mL of 20% trichloroacetic (0.5%). The mixture was heated at 95◦C for 30 min, subsequently chilled on ice for 30 min, and then the absorbance was measured at 450, 532, and 600 nm. The MDA concentration was calculated using the following equation: MDA (μmol g^{-1}FW) = $6.45(OD_{532} - OD_{600}) - 0.56OD_{450}$

4.5. Statistical analysis:

A factorial experiment was done on the basis of randomized complete design (RCD) with three replications. All measurements were conducted by the analysis of variance (ANOVA) followed by Duncan's New Multiple Range Test [47], with PROC GLM of SAS (version 9.1, SAS Institute). Pearson's correlation coefficients were carried out using SAS software. The linear and quadratic regression models for the enzyme activities in both leaves and roots were evaluated using the coefficient of determination (R-square) to explain the variance of the data portrayed by the model.

5. Conclusions

The present study demonstrates that low salinity stress (8dS/m^{-1}) does not impact rootstock, but shows there are significant increases in antioxidant enzymes and MDA content, particularly in susceptible genotypes. At moderate and higher salt levels (12dSm^{-1} and 16dSm^{-1}), all antioxidant enzymes, SOD, POD, and APX, significantly increased in leaves as well as roots. Pistachio root plays an important chromatic role in modulating the oxidative stress caused by salinity stress. Rootstock UCB-1 exhibited better intonate to the salinity stress followed by Badami, Ghazvini, KG, and Akbari. The degree of variation in salinity tolerance is correlated to the cultivar ability to correspondingly organize the antioxidant enzymes at the root level or different ratios of antioxidant enzymes and MDA content across leaf cell membranes. This study suggests that the UCB-1 and Akbari are two contrasting cultivars to salinity stress and are well suited to use for further comparative salinity tolerance studies in pistachio.

Authors Contribution Statement:

MA and NM designed the experiments. MA performed the work and wrote the first draft of the manuscript. RK reviewed and revised the manuscript. RH reviewed, edited the contents and revised the figures. MF performed statistical analyses. SM reviewed and edited the manuscript. NM overall supervised the work.

Conflict of Interest Statement:

Submitted work was not carried out in the presence of any personal, professional, or financial relationships that could be potentially are construed as a conflict of interest.

Supplementary Material:

None

Funding Disclosure:

Part of this work received funding from the University of Tabriz towards Ph. D thesis for MA. RK and RH acknowledge the support from the National Science Foundation REU grant number 1560049 to carry out the analysis of the data.

Data Availability Statements:

All datasets (generated/analyzed) for this study are included in the manuscript. All supplementary figures and tables have also been provided.

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