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# *In Vitro* Evaluation of Salinity-Induced Changes in Biochemical Characteristics and Antioxidant Enzymes in 21 Grapes Cultivars

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## ABSTRACT

**Soil salinity is one of the most important environmental constraints that reduce plant growth and productivity. This study aimed to investigate the effects of various NaCl concentrations on the physiological properties of grape cultivars. NaCl was added at three levels (0, 25, and 50 mM) to Murashige and Skoog medium under *in vitro* conditions to assess various effects on 21 grape cultivars. Effects of salinity stress were investigated on ascorbate peroxidase, catalase, and superoxide dismutase activities, as well as malondialdehyde, protein, proline, chlorophyll A and B contents, of all samples. The results showed that with an increase in salinity, the amount of antioxidant enzymes, proline content, and protein increased in cv. Rasha, suggesting that it was more tolerant than the other cultivars. Malondialdehyde and Electrolyte leakage accumulation also increased in all cultivars, but this increase was higher in salinity-sensitive cultivars, such as hybrids and wild cultivars than resistant cultivars. During salinity stress, chlorophyll content decreased, and the lowest decrease in chlorophyll content was recorded in cv. Rasha, compared to other cultivars. This research demonstrated that the resistance of cv. Rasha, H6 and H4 to salinity stress was due to its ability to adjust proline, protein content, and antioxidant enzymes.**

**Keywords:** Salinity stress, *Vitis vinifera*, Proline, Grape cv. Rasha..

## INTRODUCTION

Salt stress is one of the main factors that restrict the growth and production of horticultural crops including grapevine. Soil is considered saline when the electrical conductivity in the soil solution reaches 4 dS $m^{-1}$, which is roughly equal to 40 mM NaCl (Singh *et al*., 2004). Today, soil salinity has become a global problem, and some countries/regions are more adversely affected than others. For instance, about 15% of the crop fields around Urmia Lake in the West Azerbaijan Province of Iran are affected by salinity to varying degrees (Bybordi, 2012).The higher amount of $Na^+$ and $Cl^-$ ions under salinity stress cause toxic ionic and osmotic stresses in plants and reduce the capacity of the photosynthetic apparatus (Amini and Ehsanpour, 2005). Like other abiotic stresses, in addition to its primary effects, salinity activates oxidative stress and leads to the accumulation of active oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals

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(De Azevedo Neto *et al*., 2006). Reactive oxygen species are highly reactive and contribute to oxidative damage to lipids, proteins, and nucleic acids. Two strategies are proposed to reduce the effect of salinity stress in plants. The first strategy is based on soil reclamation through improved drainage and amendment-based interventions, including the widespread practice of leaching with fresh-water. The other is based on the selection and development of salt-tolerant crops and cultivars (Singh *et al*., 2018). However, the selection of resistant/tolerant crop cultivars to ensure better crop performance in salt-affected soils is considered a cost-efficient strategy (Owais, 2015). Plants have developed various mechanisms to cope with osmotic and ionic effects caused by salinity stress. Amino acids such as proline and proteins are osmolytes that are accumulated in higher plants in response to salinity stress (Fakhrfeshani *et al.,* 2015).

The province of West Azerbaijan covers an area of 39,487 km², or 43,660 km², including Urmia Lake. There are several productive plains all around the lake, which are important agricultural production areas. Urmia Lake has been shrinking for a long time; with an annual evaporation rate of 0.6 to 1 m that can increase salinity stress in the region (Hamzehpour and Rahmati, 2016). West Azerbaijan Province has the fourth rank in grapevine production in Iran and is ranked the first in the quality of grapes produced (Baneh *et al*., 2013). Grapevine (*Vitis vinifera*) is an economically important plant that is widely cultivated in different parts of the world. Grape is classified as a moderately sensitive plant to salinity stress (Mohammadkhani, 2018). The decrease in grape yield starts from the electrical conductivity of the saturated extract at 2 dS $m^{-1}$, and for every 1 dS $m^{-1}$ increase, the yield decreases by about 10% (Zhang *et al*., 2002). There are two strategies to deal with salinity stress in grapes. The first mechanism involves reducing transpiration and growth that occurs immediately after the onset of stress due to reduced osmotic potential of the soil solution, and the second mechanism is grape mortality that coincides with a sharp increase in toxic ion content (Na and Cl) in the leaves (Sivritepe *et al*., 2010). Due to the increase of saline soils in irrigated areas around the globe, it is important to use rootstocks that can maintain productivity in such challenging situations. The use of special rootstocks has long been suggested as a suitable method to increase the resistance of grapes to salinity. However, choosing the right rootstock depends on a variety of factors, including soil type, physical and chemical properties, pests, diseases, water availability, and environmental factors (Sivritepe *et al*., 2010).

Baneh *et al*. (2013) analyzed the effect of different concentrations of NaCl on proline content and CAT activity of four grape cultivars and observed the proline content as an osmoregulat, and CAT activity increased significantly at the highest NaCl concentration. In another study based on pistachio, the effect of different concentrations of NaCl on the content of superoxide dismutase and catalase was investigated, and the activities of both enzymes increased with increasing NaCl concentration (Goharrizi *et al*., 2019). In another study, the sensitivity to salinity stress in five Jordanian grape cultivars was evaluated, which caused a significant reduction in physiological parameters such as total chlorophyll content and leaf minerals in response to stress. This indicates the harmful effect of NaCl on plant biomass and the physiological yield of grapes (Owais, 2015).

Although the susceptibility of some grapevine cultivars to salt tolerance has been studied, not all cultivars have been studied in this respect and their potential for cultivation in saline soils is unclear. This study aimed to evaluate the salinity tolerance of 21 rootstock grape cultivars at the biochemical level by measuring proline, protein, malondialdehyde, electrolyte leakage and chlorophyll contents and antioxidant enzyme activities.

## MATERIALS AND METHODS

### Plant Material and Salinity Treatments

In the present study, the biochemical reaction of twenty-one different grapevine cultivars (Table 1) was evaluated to different NaCl levels *in vitro*. The rootstocks were prepared from the Agricultural Research Education, and Extension Organization (AREEO) and used as a source of explants. Cuttings with a diameter of half a centimetre and three nodes (three buds) were selected. Cuttings of the wild cultivars were smaller in diameter. Cuttings were grown in a greenhouse at 21 °C and 70% relative humidity, in perlite medium. Then, the terminal buds of the produced branches were cultured in MS medium containing 0.2 mg of indole butyric acid and 200 mg of activated charcoal and were transferred to the growth chamber at a temperature of 20 to 25 °C, a relative humidity of 40-50%, and the duration of 16 hours of light (800 lux) and 8 hours of darkness (Singh et al., 2004) to produce a branch 5-6 cm in size. These buds grown in an MS medium were fragmented and explanted to produce enough seedlings to apply salinity stress. The seedlings were transferred to MS culture medium containing NaCl so that three salinity levels (0, 25, and 50 mM) were applied to each cultivar, with 3 replications for each salinity level (Mozafari and Ghaderi, 2019). Each culture vessel had five samples. Finally, after 2 weeks of salinity stress, seedlings were used for experiments.

### Preparation of Extraction Buffer

To prepare a phosphate buffer of 50 mM with pH 7, firstly, the stoke 1 and 2 solutions were prepared. Solution 1 (0.68 g of potassium dihydrogen phosphate) and solution 2 (0.87 g of potassium monohydrogen phosphate) dissolved in 100 mL of distilled water, and 2 g of PVPP and EDTA was added separately to each solution. At each test time, 39 mL of solution 1 were mixed with 61 mL of solution 2, and the pH was adjusted to 6.8-7.2 using a pH meter. The preparation of extraction buffer was done by the method described in detail by Beauchamp and Fridovich (1971).

**Table 1.** Grape rootstock cultivars used in the present study.

| Cultivar | Origin | Type of Use |
|---|---|---|
| Red-Khalili | Iran | Table grape |
| QzelOuzum | Iran | Table grape |
| H1R4B13 | Iran (hybrid) | Raisin and table grape |
| *V. vinifera*ssp*sylvestris* | Iran (wild type) | - |
| Rasha | Iran | fresh use |
| Askari | Iran | fresh use Raisins |
| Chavaga | Iran | Juice and table grape |
| H6 | Interspecies hybrid | Rootstock |
| H1R14B13 | Iran (hybrid) | Raisin and table grape |
| Garmiyan | Iran | Raisin and table grape |
| Thompson Seedless | Afghanistan | Raisin and table grape |
| Suparior | | Table grape |
| Goy Maleki | Iran | Raisin and table grape |
| Flame Seedless | United state | Table grape |
| Fiesta | Italya | Raisins |
| Sylvestris | Iran (wild type) | - |
| Mam Beraima | Iran | Raisin and table grape |
| H1R18B2 | Iran (hybrid) | Raisin and table grape |
| H1R16B16 | Iran (hybrid) | Raisin and table grape |
| H4 | Interspecies hybrid | Rootstock |
| H1R22B13 | Iran (hybrid) | Raisin and table grape |

### Enzyme Extraction

In order to obtain enzyme extraction and activity assay, the samples were separately crushed with liquid nitrogen. Half g of the samples were transferred to micro tubes, and 1mL of the extraction buffer was added to them, then, they were centrifuged with 20000×g for 15 minutes at 4°C. The extract was transferred to new micro tubes and rest at -80°C. The extracts were used to measure the activity of enzymes like SOD, APX, and CAT, as well as protein content based on the protocol described in detail by Beauchamp and Fridovich (1971).

### Determination of Enzyme Activity

The activity of SOD was determined based on the enzyme's ability to inhibit the photochemical reduction of nitroblue tetrazolium via the method described in detail by Giannopolitis and Ries (1977). The reaction mixture contained 50 mM phosphate buffer with pH7, EDTA 0.1 Mm, methionine 13 Mm, nitroblue tetrazolium 7 µM, and enzyme extracts. Riboflavin was added to the reaction mixture, and the samples were homogenized by a shaker for 15 minutes at 100 rpm (Blanc samples were placed in the dark for 15 minutes). Finally, the absorbance of the samples was read at 560 nm.

$$\text{SOD activity} = \frac{100-\left[\frac{(OD\ Control-OD\ Sample)}{OD\ Control}\times 100\right]}{50} \quad (1)$$

CAT activity was assayed according to the method described by Chance and Maehly (1955). The buffers were: buffer A that contained 25 mM phosphate buffer with pH 7 and buffer B that contained buffer A plus 0.1 mM EDTA and 10mM $H_2O_2$. A blank sample was 495 µL of buffer B, and 5 µL of buffer A. Plant samples were 495 µL of buffer B and 5 µL of enzyme extracts. The enzyme activity was assayed by measuring the hydrogen peroxide by spectrophotometer at 240 nm for 1 min. Enzyme activity was calculated by molar extinction coefficient as follows 40 $mM^{-1}cm^{-1}$.

APX activity was assayed by the method described by Nakano and Asada (1981). The buffers were: buffer A that contained 25 mM phosphate buffer with pH 7, and buffer B that contained buffer A plus 0.1 mM EDTA, 0.5 mM ascorbic acid, and 10 mM $H_2O_2$. The blank sample was 490 µL of buffer B, and 10 µL of buffer A. Plant samples were 490 µL of buffer B and 10 µL of enzyme extracts. The enzyme activity was determined by measuring the ascorbate oxidation by spectrophotometer at 290 nm for 1 minute. Enzyme activity was quantified using the molar extinction coefficient for ascorbate as follow 2.8 $mM^{-1}$ $cm^{-1}$.

### Determination of Protein Content

A part of the enzyme extract was used to measure the protein content by the Lowry method. One milliliter of the reagent A including an equal mixture of sodium carbonate 20%, copper sulfate (pentahydrate) 0.1%, potassium tartrate 0.12%, sodium carbonate 10%, sodium hydroxide 0.8 N, sodium dodecyl sulfate 10%, and double-distilled water, was added to 1 mL of sample and stored in static state at 20°C for 10 minutes. Then, 0.5 mL of the reagent B, including a mixture of the Folin-Ciocalteu Phenol reagent (1 part) with double distilled water (5 parts), was added to the solution. After 30 minutes, the absorption at 750 nm was measured by a UV-vis spectrophotometer (80-2092-26, Pharmacia LKB Biochrom, Cambridge, UK). The protein content was calculated using a calibration curve ($y=0.0012x+0.0007$, $R^2=0.9845$). The standard curve was constructed for 0–100 (mg/mL) concentration of bovine serum albumin (Sigma-Aldrich, St. Louise, Missouri, USA) at 750 nm (Qados, 2011).

### Determination of Proline Content

Proline content was determined following the method described by Bates *et al*. (1973). First, 1.25 g of ninhydrin was mixed with 30 mL of glacial acetic acid and 10 mL of phosphoric acid to prepare ninhydrin reagent. The resulting solution was warmed to dissolve completely and then cooled at 4°C and stored for 24h. Half g of plant tissue was crushed in 10 mL of 3% sulfosalicylic acid to be perfectly homogenized, then, the solution was centrifuged for 5 minutes at 2000×g. The solution was mixed with 2 mL ninhydrin reagent and placed at 100ºC for 1 hour. The reaction was ended in the ice bath, and the compound was extracted with 2 mL of Toluene. The absorbance of samples was measured at 520 nm with a spectrophotometer. The proline content was calculated by an equation obtained from the calibration curve of pure proline from 10 to 100 mg/mL concentrations.

### Determination of Lipid Peroxidation

The level of lipid peroxidation was determined by measuring the amount of MDA produced by the thiobarbituric acid reaction. Exactly 0.2 g of the leaf sample was dissolved in 5 mL of trichloroacetic acid and centrifuged at 8000 g for 10 minutes. The extract was then mixed with a solution containing 0.5% thiobarbituric acid (w/v) and 20% trichloroacetic acid (w/w). The resulting mixture was heated at 95 ° C in Ben Marie for 30 minutes and immediately centrifuged at 3000 g for 10 minutes after cooling in an ice bath. Finally, the absorbance of the samples at 600 and 532 nm was measured by a spectrophotometer (UV-Vis 2100 model) (De Azevedo Neto *et al*., 2006).

### Determination of electrolyte leakage

To measure electrolyte leakage (EL), 100 mg of freshly cut leaf samples were transferred to tubes containing 10 mL of deionized distilled water. The lids of the tubes were closed and placed in a hot water bath at a temperature of 35°C. After 2 hours, the EL of the samples was measured by an electrical conductivity meter (Taina EZDO-6021, Taiwan) (EC1). The samples were then autoclaved for 20 min to completely remove tissue and release electrolytes. The tubes containing the sample were cooled to 25°C and the EL of the samples was measured again (EC2) (Dionisio-Sese and Tobita, 1998).

$$EL\ (\%) = (EC1/\ EC2) \times 100 \quad (2)$$

### Determination of Chlorophyll Content

Measurement of chlorophyll A and B content was done by using Arnon, (1949) method. For this purpose, 0.5 (g) of the samples were crushed with liquid nitrogen. Twenty (mL) of 80% acetone was added to the samples and centrifuged at 6000×g for 10 minutes. The absorbance of samples was read for chlorophyll A at 663 nm and, for chlorophyll B at 645 nm. Finally, to calculate the content of chlorophyll A, B, and carotenoids, the following formulas were used.

$$\text{Chlorophyll A} = (19.3\times A_{663} - 0.86\times A_{645}) \times V / (100\times W) \quad (3)$$

$$\text{Chlorophyll B} = (19.3\times A_{645} - 3.6\times A_{663}) \times V / (100\times W) \quad (4)$$

### Statistical Analyses

In this research, all measurements were done in triplicate, and the final results were reported as means ± SD. General linear models (GLM) procedure was used for statistical analysis of data, and Tukey test with a significance level of $P<0.05$ was used for multiple comparisons of means in the Minitab version 18.1 software (Minitab Inc., State College, Pa., USA) (Amiry *et al*., 2017). The model included the fixed effects of NaCl, grapes cultivars, and their interactions. Pearson's correlation coefficients between dependent variables and principal component analysis (PCA)

were carried out by the JMP version 10 (SAS Institute, Cary, North Carolina, USA) (Fibrianto *et al*., 2018).

## RESULTS

According to the results of the analysis of variance, the main effect of salt, cultivar, and the interaction effect of salt × cultivar was significant (P≤0.05) on SOD, APX and CAT activity, protein, proline, lipid peroxidation, electrolyte leakage, and chlorophyll A and B contents (Table 2).

SOD enzyme at high salinity level (50 mM) showed an increase in the resistant cultivars; such as H6 (6.655 unit/mg protein), H4 (5.413unit/mg protein) and Rasha (5.206 unit/mg protein) which was much higher than in salinity-sensitive cultivars like; *V. vinifera* ssp *sylvestris*, Flame Seedless, Thompson Seedless, Goy Maleki, and some hybrid varieties (Figure 1-a).

A significant increase in APX activity was observed at 50 mM NaCl, for Rasha, Chavaga, H6 and H4 cultivars, respectively, which was almost the same for H6 and H4 cultivars (Figure 1-b). In 50 mM salinity treatment, the highest enzyme activity (210%) was observed in cv. Rasha, while the least enzyme activity was observed in cv. $H_1R_{18}B_2$ (104%) indicates the higher synthesis of this enzyme under stress conditions in the resistant than sensitive cultivars.

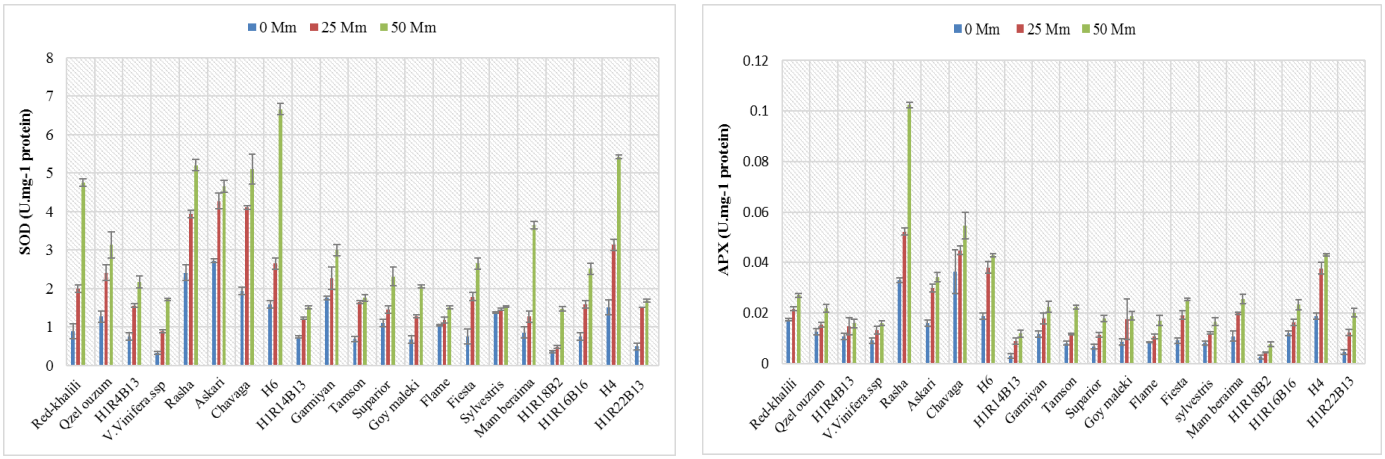
All cultivars exhibited a significant increase in CAT activity with increasing NaCl concentrations. The highest CAT activity was observed in Rasha and H6 compared to the other cultivars under all salt treatments, confirming the higher resistance of these two cultivars to salinity. After the mentioned cultivars, H4, Mam beraima, Garmiyan, Askari and Red-khalili showed a greater increase in enzyme content in compared to other cultivars studied (Figure 2-a).

The highest protein content was observed in Rasha, H6, H4 and Askari cultivars,

**Table 2.** Analysis of variance for physiological characteristics in 21 cultivars of grapes under salt stress conditions. [a]

| Source of variation | | Mean square | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | df | APX | CAT | SOD | Prolin | Protein | MDA | EL | Chlorophyll A | Chlorophyll B |
| Salt | 2 | 0.003* | $1.894\times10^{-5}$* | 39.048* | 810280.979* | 225205.858* | 0.002* | 1127.214* | 21.488* | 9.240* |
| Cultivar | 20 | 0.001* | $5.563\times10^{-6}$* | 6.287* | 770154.722* | 1000563.687* | 0.002* | 143.931* | 27.003* | 3.619* |
| Salt × Cultivar | 40 | 0.000* | $5.158\times10^{-7}$* | 0.944* | 22276.260* | 7915.702* | 0.000 | 22.490* | 1.835* | 0.443* |
| Experimental Error | 63 | $3.757\times10^{-6}$ | $3.013\times10^{-8}$ | 0.017 | 146.863 | 25.756 | $5.256\times10^{-5}$ | 0.117 | 0.012 | 0.072 |

[a] df: degree of freedom, APX: Ascorbate peroxidase, CAT: Catalase, SOD: Superoxide dismutase, MDA: Malondialdehyde, EL: Electrolyte leakage. ns: non-significant, * and **. significant at 0.05 probability level, respectively.




(a) (b)

**Figure 1.** Activities of superoxide dismutase (a) and ascorbate peroxidase (b), in 21 cultivars of grape treated with 3 levels of NaCl.

confirming their tolerance to salinity stress. Hybrid cultivars *V. vinifera* ssp *sylvestris*

and Flame Seedless showed the slightest changes in protein content, indicating their sensitivity to salinity stress (Figure 2-b). Other cultivars, including Red-khalili, Mam beraima and Garmiyan, were intermediate in terms of protein production.

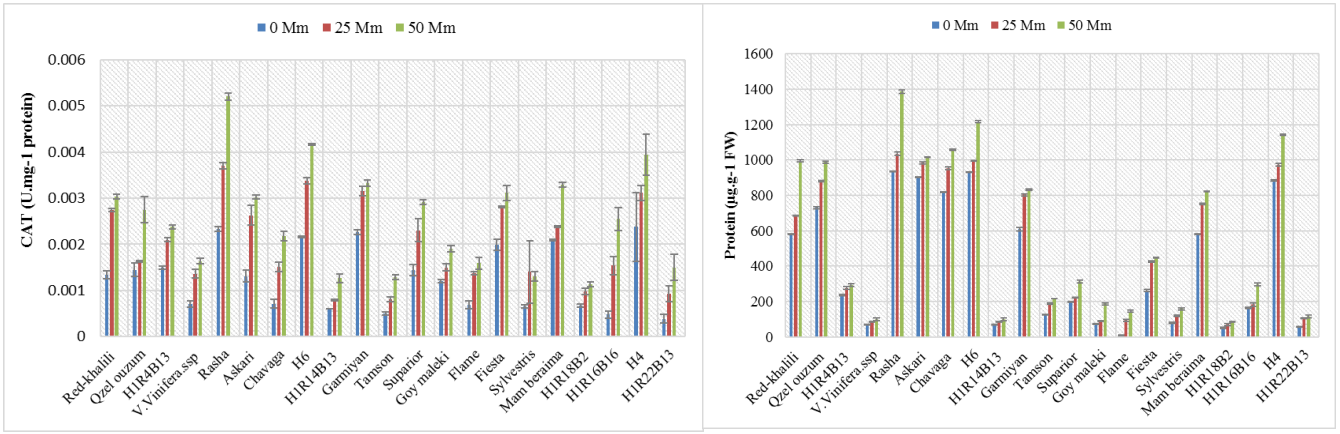
Proline amount increased by increasing salt intensity in tolerant cultivars such as Rasha, H6, H4, Chavaga and Mam beraima (Figure 3). The highest proline concentration was observed at 50 mM salinity level in Rasha, indicating and confirming high tolerance of this cultivar to salinity conditions. A high positive correlation was observed between proline and protein contents in studied cultivars under 50 mM NaCl ($r^2$=0.9576) (Table 5).

According to the results of analysis of variance, the effect of salinity on the amount of lipid peroxidation was statistically significant (P <0.05) (Table 2). With increasing NaCl in the culture medium, the amount of malondialdehyde increased in all samples, and this increase was more in salinity sensitive cultivars than other cultivars. The highest amount of malondialdehyde was observed in the highest salinity level (50 mM) in susceptible cultivars such as H1R18B2, H1R14B13, and *V.Vinifera*.ssp (Table 3).

The extent of damage to the cell membrane was indirectly measured by measuring the conductivity of solute leakage from the cells. Electrolyte leakage increased in all samples with increasing salinity level compared to control plants, which was higher in susceptible cultivars than resistant cultivars. No significant change was observed in electrolyte leakage in tolerant cultivars, from zero to 50 mM salinity, but in susceptible cultivars (H1R18B2), the amount of electrolyte leakage at 50 mM salinity level showed an increase of 12.34% (Table 4).

The effects of salinity stress on chlorophyll A and B are shown in Figures 4-a and -b, respectively. This study indicated that the content of chlorophyll A and B decreases with increasing salinity levels (P<0.05). However, chlorophyll levels in some samples were generally lower than other samples, indicating different susceptibility of cultivars to salinity stress (Figures, 4-a and -b).

Chlorophyll A and B decreased in all cultivars with increasing salinity levels, which was very significant in salinity-sensitive cultivars. The decrease in chlorophyll A levels in the tolerant and moderately tolerant cultivars did not differ much, and chlorophyll A changes in the resistant cultivars such as H6 and semi-susceptible cultivars such as Askari were almost similar; and only the two very




(a) (b)

**Figure 2.** Activities of catalse (a) enzymes and content of protein (b), in 21 cultivars of grape treated with 3 levels of NaCl.

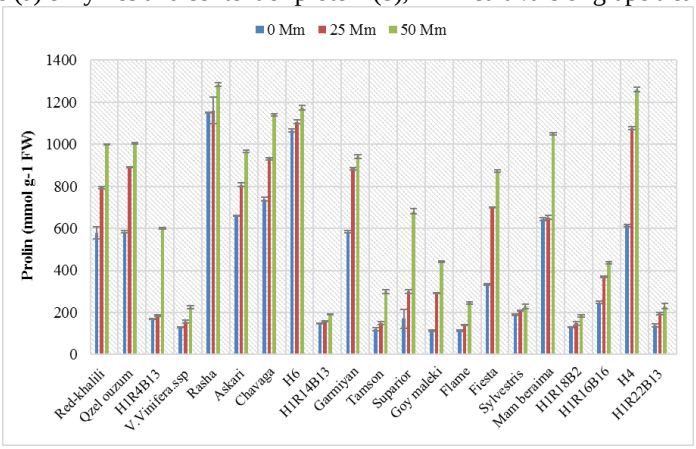


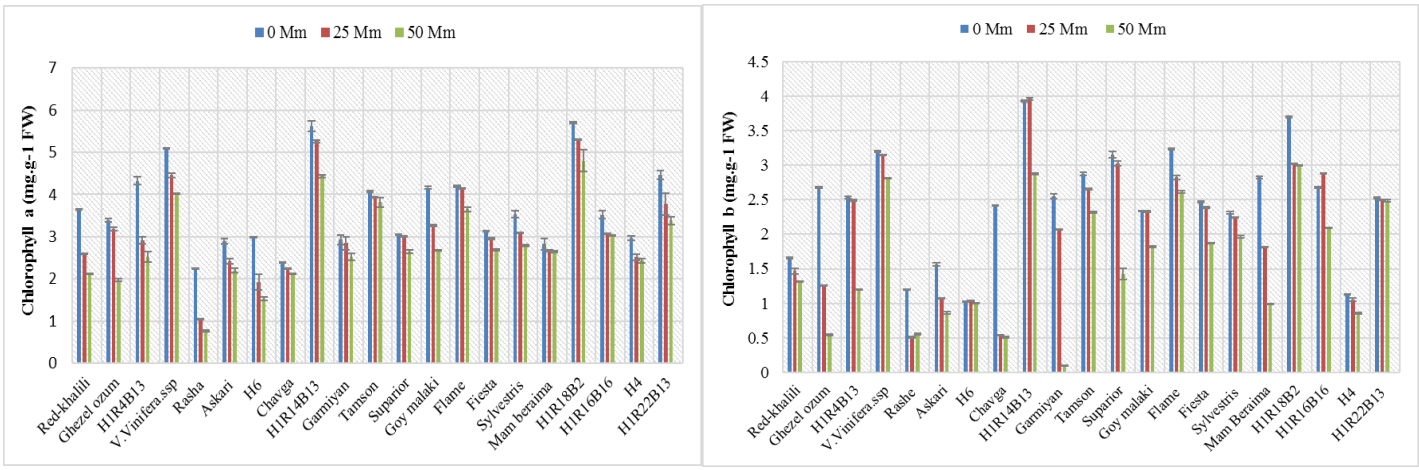

**Figure 3.** Content of proline in 21 cultivars of grape treated with 3 levels of NaCl.

**Table 3**. Content of malondialdehyde in 21 cultivars of grape treated with 3 levels of NaCl.

| Cultivars | Salinity stress | | |
|---|---|---|---|
| | 0 mM | 25 mM | 50 mM |
| Red-khalili | 0.024±.003 | 0.025±0.000 | 0.033±0.003 |
| Qzel ouzum | 0.023±0.003 | 0.029±0.007 | 0.032±0.003 |
| H1R4B13 | 0.018±0.008 | 0.031±0.005 | 0.038±0.002 |
| V.Vinifera.ssp | 0.065±0.000 | 0.071±0.010 | 0.074±0.005 |
| Rasha | 0.007±0.002 | 0.015±0.003 | 0.017±0.004 |
| Askari | 0.016±0.005 | 0.020±0.010 | 0.034±0.009 |
| H6 | 0.020±0.005 | 0.018±0.005 | 0.023±0.001 |
| Chavaga | 0.023±0.004 | 0.024±0.000 | 0.030±0.000 |
| H1R14B13 | 0.059±0.015 | 0.070±0.001 | 0.090±0.003 |
| Garmiyan | 0.022±0.001 | 0.027±0.005 | 0.031±0.013 |
| Tamson | 0.040±0.004 | 0.044±0.000 | 0.066±0.004 |
| Suparior | 0.025±0.005 | 0.031±0.003 | 0.038±0.000 |
| Goy maleki | 0.018±0.005 | 0.022±0.000 | 0.040±0.001 |
| Flame | 0.032±0.015 | 0.041±0.000 | 0.069±0.001 |
| Fiesta | 0.023±0.010 | 0.032±0.011 | 0.034±0.010 |
| Sylvestris | 0.042±0.001 | 0.049±0.006 | 0.056±0.000 |
| Mam beraima | 0.021±0.001 | 0.022±0.003 | 0.027±0.005 |
| H1R18B2 | 0.071±0.003 | 0.093±0.026 | 0.096±0.004 |
| H1R16B16 | 0.026±0.011 | 0.035±0.003 | 0.051±0.010 |
| H4 | 0.021±0.001 | 0.022±0.000 | 0.023±0.001 |
| H1R22B13 | 0.028±0.008 | 0.058±0.000 | 0.057±0.005 |

**Table 4.** Content of electrolyte leakage in 21 cultivars of grape treated with 3 levels of NaCl.

| Cultivars | Salinity stress | | |
|---|---|---|---|
| | 0 Mm | 25 Mm | 50 Mm |
| Red-khalili | 60.42±0.20 | 70.14±0.26 | 70.64±0.52 |
| Qzel ouzum | 64.37±0.11 | 72.83±0.24 | 73.98±0.48 |
| H1R4B13 | 58.97±0.07 | 66.06±0.07 | 86.18±0.11 |
| V.Vinifera.ssp | 71.55±0.26 | 75.62±0.54 | 78.26±0.38 |
| Rasha | 60.81±0.48 | 64.95±0.18 | 65.85±0.21 |
| Askari | 56.23±0.47 | 67.97±0.28 | 69.97±0.44 |
| H6 | 65.76±0.42 | 68.41±0.19 | 69.39±0.12 |
| Chavaga | 63.00±0.30 | 65.50±0.38 | 67.22±0.80 |
| H1R14B13 | 76.19±0.06 | 78.61±0.50 | 88.64±0.67 |
| Garmiyan | 65.07±0.08 | 68.89±0.18 | 70.25±0.13 |
| Tamson | 66.64±0.20 | 69.53±0.43 | 79.07±0.30 |
| Suparior | 62.15±0.25 | 65.70±0.05 | 75.45±0.02 |
| Goy maleki | 68.44±0.14 | 69.59±0.59 | 74.13±0.01 |
| Flame | 69.26±0.51 | 70.23±0.22 | 82.09±0.37 |
| Fiesta | 60.25±0.18 | 62.84±0.28 | 80.32±0.22 |
| Sylvestris | 61.29±0.43 | 73.86±0.32 | 79.94±0.10 |
| Mam beraima | 67.05±0.19 | 68.98±0.27 | 71.88±0.60 |
| H1R18B2 | 68.90±0.46 | 83.16±0.24 | 93.29±0.08 |
| H1R16B16 | 61.87±0.34 | 68.33±0.62 | 73.68±0.29 |
| H4 | 59.75±0.37 | 66.30±0.58 | 67.06±0.08 |
| H1R22B13 | 65.38±0.07 | 71.20±0.05 | 86.52±0.18 |




(a) (b)

**Figure 4.** Content of chlorophyll A (a) and chlorophyll B (b) in 21 cultivars of grape treated with 3 levels of NaCl.

sensitive hybrid cultivars H1R14B13 and H1R18B2 showed significant changes. However, the reduction in chlorophyll B levels was negligible for the resistant cultivars Rasha, H6, H4 compared to semi-sensitive and sensitive cultivars (Figures 4-a and -b).

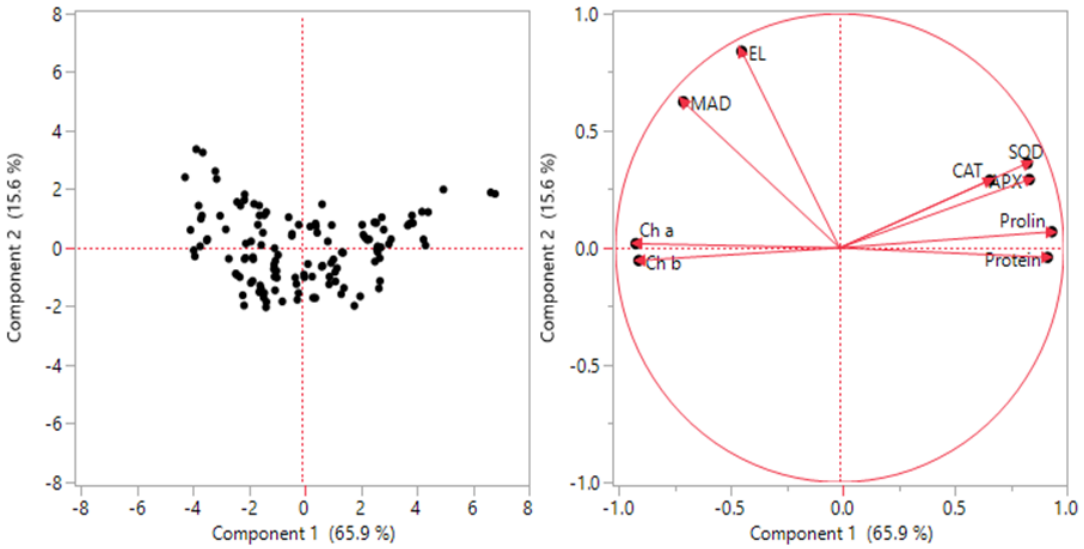
Principal component analysis (PCA) was used to determine an overall variance in all analyzed traits (65.9% and 15.6% for PC1 and PC2, respectively). APX, CAT, and SOD showed a positive correlation to PC1. MDA, EL, Chlorophyll A, and Chlorophyll B were positively correlated to PC2; whereas, protein and proline were negatively correlated to the same (Figure 5).

The Pearson matrix underlined the inter-correlations among the analyzed parameters.

**Table 5.** Simple correlation coefficients among physiological characteristics in 21 cultivars of grapes under salt stress conditions. [a]

| Character | APX | CAT | SOD | Protein | Prolin | MAD | EL | Ch a | Ch b |
|---|---|---|---|---|---|---|---|---|---|
| Normal condition (0 mM NaCl) | | | | | | | | | |
| APX | 1 | | | | | | | | |
| CAT | 0.402594 | 1 | | | | | | | |
| SOD | 0.648839 | 0.502928 | 1 | | | | | | |
| Protein | 0.78129* | 0.611359 | 0.74038* | 1 | | | | | |
| Prolin | 0.88707* | 0.57596 | 0.74636* | 0.91115* | 1 | | | | |
| MAD | -0.50557 | -0.47171 | -0.53364 | -0.55985 | -0.54518 | 1 | | | |
| EL | -0.62033 | -0.35186 | -0.40092 | -0.59038 | -0.58096 | 0.595206 | 1 | | |
| Ch a | -0.64168 | -0.68133 | -0.73463 | -0.7134 | -0.73899 | 0.74542* | 0.71599* | 1 | |
| Ch b | -0.78692 | -0.43692 | -0.61502 | -0.76414 | -0.72067 | 0.663912 | 0.71414* | 0.667392 | |
| Salt stress conditions (25 Mm NaCl) | | | | | | | | | |
| APX | 1 | | | | | | | | |
| CAT | 0.55191 | 1 | | | | | | | |
| SOD | 0.83597* | 0.341059 | 1 | | | | | | |
| Protein | 0.80592* | 0.466155 | 0.81898* | 1 | | | | | |
| Prolin | 0.85478* | 0.512294 | 0.79994* | 0.95416* | 1 | | | | |
| MAD | -0.61221 | -0.33786 | -0.61225 | -0.66284 | -0.66465 | 1 | | | |
| EL | -0.55876 | -0.30794 | -0.59999 | -0.56837 | -0.64326 | 0.81242* | 1 | | |
| Ch a | -0.81324 | -0.44787 | -0.74592 | -0.77085 | -0.79268 | 0.85464* | 0.77727* | 1 | |
| Ch b | -0.84689 | -0.42976 | -0.85215 | -0.89803 | -0.87844 | 0.691858 | 0.608779 | 0.84030* | |
| Salt stress conditions (50 Mm NaCl) | | | | | | | | | |
| APX | 1 | | | | | | | | |
| CAT | 0.81376* | 1 | | | | | | | |
| SOD | 0.74200* | 0.625331 | 1 | | | | | | |
| Protein | 0.75493* | 0.74887* | 0.93805* | 1 | | | | | |
| Prolin | 0.689993 | 0.77601* | 0.90063* | 0.95763* | 1 | | | | |
| MAD | -0.57421 | -0.68406 | -0.71029 | -0.78083 | -0.8741 | 1 | | | |
| EL | -0.60939 | -0.67755 | -0.74352 | -0.8166 | -0.81855 | 0.85790* | 1 | | |
| Ch a | -0.75423 | -0.73968 | -0.77322 | -0.83184 | -0.84706 | 0.90748* | 0.86964* | 1 | |
| Ch b | -0.55065 | -0.69724 | -0.71244 | -0.84321 | -0.87926 | 0.87137* | 0.86526* | 0.86126* | |

[a] APX: ascorbate peroxidase, CAT: catalase, SOD: superoxide dismutase, MDA: Malondialdehyde, EL: Electrolyte leakage, Ch a: Chlorophyll A, Ch b: Chlorophyll B; * Significant at 0.05 probability level.




(a) (b)

**Figure 5.** (a) Score plot and (b) Loading plot of principal component analysis of different grapes cultivars under salt stress.

As the salinity level increased, the correlation between the two enzymes APX and CAT increased from $r^2$=0.40259 in samples without salinity stress to $r^2$=0.81376 at a high salinity level (50 mM). This suggests the high $H_2O_2$ scavenging activity of these two enzymes. Chlorophyll B was negatively correlated with proline ($r^2$=-0.87926), and protein ($r^2$= -0.84321), which indicates that they strongly affected the chlorophyll content during salt stress (Table 5).

According to the comparison of 21 cultivars which were investigated in this study, Rasha, H6 and H4 were classified as resistant cultivars. Also, Chavga, Askari, Red-khalili, Ghezel ozum, Garmiyan, Mam Beraima, Goy Maleki and Fiesta cultivars were classified as semi-resistant cultivars. Finally, cultivars like Suparior, Thompson seedless, Flame seedless, Suparior, H1R4B13, H1R16B16, H1R22B13, H1R14B13, H1R18B2 and *V. vinifera* ssp *sylvestris* showed sensitivity to salinity stress compared to other cultivars studied in this study.

## DISCUSSION

Increasing salinity in the soil is a serious threat to growth of grapes, fruit productivity, and quality (Nakano and Asada, 1981). The effect of salinity stress has been investigated in grape (Baneh *et al*., 2013), mango (Elsheery and Cao, 2008), Pistachio (Goharrizi *et al*., 2019), and walnut (Karimi *et al*., 2020). The results of this study were in agreement with the previous findings.

There are efficient systems for removing oxygen species in plants to protect them against oxidative burst. Antioxidant enzymes, one of the most important components of the defense system, play an important role in defense mechanisms (Zhu *et al*., 2004). Increasing antioxidant enzymes activity under salinity conditions has been reported in various plants such as grape (Baneh *et al*., 2013), walnut (Karimi *et al*., 2020), and Pistachio (Goharrizi *et al*., 2019). In all cultivars, by increasing the salinity concentration in the culture medium, CAT, APX and SOD content increased significantly ($P<0.05$).

SOD is a major scavenger of superoxide $O_2^{\bullet}$-, and its enzymatic action results in the formation of $H_2O_2$ and $O_2$, thereby it moderates the risk from free radicals such as $OH^{\bullet}$ part, which causes serious damage to DNA and membranes proteins (Singh *et al*., 2010). Therefore, SOD is considered a key enzyme in maintaining normal physiological status and coping with oxidative stress by regulating ROS levels. In response to salinity stress, the activity of the SOD enzyme increased similar to those reported in grape (Soylemezoglu *et al*., 2009; Baneh *et al*., 2013) and maize (Owais, 2015), suggesting that the salt-tolerant cultivar has a better $O_2^{\bullet}$- radical scavenging ability. In this study, three cultivars, H6, H4 and Rasha, respectively, showed the highest enzyme production at a salinity level of 50 mM, which indicates the better ability of $O_2^{\bullet}$- radical inhibition by these cultivars. $H_2O_2$ as the final product of the superoxide dismutase is a toxic compound, and it must be converted to $H_2O$ destroyed during the reaction. In plants, enzymes such as APX and CAT can catalyze the conversion of hydrogen peroxide to water and oxygen (Singh *et al*., 2010).

Results of the present study revealed that parallel with increasing salinity levels in the culture medium, the amount of APX and CAT in all studied cultivars increased. Similar results were observed in walnut (Karimi *et al*., 2020), grapevine (Soylemezoglu *et al*., 2009), and lentil (Bandeoğlu *et al*., 2004) under abiotic stresses. In fact, it can be said that stimulation of SOD activity, which is due to salinity stress, has increased the enzymes of APX and CAT in resistant cultivars such as Rasha, H6, and H4 compared to sensitive cultivars and this indicates that the $H_2O_2$ scavenging mechanism has been less effective in sensitive cultivars such as hybrids. Therefore, our results show that the activity of APX and CAT enzymes are

coordinated with the activity of SOD enzyme and they play an important role in $H_2O_2$ scavenging. Comparing the activity of the two enzymes APX and CAT, it was observed that CAT has a much higher $H_2O_2$ scavenging activity than the APX enzyme. Therefore, it could be hypothesized that CAT is the most important among the $H_2O_2$ scavenging enzymes in leaves (Misra and Gupta, 2006). Similar results were observed in grapevine (Baneh *et al*., 2013), Pistachio (Goharrizi *et al*., 2019), and *Cartharanthusraseus* (Misra and Gupta, 2006).

Under salinity conditions, plants accumulate a form of nitrogen through protein accumulation, which may play a role in osmotic regulation when stress is over. The reason for the increase in soluble proteins is the synthesis of osmotic proteins such as proteins or structural proteins involved in the modification of the cell wall (Qados, 2011). In addition to the osmotic regulation role of proteins, some of them like CAT and APX enzymes, due to antioxidant activity, play an important role in detoxifying oxygen-reactive species and tolerating abiotic stress (Fibrianto *et al*., 2018). At high concentrations of NaCl, the amount of protein in all cultivars increased, but this increase was less in sensitive cultivars such as *V.Vinifera*.ssp and hybrids like H1R18B2 and H1R14B13, this may be due to a decrease in protein synthesis at stress condition. Similar results were reported in strawberry (Keutgen and Keutgen, 2003) and *Lycopersicon esculentum* (Amini and Ehsanpour, 2005).

Under salinity stress, plants produce compatible solutes such as proline in addition to antioxidant compounds that not only act as osmotic buffers but also maintain the function of macromolecules by inhibiting ROS (Soylemezoglu *et al*., 2009). Proline is one of the most important osmoprotectants in plants and its accumulation increases when plants are encountered with salt-stressed conditions (Bandeoğlu *et al*., 2004). Sumithra *et al*. (2006) reported a positive relationship between proline accumulation and salinity stress, which was similar to the result of our study. The lowest amount of proline was observed in the control samples, while its highest value was observed in samples under 50 mM salinity, of course, depending on the cultivar. Higher accumulation of proline in cultivars such as Rasha, H6, H4, Chavaga than H1R18B2 indicates that these cultivars had a better potential to maintain osmotic balance under salt stress. Sabbagh *et al*. (2014) reported that high proline content might be influenced by several factors, including the expression of genes that encodes the expression of critical enzymes for proline synthesis, a decrease in the conversion rate of proline oxidation, and a decrease in the use of proline in protein synthesis. Increased proline accumulation in species under salinity stress, including pistachio rootstocks (Rahneshan *et al*., 2018) and mango (Elsheery and Cao, 2008), has been reported in vitro, which is consistent with the results of this study.

Numerous reports have been published on the increase of malondialdehyde (Zhu *et al*., 2004; Soylemezoglu *et al*., 2009) in plants. Malondialdehyde is the product of lipid peroxidation, which is considered as an indicator for measuring the peroxidation of fats and membrane damage in non-biological stresses. Therefore, cell membrane stability is widely used to identify salinity-tolerant and salinity-sensitive cultivars (De Azevedo Neto *et al*., 2006). According to the results of this study, the content of malondialdehyde in resistant genotypes increased to a lesser extent, which is probably due to increased activity of antioxidant enzymes that reduce $H_2O_2$ levels and membrane damage (Soylemezoglu *et al*., 2009). This condition was seen in cultivars such as Rasha, and H6, which produced more antioxidant enzymes under stress condition.

Electrolyte leakage increased in all samples with increasing salinity level, which was higher in susceptible genotypes such as H1R18B2, H1R14B13, and Flame than in resistant genotypes. It has been reported that

stress may alter the chemical composition and organic acids and physical structure of biological membranes and plasma membranes and lead to oxidative damage in plants, which has a direct effect on electrolyte leakage (Mahlooji et al., 2018). Similarly, an increasing trend of electrolyte leakage has been reported in salinity-sensitive cucumber cultivars compared to salinity-tolerant cultivars (Khan et al., 2013). This phenomenon has been observed in several studies such as pomegranate (*Punica granatum*) (Jannatizadeh, 2019), banana (*Musa sapientum*) (Liu et al., 2019) and litchi fruit (Ali et al., 2016). The results were consistent for the 21 grape genotypes studied.

Chlorophyll content decreased in all samples with increasing salinity levels, which was lower in resistant samples than in susceptible samples. The results of this study showed a significant decrease in chlorophyll A and B levels under salt stress conditions, which are in agreement with previous results on grape (Mozafari and Ghaderi, 2019), thymus (Bistgani et al., 2019), and rice (Ali et al., 2004). The reduction of chlorophyll content in plants under salt stress can be for the following reasons: i) inhibition of chlorophyll synthesis and its decomposition by the chlorophyllase enzyme, which has been considered as a sign of oxidative burst, and ii) reduction in d-aminolevulinic acid synthesis as a chlorophyll biosynthesis precursor, due to a decrease in the amount of Aminolevulinic acid (Bistgani et al., 2019). Another reason for the reduction of chlorophyll synthesis in salt stress is preventing the biosynthesis of various chlorophyll crystals, which is probably due to the inhibitory effect of the accumulation of various salt ions (Ali *et al*., 2004).

## CONCLUSION

When choosing grape rootstocks, a wide range of internal and external factors, including purposes of use, metabolites, environmental conditions, and stress tolerance, must be considered to produce grapes with high production performance, quality, and marketability. Under field conditions, especially in arid areas with nutrient-poor saline soils, abiotic stresses have different effects on water and nutrient uptake, homeostasis, metabolic pathways, and physiological characteristics of grapes. In these conditions, selection of tolerant rootstocks can be used to improve the absorption of nutrients and water as well as the quality characteristics of the produced grapes. Our data suggests that certain types of grapes, for example, Rasha, can be selected as a salt-resistant cultivar and used as a resistant rootstock. Rasha, H6 and H4 had the highest antioxidant enzymes, while the hybrid cultivars had the lowest enzymes. The APX enzyme was more affected in different grape varieties. It can be concluded that cultivars more resistant to salinity stresses have more antioxidant enzymes, proteins, and proline, which can be used to identify more resistant cultivars. However, understanding the behavior of grapes in different environmental conditions and its relationship with the combined effects of rootstock and scion composition, planting, training and harvesting of vineyards on fruit yield, nutrient composition, and quality characteristics needs more studies. As a result, the study of the effect of biostimulants on grape cultivars in different environmental conditions can also provide valuable information to improve the yield, content of nutrients, and quality of the produced grapes.

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# ارزیابی درون شیشه ای تغییرات ناشی از شوری در خصوصیات بیوشیمیایی و آنزیم های آنتی اکسیدان 21 رقم انگور

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## چکیده

شوری خاک یکی از مهمترین محدودیت‌های محیطی است که رشد و بهره‌وری گیاه را کاهش می-دهد. این مطالعه با هدف بررسی تأثیر غلظت‌های مختلف NaCl بر خصوصیات فیزیولوژیکی ارقام انگور انجام شد. NaCl در سه سطح (0 ، 25 و 50 میلی مولار) برای ارزیابی اثرات مختلف بر روی 21 رقم انگور به محیط موراشینگ اسکوک در شرایط درون شیشه‌ای اضافه شد. اثرات تنش شوری بر روی فعالیت‌های آسکوربات پراکسیداز، کاتالاز و سوپراکسید دیسموتاز، و همچنین مالون دی آلدئید، پروتئین، پرولین، کلروفیل A و B، از همه نمونه‌ها بررسی شد. نتایج نشان داد که با افزایش سطح شوری، مقدار آنزیم‌های آنتی اکسیدانی، پرولین و پروتئین در رقم رشه افزایش می‌یابد که نشان دهنده مقاومت این رقم نسبت به سایر ارقام می‌باشد. تجمع مالون دی آلدئید نیز در همه ارقام افزایش یافت، اما این افزایش در ارقام حساس به شوری مانند هیبریدها و ارقام وحشی بیشتر از ارقام مقاوم بود. در طی تنش شوری، مقدار کلروفیل در تمامی ارقام کاهش یافت، که این کاهش در سایر ارقام به مقدار بیشتری مشاهده شد در حالی که کمترین کاهش در مقدار کلروفیل در رقم رشه دیده شد. این تحقیق نشان داد که مقاومت ارقام رشه، H6 و H4 به تنش شوری به دلیل توانایی آنها در تنظیم محتوای پرولین، پروتئین و آنزیم های آنتی اکسیدانی می باشد.