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THE EFFECT OF PLANT DENSITY, VARIOUS ORGANIC AND NANO-FERTILIZERS ON ACCUMULATION AND ACTIVITY OF PROTECTIVE MOLECULES IN *CARTHAMUS TINCTORIUS* L.

SUMMARY

Agronomic management such as plant density and plant nutrition can affect the final performance of the plant through the alternation in molecular activity. A field experiment was designed to evaluate the effect of fertilizers (C: control, AM_{10}, and AM_{20}: application of 10 and 20 t ha^{-1} of animal manure, NPK, nano Fe+Zn fertilizers) and inter-row spacing (IS: 40 and 60 cm) on the accumulation and activity of some protective molecules in safflower plants in the Hamadan region-west of Iran. The application of all fertilizers significantly increased catalase activity compared to control conditions. Planting in narrower rows and applying nano Fe+Zn resulted in the highest ascorbate peroxidase activity and the lowest activity was recorded under NPK + IS_{60}. The consumption of micronutrients increased the activity of guaiacol peroxidase about 3 times compared to the control. The highest activity of total superoxide dismutase was obtained by using NPK+IS_{60}. The highest amount of protein in leaves was recorded in AM_{20} + IS_{60} and NPK+IS_{60} application conditions. The lowest amount of hydrogen peroxide was observed in plants grown under AM_{10} + IS_{60} conditions. Consumption of AM_{20} reduced the level of malondialdehyde by 42% compared to the control condition. However, the consumption of high levels of animal manure decreased the amount of proline in the leaves by 2 times compared to the control. Altogether, the results showed that lower planting density and the use of high levels of organic fertilizer, NPK, and nanostructured micronutrients had a significant effect on increasing antioxidant activity and improving the defense function of safflower

Keywords: antioxidants, ascorbate peroxidase, catalase, guaiacol peroxidase, proline

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INTRODUCTION

Safflower is an annual plant from the Asteraceae family that is grown as an oilseed, livestock feed, food additive, and also for its medicinal properties. Safflower seems to be a relatively low-expectation plant and is resistant to water shortage conditions (Zanetti *et al*. 2022). Although safflower is one of the oldest cultivated plants, during the past decades it has been less noticed by farmers and has been considered an under-utilized or forgotten plant (Emongor, 2010). It appears that the main reason for the low reception of safflower by farmers and its remaining as a minor crop is the low availability of managing information, and technical instructions for cultivation, as well as the lack of high yielding varieties (Emongor et al. 2017).

Although the root system of safflower is relatively deep and can use the moisture stored in one-meter-deep soil, many limitations in the soils of semi-arid areas severely reduce this ability of safflower (Singh *et al*., 2016). High soil pH, low organic matter, nutrient deficiencies or unavailability of nutrients, small effective rainfall during the active growth of the crop, and low depth of agricultural soil are some of the soil limitations that undesirably affect the performance of safflower in semi-arid areas. Therefore, nutritional management should be seriously considered (Chaudhary *et al*., 2023). Application of 20 t ha^{-1} animal manure along with 80 kg ha^{-1} of nitrogen caused a significant increase in vegetative growth and seed production in safflower grown in Baneh region (Fattahi *et al*.., 2023). The results of Maghsoud et al. (2024) showed that the use of organic fertilizers alone or in combination with chemical fertilizers improved the relative water content of leaves, and increased the amount of chlorophyll, and the concentration of soluble proteins in leaves. However, the application of organic fertilizer alone caused a decrease in carotenoid pigments, the concentration of soluble sugars, and the free proline content. Although some previous experiments have investigated and reported the effect of using different fertilizers on safflower in semi-arid regions, there is still not much information about the effect of nutritional management on intracellular processes such as the activity of antioxidant enzymes.

On the other hand, plant density per surface unit or inter-row spacing can affect plant performance by affecting the access to light, water, and nutrient resources in the soil (da Silva *et al*. 2024).

Adjusting plant density can provide the basis for the emergence of genetic potential and maximum yield. However, choosing the optimal plant density is influenced by several factors. Optimum density varies according to plant characteristics, horizontal and longitudinal growth rate, load resistance and soil condition, climatic conditions, soil fertility, altitude of the area above sea level, number of sunny hours, and evapotranspiration potential (Zhang *et al.* 2021). Safflower has the ability of lateral growth and the production of secondary branches, and this increases its flexibility in different densities, but it is not yet clear in which density the best yield can be achieved (Neuhaus, 2012). On the other hand, there is a negative compensatory relationship between the grain yield components. There is, and it seems that increasing the number of plants per unit area causes a decrease in the yield of a single plant due to the increase in intra-species competition (Kouchakkhani *et al*. 2024). However, there is still not much information about the effect of plant density on cellular and molecular activities. Evaluation of nutrition management and plant densities in different conditions can make the selection of safflower planting methods easier and more scientific. Agronomic management may cause redox changes in plant cells and hence lead to changes in the activity of antioxidant enzymes (Darvizheh et al. 2019). The use of nanoparticles has opened a new window to agricultural science. Foliar application of zinc nanoparticles was able to somewhat mitigate the damaging effects of drought stress in safflower compared to bulk particles (Ghiyasi *et al*. 2023). However, the effect of the use of beneficial nanoparticles on enzymatic antioxidant systems has not yet been well studied under the semi-arid region. In addition to antioxidant enzymes, proline as an Osmo-protectant and regulator of cell redox status plays a role in reactive oxygen species (ROS) scavenging (Atta *et al.,* 2024). Safflower is one of the forgotten plants, and any information about the effect of management methods such as density and fertilizer application on the functioning of its defense systems can be effective in improving agronomic methods. The present experiment aimed to investigate the effect of different inter-row spacing and application of different fertilizers (organic, chemical, and nano-structured) on the activities of antioxidant enzymes and some biomarkers concentration safflower grown in the semi-arid region in the west of Iran.

MATERIAL AND METHODS

Site description

A field experiment was conducted in the Damaq-Hamadan region in the west of Iran (35°26' N 48°49' E and the height from sea level was 1848 m) during 2022-2023. The mentioned area has a semi-arid-cold climate, the amount of rainfall during the growing season is 146 mm and the average annual temperature is 16 ˚C. Previous investigations of the soil of the studied location at a depth of 0-30 cm indicated that soil texture was loamy clay and contained 2727 mg kg^{-1} of K, 12 mg kg^{-1} of absorbable P, 0.14% of total N, and 0.43% of organic carbon. Further chemical properties of the soil were pH: 7.62, percentage of neutralizing materials: 8.38, electrical conductivity: 1.41 ds m-1, calcium carbonate: 13%, and cation exchange capacity: 19.8 Cmolc kg^{-1}. Micronutrients such as zinc and iron were 0.47 ppm and 0.61 ppm, respectively. The experiment was conducted to investigate the effect of inter-row spacing and the application of different fertilizers on some molecular activities in safflower leaves. The test was done in a factorial manner (2×5) based on an arbitrary block design with 3 repetitions. The first factor included the inter-row distances of 40 cm (IS_{40}) and 60 cm (IS_{60}). The second factor included the application of different fertilizers (C: control, AM_{10}, and AM_{20}: application of 10 and 20 t ha^{-1} of animal manure, NPK, nano Fe+Zn). Zinc oxide and iron oxide nanoparticles with 99% purity were obtained from Nano Pishgaman Company, Mashhad, Iran. X-ray diffraction and scanning electron microscope images indicated that the particle sizes were between 20 and 100 nm.

Implementation of treatments

Primary plowing was done during October 2022 with a reversible mold board plow, and the secondary plowing, and seedbed preparation were carried out at the end of March 2023. After plotting the field area, manure was used with the mentioned amounts and mixed with the soil with a depth of 10-15 cm through a rotavator. Then, the surface of the land was made into ridge and furrow form by furrower with the mentioned inter-row intervals. Seeds of Safeh variety were planted manually on the top of the ridge on April 7, 2023, with 5 cm inter-row spacing. NPK fertilizer included 100 kg ha^{-1} of nitrogen through urea fertilizer, 60 kg ha^{-1} of phosphate through triple superphosphate fertilizer, and 40 kg ha^{-1} of potassium through potassium sulfate. One-third of the nitrogen was consumed during planting and the rest during the stem elongation stage (BBCH scale: 31), and the beginning of heads emergence (BBCH scale= 50; Flemmer *et al*., 2015). Phosphorus and potassium fertilizers were used during planting. Micronutrient nanostructured fertilizers with the recommended amount of 2 kg ha^{-1} were used during planting.

At the end of the main head emergence stage, some physiological characteristics such as protein and proline content as well as the activity of some reactive oxygen species scavenging enzymes were evaluated by sampling from the upper developed leaves. The harvested leaves were placed inside the aluminum foil and were immediately transferred to the tank containing liquid nitrogen. 0.5 g of the leaves was digested in a ceramic mortar using some liquid nitrogen. The digested sample was poured into micro tubes and 2 ml of PBS (pH: 7) was added. Then the microtubes were centrifuged for 18 min at 20,000 rpm. Protein measurement was done by the Coomassie protein assay method (Bradford 1976).

Estimation of proline

The method of Bates et al. (1973) was used to measure proline. 0.5 g of the digested leaf sample was mixed with 10 ml of sulfosalicylic acid 3% and centrifuged for 10 min at 4 ˚C and 12000 rpm. Then 2 ml of supernatant phase was mixed with 2 ml of ninhydrin and galaceal acetic acid. After placing the desired solution in a bain-marie bath, the solution was mixed with 4 ml of toluene and vortexed for 20 sec. Finally, the proline concentration of the samples was calculated using a spectrophotometer and the standard curve of pure proline at a wavelength of 520 nm.

Measuring the activity of antioxidant enzymes

The activity of guaiacol peroxidase enzyme was measured by the method of Chakmak and Horst (1991). To measure guaiacol peroxidase, after extracting cell contents by extraction buffer, 50 µl extracted solution, 600 µl of Phosphate-buffered saline (PBS) (pH=7), 600 µl of guaiacol, and 600 µl of hydrogen peroxide to the reaction mixture. After reading the amount of absorbance in the solution without the plant sample at 470 nm wavelength by spectrophotometer, the absorbance changes were read at 0 and 60 seconds at 470 nm wavelength.

Catalase enzyme activity was measured by the method of Mishra et al. (1993). The reaction mixture included 750 µl of hydrogen peroxide, 2 µl of PBS (pH=7), 75 µl of plant-extracted solution was prepared. From the resulting mixture, the plant sample component was blanked at a wavelength of 240 nm, and the absorbance was read at 0 and 60 seconds at a wavelength of 240 nm.

To measure the ascorbate-peroxidase enzyme, 700 microliters of ascorbate, 700 µl of PBS (pH=7), 500 µl of ETDA, and 700 µl of hydrogen peroxide were added to 50 µl of plant sample. Then 500 µl of water was added to the reaction mixture. After reading the amount of absorbance in the solution without the plant sample at 290 nm wavelength by spectrophotometer, the absorbance changes were read at 0 and 60 seconds at 290 nm wavelength (Dzung *et al*., 2011).

To measure hydrogen peroxide concentration, 5 ml of trichloroacetic acid 1% was added to 0.5 g homogenized leaf sample, and centrifuged at 1200 rpm for 15 min. Then the absorbance was measured at 390 nm for the reaction complex containing 0.5 ml supernatant, 0.5 ml of 10 mM PBS and 1 ml of 1 M Kl (Velikova *et al*., 2000). The method by Rao and Sresty (2000) was used to measure malondialdehyde content.

Statistical analysis

Data analysis was performed based on one-way ANOVA. Before statistical analysis, a data normality test was performed and statistical analysis was performed through SAS software (v. 9.1). The least significant difference test was used to compare the means. Box plots were drawn through SPSS (v. 20). Component analysis (PCA), and clustering of combined treatments were executed by Statistica (v. 10.1) software.

RESULTS AND DISCUSSION

The results of the analysis of variance are shown in Table 1. The activity of all antioxidant enzymes as well as the concentration of biomarkers such as proline and malondialdehyde were significantly affected by the use of fertilizers. The effect of fertilizer application was much more evident than the effect of inter-row distances on the evaluated components. The effect of fertilizer application was significant at the statistical level of 1% and the mutual effects of FIRS at the level of 5% on the activity of ascorbate peroxidase (APX) enzyme. The highest activity of APX was seen under the condition of Nano Fe+Zn + IS_{40} and in plants grown with C+IS_{40}. However, the lowest activity was recorded under the condition of the effect of fertilizer application was significant at the statistical level of 1% and the mutual effects of F×IS at the level of 5% on the activity of ascorbate peroxidase (APX) enzyme.

However, the lowest activity was recorded under the condition of application of NPK+IS_{60}. The comparison between fertilizer levels also showed that the highest activity of APX was recorded in plants grown with the application of nano-structured micronutrients and AM_{10}. The lowest activity of APX was related to plants grown under NPK application conditions (Figure 1).

Table1. Fertilizer application and inter-row spacing on antioxidant enzymes and proline concentration safflower (*Carthamus tinctorius* L.) leaves.

		APX	CAT	GPX	TSOD	PRO	H_2O_2	MDA	PLN
Inter-row spacing	IS_{40}	0.130a	0.057a	0.382a	3.15a	11.87a	1.206a	215.05a	249.59a
	IS_{60}	0.121a	0.065a	0.360a	3.80a	12.75a	0.96b	200.51a	234.95a
Fertilizers	C	0.138a	0.020b	0.247c	2.35c	10.75c	1.24a	264.79a	303.17a
	AM_{10}	0.140a	0.065a	0.260c	2.59c	11.46bc	1.15a	180.92c	298.57a
	AM_{20}	0.121ab	0.069a	0.418b	3.22bc	13.44ab	0.76b	152.12c	170.01c
	NPK	0.089b	0.074a	0.362bc	5.32a	13.61a	1.09a	213.31b	216.18b
	Nano-Fe+Zn	0.143a	0.080a	0.571a	3.89b	12.63ab	1.18a	227.79b	223.42b
	C+ IS_{40}	0.154a	0.015c	0.27de	1.53e	10.82b	1.37a	281.92a	14.67a
	C+ IS_{60}	0.121abcd	0.026bc	0.22e	3.19cd	10.69b	1.11abc	247.65ab	291.67ab
	AM_{10}+ IS_{40}	0.147ab	0.068a	0.25de	2.54de	10.75b	1.24ab	188.74e	305.64a
	AM_{10}+ IS_{60}	0.132abcd	0.062ab	0.25de	2.66cde	12.18ab	1.06bc	173.1de	291.51ab
	AM_{20}+ IS_{40}	0.105bcd	0.058ab	0.46bc	2.92cde	11.93ab	0.85cd	156.46e	184.64de
	AM_{20}+ IS_{60}	0.137abc	0.081a	0.37bcde	3.53bcd	14.31a	0.68d	147.78e	155.38e
	NPK+ IS_{40}	0.091cd	0.075a	0.41bcd	4.69ab	13.59a	1.25ab	218.99bc	195.99cde
	NPK+ IS_{60}	0.086d	0.074a	0.31cde	5.96a	13.64a	0.93cd	207.64bcd	236.37cd
	Nano Fe+Zn+ IS_{40}	0.157a	0.072a	0.50ab	4.09bc	12.30ab	1.31ab	229.15bc	24.02bc
	Nano Fe+Zn+ IS_{60}	0.129abcd	0.087a	0.63a	3.68bcd	12.96ab	1.058bc	226.43bc	199.81cde
					statistical significance				
IS		*NS*	*NS*	*NS*	*	*NS*	**	*NS*	*NS*
F		**	*	**	**	*	*	**	*
IS×F		*	*	*	**	*NS*	**	**	*NS*

IS_{40}: Inter-row spacing 40 cm, IS_{60}: Inter-row spacing 60 cm, C: no fertilizer application or control, AM_{10}, and AM_{20}: application of 10 and 20 t ha^{-1} animal manure, NPK: chemical macronutrient fertilizer containing 3 main elements nitrogen, phosphorus, and potassium, Nano-Fe+Zn: nano-structured zinc and iron chelated on EDTA (ethylenediaminetetraacetic acid), APX: ascorbate peroxidase enzyme activity (µmol min^{-1} mg^{-1} protein), CAT: catalase (µmol min^{-1} mg^{-1} protein), GPX: guaiacol peroxidase (µmol min^{-1} mg^{-1} protein), TSOD: total superoxide dismutase (µmol min^{-1} mg^{-1} protein), PRO: protein concentration (mg g^{-1} FW), H_2O_2: concentration of hydrogen peroxide (nmol g^{-1} FW), MDA: malondialdehyde content (nmol g^{-1} FW), PLN: Proline content (µmol g^{-1} FW). In each column means with different letters have statistically significant differences at the 5% level ($P \leq 0.05$). *: significant at 0.05 statistical level, **: significant at 0.01 level, NS: not statistically significant.

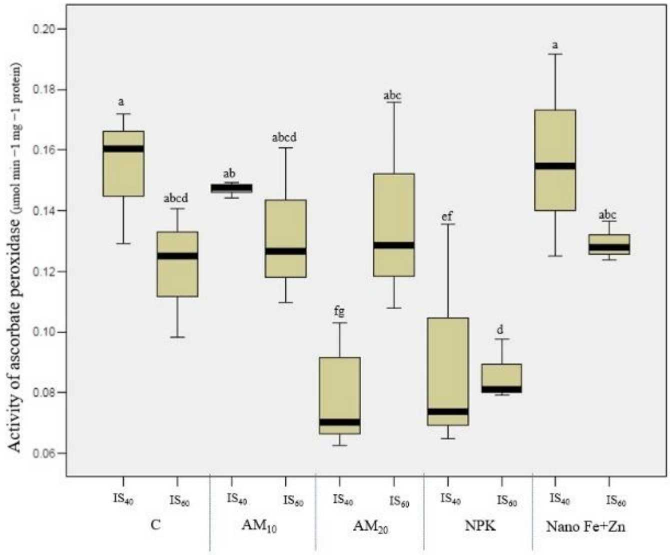

Figure 1. The effect of application of different fertilizers and spacing between planting rows on the activity of ascorbate peroxidase enzyme in safflower in the Hamadan region, west of Iran.

The evaluation of CAT enzyme activity showed that the main effect of fertilizer and the mutual effect of F×IS was significant at the 5% level. Consumption of all fertilizers increased CAT activity by about 4 times compared to the control. However, there was no significant difference between the used fertilizers in terms of CAT activity. The application of NPK and nano-structured micronutrient fertilizers in both inter-rows spacing caused the highest CAT activity.The interaction effect of F×IS was significant for guaiacol peroxidase (GPX) activity. The comparison of mean GPX between fertilizer levels indicated that the application of nanostructured fertilizers and AM_{20} increased the activity of the mentioned enzyme by 231% and 169% compared to the control (Figure 2). However, the highest GPX activity was recorded with the application of iron and zinc nanostructured fertilizers (0.63). The lowest activity of GPX was recorded in plants grown under no fertilizer application and with 40 cm inter-row spacing (0.22).

The interaction effects of IS*F were significant for the activity of total superoxide dismutase isozymes (TSOD). The highest TSOD activity was recorded in plants grown with the application of NPK fertilizers at 60 cm (5.69) inter-row spacing, and the plants grown under NPK+IS_{40} conditions were in second place. Increasing the inter-row distances in the condition of not using fertilizer, AM10, and NPK utilization significantly increased the activity of TSOD. This increase in the control condition was about 208%. However, under the conditions of application of micronutrient nanostructured fertilizers, the reduction of inter-row distances caused an 11% increase in TSOD activity (Figure 3).

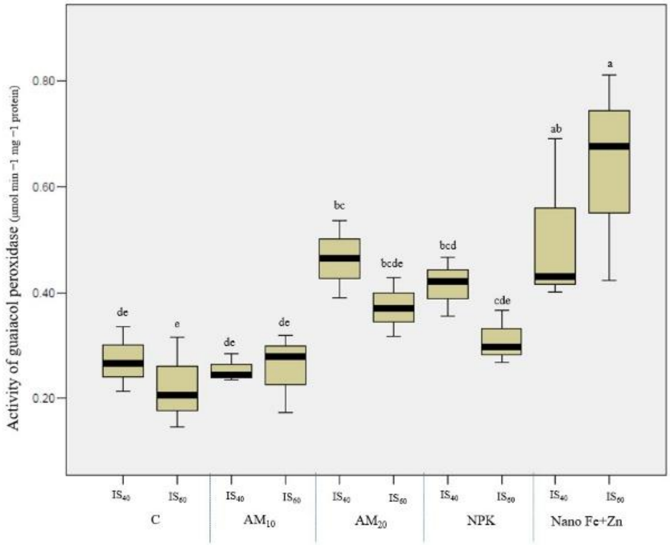

Figure 2. Mean comparison of guaiacol peroxidase activity in safflower leaves under different inter-row spacing and various fertilizers application.

Fertilizer use had a significant effect on protein content. The use of NPK, AM_{20}, and nanostructured fertilizers increased this component by 27%, 25%, and 17% over the control, respectively. The evaluation of the concentration of hydrogen peroxide showed that the mutual effects of F×IS were statistically significant at the level of 1%. Increasing the distance between rows from 40 to 60 cm increased the amount of this reactive oxygen species by 21%.

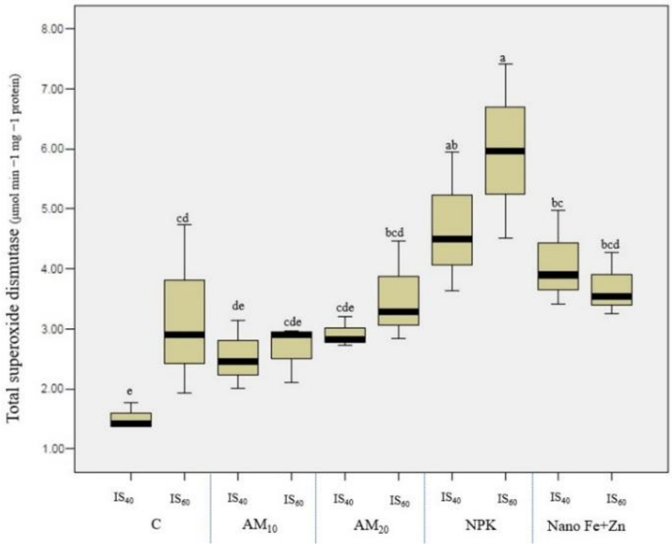

Figure 3. Evaluation of the effect of application of different fertilizers and inter-row distances on total superoxide dismutase activity of safflower in the Hamadan region in western Iran.

The highest amount of hydrogen peroxide was recorded in plants grown under the condition of not using fertilizer + IS_{40}. The plants cultivated with the application of micronutrient nanostructured fertilizers (1.31 nmol g^{-1} FW), NPK + IS_{40} (1.25 nmol g^{-1} FW), and AM_{10} + IS_{40} (1.24 nmol g^{-1} FW) were in the next position (Figure 4).

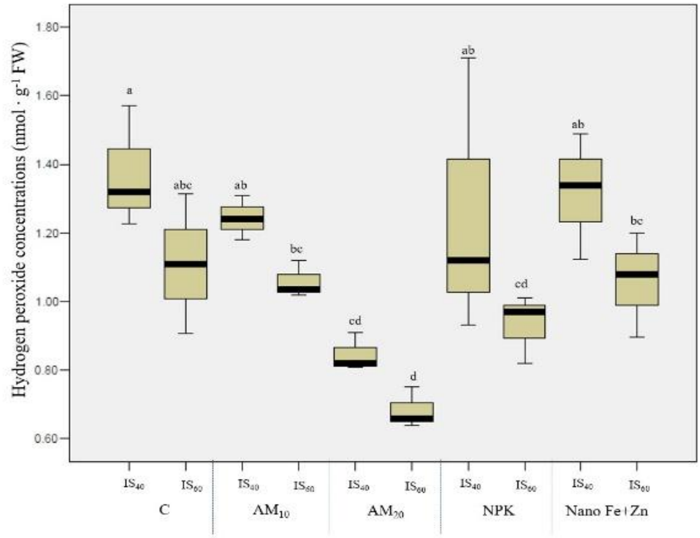

Figure 4. Hydrogen peroxide concentration in safflower leaves under different conditions of fertilizer application and inter-row spacing in the Hamadan-Iran region.

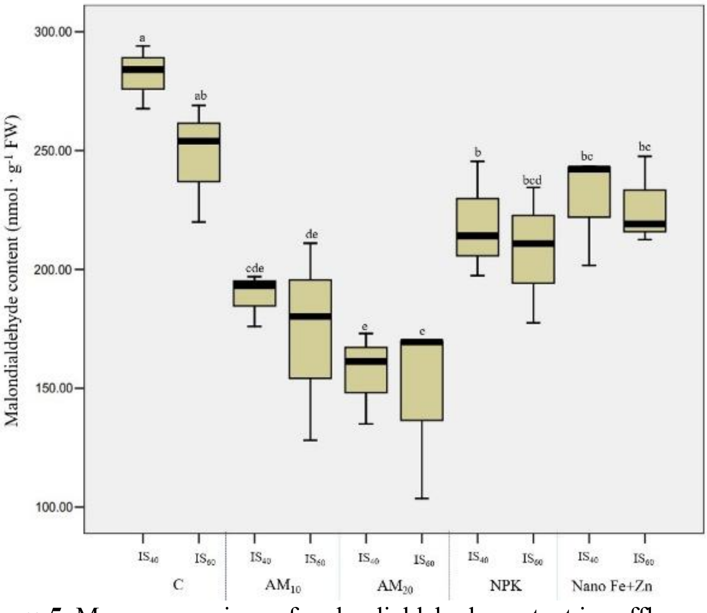

Figure 5. Mean comparison of malondialdehyde content in safflower leaves under different conditions of fertilizer management and different Inter-row spacing.

The interaction effect of F×IS for malondialdehyde (MAD) was statistically significant at the 1% level. Although the effect of inter-row distances on this component was not significant, the application of fertilizers caused a significant reduction in this component compared to the control.

The lowest amount of MAD was recorded in plants grown with the application of AM_{20} (152.12 nmol g^{-1} FW). The comparison of combined treatment is shown in Figure 5. The highest amount of MAD was recorded in plants grown under no fertilizer application + IS_{40} (281.65 nmol g^{-1} FW FW) and the lowest amount was related to plants grown under the application of AM_{20} (151). It is interesting to note that the application of animal manure caused a significant decrease in MAD. The evaluation of proline content indicated that the effect of fertilizer application at the statistical level of 5% on this biomolecule was significant ($p<0.05$). The highest amount of proline was recorded in plants grown under control conditions (303 µmol g^{-1} FW), and AM_{10} (298 µmol g^{-1} FW FW). The lowest amount was related to plants grown under application of AM_{20} (170.1 nmol g^{-1} FW).

Clustering of treatment combinations showed that no fertilizer application or low-level manure application at both row spacings had similar effects on the studied traits and were placed in cluster 1. This group showed weak performance for most traits. However, the application of NPK, nano-micronutrient and AM_{20} fertilizers, especially at 60 cm row spacings, stimulated the activity of protective molecules more (Figure 6).

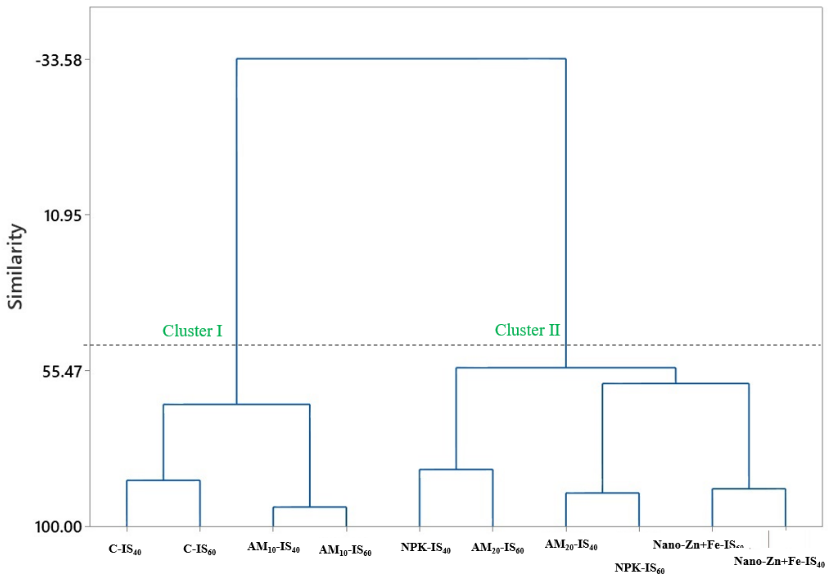

Figure 6. Clustering of treatment combinations based on similarity in their reactions to the application of fertilizers and inter-row spacing of safflower in Hamedan-Iran.

Inter-angle correlations indicated that catalase activity had a significant positive correlation with protein content. The greatest effect on catalase enzyme was not recorded in AM_{20}+ IS_{40} conditions. However, a negative correlation was observed between catalase enzyme activity and ascorbate peroxidase activity with an angle of about 180 degrees (Cos 180° = -1). The correlation between the total activity of superoxide dismutase isozymes and guaiacol peroxidase was positive and significant. The highest activity of these enzymes was recorded under the conditions of application of NPK and Fe and Zn-nanostructured fertilizers + IS_{60}. Regarding the 90-degree angle between malondialdehyde or hydrogen peroxide with protein and catalase content, no significant relationship was seen between them (Cos 90° = 0). A correlation between hydrogen peroxide and malondialdehyde was observed. Proline content showed a significant correlation with ascorbate peroxidase and the highest values of the mentioned components were recorded under AM_{10} conditions.

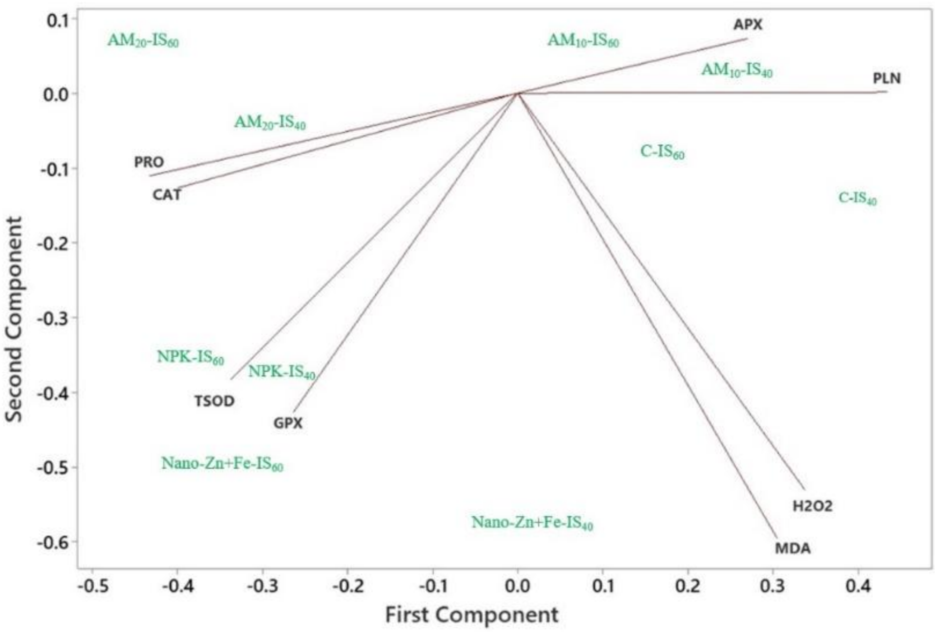

Figure 7. Principal component analysis (PCA) plot to interpret the distribution of treatment compounds and their effectiveness on the investigated molecular traits in safflower under the conditions of application of different fertilizers and various inter-row distances.

Although safflower is a plant that is relatively low expected and resistant to adverse environmental conditions such as drought and heat, a brief overview of the soil condition in the region indicated that the deficiency of micronutrients as well as macronutrient elements was evident. In addition to the low of organic matter and high EC was also other limitations of the soil, which probably causes significant difficulties in the release and absorption of elements in the rhizosphere

(Zgallai *et al*., 2024). These functions finally regulate the intracellular redox state and cause an acceptable performance of the plant. These basic conclusions are consistent with finding of Maghsoudi *et al*., 2024 who reported that the protein content, the content of chlorophyll pigments and carotenoids, the efficiency of photosystem II and the proline content were improved by the integrated application of organic and chemical fertilizers.

Although in the present study the main effect of plant density or inter-row distances on the evaluated physiological components was not significant, the mean comparison of the combined treatments often indicated the superiority of plants grown under 60 cm inter-row distances. It seems that the effect of fertilizer concentration and application on intracellular functions depends to a large extent on other conditions such as soil conditions, climatic conditions (annual variables) and even plant genetics. However, in the rice plant, the evaluation of ascorbate peroxidase, superoxide dismutase, peroxidase, and catalase showed that the application of nitrogen increased the activity of the mentioned enzymes, however, the increase in plant density in some years caused a decrease and, in some years, caused an increase in antioxidant activities (Wang *et al.,* 2022). As the authors mentioned before, the physiological responses of the plant are the result of the functioning of the intracellular pathways, many of these pathways are cross-talking with each other, and this makes the examination and identification of key points affected by the treatments more complicated. However, the use of omics methods can be a great help in the detailed description of intracellular mechanisms against the application of fertilizers and agricultural management treatments.

Malondialdehyde (MDA) can be a suitable indicator for evaluating the amount of oxidative damage and oxidation of membrane lipids (Zhang *et al*., 2021). In the present study, the use of animal manure significantly reduced the amount of MDA. It seems that by improving the physical and chemical conditions of the soil, the use of animal manure has improved the functioning of intracellular biochemical pathways and reduced electron deviation and production of reactive oxygen species. However, increasing the activity of antioxidant enzymes and improving the integrity of the membrane can also be one of the reasons for the reduction of MDA.

Although NPK chemical fertilizers and micronutrient nanostructured fertilizers also had a significant effect on the activity of antioxidant enzymes and other biological molecules evaluated by providing part of the nutritional requirement, the effect of farmyard manure was more prominent. The physical and chemical conditions of the soil in the investigated area were poor, and on the other hand, the low rainfall and its asymmetric distribution aggravated the dire soil conditions. In such a situation, it seems that improving the physical, chemical and biological conditions of the soil through the use of animal manures should be considered as a management priority. The scavenging of reactive oxygen species is a complex network with countless crosstalk with many biochemical pathways. Therefore, the increase or decrease of hydrogen peroxide can be caused by the operation of other different pathways, such as signaling (Fatima *et al*., 2023). Therefore, the interpretation of such data should be considered with caution and doubt. A comprehensive interpretation of the impact of agricultural management such as the use of fertilizers and planting density requires the evaluation of different antioxidant networks (including enzymes and molecules with scavenging properties) within cells, obtaining sufficient information on genetic backgrounds and gene expression, translation of transcripts, and the accumulation of proteins is due to the use of fertilizers.

CONCLUSIONS

The finding of the experiment revealed that plant density and providing nutrients from various fertilizer sources can affect the activity of antioxidant enzymes and accumulation of some protective molecules in safflower. The activity of antioxidant enzymes increased with the application of NPK fertilizers and nanostructured micronutrient fertilizers. On the other hand, the lowest malondialdehyde content and plasma membrane integrity was recorded under the conditions of organic fertilizer application. The results showed that application of AM_{20} was able to stimulate the activity of most protective molecules significantly compared to other fertilizer treatments. The effect of fertilizer treatments was more evident at lower densities with row spacings of 60 cm.

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