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Effect of Drought Stress on Superoxide Dismutase Activity in Two Species of *Haloxylon aphyllum* and *Haloxylon persicum*

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Abstract: Superoxide dismutase activity changes were studied at different periodic tensions using of spectrophotometric measurement of decline in NitroBlue Tetrazolium reduction to Blue Formazan at 560 nm in *Haloxylon aphyllum* and *Haloxylon persicum*. The aim of this study was to investigate the changes of superoxide dismutase in applied drought stress in two species of Haloxylon. The results showed that the effect of drought stress on increase in superoxide dismutase activity was significant ($p \leq 0.01$) in two haloxylon species. Drought increased enzyme activity at severe tensions. When two haloxylon species were placed under 7 and 14 days no-watering treatments (mild tensions), the enzyme activity was more than its activity in control treatment and less than one in 21 and 28 days no-watering treatments (severe tensions). The enzyme activity in branchlets of *Haloxylon aphyllum* under 21 and 28 days no-watering treatments was 20.2 and 29.5% more than its activity under control treatment respectively. This activity in *Haloxylon persicum* was 21.6 and 31.4% more than its activity under control treatment, respectively. With the advent of drought, superoxide dismutase activity increased in two species of haloxylon. The increasing of superoxide dismutase activity in time of dryness advent in *Haloxylon aphyllum* was more than *Haloxylon persicum*, which can be raised as an acceptable factor and vindicator in being more resistant of *Haloxylon aphyllum* to environmental drought.

Key words: Superoxide dismutase, drought stress, haloxylon, reactive oxygen species, antioxidant

INTRODUCTION

Haloxylon persicum and *Haloxylon aphyllum* species are considered as resistant species to drought in desert and semi-desert areas of Iran (Arabzadeh, 2011, 2012a, Arabzadeh *et al*., 2009). Hand-planted haloxylons surrounding of Kerman with approximately 160 mm of rainfall per year and four-month dryness period at summer and hand-planted haloxylons of Narmasheer (in Bam of Kerman) with rainfall less than 90 mm per year and at least four months dryness period at summer are including these areas (Arabzadeh, 2009; Arabzadeh *et al*., 1992).

Drought is the most destructive factor among the environmental stresses that affecting the agricultural production (Bhardwaj and Yadav, 2012; Vinocur and Altman, 2005; Abd El-baky *et al*., 2003). Stress is the result of an unusual metabolism that can occur in form of growth failure, plant death or death of a part of the plant (Levitt, 1980). It seems that the susceptibility of plants to environmental stresses (especially drought and salinity stresses), the superoxide dismutase enzyme activity and its value changes and expression of its increasing gene in the cell environment depend on type and amount of applied stress (Arabzadeh, 2009). In all environmental conditions, plants are exposed to different types of stress (such as drought stress), which increase ROS (Reactive Oxygen Species) production (Abd El-baky *et al*., 2003). One of these (ROS) is superoxide radical which can threaten the plant tissues (Misra *et al*., 2006; Hippeli *et al*., 1999). ROS are removed by enzymatic antioxidant system consisting of superoxide dismutase and several other enzymes (Bhardwaj *et al*., 2007).

Superoxide dismutase is an antioxidant that reduces the oxidative destruction which is caused by environmental stresses in plants (Gill, *et al*., 2011; GholamhoseinianNajar *et al*., 2009; Lee *et al*., 2007; Tang *et al*., 2006; Gupta *et al*., 1993). Superoxide dismutase which increases under water stress (Saleh *et al*., 2007; Li *et al*., 2006) has an important role in the defense against damage by oxygen radicals in the chloroplasts (Kardish *et al*., 1994). Overexpressing

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cytosolic superoxide dismutase in plants is responsible for tolerance against water stress (Faize *et al*., 2011).

The activity of SOD is increased after changing in plants antioxidant potential (Sivakumar and Panneerselvam, 2011). One of the most important factors that affects on changing antioxidant potential of plants is drought stress (Arabzadeh, 2009). Salinity and drought stresses affect the metabolism of plant cells leading to severe crop damage and loss of yield (Arabzadeh, 2012a, 2009; Misra *et al*., 2006). Antioxidative enzymes system is activated by plants to reduce the destructive effects of stress for plants protecting from oxidative stress (Singh *et al*., 2008). Antioxidant enzymes such as superoxide dismutase are important in plant drought resistance (Bhardwaj and Yadav, 2012; Kwon *et al*., 2002). Antioxidative enzymes like SOD are very valuable in plants (Sivakumar and Panneerselvam, 2011). The basis of studies in survey of stress effects on oxidants and antioxidants is oxygen radical (O_2^{0-}).

Because haloxylons are used in the biological stabilization of sandy hills and sandy land as pioneer species, the need to examine the effects of induced water (drought) stress on some physiological characteristics of their seedlings was felt. In this study the effects of drought stress on superoxide dismutase has been studied in two species of *H. aphyllum* and *H. persicum*.

MATERIALS AND METHODS

This research was conducted from mid-2009 to May 2010 in Natural Resources Research Department of Agriculture and Natural Resources Researches Center of Kerman province.

Experimental design type: The obtained data were reviewed within the format of a Randomized Complete Design. Analysis of data was made by using two-side variance analysis and comparison of averages was made by using Duncan's test. The rate of each data in the tables of comparing averages was the result of at least five measuring.

Induction of drought stress to seedlings of two *Haloxylon* species: Annual seedlings from seeds of *Haloxylon* species were treated with periodic drought stresses (at 6 periods or replicates) as 7, 14, 21 and 28 no-irrigated days after taking a month to comply with greenhouse conditions.

Preparing extracts from branchlets of two *Haloxylon* species: For preparing of branchlet extract to distinguish species and drought stress treatments (control, 7, 14, 21 and 28 days without irrigation) was used one hundred grams dry matter of branchlets of haloxylon different treatments (including control, 7, 14, 21 and 28 days without irrigation separately). The dry matters were homogenized in 0.01 M phosphate buffer with pH 7 firstly. Then, the samples were centrifuged in 3000 g for 10 min and in 3500 g for 30 min, respectively. The clear supernatant solution (crude extract) was used for testing.

Preparation of phosphate-citrate-borate buffer (T.S. Buffer): This buffer can tolerate a wide range of pH. To prepare 200 mL of the buffer with concentration of 0.1 M amounts of 20, 40, 20 and 80 mL from phosphoric acid 1 M, boric acid 0.5 M, citric acid 1 M and H_2O (double distilled) was mixed together respectively and then using NaOH was prepared solutions with the proper pH. Finally, the volume of the solution using double-distilled water was brought to 200 mL.

Measurement of protein content in the sample: The Lowry method (Lowry *et al*., 1951) was used to measure protein in the extract. The three solutions A, B and C were prepared as follows:

- **A:** Na_2CO_3 10 g
- **B:** NaK tartrate 1.35 g
  NaOH (0.1 M) to 500 mL H_2O (double distilled water) to 50 mL
- **C:** $CuSO_4$ 0.5 g
  H_2O (Double distilled water) to 50 mL

These solutions were, as stored and each day of testing were mixed relative to the following: A: 4.9, B: 0.05, C: 0.05 mL.

To obtain the protein content in the unknown sample, the bovine serum albumin was used as standard protein. Initially, serial concentrations were prepared from standard protein with the amplitude 20-400 μg mL^{-1}. Then, the amount of 0.5 mL of each prepared concentrations of standard protein was put in separate tubes. Then the values 5, 10, 15, 20 and 30 μL of unknown samples were added to the pipes and their volumes increased to 0.5 mL with distilled water. The control (blank) tube had only 0.5 mL of double distilled water. Completing the first stage of preparation, the tubes were numbered, respectively. Now, all the tubes had 0.5 mL of the sample. In the next step, 5 mL of the mixture of A and B and C (with ratios listed) were added to all tubes from first to last, respectively. After adding this mixture to the first tube, 10 min incubation was performed at room temperature.

After a period of time, 0.5 mL of Folin reagent (1 M), which was diluted equally with distilled water, was added to all tubes (from first to last tube). Since the addition of Folin reagent to the first tube, 30 min incubation was performed at laboratory temperature until the reaction occurs. After 30 min, initially the device zero was set using the control tube and then the absorption rate was plotted against concentration of standard protein. Then the protein concentration in unknown sample was determined knowing its absorption and its matching with the standard curve, based on μg mL^{-1}. Standard curve was prepared each day of testing.

Measurement of SODs activity: Measuring the activity of this enzyme takes usually place based on substrate disappearance or products formation. Dismutation reaction that is catalyzed by SOD can not easily be measured by these criteria. The largest complexity comes from the nature of substrate "$O_2^{\circ-}$" that is unstable and disappears spontaneously. Also its half-life is deeply short and or shortening by intermediate metal ions that may be in the reaction.

Indirect method was used to measure the activity of SODs. In this method, the enzymatic reactions are used to produce $O_2^{\circ-}$ and a reagent that could be able reacts with it. Reagents that are also hunting $O_2^{\circ-}$, are used at high concentrations and can basically trap the entire generated $O_2^{\circ-}$. SOD present in the samples prevented the use of $O_2^{\circ-}$ by the reagent. Thus the reaction between $O_2^{\circ-}$ and reagent is changed. Methods that the formation of reaction product between $O_2^{\circ-}$ and a reagent is inhibited by SOD are known as Indirect Negative Assays and methods that the formation of reaction product between $O_2^{\circ-}$ and a reagent is increased by SOD are known as Indirect Positive Assays (Bannister and Calabrese, 2006; Van Breusegem *et al*., 2001; Misra and Fridovich, 1977a, b; Beauchamp and Fridovich, 1971; Massey *et al*., 1969).

RESULTS AND DISCUSSION

Measurement of superoxide dismutase activity in enzyme extracts of two species of *Haloxylon* to determine the best time of superoxide dismutase activity: SOD enzyme activity assay to determine the best time of enzyme activity was performed using Nitroblue Tetrazolium (NBT) reduction to Blue Formazan (BF). In the control tubes (no extract), superoxide radicals generated during the reaction was lead to the reduction of nitroblue tetrazolium. This reduction was followed the absorption changes in the wavelength of 560 nm. With the addition of extract (0.1 mg) to the reaction mixture, the reduction of nitroblue tetrazolium to blue formazan after 6, 12, 18 and 24 min of added riboflavin, decreased significantly ($p \leq 0.01$) to 37.2, 33.8, 26.6 and 25.9%, respectively in *H. aphyllum* and decreased (at the same significance level) to 39.6, 35.7, 28.3 and 27.7%, respectively in *H. persicum*. The reduction was implicated the presence and function of superoxide dismutase in extract of both species of haloxylon (Table 1 and 2).

As seen with increasing amounts of extract in reaction mixture and after 6 min of the addition of riboflavin to the mixture, enzyme activity increased; that way, the highest SOD activity for 1.6 mg of extracts protein, was observed up to 23.87 IU (mg pro.)$^{-1}$ in *H. aphyllum* and up to 20.25 IU (mg pro.)$^{-1}$ in *H. persicum* and the lowest SOD activity for 0.05 mg of protein of extract, was seen up to 7.74 IU (mg pro.)$^{-1}$ in *H. aphyllum* and up to 6.90 IU (mg pro.)$^{-1}$ in *H. persicum*. The maximum activity of enzyme was more than three times of minimum activity of enzyme in *H. aphyllum* and was slightly less than three times of minimum activity of enzyme in *H. persicum* (Table 3).

The enzyme activity increased again with time and after 12 min of adding riboflavin to the reaction mixture. So that the enzyme maximum activity for 1.6 mg of extracts protein was 35.98 and 32.42 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. The enzyme maximum activity was more than 3.5 times of the minimum activity in both species of *Haloxylon* (Table 4).

Table 1: Reduction of nitroblue tetrazolium (NBT) to blue formazan (BF) at wavelength 560 um; after 6, 12, 18 and 24 min of additing Riboflavin (0.12 mM) to the reaction mixture in *Haloxylon aphyllum*

	OD (560 nm)	
Time (min)	PBE	ABE
0	0	0
6	0.412±0.025	0.656±0.019
12	0.613±0.029	0.925±0.024
18	0.690±0.023	0.940±0.025
24	0.701±0.031	0.945±0.020

With volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH), EDTA: Ethylene diamine tetraacetic acid (0.007 mM), Potassium cyanide (0.02 mM), Nitroblue tetrazolium (0.05 M) with presence 0.1 mg branchlet extract (PBE) and absence branchlet extract (ABE) of *Haloxylon aphyllum*

Table 2: Reduction of NitroBlue Tetrazolium (NBT) to Blue Formazan (BF) at wavelength 560 um; after 6, 12, 18 and 24 min of additing Riboflavin (0.12 mM) to the reaction mixture in *Haloxylon persicum*

	OD (560 nm)	
Time (min)	PBE	ABE
0	0	0
6	0.327±0.031	0.616±0.028
12	0.569±0.027	0.885±0.025
18	0.646±0.033	0.901±0.026
24	0.657±0.026	0.909±0.023

With volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH), EDTA: Ethylene diamine tetraacetic acid (0.007 mM), Potassium cyanide (0.02 mM), NitroBlue Tetrazolium (0.05 M) with presence 0.1 mg PBE: Branchlet extract, ABE: Absence branchlet extract of *Haloxylon persicum*

Table 3: Superoxide dismutase activity in the presence of varying amounts of enzyme extract of branchlets of two *Haloxylon* species, After 6 min of adding Riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

Mg protein in reaction mixture	SOD activity [u (mg protein^{-1})]	
	Haloxylon persicum	*Haloxylon aphyllum*
0	0	0
0.05	06.90±0.62	07.74±0.63
0.1	08.25±0.68	09.12±0.66
0.2	11.31±0.73	12.75±0.78
0.4	15.46±0.69	18.95±0.65
0.8	17.11±0.66	21.30±0.75
1.2	19.51±0.59	23.11±0.68
1.6	20.25±0.73	23.87±0.77

EDTA: Ethylene diamine tetraacetic acid, (0.007 mM), Potassium cyanide (0.02 mM) and nitroblue, Tetrazolium (0.05 M)

Table 4: Superoxide dismutase activity in the presence of varying amounts of enzyme extract of branchlets of two *Haloxylon* species, After 12 min of adding Riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

Mg protein in reaction mixture	SOD activity [u (mg protein^{-1})]	
	Haloxylon persicum	*Haloxylon aphyllum*
0	0	0
0.05	09.01±0.70	09.96±0.79
0.1	10.30±0.64	11.31±0.68
0.2	13.47±0.69	14.90±0.65
0.4	21.10±0.78	25.82±0.70
0.8	27.72±0.68	31.93±0.77
1.2	31.86±0.58	35.42±0.69
1.6	32.42±0.87	35.98±0.71

EDTA: Ethylene diamine tetraacetic acid, (0.007 mM), Potassium Cyanide (0.02 mM) and nitroblue tetrazolium (0.05 M)

Table 5: Superoxide dismutase activity in the presence of varying amounts of enzyme extract of branchlets of two *Haloxylon* species, After 18 min of adding riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

Mg protein in reaction mixture	SOD activity [u (mg protein^{-1})]	
	Haloxylon persicum	*Haloxylon aphyllum*
0	0	0
0.05	09.72±0.62	10.85±0.57
0.1	11.19±0.66	12.28±0.69
0.2	14.32±0.75	15.81±0.72
0.4	21.87±0.70	26.67±0.63
0.8	28.63±0.61	32.81±0.55
1.2	32.61±0.69	36.10±0.74
1.6	33.10±0.79	36.59±0.76

EDTA: Ethylene diamine tetraacetic acid, (0.007 mM), Potassium cyanide (0.02 mM) and Nitroblue tetrazolium (0.05 M)

Table 6: Superoxide dismutase activity in the presence of varying amounts of enzyme extract of branchlets of two *Haloxylon* species, After 24 min of adding riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

Mg protein in reaction mixture	SOD activity [u (mg protein^{-1})]	
	Haloxylon persicum	*Haloxylon aphyllum*
0	0	0
0.05	10.29±0.66	11.31±0.74
0.1	11.71±0.81	12.80±0.85
0.2	15.06±0.76	16.30±0.72
0.4	22.81±0.79	27.19±0.65
0.8	29.32±0.68	33.37±0.78
1.2	33.37±0.77	36.63±0.73
1.6	33.86±0.78	37.11±0.66

EDTA: Ethylene diamine tetraacetic acid, (0.007 mM), Potassium cyanide (0.02 mM) and nitroblue tetrazolium (0.05 M)

The enzyme activity increased after 18 min of riboflavin added to the reaction. So that the enzyme minimum activity for every 0.05 mg of extracts protein in the reaction mixture was 10.85 and 9.72 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. The enzyme activity increased with increasing of extract amount in the reaction mixture, so that for the 1.6 mg of extract protein, its activity increased to 36.59 and 33.10 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively (Table 5).

In continuing and after 24 min of testing time, the enzyme activity for the 0.05 mg of extract protein increased to 11.31 and 10.29 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. Gradually with increasing the amount of extract in reaction mixture, the rate of enzyme activity rose. The highest of enzyme activity up to 37.11 and 33.86 IU (mg pro.)$^{-1}$ was observed in the presence of 1.2 mg of extract protein that belonged to *H. aphyllum* and *H. persicum*, respectively (Table 6).

Table 3 to 6 show the special activity [IU (mg pro.)$^{-1}$] of superoxide dismutase in the presence of varying amounts of enzyme extract of branchlets of two haloxylon species (no drought stress) after 6, 12, 18 and 24 min after addition of riboflavin to the reaction mixture. This activity has been created (due to having SOD) based on the property of NBT reduction by O_2^{0-}. It should be noted that the results obtained in this section and the next sections have been tested in at least three times. Six min after the addition of riboflavin to the reaction mixture, the optimum activity of SOD for the 1.6 mg of extract protein was 23.87 and 20.25 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively (Table 3). The enzymatic activity after 12 min for the same amount of protein reached to 35.98 and 32.42 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively (Table 4). After 18 min, this activity increased to 36.59 and 33.10 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively (Table 5). After 24 min of adding riboflavin to the reaction mixture, the enzymatic activity increased

to 37.11 and 33.86 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively (Table 6).

Thus, in both species of haloxylon, the highest activity of enzyme was obtained after 24 min of adding riboflavin to the reaction mixture. Table 1 and Table 2 express the same subject too. These tables show that the decrease in reduction of NBT to blueformazan occurred at 6 to 24 min after adding 0.1 mg of extract protein to the reaction mixture. This is represents an increase in enzyme activity.

Not mentioned special time for measuring enzyme activity in several papers and this time in different laboratory conditions can be variable considered (Winterbourn *et al*., 1975). According to the obtained results (Table 3-6) the highest enzyme activity was observed 24 min after adding riboflavin to the reaction mixture, which was the best time to measure SOD activity in this series of tests.

Effect of dryness on superoxide dismutase activity in branchlets of two haloxylon species in different drought stress intensities (control, 7, 14, 21 and 28 days lack of irrigation).

Effect of drought stress on the changes of superoxide dismutase activity was performed using 5 levels of drought stress (from 0 to 28 days without irrigation). Evaluation of superoxide dismutase activity in the presence of varying amounts of enzyme extract of branchlets of two haloxylon species in zero drought stress (control), after 24 min of adding riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 mM, pH equal to 7.8), EDTA (0.007 mM), potassium cyanide (0.02 mM) and nitroblue tetrazolium (0.05 mM) suggests that optimom activity of SOD in branchlets without stress (control), for the 0.05 mg of extract protein in the reaction mixture was 11.31 and 10.29 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. With increasing the amount of extract in reaction mixture, enzyme activity increased, so that in the 1.6 mL of protein extract in the reaction mixture reached to 37.11 and 33.86 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively.

In treatment of seven days lack of irrigation, SOD optimom activity for the 1.6 mL of extract protein in reaction mixture was 39.57 and 36.27 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. The lowest enzyme activity for the 0.05 mg extract protein was 13.81 and 12.74 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively that were 25.76 units lower compared with the enzyme optimom activity (Table 7).

Fourteen days treatment of drought stress showed that the optimom activity of SOD for the 1.6 mg of extract protein in reaction mixture was 42.60 and 39.30 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. The lowest enzyme activity for the 0.05 mg extract protein was 16.90 and 15.81 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively (Table 8).

In treatment of 21 days of drought stress, SOD optimom activity for the 1.6 mL of extract protein in reaction mixture was 46.49 and 43.19 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. By reducing the amount of extract, the enzyme optimom activity fell and for the 0.05 mg of extracts protein decreased to 20.29 and 19.70 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively (Table 9).

At 28-days treatment of drought stress, SOD optimom activity for the 1.6 mg of extract protein in reaction mixture increased to 52.66 and 49.36 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. The lowest enzyme activity for the 0.05 mg of extract protein was 26.96 and 25.87 IU (mg pro.)$^{-1}$ that was seen in *H. aphyllum* and *H. persicum*,

Table 7: Superoxide dismutase activity in the presence of varying amounts of enzyme extract of branchlets of two *Haloxylon* species, At drought stress 7 days without irrigation after 24 min of adding Riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

	SOD activity [u (mg protein^{-1})]	
Mg protein in reaction mixture	*Haloxylon persicum*	*Haloxylon aphyllum*
0	0	0
0.05	12.74±0.74	13.81±0.77
0.1	14.19±0.68	15.32±0.71
0.2	17.47±0.65	18.29±0.64
0.4	25.27±0.71	29.66±0.62
0.8	31.76±0.67	35.82±0.75
1.2	35.79±0.72	39.11±0.65
1.6	36.27±0.76	39.57±0.74

EDTA: Ethylene diamine tetraacetic acid, (0.007 mM), Potassium cyanide (0.02 mM) and nitroblue tetrazolium (0.05 M)

Table 8: Superoxide dismutase activity in the presence of varying amounts of enzyme extract of branchlets of two *Haloxylon* species, At drought stress 14 days without irrigation after 24 min of adding Riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

	SOD activity [u (mg protein^{-1})]	
Mg protein in reaction mixture	*Haloxylon persicum*	*Haloxylon aphyllum*
0	0	0
0.05	15.81±0.81	16.90±0.86
0.1	17.25±0.76	18.41±0.73
0.2	20.52±0.66	21.85±0.62
0.4	28.31±0.74	32.71±0.71
0.8	34.81±0.79	38.89±0.69
1.2	38.81±0.61	42.18±0.75
1.6	39.30±0.77	42.60±0.83

EDTA: Ethylene diamine tetraacetic acid (0.007 mM), Potassium cyanide (0.02 mM) and NitroBlue tetrazolium (0.05 M)

Table 9: Superoxide dismutase activity in the presence of varying amounts of enzyme extract of branchlets of two *Haloxylon* species, At drought stress 21 days without irrigation after 24 min of adding Riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

	SOD activity [u (mg protein^{-1})]	
Mg protein in reaction mixture	*Haloxylon persicum*	*Haloxylon aphyllum*
0	0	0
0.05	19.70±0.73	20.79±0.78
0.1	21.10±0.65	22.29±0.73
0.2	24.40±0.79	25.70±0.81
0.4	32.17±0.64	36.59±0.69
0.8	38.65±0.71	42.71±0.77
1.2	42.69±0.68	46.08±0.66
1.6	43.19±0.77	46.49±0.75

EDTA: Ethylene diamine tetraacetic acid, (0.007 mM), Potassium cyanide (0.02 mM) and NitroBlue tetrazolium (0.05 M)

Table 10: Superoxide dismutase activity in the presence of varying amounts of enzyme extract of branchlets of two *Haloxylon* species, At drought stress 28 days without irrigation after 24 min of adding Riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

	SOD activity [u (mg protein^{-1})]	
Mg protein in reaction mixture	*Haloxylon persicum*	*Haloxylon aphyllum*
0	0	0
0.05	25.87±0.96	26.96±0.93
0.1	27.27±0.84	28.46±0.87
0.2	30.57±0.87	31.87±0.81
0.4	38.34±0.89	42.76±0.86
0.8	44.82±0.74	48.88±0.89
1.2	48.86±0.83	52.25±0.79
1.6	49.36±0.79	52.66±0.75

EDTA: Ethylene diamine tetraacetic acid, (0.007 mM), Potassium cyanide (0.02 mM) and NitroBlue tetrazolium (0.05 M)

respectively that were 25.70 units (in *H. aphyllum*) and 23.50 units (in *H. persicum*) lower compared with the enzyme optimom activity (Table 10).

Evaluation of drought stress treatments (control, 7, 14, 21 and 28 days without irrigation) (Table 11) showed that the enzyme activity increased with increasing stress levels. The highest SOD activity in both species of haloxylon was observed in the treatment of 28 days without irrigation, which its value was equal to 52.66 and 49.36 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. The increasing of enzyme activity from control treatment to treatment of 14 days without irrigation was with softer process and from treatment of 14 days without irrigation to treatment of 28 days lack of irrigation was with a more intense process in both species of haloxylon. So that the amount of increasing from control treatment to treatment of 14 days without irrigation was 5.5 IU (mg pro.)$^{-1}$ in both species of haloxylon. This amount of increasing from treatment of 14 days without irrigation to treatment of 28 days without irrigation was equivalent to 10 units per one mg protein of extract in both species of haloxylon. The lowest SOD activity in both species of haloxylon was related to the

Table 11: Comparison of superoxide dismutase activity in the presence of the highest amount of enzyme extract (1.6 mg protein in the reaction mixture) of branchlets of two *Haloxylon* species in the 5 levels of tension

Different treatments of drought stress	SOD activity [u (mg protein^{-1})]	
(days without irrigation)	*Haloxylon persicum*	*Haloxylon aphyllum*
0 (Control)	33.86±0.78	37.11±0.66
7	36.27±0.76	39.57±0.74
14	39.30±0.77	42.60±0.83
21	43.19±0.77	46.49±0.75
28	49.36±0.79	52.66±0.75

control treatment. The amount of activity was equivalent to 37.11 and 33.86 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. The highest SOD activity in both species of haloxylon was belonging to the treatment of 28 days without irrigation. The amount of activity was equivalent to 52.66 and 49.36 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively.

Despite abundant researches about drought stress and its effect on various plants, it seemed that in the case of valuable plants such as haloxylon must be done more studies. So a part of the studies was devoted to the effects of drought stress on superoxide dismutase activity in branchlets of *H. aphyllum* and *H. persicum*.

Numerous studies in different plant systems show the link between environmental stresses such as drought with performance of antioxidants such as superoxide dismutase. In most of these results, the rate of SOD activity and enzyme mRNA is also altered. The review of two haloxylons under treatment of drought stress showed that, these plants were resistant to drought and drought caused major changes in their metabolism of SOD enzyme. Haloxylon tension under different periods of drought (7, 14, 21 and 28 days) was led to an increase in SOD activity at severe tensions. When haloxylon species were under mild drought stress (7 and 14 days without irrigation), SOD activity was more than control treatment and less than treatments of 21 and 28 days lack of irrigation (severe stress), respectively. SOD activity in *H. aphyllum* seedlings under drought stress of 21 and 28 days was, respectively 20.2 and 29.5% more than its activity in control treatments. This activity in *H. persicum* seedlings under drought stress of 21 and 28 days was, respectively 21.6 and 31.4% more than the activity in control treatments (Table 11).

The increasing of SOD activity under drought stress can be caused by changes in the synthesis and further accumulation of active enzymes. It is not clear that drought stress, increased activity of SOD-encoding gene or has had an impact on enzyme activity. Researches in different species of plants have shown that drought stress increases SOD activity in the plants tolerant to

water deficit, as in the sensitive varieties is led to decrease of enzyme activity. The rate of changes of SOD activity in addition to type and the inherent characteristics of plants depends on to factors such as tissues and stages of plant development and in particular severity and duration of drought stress. SOD total activity in maize leaves was inversely correlated with the severity of stress. So that high stress intensities caused a severe reduction in plant growth and subsequently, the plant was wilted (Menezes-Benavente *et al*., 2004). In drought-resistant varieties of peas the activity of chloroplast Cu/Zn SOD increased up to 1.7 times after a 15-days drought stress and under 20-days drought stress, Fe SOD showed greater increase (about 3.5 times). It seems that increasing of drought tolerance rate needs to special isozymes induction of SOD in specific organs. In drought-sensitive varieties of this plant, with increasing of stress intensity not only chloroplast SOD activity did not increased but also cytosol and mitochondrial SOD activity decreased too (Gomez *et al*., 2004).

Hydrophilic properties of *Beta vulgaris* (the plant is susceptible to drought) are limited its cultivation in the areas under drought stress. In drought-sensitive varieties of this plant, SOD activity decreased with increasing stress intensity, while increased in varieties that were tolerant to drought (Bor *et al*., 2003). Decreasing of SOD activity was also observed in the drought-sensitive varieties of rice. Ability of these varieties in the radical removal of O_2^{0-} decreased during drought and ROS accumulated and degradation of the membranes was done subsequently (Dionisio-Sese and Tobita, 1998).

This series of researches at transgenic plants topics is very important, so that gene too expression leads to more tolerance of oxidative stress in plants (Malan *et al*., 1990). Of course, the ability or capacity of drought tolerance in plants is not determined only by SOD and activity of other enzymes such as ascorbate peroxidase, catalase and glutathione reductase with non-enzymatic antioxidants have also an effective role.

Effect of different concentrations of two inhibitors of potassium cyanide and sodium azide on the superoxide dismutase activity in branchlets of two *Haloxylon* species: The enzyme activity in the presence of 0.2 mg of extract protein in the reaction mixture and the absence of inhibitor was equivalent to 16.30 and 15.06 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. The enzyme activity was gradually reduced with increasing concentration of KCN in the reaction mixture. The activity in concentrations of 10, 100 and 1000 mM of KCN declined up to 50.2, 76.1 and 92.6% in both species of haloxylon, respectively. And finally, the enzyme was fully controlled in concentrations of 0.01 and 0.1 M and showed no activity (Table 12).

It also with doubling the amount of extract in reaction mixture (0.4 mg), the enzyme activity was reduced, such that the rate of it in reaction mixture without KCN was equal to 27.19 and 22.81 IU (mg pro.)$^{-1}$ in *H. aphyllum* and *H. persicum*, respectively. At concentrations of 10, 100 and 1000 mM of KCN, the enzyme activity was reduced to 33.4, 55.6 and 70.4%, respectively in both species of haloxylon and eventually in the two concentrations of 0.01 and 0.1 M of KCN and unlike previous results, the enzyme was not fully inhibited and its activity decreased up to 80.3 and 81.2% in both species of haloxylon (Table 13).

Potassium cyanide is one of the superoxide dismutase inhibitors, which in many cases has been pointed to its inhibitory role (Goldberg and Stern, 1976; Tyler, 1975; Weisiger and Fridovich, 1973). Due to the variable sensitivity of types of SOD isozyme to different inhibitors, using them as a useful tool for identifying the isozyme type in the extracts is very useful. Cyanide ion (CN-) with forming the copper-cyanide complexes leading to competitive inhibition of Cu/Zn SOD. The cyanide ion is used for detecting this type of SOD from others which are not sensitive to it. Effect study of different

Table 12: Superoxide dismutase activity in the presence of varying amounts of Potassium Cyanide and 0.2 mg enzyme extract of branchlets of two haloxylon species, After 24 min of adding Riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

KCN (m) in reaction mixture	SOD activity [u (mg protein 1)]	
	Haloxylon persicum	Haloxylon aphyllum
0	15.06±0.354	16.30±0.241
0.00001	07.49±0.382	08.12±0.285
0.0001	03.71±0.361	03.90±0.253
0.001	01.11±0.326	01.20±0.271
0.01	00.00±0.338	00.00±0.222
0.1	00.00±0.375	00.00±0.263

EDTA: Ethylene diamine tetraacetic acid, (0.007 mM), Potassium cyanide (0.02 mM) and NitroBlue tetrazolium (0.05 M)

Table 13: Superoxide dismutase activity in the presence of varying amounts of potassium cyanide and 0.4 mg enzyme extract of branchlets of two haloxylon species, After 24 min of adding Riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

KCN (m) in reaction mixture	SOD activity [u (mg protein 1)]	
	Haloxylon persicum	Haloxylon aphyllum
0	22.81±0.589	27.19±0.531
0.00001	15.20±0.512	18.12±0.501
0.0001	10.13±0.555	12.08±0.545
0.001	06.75±0.593	08.05±0.572
0.01	04.50±0.523	05.36±0.585
0.1	04.19±0.588	05.11±0.611

EDTA: Ethylene diamine tetraacetic acid (0.007 mM), Potassium cyanide (0.02 mM) and NitroBlue tetrazolium (0.05 M)

concentrations of KCN on SOD activity of two under-stressed haloxylon species showed that the amount of enzyme activity was reduced with increasing of inhibitor concentration in the reaction mixture. So that the enzyme was completely inhibited at concentrations of 0.01 and 0.1 M of KCN (Table 12).

According to these results were obtained in the presence of 0.2 mg extracts protein in the reaction mixture, it can be inferred that the isozyme in extracts of both species of haloxylon is Cu/Zn SOD, that shows sensitivity to inhibition by KCN. With doubling the amount of extract and repeating the experiment, this time enzyme activity also decreased with increasing inhibitor concentration in the reaction mixture. But unlike previous results, the enzyme was not completely inhibited at concentrations of 0.01 and 0.1 M of KCN (Table 13). It can be supposed to justify this condition, when the amount of extract was doubled in the reaction mixture, another isozyme of the SOD which was not sensitive to (CN-) was activated. The isozyme did not show itself in less amount of the extract, or was less active. Sodium azide inhibitor was used for probable detection of the isozyme.

The enzyme activity was observed up to 16.30 and 15.06 IU (mg pro.)$^{-1}$ [in the presence of 0.2 mg of extract protein and the absence of inhibitor (sodium azide) in the reaction mixture] in *H. aphyllum* and *H. persicum*, respectively. In concentration of 10 mM of sodium azide, the enzyme activity decreased up to 16 and 17.4% in *H. aphyllum* and *H. persicum*, respectively. With increasing of sodium azide concentration in the reaction mixture, the enzyme activity was not inhibited and gradually increased. So that its activity at concentrations of 0.01 and 0.1 M of azide increased up to 1.3 times and 1.5 times compared with the control (without inhibitor) in both species of haloxylon. This result is the indicator of sodium azide dual behavior with other components of the reaction mixture. So that at high concentrations was increased SOD activity (Table 14).

Table 14: Superoxide dismutase activity in the presence of varying amounts of sodium Azide and 0.2 mg enzyme extract of branchlets of two *Haloxylon* species, After 24 min of adding Riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

	SOD activity [u (mg protein^{-1})]	
Azide (m) in reaction mixture	*Haloxylon aphyllum*	*Haloxylon persicum*
0	16.30±0.414	15.06±0.395
0.00001	13.69±0.432	12.45±0.415
0.0001	15.67±0.385	14.43±0.380
0.001	17.60±0.426	16.36±0.401
0.01	21.12±0.416	19.88±0.425
0.1	23.62±0.397	22.37±0.388

EDTA: Ethylene diamine tetraacetic acid (0.007 mM), Potassium cyanide (0.02 mM) and NitroBlue tetrazolium (0.05 M)

With doubling the amount of extract (0.4 mg) in reaction mixture, this time, the low concentration of sodium azide was increased the enzyme activity. So that, the amount of the activity in the reaction mixture without sodium azide, was equal to 27.19 and 22.81 in *H. aphyllum* and *H. persicum*, respectively. At concentration of 10 mM of sodium azide, the enzyme activity decreased up to 37.9 and 45.2% in *H. aphyllum* and *H. persicum*, respectively. With increasing of sodium azide concentration in the reaction mixture, enzyme activity was not inhibited and gradually increased, so that at concentration of 1000 mM of sodium azide was equal with control (without inhibitor) sample. At concentrations of 0.01 and 0.1 M of sodium azide, the enzyme activity increased up to 1.5 and 1.8 times in both species of haloxylon (Table 15).

Sodium azide inhibited all isozymes of SOD partially but was also observed differences in the rate of their inhibition. Azide acts according to the order of Cu/Zn<Mn<Fe SOD. Thus, the lowest and the highest sensitivity to inhibition by azide pertain to Cu/Zn SOD and Fe SOD, respectively (Meier *et al*., 1998). Several studies have shown that Fe SOD inhibition by azide is pH dependent. So that at low pH (6.8), N_3-binds to the sixth binding position of Fe+3 (the binding site of O_2^{0-}) in the active position. At this pH, the place is empty and at alkaline pH the rate of enzyme inhibition is less with the place being occupied by water molecules or hydroxyl anion (Meier *et al*., 1998).

The effect of different concentrations of NaN_3 on superoxide dismutase activity in the presence of 0.2 mg of branchlets extract of two haloxylon species, showed that for every ten mM of azide, the enzyme activity decreased up to 16 and 17.4% in *H. aphyllum* and *H. persicum*, respectively. The enzyme activity gradually increased with increasing of inhibitor concentration in the reaction mixture. The increasing of enzyme activity was possibly due to azide interference in the reaction (Table 14).

Studies have shown that sodium azide is a very active compound which researchers are reminded of it as

Table 15: Superoxide dismutase activity in the presence of varying amounts of sodium Azide and 0.4 mg enzyme extract of branchlets of two *Haloxylon* species, After 24 min of adding Riboflavin (0.12 mM) to the reaction mixture the volume of 3 mL and containing sodium phosphate buffer (0.067 M, 7.8 pH)

	SOD activity [u (mg protein^{-1})]	
Azide (m) in reaction mixture	*Haloxylon aphyllum*	*Haloxylon persicum*
0	27.19±0.666	22.81±0.672
0.00001	16.89±0.679	12.50±0.644
0.0001	21.19±0.642	16.79±0.665
0.001	27.08±0.696	22.77±0.681
0.01	40.62±0.605	34.15±0.649
0.1	48.74±0.688	40.98±0.657

EDTA: Ethylene diamine tetraacetic acid (0.007 mM), Potassium cyanide (0.02 mM) and NitroBlue tetrazolium (0.05 M)

suppressive (quencher) of ROS (Maisch *et al*., 2005). Therefore, azide at higher concentrations of 10 mM helps to SOD in the removal of superoxide anion with reducing or suppressing (quenching) $Q_2^{\circ-}$ radical and thus increases the activity of SOD. With doubling the amount of extract (0.4 mg) and repeating the experiment and in the presence of 10 mM azide, SOD activity decreased up to 37.9% and 45.2% in *H. aphyllum* and *H. persicum*, respectively. So, compared to the previous experiment (0.2 mg extract), the enzyme activity was inhibited up to 2.4% in both species of haloxylon (Table 15). In this amount of extracts (0.4 mg), as the last case, concentrations more than 10 mM azide led to increased activity of SOD. In the less amount of extract which enzyme was only inhibited up to 16% and 17.4% in *H. aphyllum* and *H. persicum*, respectively, we expected that the isozyme is Cu/Zn SOD, which has the least sensitivity to azide. While with doubling the amount of extract, another isozyme that is more sensitive to azide, was activated. It was Fe SOD.

CONCLUSION

Dryness stimulated superoxide dismutase and increased its activity. This increase was commensurate with the extent of drought or severe drought and had a direct correlation with it. The activity of this enzyme in *H. aphyllum* was more than *H. persicum*. Perhaps it could be said that one of the reasons of more resistance of *H. aphyllum* (compared with *H. persicum*) to drought stress (Arabzadeh, 2012b) is higher activity of superoxide dismutase in this species.

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