Antioxidant capacity response of Bakhtiari savory (*Satureja bachtiarica* Bunge.) to plant density and organic fertilizers in dryland farming conditions

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Abstract

Antioxidant enzymes synthesis in plants is an essential process for increasing their tolerance to abiotic stresses. Plant cells and their organs are protected by antioxidant enzymes with removing reactive oxygen species (ROS). To investigate the effect of plant density and organic fertilizers on the antioxidant enzymes activity including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), polyphenol oxidase (PPO), ascorbate peroxidase (APX), and electrolyte leakage (EL) of Bakhtiari savory (*Satureja bachtiarica* Bunge.*)*, a field experiment was carried out at dryland farming conditions in Research Station of Forests and Rangelands of Homand, Damavand, during 2018 and 2019. The results indicated that organic substrates and plant density significantly changed antioxidant enzyme activities ($P \leq 0.05$). Under these conditions, cattle manure reduced the antioxidant enzymes of APX, SOD, PPO, and EL. High plant density resulted in less SOD, POD, APX, CAT, and EL compared with low plant density. Compared with control (nonorganic fertilizer), cattle manure decreased the activity of SOD, CAT, PPO, and EL by 8, 14, 8, and 28%, respectively in the second-year plants. High plant density decreased the activity of SOD, POD, APX, and CAT by 12, 25, 14, and 23%, respectively. The present study can suggest the use of cattle manure and high plant density to reach the optimal growth under dryland farming conditions through controlling antioxidant enzymes production.

Keywords: Bakhtiari savory, catalase, cattle manure, dismutase, electrolyte leakage, superoxide

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Introduction

Bakhtiari savory (*Satureja bachtiarica* Bunge.) is an endemic medicinal plant of Lamiaceae family in Iran with a wide distribution in the western, southwestern, and central regions of the country (Jamzad, 2009). Bakhtiari savory is considered as a valuable medicinal plant, and its flowering branches are widely used in the food and pharmaceutical industries. It is appropriate for growing in harsh environmental conditions such as

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dryland farming. The possibility of dryland farming is an excellent advantage for areas with limited groundwater levels. A large part of Iran's arable land has been allocated to dryland farming planting, which unfortunately has little performance due to non-compliance with dryland farming system principles and proper management. Recently, with the global changes and increasing the environment temperature, the rainfall is limited in most parts of the world and the importance of dry farming is much highlighted (Yirdaw et al., 2017). Due to the fact that the annual plants include high price for cultivation and need more labor forces, cultivating perennial medicinal plants as an alternative could be the best practice. The establishment of perennial medicinal plants can prevent the soil erosion caused by agricultural activities like repeated annual plow (Lebaschi and Sharifi, 2016).

One of the main principles of sustainable agriculture is the use of organic substances to mitigate adverse effects of chemical fertilizers (Emami Bistgani et al., 2018). Organic fertilizers are useful for plants, micro and macro organisms in the soil, and also can ensure human health (Sharma and Singh, 2002; Basak et al., 2019).

While it is believed that mineral fertilizers may reduce the antioxidant levels of the plant, organic fertilizers have already been established to be capable of reducing the antioxidant content of plants (Sinkovi et al., 2015). In dryland farming conditions, plant density is an effective strategy in improving plant yield, which depends on climate and rainfall. At optimal density, plants use maximum value of environmental factors such as light, moisture, and nutrients. Plant density per unit area is one of the most critical factors in creating competition among crops, especially for water and nutrients. The result of proper density is reduced stress and increased yield of medicinal plants in dryland farming systems. Therefore, achieving proper density is very important in different areas of dryland farming system (Blaise et al., 2021).

When the plants are exposed to environmental stresses such as water deficit, antioxidant potential including enzymatic and non-enzymatic systems protect plant physiological pathways like photosynthesis through minimizing the oxidative damages to reach the optimal growth (Desikan et al., 2005; Ahanger et al., 2017). Plants' adaptation to drought stress is complicated as it is affected by internal stress tolerance mechanisms and environmental factors (Shamim et al., 2009). The plants' antioxidant activity increases over time due to their physiological activity during the time when the they are exposed to environmental parameters such as humidity, temperature, and light (Naghuib et al., 2012).

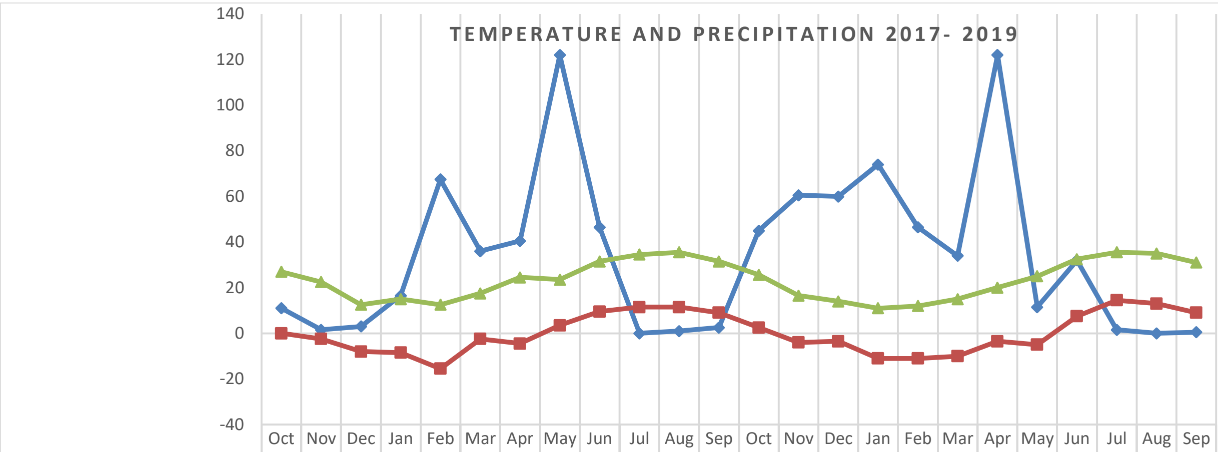
The physio-chemical mechanism of plants is an effective strategy to cope with different environmental changes. Antioxidant change is a powerful mechanism to protect plants under biotic and abiotic stress. Variations in antioxidant parameters such as reduced catalase (CAT) and superoxide dismutase (SOD) activities have been reported in *Satureja mutica* under cattle manure and high plant density (Saki et al., 2020). However, the useful effects of organic material on fortifying the antioxidant capacity have been reported in *Satureja macrantha* (Bakhtiari et al., 2020), *Satureja mutica* (Saki et al., 2019), and *Cymbopogon flexuosus* (Basak et al., 2020). Up to now, little is known about the antioxidant response of Bakhtiari savory to organic materials and plant density. Therefore, the aim of the present study was to evaluate the antioxidant enzyme activity and EL of Bakhtiari savory plants under different plant densities with application of organic substance at dryland farming conditions.

Materials and Methods

Plant material and growth condition

This experiment was conducted at Homand Rangeland Research Station (52° 20″ E and 35° 42″ N, 1960 asl) in Damavand district, Iran, during 2018-2019. The mean annual temperature is 12 ℃ with minimum and maximum temperatures as -16 ℃ in January-February and 35 ℃ in July-August, respectively. The mean annual precipitation was 340 mm (Fig. I).

Bakhtiari savory seeds were obtained by Research Institutes of Forests and Rangelands (RIFR), Iran. The seeds were planted in plastic trays containing a mixture of vermiculite and peat moss at a ratio



	Oct 2017	Nov 2017	Dec 2017	Jan 2017	Feb 2017	Mar 2017	Apr 2018	May 2018	Jun 2018	Jul 2018	Aug 2018	Sep 2018	Oct 2018	Nov 2018	Dec 2018	Jan 2018	Feb 2018	Mar 2018	Apr 2019	May 2019	Jun 2019	Jul 2019	Aug 2019	Sep 2019
Precipitation mm	11	1.5	3	16.5	67.5	36	40.5	122	46.5	0	1	2.5	45	60.5	60	74	46.5	34	122	11.5	32	1.5	0	0.5
Min temperature 0C	0	-2.5	-8	-8.5	-15.5	-2.5	-4.5	3.5	9.5	11.5	11.5	9	2.5	-4	-3.5	-11	-11	-10	-3.5	-5	7.5	14.5	13	9
Max temperature 0C	27	22.5	12.5	15	12.5	17.5	24.5	23.5	31.5	34.5	35.5	31.5	25.6	16.5	14	11	12	15	20	25	32.5	35.5	35	31

Fig. I. ambient thermic and annual percipitation diagram during 2017- 2019

of 1:1 in the greenhouse conditions. Two-month old and uniform seedlings were planted in the open field in Homand Damavand Rangeland Research Station. The soil properties of the experimental field are presented in Table 1.

Treatment details

This research was done as a split-plot based on completely randomized design (CRD) in three replicates. The main plot was organic fertilizer at three levels (cattle manure: 30 tons ha^{-1}, wheat straw enriched with ammonium sulfate: 10 tons ha^{-1}, and control: no fertilizer application). The subplot was plant density in three levels (low density: 26666 plant ha^{-1}, medium density: 40000 plant ha^{-1}, and high density: 80000 plant ha^{-1}). During the two-year experiment, we did not use any pesticide, and the weeds were removed manually. Plants were harvested at flowering stage above the soil surface in September of both years. The chemical analyses of cattle manure properties are presented in Table 2.

Enzyme extraction

To extract and measure the activity of enzymes, leaf samples were milled in the presence of liquid nitrogen. One mL phosphate buffer was added to 0.5 g of powdered leaf. Then, they were centrifuged for 15 min with 14000 rpm at 4 ℃. This extract was used to measure SOD, POD, CAT,

Table 1
Soil properties used in the case study in two soil depth (0-20 cm and 20-40 cm)

Year	Depth	pH	EC (dS.m^{-1})	OC (%)	N (%)	P (mg/kg)	K (mg/kg)	Sand (%)	Silt (%)	Clay (%)
2018	0-20 cm	8.1	0.8	1.1	0.97	18	270	21	46	33
	20-40 cm	8.3	0.4	1.1	0.98	17	255	21	42	37
2019	0-20 cm	8.1	0.9	1.3	0.96	21	273	20	47	33
	20-40 cm	8.2	0.4	1.2	0.95	19	259	21	43	36

Table 2
Chemical analysis of cattle manure properties

Fertilizer	Ash%	DM%	S%	N%	P%	K%	Ca%	OM%	Total K (mg/kg)	Total P (mg/kg)	EC (ds m^{-1})	pH
Cattle manure	14	92	1.78	2.1	1	0.5	1.2	39	2583.97	1299.5	16.4	8.2

PPO, and APX activities. All assays were done at 25 ℃ using a spectrophotometer (T80$^+$, PG Instrument., UK).

Superoxide dismutase enzyme (SOD)

The SOD activity was measured according to the methods of Beauchamp and Fridovich (1971) at 560 nm. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 200 mM methionine, 1.125 mM NBT, 1.5 mM EDTA, 75 µM riboflavin, and 0-50 µL of the enzyme. The reaction was conducted in test-tubes at 25 ℃ under illumination supplied by fluorescent lamps. The SOD activity was shown as U g^{-1} FW.

Peroxidase enzyme (POD)

The activity of POD was calculated by the oxidation of guaiacol in the presence of H_2O_2 at 470 nm (Chance and Maehly, 1955). The reaction mixture contained 100 µL of crude enzyme extract, 500 µL of 5 mM H_2O_2, 500 µL of 28 Mm guaiacol, and 1900 µL of 50 mM potassium phosphate buffer (pH 7.0). Finally, the enzymatic activity was reported as U g^{-1} FW.

Catalase enzyme (CAT)

The CAT activity was measured by the method of Beers and Sizer, (1952) at 240 nm. The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer (pH 7.0), 400 µL of 15 mM H_2O_2, and 40 µL of enzyme extract. The enzymatic activity was reported as U g^{-1} FW.

Polyphenol oxidase enzyme (PPO)

The activity measurement of PPO was performed using a 4-methylcatechol substrate. Three hundred (300) µL of enzyme extract was added to 300 µL 4-methyl catechol 0.5 M, and 2400 µL phosphate buffer solution (0.1 M, pH=7.2). Absorbance was read at 420 nm according to Luh and Phithakpol, (1972), and its activity was reported as U / 100 g^{-1} FW.

Ascorbate peroxidase enzyme (APX)

The APX activity was measured according to Nakano and Asada (1980) at 290 nm. The reaction mixture contained 50 mM (pH 7.0) potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H_2O_2, and 100 µL of the enzymes extract. The enzyme activity was finally reported as U g^{-1} FW.

Electrolyte leakage measurement (EL)

Measurement of electrolyte leakage (EL) was performed by the method of Lutts et al. (1995). For this purpose, fresh leaves were thoroughly washed with distilled water. From each leaf, 2 discs with a diameter of 1 cm were prepared and moved to Erlenmeyer flasks containing 10 ml of double-distilled water at 25 ℃. It was placed on a shaker and after 24 hours, the EC of samples was measured by a digital EC meter (EL_1 µS/cm). Containers containing the sample were placed in an autoclave for 1 hour at 120 ℃, the EC of the samples was measured (EL_2 µS/cm), and finally the EL was calculated with the following formula:

$$EL (\%) = (EL_1\ \mu S/cm\ /\ EL_2\ \mu S/cm) \times 100$$

Statistical Analysis

Data analysis was performed using SPSS software (version 24) and graphs were prepared through Excel. Mean comparisons (n=3) were made by Duncan's multiple range test.

Results

Superoxide dismutase enzyme (SOD)

The effect of organic fertilizer and plant density on the SOD activity was significant (P≤0.05, Table 3). Compared with control, cattle manure decreased SOD activity by 9%. However, the main changes of SOD activity were observed under plant density, in which low plant density increased SOD activity by 14% as compared to high plant density (Table 4).

Peroxidase enzyme (POD)

The POD activity significantly changed under year and plant density (P≤0.05, Table 3). The second-year plants showed higher POD activity relative to the first-year ones. The highest POD activity was obtained in plants experiencing low plant density (0.0079 U g^{-1} FW). However, there was no significant difference between high and medium plant density.

Table 3
Analysis of variance of physiological traits of medicinal plant Bakhtiari savory

S.O.V	df	SOD	POD	CAT	APX	PPO	EL
year	1	17.454^{ns}	0.0000602*	1.602*	0.296^{ns}	0.0056019*	130.667**
rep(Y)	4	2.686	0.0000046	0.098	2.574	0.0004685	4.722
Fertilizer	2	72.116*	0.0000005^{ns}	0.496^{ns}	200.907**	0.0003907*	122.389**
fer*year	2	14.857^{ns}	0.0000002^{ns}	2.629*	3.352^{ns}	0.0007796**	81.167**
E(fer)	8	8.573	0.0000023	0.474	4.435	0.0000519	4.583
density	2	250.934**	0.0000161**	16.172**	191.907**	0.0011352**	16.667*
den*year	2	14.279^{ns}	0.0000021^{ns}	1.635*	21.907*	0.0004241*	6.222^{ns}
den*fer	4	11.500^{ns}	0.0000037^{ns}	0.934^{ns}	3.991^{ns}	0.0002157^{ns}	0.972^{ns}
Year*fer*den	4	3.010^{ns}	0.0000029^{ns}	0.339^{ns}	18.546*	0.0001602^{ns}	5.472^{ns}
E	24	7.736	0.0000025	0.430	5.315	0.0001102	3.880
% C.V	-	5.97	23.59	9.89	6.23	13.12	7.12

*, **, and ns mean significant at 5%, 1%, and non-significant, respectively. SOD: Superoxide dismutase, POD: Peroxidase, CAT: Catalase, PPO: Poly-phenol oxidase, APX: Ascorbate Peroxidase, EL: Electrolyte leakage

Catalase enzyme (CAT)

Organic fertilizer and plant density significantly affected CAT activity (P≤0.01, Table 3). Applying cattle manure to the soil reduced CAT activity by 14% compared with control in the second-year plants (Table 5). The highest level of CAT activity (8.17 U g^{-1} FW) was observed at low plant density during the second year (Table 6).

Ascorbate peroxidase enzyme (APX)

The interaction effect of organic fertilizer, plant density, and year was significant on APX enzyme activity (P≤0.05, Table 3). Cattle manure reduced APX activity and so did the plant density. The lowest APX activity (31.3 U g^{-1} FW) was observed in the first-year plants treated with cattle manure at medium and high plant densities (Fig. II).

Polyphenol oxidase enzyme (PPO)

The effect of organic fertilizer and year as well as the effect of density and year were significant on PPO enzyme activity, (P≤0.05, Table 3). The highest PPO enzyme activity (0.095 U g^{-1} FW) was obtained in cattle manure treatment and in the second year; however, the lowest PPO enzyme activity was observed in the cattle manure in the first-year plants (Table 5). For plant density, we observed the minimum PPO enzyme activity (0.057 U g^{-1}) FW in high plant density in the first year. In contrast, the second-year plants under medium plant density had the maximum PPO enzyme activity (0.097 U g^{-1} FW) (Table 6).

Table 4
Comparison of mean physiological traits of Bakhtiari savory under the influence of the main study factors

Treatment	SOD U g^{-1} FW	POD U g^{-1} FW
Fertilizer		
Cattle Manure	44.42^{b}	0.0068^{a}
Wheat straw	46.84^{a}	0.0066^{a}
Control	48.40^{a}	0.0069^{a}
Plant Density		
High density	44.62^{b}	0.0062^{b}
Medium density	44.18^{b}	0.0063^{b}
Low density	50.86^{a}	0.0079^{a}

In each column, similar letters indicate no significant difference among the means (by using Duncan at the 5% level); SOD: Superoxide dismutase, POD: Peroxidase, CAT: Catalase, PPO: Poly-phenol oxidase, APX: Ascorbate Peroxidase, EL: Electrolyte leakage

Table 5
The interaction effect of year and fertilizer on physiological traits of Bakhtiari savory

Year	fertilizer	CAT U g^{-1} FW	PPO U g^{-1} FW	EL %
2018	Cattle Manure	6.70 ab	0.060 e	28.66 a
	Wheat Straw	6.43 ab	0.071 d	29.55 a
	Control	6.23 b	0.078 cd	29.44 a
2019	Cattle Manure	6.17 b	0.092 ab	20.66 b
	Wheat Straw	7.06 a	0.083 bc	29.22 a
	Control	7.15 a	0.095 a	28.44 a

CAT: Catalase, PPO: Poly-phenol oxidase, EL: Electrolyte leakage).

Electrolyte leakage (EL)

The effect of organic fertilizer and year on EL was significant (P≤0.01, Table 3). Cattle manure led to the lowest EL (20.6%) in the second-year plants.

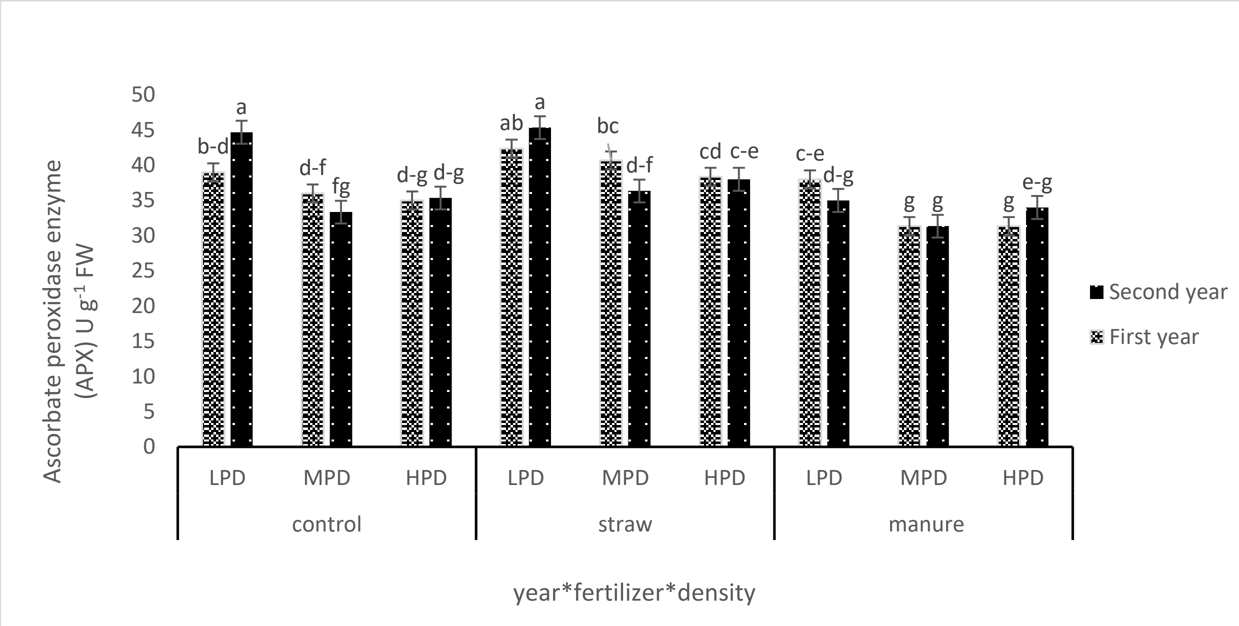


Fig. II. The interaction of organic fertilizer, plant density, and year on APX (Ascorbate Peroxidase enzyme) of medicinal plan Bakhtiari savory; control: no application, manure: cattle manure, straw: enriched wheat straw, LPD: low plant density, MPD: medium plant density, HPD: high plant density

Table 6
The interaction of year and plant density on physiological traits of Bakhtiari savory

Year	Plant Density	CAT U g^{-1} FW	APX U g^{-1} FW	PPO U g^{-1} FW
2018				
	High Density	5.86 c	34.88 b	0.057 d
	Medium Density	6.23 c	36.00 b	0.080 bc
	Low Density	7.26 b	39.77 a	0.072 c
2019				
	High Density	6.27 c	35.77 b	0.080 ab
	Medium Density	5.94 c	33.66 b	0.097 a
	Low Density	8.17 a	41.66 a	0.084 b

CAT: Catalase, APX: Ascorbate Peroxidase PPO: Poly-phenol oxidase

Table 7
The correlation between the studied traits

	SOD	POD	CAT	APX	PPO
POD	0.213				
CAT	0.603**	0.318*			
APX	0.616**	0.264	0.577**		
PPO	-0.004	0.249	0.060	-0.004	
EL	0.467**	-0.172	0.280*	0.481**	-0.290*

SOD: Superoxide dismutase, POD: Peroxidase, CAT: Catalase, PPO: Poly-phenol oxidase, APX: Ascorbate Peroxidase, EL: Electrolyte leakage

However, the highest EL (29.55%) was observed under enriched wheat straw treatment and during the first year (Table 5). Finally, the correlation coefficient between EL and SOD was positive and significant (P≤0.01, Table 7).

Discussion

Cattle manure and high plant density were able to reduce antioxidant enzyme activities. Decreased SOD enzyme in cattle manure treatment showed that cattle manure due to its high water availability and improvement of nutrient uptake reduces drought stress in plants (Saki et al., 2019). SOD is activated as the first line of antioxidant system defense against ROS. Under abnormal conditions, SOD plays a critical role in catalyzing HO_2 (ROS) into H_2O_2 (Gratao et al., 2005). Increased SOD activities under drought have been reported in various medicinal plants (Cheng et al., 2018; Saki et al., 2019; Bidabadi et al., 2020). Decreased SOD enzyme at high plant density shows that this practice compared to low plant density increased the plant canopy per unit area and reduced weed growth due to plant shading and eventually preserved soil moisture. This leads to increased plant tolerance to stress caused by sunlight particularly at low plant density. When the plants are exposed to environmental stresses such as shortage of moisture, antioxidant enzymes activity protect plant photosynthesis against oxidative damage (Bidabadi et al., 2020). In these conditions, SOD plays an important role in stress tolerance of plants through protecting membrane integrity from oxidase damage induced by abiotic stress (Wang et al., 2009).

The increase in POD enzyme level in control shows that the lack of organic fertilizer application reduced the plant's resistance against environmental stress and increased POD enzyme activity in dryland farming system conditions. It has been reported that supplying the plants with organic substrates can fortify their resistance against different abnormal conditions through changing the antioxidant enzymes activity (Dumas et al., 2003). Decreased antioxidant activity with organic fertilizers may be due to the availability of moisture and other elements including micronutrients (Feizabadi et al., 2021). Kumar et al. (2010) showed a decreased in POD activity of corn plants when organic fertilizer was used. Increased POD activity in low plant density can be due to less canopy level per unit area and increased weed growth compared to high plant density (Boutasknit et al., 2020). It seems that in dryland farming conditions, increased planting density due to competition for the absorption of moisture and soil nutrients leads to enhanced oxidative stress in the plant (Eskandari et al., 2010).

The highest CAT enzyme activity was observed in treatments of control and enriched wheat straw in the second year and also in low plant density treatment in the second year. Non-manure application and low plant density reduced the plant's resistance to environmental stress in dryland farming conditions. CTA can catalyze changing H_2O_2 into H_2O and oxygen (Qi et al., 2018). Increasing the activity of CAT enzyme is an adaptability strategy to prevent cell damage in plants via reducing the H_2O_2 produced by cellular metabolism (Gill and Tuteja, 2010). At low concentrations of H_2O_2, it is eliminated by other peroxidases collaboration such as glutathione and ascorbate (Mhamdi et al., 2010).

The results showed APX activity in cattle manure was less than in enriched wheat straw and control. In addition, APX activity reduced by increasing the plant density and the minimum activity of APX was observed at high plant density. Application of cattle manure and high plant density provides moisture and soil nutrients. It also improves the plant's ability to tolerate drought stress in dryland farming conditions, reducing the content of this enzyme. When plants are exposed to environmental stresses such as drought, antioxidant enzymes protect their photosynthesis against oxidative damage (Desikan et al., 2005). Similar to our results, Abbas et al. (2020) showed the remarkable reduction of antioxidant enzymes activities such as APX in maize plants when organic fertilizers were used as soil amendment.

The amount of PPO activity significantly decreased with cattle manure application. This can be due to the optimal moisture and nutrients uptake under organic fertilizers. Moreover, high plant density reduced PPO activity significantly. This is probably due to the plant shading and the prevention of light intensity in plants (Saki et al., 2019). In dryland farming conditions, the PPO activity increases due to the lack of moisture. The shortage of soil moisture leads to increased ROS accumulation and PPO can scavenge these radicals as an antioxidant defense system. The decreased PPO under organic fertilizer was reported on garlic plants (Boutasknit et al., 2020), which is consistent with our results.

The EL decreased in Bakhtiari savory with the cattle manure application. It seems that using cattle manure through improving the physical and chemical conditions of soil, such as increasing its water holding capacity, makes a desirable situation for plants and leads to reduced EL. in addition, the better plant density had significant a role on reduction of EL in Bakhtiari savory plants. Therefore, the lowest EL was obtained in cattle manure and high/medium densities. The improvement of EL under organic fertilizers have been previously reported by Bidabadi et al. (2017) and Zhu et al. (2017).

Conclusion

This study showed that in dryland farming conditions, the activity of antioxidant enzymes such as SOD, POD, CAT, and PPO, and also the amount of EL increased. Increasing the activity of antioxidant enzymes to neutralize and scavenge ROS produced due to water shortage stress was a resistance mechanism in plants. The minimum changes in the level of theses enzymes activities in plants treated with cattle manure and high plant density showed that these agricultural practices are able to make suitable environmental conditions and eventually reduce the need to produce antioxidant enzymes. Hence, we can recommend the use of cattle manure and high plant density to overcome environment variations through controlling antioxidant enzymes production to reach the optimal growth under dryland farming conditions.

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