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Interaction of ascorbic acid and 24- epibrassinolide on the level of protein and activity of antioxidant enzymes of catalase and peroxidase on jiropt bean plants (Phaseolus acutifolius) under salinity condition

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***Abstract*---**A group of strong antioxidants, such as ascorbic acid and 24- epibrassinolide have significant environmental effects on plant growth and development. They can cause an increase in plant tolerance to environmental stresses. This research is performed to study the interaction of ascorbic acid and 24- epibrassinolide on the amount of protein and activity of antioxidant catalase enzymes and peroxidase in jiropt bean plants (Phaseolus acutifolius) under salinity. The experiments were performed completely as randomized factorial designs with three concentration of NaCl (0,5,10 %),three concentration of ascorbic acid (0,10,15µm) and Three concentration of 24- epibrassinolide(0,5,10 µm)each one with three replications in greenhouse controlled conditions.The results showed that salinity has a significant and cumulative effect on the protein content and activity of antioxidant enzymes of catalase and peroxidase. The destructive Ascorbic acid-modified effects salt and increases protein content and activity of catalase and peroxidase enzymes, 24-epibrassinolide modified salt effects and caused more increase in protein content and activity of antioxidant enzymes compared to salt treatment, the interaction between ascorbic acid and 24- epibrassinolide on

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modifying the salt effects were observed in bean plants. Based on the results obtained from this research, the use of ascorbic acid and 24-epibrassinolide can be recommended to decrease the harmful effects of sodium chloride on jiroft bean plants.

***Keywords*---**NaCl, ascorbic acid, 24- epibrasinolide, jiroft bean plants, Phaseolus acutifolius.

Introduction

Different environmental stresses include biotic and abiotic stresses, result in producing oxygen-free radicals. Among these stresses are salinity, drought, severe light, and even illness-producing factors (Karpinski et al,2003).salinity is considered important abiotic stress and it consist of a significant effect on the growth, yield, and biochemical and physiological processes of plants. through the creation of ionic poison, the shortage of nutrition and oxidative stress affect plant life(Meloni et al, 2003). Like other environmental stresses, salinity results in accumulating active oxygen species such as superoxide ion, hydrogen peroxide, and hydroxyl radical in the cell and causes damages to the membrane, proteins, and nucleic acid(Noctor and foyer,1998; Becana et al,1998). In-plant cells, chloroplasts, mitochondria, and peroxisomes are the main producers of oxygen free radicals within the cell (Salin,2003). Antioxidants are chemical compounds that in trace amounts rapidly react to oxygen free radicals and cause a halt in the process (Krishna,2003). The antioxidant system includes antioxidant compounds with low molecular weight such as ascorbate, glutathione, tocopherols, carotenoids together with an antioxidant enzyme such as superoxide dismutase, ascorbate peroxidase, catalase, glycols peroxidase (Noctor and foyer,2003).

Ascorbic acid is known as vitamin C that is an antioxidant that is soluble in water and serves as the primary substrate in the cycle of removing hydrogen peroxide poisons and also plays a role as a secondary antioxidant in regenerating alpha-tocopherol and other lipophile antioxidants. In addition, it is also found in cytosule,vacuel,chloroplast, mitochondria and cell wall(Rautenkranz et al,1994; Smirnoff,1996).It can be found by using biologic regulators of plant growth, salt tolerance increases in many plants. In this regard, using brassinosteroids could increase plant tolerance in a variety of environmental stresses such as salinity, cold, and heat. This increase generally depends on the production and transcription of anti-stress genes such as heat-shock, indicating the increase of transcription of genes responsible for response to stress to raise the stress tolerance in the plant treated by brassinosteroids (Clous et al,1992). External application of brassinosteroids improve the activity of none-enzymatic antioxidants such as alkaloids, carotenoids, flavonoids, ascorbate, tocopherol, and gelatin, so that it can protect the plants under stress(Nunez et al,2004; Ozdimer et al;2004).salinity activate protective genes in the plant, Consequently, it produces and increases HSP proteins, on the other hand, the level of enzymes such as Rubisco, phosphofructokinase, Invertase and Sucrose Synthase in the shoots changes(Horvath et al,2007). The report, about two cultivars of rice in salinity, shows protein levels in these two cultivars increase (Demiral,2005).

In the experiment performed on wheat, it was found that in a cold-adapted plant, the level of some proteins increased(keles and Oncel,2004). The system of detoxifying active oxygen is divided into classes of enzymatic and nonenzymatic in all plants(foyer et al,1994).In response to increasing the production of oxygen free radicals, the capacity and activity of the defensive system increase (Gressel and Galum,1994). The first enzyme scavenging oxygen active species is superoxide dismutase which converts superoxide radicals to hydrogen peroxide, this radical is eliminated by ascorbate peroxides(Asada,1987; Asada,1999; Salin,1991). Also hydrogen peroxide is removed by catalase enzyme(Anderson,1995; Comba,1998). Changing the level of activity of antioxidant enzymes in salinity conditions is also reported(Hernandez et al,2000).In a research, it was reported that there is an increase in the activity of antioxidant enzymes like catalase and peroxidase in the leaves of colza under salinity(Ashraf and Ali,2008; Lestari, K., et. al., 2020; Sargazi, M., et. al., 2020). Therefore this study aims to study the role of jiroft bean plants in human nutrition. The protective role of ascorbic acid and 24-epibrassinolide and also their interaction in antioxidative stress resulting from. NaCl.

Materials and Methods

The soil was taken from a surface layer (0-30cm) from the Narmashir region located in 30km so that Bam and it was analyzed.

Table1
Physical and chemical analysis of the employed soil

Soil tissue	Sodium mg.kg	Manyasium mg.kg	Calcium mg.kg	Cholor mg.kg	Sulphate mg.kg	Hydrogen carbonate	Potassium mg.kg	Phosphor mg.kg	Organic carbon OC	Satured mud acidity	Electrical conduction EC(ds.m)
Lumic-sand	14	11.2	18.8	28	10	4	200	4	o.23	7.7	3.7

Plant material and culture condition

Seeds of jiroft bean plants (*Phaseolus acutifolius*) were employed, seeds were prepared from Bam research. After separating uniformly sized seeds, they were disinfected with 5% sodium hypochlorite and then rinsed with distilled water, they were transported to the pots containing the soil with sand- lume texture. For each treatment, 3pots were considered as three replications and 4 seeds were planted in each pot. After planting the seed, they were allowed to Germinate and the seedlings to grow under greenhouse controlled conditions with a photoperiod of 14 /10h (dark / light) with light intensity wan13kilo lux,65%humidity and 28±2 /18±2 temperature. (day and night)

Preparing the solution of 24-epibrassinolide and ascorbic acid

100mg 24- epibrassinolide (Mw=480.7) was bought from Sigma (USA).10mg of stock solution with a concentration of(0.2083×10^{-3}M) was made using ethanol and was stored in 4c. Then an optimal volume of stock solution was prepared by the addition of distilled water. Required L-ascorbic acid (Mw= 176.3) was bought from Merck company, Germany. Then,10 and 15 µm solutions were made.

Treatment of the Plants

3 weeks after seeds germinated, seedlings were irrigated using distilled water. Then the pots were treated with solutions of salt, ascorbic acid, and 24-epibrassinolide.

Table 2
The treatments used in the experiment of jiroft bean plants under controlled greenhouse conditions

Treatment, No	Concentrations of NaCl	Concentrations of 24-epibrasinolide	Concentrations of Ascorbic acid
1	0	0	0
2	0	0	10
3	0	0	15
4	0	5	0
5	0	10	0
6	0	5	10
7	0	5	15
8	0	10	10
9	0	10	15
10	5	0	0
11	10	0	0
12	5	5	0
13	5	10	0
14	10	5	0
15	10	10	0
16	5	0	10
17	5	0	15
18	10	0	10
19	10	0	15
20	5	5	10
21	10	5	10
22	5	10	10
23	10	10	10
24	5	5	15
25	10	5	15
26	5	10	15
27	10	10	15

Treatments were performed for 3 weeks with 4 replications, 7days after treatment in the eight-leaf stage, the harvesting commenced and the plants were removed from pots. After rinsing and drying the plants, roots and shoots were separated and the samples were wrapped in aluminum foil and then frozen in liquid nitrogen. They were stored at the temperature of-80°C.For chemical analysis.

Extracting protein extract

To extract the protein extract to test the proteins and enzymes,0.2g of the green leaf was harvested and ground with 4cc of potassium phosphate buffer 0.1molar with pH =7.5 cold mortar and then it was transformed to homogenous. The homogenous mixture was passed through filter paper and centrifuged with 16000rpm for 15minutes .then a higher phase was used as protein extract to test enzymatic activity(kar and mishra,1978).

Assaying catalase enzyme

To assay the activity of catalase enzyme,0.2ml of extracting solution is mixed with 2.5ml phosphate buffer(pH=7.5) and 0.3ml of oxygen water(3%), and absorption level is measured in spectrophotometer system in wavelength of 350nm(Chance and Maely,1995)

Assaying catalase enzyme

Assaying the activity of peroxidase enzyme was determined according to the method of Ghanati et al. Enzymatic activity was initiated by adding appropriate amounts of enzymatic extract to the mixture of 0.1molar potassium phosphate buffer (pH=7), glycol with a concentration of 28mM, and hydrogen peroxide with a concentration of 5 mm that were poured in spectrophotometer dish and for one minute the variation in the wavelength of 470nm was studied. Enzymatic activity was expressed in mg protein.

Assaying The protein concentration of leaf solution

The amount of leaf protein was determined according to the Bradford method (1976). After complete mixing 1ml of Bradford solution with 100µl enzymatic extract was placed in a spectrophotometer and the absorption was recorded in wavelength of 595nm. protein concentration was calculated in mg /g fresh tissue by using a standard curve that was prepared by using bovine serum albumin (BSA) as standard. Its unit was expressed in mg protein per g wet weight.

Statistical operations

In the experiments, 3 replication were considered per treatment. Four plants were placed per replication. In order to determine the significant effect of the treatments of ascorbic acid and 24-epibrassinolide and salinity on the protein level of solution and activity of catalase and peroxidase statistical analysis was performed. The analysis of data resulting from assaying these parameters was performed through completely randomized design under ANOVA and by SPSS software (ver.20) and data means were compared by Duncan test and by using SAS software.

Results

Changes in protein content

The results of this research indicated that different concentrations of salinity increase the protein content of plants compared to the control plant. This increase was not significantly different from control plants. Addition of ascorbic acid to plant without salinity results in a significant increase of protein content compared to controls. The addition of 24-epibrassinolide without salinity cause an increase in protein content relative to control plants so this increase was significantly different from the control plant in high concentrations of 24-epibrassinolide. Simultaneous addition of 24-epibrassinolide and ascorbic acid to the plant also caused a significant increase in protein content compared to the control plant, so a significant difference was observed between leaf protein content in these treatments and control plants. The addition of ascorbic acid and salt caused an increase in protein content compared to salt treatments alone, but this increase was not different from salt treatments in low concentrations of ascorbic acid, while the difference was significant in a high concentration of ascorbic acid($P<0.05$). The addition of 24-epibrassinolide and salt also increased protein content but in the low concentration of 24-epibrassinolide, this increase was not significantly different from salt treatments. But in a higher concentration of 24-epibrassinolide, protein content increased significantly different from salt treatments alone ($P<0.05$). Simultaneous treatment of ascorbic acid and 24-epibrassinolide under salinity result in increasing protein content compared to 5 and 10 percent salt except in the treatment (10% salt+5 µm 24-epibrasinolide+10 µm ascorbic acid) and the treatments (10%salt+10 µm 24-epibrasinolide+10 µm ascorbic acid) were not significantly different from the same concentration of salt (Diagram1).

Table 3
ANOVA of the square mean of salt and ascorbic acid and 24-epibrassinolide effect on the content of protein and activity of enzymes of catalase and peroxidase in jiroft bean plants (*Phaseolus acutifolius*)

peroxidase	catalase	protein	Freedom degree	Variation Sources
**11.74	**0.067	**0.86	2	salt
0.007ns	**0.002	**14.49	2	Ascorbic acid
**0.192	**0.009	**3.45	2	24-epibrasinolide
**0.342	**0.002	**1.68	4	Salt ×Ascorbic acid
**0.151	**0.002	**0.66	4	Salt× 24-epibrasinolide
**1.35	**0.003	**0.65	4	Ascorbic acid × 24-epibrasinolide
**0.859	**0.003	**0.62	8	24-epibrasinolide × Ascorbic acid× salt
0.030	0.00007	0.155	54	Tottal rror
			80	Total
13.3%	6.6%	12.7%		%Variation coefficient

ns is a lack of signification difference
and * are signification in 1 and 5% respectively**

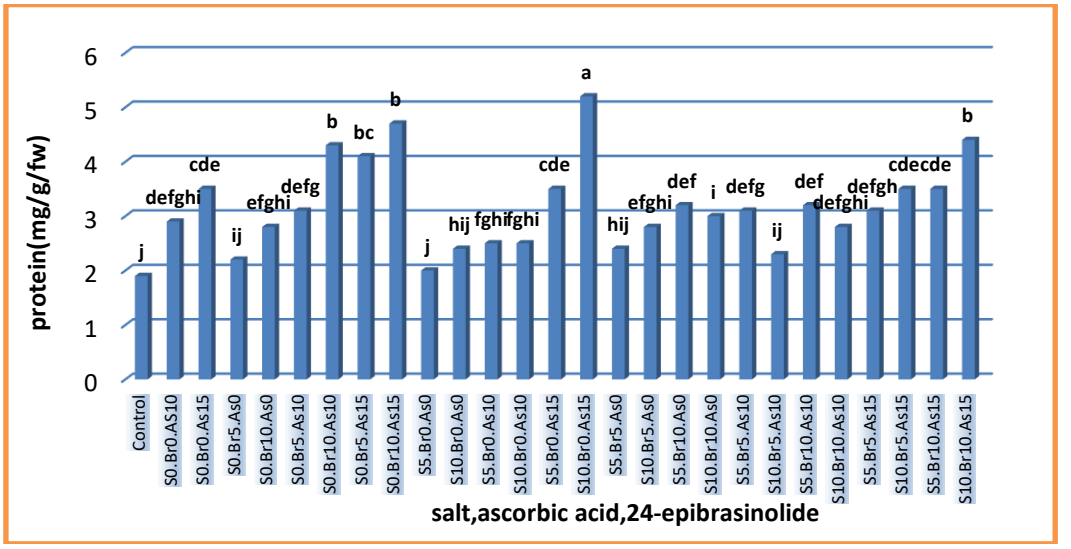

Diagram 1. Variation of protein content in jiroft bean plants under different conditions

Variations of the content of catalase enzyme activity

The results showed that with increasing salinity, The content of the activity of catalase increased significantly compared to the control sample. The addition of ascorbic acid to plant increased the activity of catalase. But this increased the activity of enzyme activity was not different from the control plant. The treatment of 24-epibrassinolide without salinity increases the activity of catalase. Only in higher levels of 24-epibrassinolide (10 µm), this increase is significantly different from the control plant. Simultaneous treatment of 24-epibrassinolide and ascorbic acid on the plant also increases enzyme activity compared to the control plant. So that the difference was observed between the content of the activity of catalase in leaf in these treatments compared to control plant. According to the results, the treatments containing salt and ascorbic acid showed a significant increase($P<0.01$) in the content of catalase activity. The treatments containing salt and 24-epibrassinolide showed an increase in catalase activity($P<0.01$). Simultaneous treatments of ascorbic acid and 24-epibrassinolide under salinity result in a significant increase in catalase activity compared to 5 and 10% salt, except in these treatments (5% salt+5 µm 24- epibrassinolide +10 µm ascorbic acid),(10 % salt+5 µm 24- epibrassinolide +10µm ascorbic acid) and(5% salt+5 µm 24- epibrassinolide +15 µm ascorbic acid) which were not significantly different from the same concentration salinity (Diagram2).

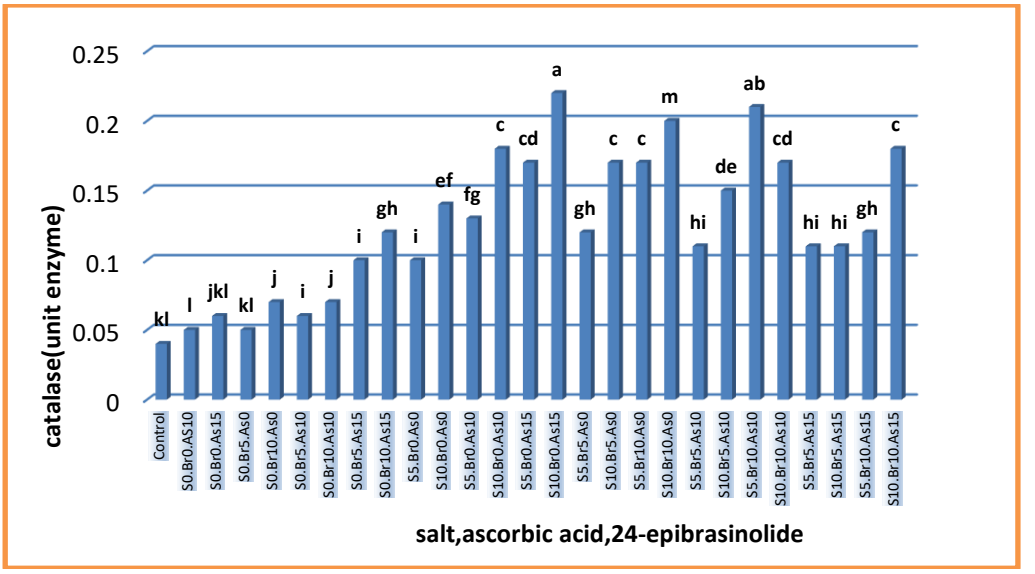

Diagram 2. variation of catalase enzyme activity level in jiroft bean plant in different condition

Variations of Level of peroxidase enzyme activity

The results indicated that with an increase of salinity, the concentration of peroxidase activity significantly increased compared to control plants($P<0.01$), the addition of ascorbic acid to jiroft bean plants without salinity did not create change in the activity of peroxidase. The treatments of 24-epibrassinolide increased the peroxidase activity but this increase did not show any difference to the control plant. The simultaneous treatments of ascorbic acid and 24-epibrassinolide also increased the activity of peroxidase. But only in the treatments(10µm 24- epibrassinolide +10 µm ascorbic acid) and(5µm 24-epibrassinolide +15 µm ascorbic acid), this increase was significantly different from the control plant. According to the results, the treatments containing salinity and ascorbic acid showed a significant increase in peroxidase activity compared to salinity treatments($P<0.01$). The treatments containing salinity and 24-epibrassinolide increased peroxidase activity compared to salinity treatments($P<0.01$). The simultaneous treatments of ascorbic acid and 24-epibrassinolide under salinity increase activity of peroxidase compared to 5 and 10% salinity and this increase had a significant difference to the same concentration salinity only in the following treatments:(5% salt+5 µm 24-epibrassinolide +10 µm ascorbic acid), (10% salt+5 µm 24- epibrassinolide +10 µm ascorbic acid), (10% salt+5 µm 24- epibrassinolide +15 µm ascorbic acid). (Diagram3).

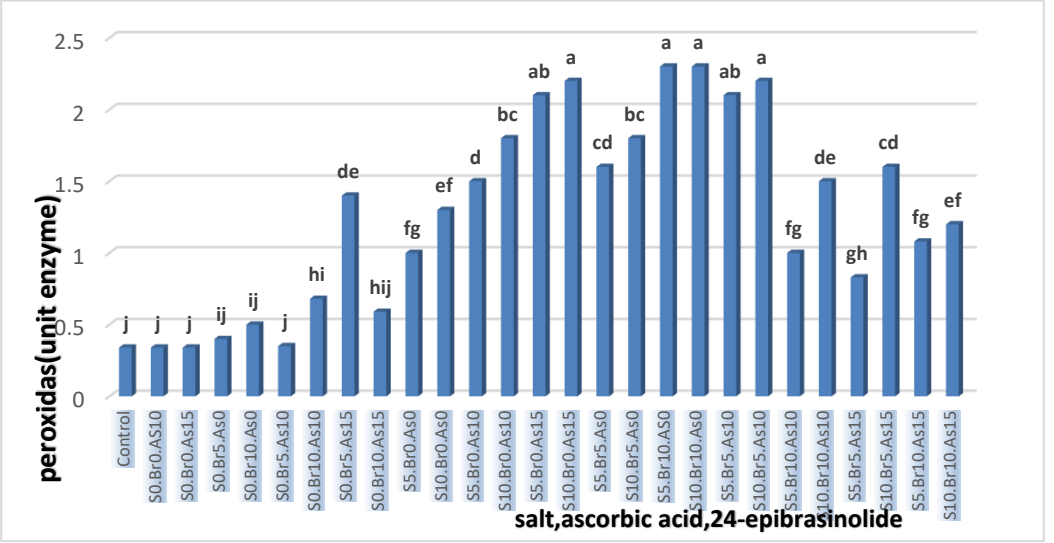

Diagram 3. variations of peroxidase enzyme activity content in jiroft bean plants under different conditions.

Discussion

Ascorbic acid and 24-epibrassinolide without salinity increase the protein content of plants. The simultaneous treatments of ascorbic acid and 24-epibrassinolide also increase protein content. In the plants treated by brassinosteroids, the protein, and nucleic acid synthesis were studied, and an increase in the activity of polymerase DNA, RNA, and increase in biosynthesis, DNA, RNA, and protein was observed(Clous and sass 1998; Jaising et al,1993; Li,2003).In a report, it was mentioned that plants treatment by brassinosteroid had a strong effect on mRNA transcription and increased protein content in the *phaseolus aureus*, *phaseolus Vulgaris* plants(Kalinich et al,1985).Probably ascorbic acid also has effects on gene expression and finally a synthesis of these compounds because as an important co-factor, this particular vitamin plays role in the biosynthesis and activity of many proteins such as enzymes and hormones. It is reported that ascorbic acid increases the protein content in two cultivars (MH-97, Wheat cultivars,S-24) (Rehman et al,2008).salinity increased the soluble protein content NaCl in Jiroft bean plants. This increase in protein content is directly related to an increase of concentrations due to the synthesis of some proteins or increasing HSP proteins that serve as protective when encountering stress in plants.In a report, salinity in Radish resulted in accumulating proteins with a molecular weight of 22KDa and an isoelectric point of 7 in the leaves(Lopez and Delgado,2000).

According to the diagrams, adding ascorbic acid to salt treatments could eliminate undesirable effects of salt, ascorbic acid is a strong antioxidant that is inhibited by reducing free radicals. It is combined with different kinds of active oxygen where it reduces them, also increases the activity of antioxidant enzymes against oxidative stress, and causes the stability of protein bands that results in increasing and preventing protein destruction(Farouk,2011).There is some report related to increased protein content in the plants sprayed by ascorbic acid, for

example, we can refer to the reports on the soy(Sheteawi,2007), *Brassica napus* L (Dolatabadian et al,2008), and pea *Pisum sativum* L(Beltagi,2008).Also, the results indicated that the treatment of 24-epibrassinolide also could reduce the effect of stress on the protein content of plants and can protect their structure. Increasing protein is probably because of the 24-epibrassinolide effect on gene expression of some proteins such as HSP and mutins that protect plants against stresses and the genes responding to stress that is activated by brassinosteroids which can encode $cp_{s,}$ organic acid,smulits, and proteins protecting against stress. Recently, the researchers identified six genes that are differently expressed during stress and treatment by EBL and It is clear that these six genes are related to Mterf protein, protein-rich of 22 glycin, myrosinase,3-ceto acyl-thiolase coenzyme, and a copy similar to polyprotein.

Therefore, brassinosteroids result in a change of gene expression under stress conditions (Gendron and Wang, 2007). Increasing protein synthesis is observed in different plants treated by brassinosteroids, As mentioned, 24-epibrassinolide results in increasing HSP proteins under salinity that increases plant resistance to stress(Pustovoitov et al,2000).The results of this research indicate that the number of soluble proteins exhibits more increase in the plants treated by ascorbic acid,24-epibrassinolide, and salinity compared to the plants treated only by salt. It seems that these two antioxidants protect protein structures by neutralizing poisonous radicals such as superoxide, hydrogen peroxide, and hydroxyl or increase these compounds by affecting the gene expression of some proteins such as mutins.The results of this research show that the addition of ascorbic acid and 24-epibrassinolide increases catalase activity but this increase was not different from control, while addition ascorbic acid does not create any change in peroxidase activity but 24-epibrassinolide increases activity of this enzyme, but this increase was not different from control. Simultaneous addition of the ascorbic acid and 24-epibrassinolide increases the activity of these two enzymes .most likely, the increase of enzyme activity in some treatments is because of the positive effect of the two compounds. A report(Rehman et al,2008) mentioned that ascorbic acid increases the activity of catalase and peroxidase. It is reported that in the plant,24-epibrassinolide(*Pisum sativum* L) increases the activity of catalase, peroxidase, and peroxidase in jiroft bean plants. Antioxidant enzymes are considered as the fastest units to encounter oxygen attack because of which salinity increases (Dirk and Montago,2002).

Catalase is one of the important scavengers of H_2O_2 that does this by converting H_2O_2 to water and O_2, catalase with peroxidase ascorbate is a scavenger of H_2O_2 and consequently regulator of H_2O_2 content in the cell(Dixit et al,2001). In this field that salt increase caused an increase in antioxidant enzymes, there are various reports, for example, drought, high temperature, and salinity increase the activity of SOD, APX, CAT, GR in wheat genotypes.(Sairam et al,2001).Ashraf and Ali (2008) found that under salinity the activity of antioxidant enzymes such as catalase and peroxidase increase in *Brassica napus* L leaves.One role of ascorbic acid is to increase the activity of enzymes of the gelatin-ascorbate cycle in mitochondria and peroxisome(Jimenez et al,1997) and consequently to increase H_2O_2 scavenger and catalase activity that protect macromolecule structures such as proteins(Dixit et al,2001).Similar to these results, the effect of ascorbic acid on increasing the activity of enzymes resulting from oxidative stress in different

varieties of cotton is reported(Rajguru and Stewart,1999).In another report in the plant of caraway, the amount of catalase increased with an increase of salinity concentration, but the plants simultaneously affected by NaCl and ascorbate exhibited a higher amount of catalase(Ghorbanli et al,1389).

Under salinity addition 24-epibrassinolide in the jiroft been increased catalase and peroxidase, the researchers indicated that brassinosteroids change enzymatic and no enzymatic antioxidant activity, when the plants are treated by epibrassinolide, the activity of superoxide dismutase, catalase, peroxidase and the contents of carotenoids and ascorbic acid(Li et al,1998).Brassinosteroids have the potential to control the activity of various antioxidant enzymes such as superoxide dismutase, peroxidase, and catalase in the plants under stressful media(Bajguz and Hayet,2009).In rice plants under salinity, the treatment of brassinosteroids results in increasing the activity of catalase and peroxidase enzymes(Nunez et al,2004).The treatment of Chlorella Vulgaris species by brassinolide increases the activity of catalase, ascorbate peroxidase and ascorbic acid, and gelatin and carotenoid under salinity (Bajguz and Hayat,2009).In this research, simultaneous applying of ascorbic acid and 24-epibrassinolide affected salinity modifying only in some treatments and resulted in other treatments, positive interaction was not observed.

Conclusion

A group of strong antioxidants such as ascorbic acid and 24-epibrassinolide had significant effects on gene expression of stress protein and antioxidant enzymes that cause bean resistance to salt. Therefore, as effective antioxidants, these compounds protect bean plants against salt. Confirming applying these compounds in agriculture requires further research.

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