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Relationship between fertilization and planting depths on antioxidant activity in saffron (*Crocus sativus* L.)

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ABSTRACT

As saffron is planted in autumn, freezing stress can damage the cell membrane, reducing the corm's growth. These effects justify the necessity of studying agronomic strategies to promote plant tolerance to freezing stress. This study evaluated the impact of fertilization and planting depth on some antioxidant activities in saffron by using split plots in a randomized complete block design with three replications in 2020 and 2021. The fertilization includes 1- Nitrogen, potassium, and phosphate fertilizers based on soil test (NPK), 2- Biofertilizers (BIO) (nitrogen-fixing bacteria, phosphates, and potassium soluble bacteria), 3- Chemical fertilizers (CHE) (calcium, microelements, potassium and amino acids) and 4- Combined method (BIO- CHE) and planting depth (10 and 15 cm). Catalase activity levels were found to be higher using nitrogen, potassium, and phosphate fertilizers in 2020 and biofertilizer at a depth of 15 cm in 2021. In comparison to nitrogen, potassium, and phosphate fertilizers, with planting at a depth of 10 cm, this was an increase of 96%. Superoxide dismutase enzyme activity was highest in 2020 from nitrogen, potassium, and phosphate fertilizers and in 2021 from chemical fertilizers. When compared with nitrogen, potassium, and phosphate fertilizers and a planting depth of 10 cm, the increase was 38.7%. Chemical fertilizers resulted in a higher dry weight of corm in 2020, while in 2021, the combined method and 10 cm planting depth produced the highest dry weight. In 2020, corm dry weight correlated significantly with leaf catalase, leaf polyphenol oxidase, and superoxide dismutase activities in the mother corm, while inversely correlated with leaf proline and mother corm polyphenol oxidase activities. As a general recommendation, use chemical fertilizers and plant 15 cm deep to reduce winter freeze stress.

1. Introduction

Traditionally, saffron (*Crocus sativus* L.) has been referred to as "red gold" since it is one of the most valuable plants in the world (Leone et al., 2018). Saffron grows in arid and semi-arid regions with high altitudes, low annual rainfall, cold winters, and hot summers (Kafi, 2006; Khilare et al., 2019). Because saffron is triploid, it is only propagated vegetatively by corms (Gresta et al., 2009). Each year, each corm (mother corm) produces other corms (daughter corm) depending on its size and cultivation conditions (Landi, 2007). As the daughter corm grows, the mother corm decomposes. A saffron's yield depends on the growth of daughter corms in the previous season (Renau-Morata et al., 2012). In general, the storage of nutrients in the mother corm determines the growth of saffron. In other words, a larger mother corm provides more energy to the growing daughter corms (Douglas et al., 2014). Many factors affect the size of a daughter corm, including climatic conditions, physical and chemical properties of soil, corm density, planting depth and nutrients.

The effects of chemical fertilizers on the environment and on human health are adverse (Ju et al., 2018), while biofertilizers (plant growth promoting bacteria (PGPR)) are natural fertilizers containing free-living bacteria that enrich the soil with essential nutrients without harming the environment or human health. Therefore, biofertilizers are important components of sustainable agriculture since they reduce the need for mineral fertilizers ((Rezaei et al., 2018). Moreover, PGPR cause (a) stimulates plant growth by the production and release of phytohormones such as indole acetic acid, cytokinins, and gibberellins; (b) enhance symbiotic N_2-fixation; (c) solubilize inorganic phosphate and the mineralization of organic phosphate and/or other nutrients; and (d) resist, tolerate or compete with detrimental microorganisms (Lugtenberg and Kamilova, 2009). Consequently, PGPR improves the growth and yield of the plant (Zhang and Kong, 2014).

It has been shown that PGPR reduces oxidative stress in bean (*Phaseolus vulgaris* L.) plants by increasing the activity of antioxidant enzymes (such as superoxide dismutase, catalase, peroxidase, and

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Nomenclature

PGPR	Plant growth-promoting bacteria.
ROS	Reactive oxygen species.
SOD	Superoxide dismutase.
POD	Peroxidase.
CAT	Catalase.
PPO	Polyphenol oxidase.
NPK	Nitrogen, phosphate, and potassium fertilizers.
BIO	Biofertilizers.
CHE	Chemical fertilizers.
BIO-CHE	Combined method including biofertilizers and chemical fertilizers.
RWC	Relative water content.
MSI	Membrane stability index.
H_2O_2	Hydrogen peroxide.
O_2	Oxygen.
H_2O	Water.
O^{-2}	Superoxide anion.
CO_2	Carbon dioxide.

glutathione reductase) (Tiryaki et al., 2019). When wheat (*Triticum aestivum* L.) is subjected to low-temperature stress conditions, using nitrogen (urea) reduces the levels of soluble sugar, proline, and abscisic acid (Li et al., 2022). Under cold stress conditions, wheat, and barley (*Hordeum vulgare* L.) plants treated with boron and plant growth-promoting bacteria exhibit higher antioxidant enzyme activities (catalase, peroxidase, and superoxide dismutase) (Turan et al., 2013).

Planting depth is the first and most crucial factor for proper root growth. Plants with broader roots can absorb the elements better and more. The planting depth can affect the severity of the cold to plant. When plants grow too high or too low in-depth, nonuniform germination and emergence occur, reducing yields and resource wastage (Wato, 2019). Planting depth not only affects the yield of saffron but also affects the amount of corm production. Plants grown at low depths have more buds than those grown at higher depths, creating more daughter plants (Negbi, 1990). But the low planting depth due to the growth of this plant in the cold seasons of the year, the risk of freezing damage, drought stress, and plant extinction.

Low temperature is one of the main factors limiting plant growth (Chen et al., 2014). Freezing stress, in addition to affecting plant growth and development, significantly limits the geographical distribution of plants (Liu and Zhou, 2018). When plants are exposed to freezing stress, their physiological responses are altered, as well as their cell membrane lipid composition and concentrations of proteins and metabolites (Kazemi-Shahandashti and Maali-Amiri, 2018). Temperature stress also accelerates generating and accumulation of reactive oxygen species (ROS) (Xu et al., 2008). High levels of ROS are harmful to all cellular compounds, adversely affect cellular metabolic processes, and increase lipid peroxidation (Mishra et al., 2011; Zhang et al., 2019). Plants reduce ROS damage by producing enzymatic and non-enzymatic antioxidants (Zhang et al., 2019). Some of the enzymes that remove ROS include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and polyphenol oxidase (PPO) (Sharma et al., 2012). The non-enzymatic response of plants to stresses involves the accumulation of osmolites such as soluble carbohydrates, proline, soluble proteins, etc., which are responsible for osmotic regulation under stress (Hasanuzzaman et al., 2019). Even though saffron is grown in temperate and arid regions, the production of daughter corms occurs in the cold half of the year. Therefore, saffron spends most of its growing season under autumn cold and winter freeze conditions, limiting its development. Intensive cold in some years affects the saffron's survival, growth, and development (Koocheki and Seyyedi, 2019a). Studying the effects of freezing stress on saffron has received less attention in previous studies. Antioxidants are thought to play a role in resistance to freezing stress. Despite this, a comprehensive study has yet to examine saffron's enzymatic and non-enzymatic antioxidants. Several factors, such as fertilization and planting depth, can influence the tolerance of plants to cold stress. We

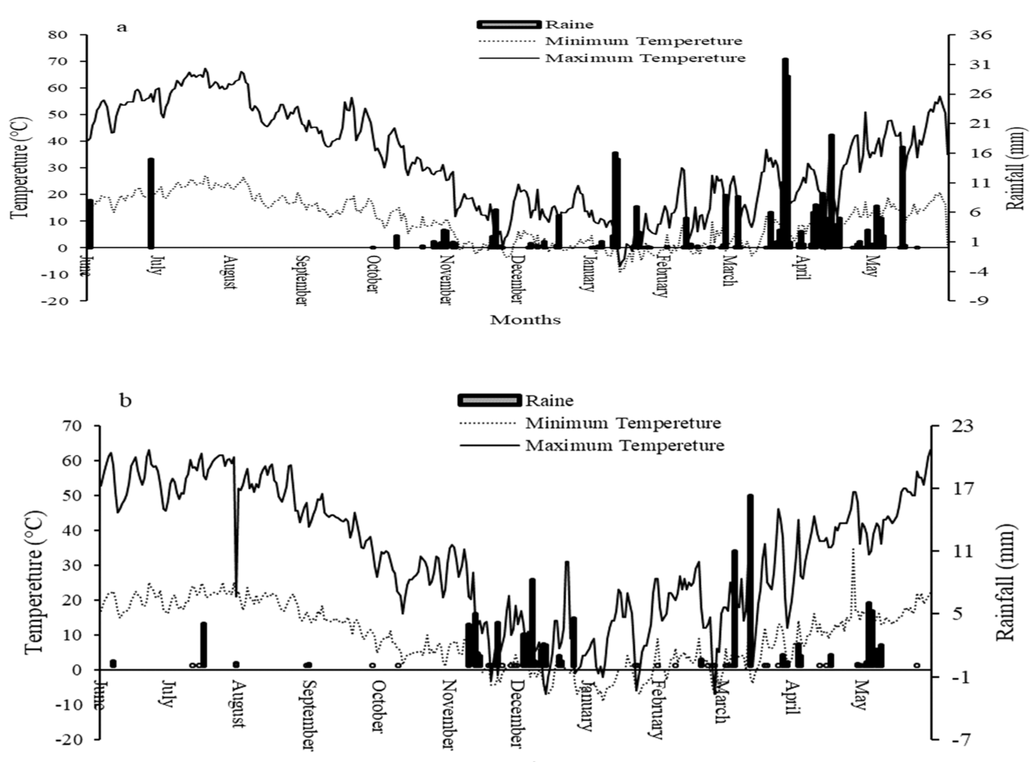

Fig. 1. Minimum and maximum daily temperature and rainfall during the saffron growing season in 2019–2020 (a) and 2020–2021 (b) in Mashhad.

Table 1
Physical and chemical properties of the soil at the test site.

Year	Soil texture	Organic carbon (%)	Nitrogen (%)	Available phosphorus (ppm)	Available potassium (ppm)	EC (dS/m)	pH
2019–2020	Silt loam	0.66	0.072	13.5	138	3.45	8.12
2020–2021	Silt loam	0.67	0.089	11.07	130	3.97	8.12

propose that the planting depth of 15 cm and the Biofertilizers and Chemical Fertilizers (BIO-CHE) nutritional program are the most resistant to freezing stress. Therefore, the following questions have been investigated as part of this study's objectives:

1. What changes occur in the cell membrane and the enzymatic and non-enzymatic antioxidants in saffron leaves and mother corms?
2. Does fertilization improve saffron's resistance to cold stress?
3. What is the recommended planting depth for ridge-furrow planting saffron? 10 or 15 cm

2. Materials and methods

2.1. Site description

The present study was conducted during the growing seasons of 2019–2020 and 2020–2021 in the Faculty of Agriculture, the Ferdowsi University of Mashhad, Iran (latitude: 36 degrees north; longitude: 59 degrees east; altitude: 985 m). The meteorological information of the test site is presented in Fig. 1 (Anon, 2021). Details of the physical and chemical properties of the test soil are given in Table 1.

Fertilization and planting depth were arranged in split plots based on a randomized complete block design (RCBD) with three replications in two consecutive years. Nutrition program (main plot) in four levels: 1- Nitrogen, phosphate, and potassium fertilizers based on soil test (NPK), 2- Biofertilizers (BIO) (Nitrogen-fixing bacteria (NFB)), phosphate solubilizing bacteria (PSB), and potassium solubilizing bacteria (KSB), 3- Chemical fertilizers (CHE) (calcium fertilizers, microelements, potassium, and amino acids) and 4- Combined method (BIO-CHE) and planting depth (10 and 15 cm) (subplot).

2.2. Agronomic practices

Each experimental plot had eight rows four meters long, and the distance between the rows was 50 cm. The final density was 75 plants per square meter. A distance of one and a half meters was considered between the blocks to eliminate the possible effects of the treatments. In the NPK treatment before planting corms, nitrogen fertilizer (urea form (46%, N) in the amount of 150 kg /ha twice, one before flowering and the other one, one month after flowering in each stage 50%), phosphate (form Triple superphosphate (46%, P_2O_5) in the amount of 50 kg /ha) and potassium (in the form of potassium sulfate (including potassium (K_2O)) at least 50% by weight, sodium (NaCl) at most 2% by weight, sulfur (S) at least 17.5% by weight and chlorine (CL) up to 2.5% by weight (175 kg /ha)) were dispersed on the soil surface based on soil test and then mixed with soil.

Nitrogen-fixing bacteria, including the genus *Bacillus* sp. *Azospirillum* sp. *Azotobacter* sp., (with a population of 10^7 per ml under the brand name Nitrobacter Dayan). Phosphate and potassium solubilizing bacteria, including the genera *Bacillus* sp. and *Pseudomonas* sp., (with a population of 10^7 per ml under the brand name Phospho and Potas Power Bacteria Dayan). Biofertilizers were applied at seven l/ha at the same time as the first irrigation (before flowering).

Calcium fertilizer include calcium (19% W/ V, CaO) and nitrogen (10% W/ V, N), microelements including sulfur (3.2% W/ V, S), iron (0.6% W/ V, Fe EDTA), manganese (1.3% W/ V, Mn), zinc (1.36% W/ V, Zn), copper (0.36% W/ V, Cu), boron (1.48% W/ V, B), molybdenum (0.013% W/ V, Mo) and vitamins B and C, potassium including potassium (54% W/ V, K_2O) and phosphorus (45% W/ V, P_2O_5) and amino acids (containing 21 types of amino acids). Daneshbonian Khoshe Parvarane Zist Fanavar Company provides all these products.

Nutritional with CHE was applied in three stages. In the first stage, calcium (three l/ ha) + amino acids (five l/ ha) at the same time as the first irrigation (before flowering). In the second stage, potassium (three l/ ha) + amino acids (three l/ ha) + microelements (two l/ ha) were applied simultaneously with the second irrigation (after flowering). In the third stage of foliar application of potassium with amino acid (one l/ 1000) on 12 March 2020. In BIO-CHE treatment, both biofertilizers and chemical fertilizers were used. All fertilizers were applied every year. Saffron corms (5–8 g) were planted on 15 July 2019. In both years, weed management was done by hand.

2.3. Properties to be studied

Each year, after winter in March, sampling was performed, and the samples were transferred to the laboratory. The following characteristics were measured in the leaf and mother corm:

Relative water content (RWC).

Relative water content was calculated using Eq. (1) based on the difference between fresh weight (FW), turgid weight (TW) and dry weight (DW) (Pieczynski et al., 2013):

$$RWC = (FW - DW)/ (TW - DW)\ 100 \quad (1)$$

Membrane stability index (MSI).

The membrane stability index was obtained from Eq. (2) based on the primary electrical conductivity (EC1) of 24 h in distilled water and the secondary electrical conductivity (EC2) after autoclaving (Ilík et al., 2018):

$$MSI= ((EC1/ EC2) \times 100) - 100 \quad (2)$$

Proline content.

Proline content was measured according to the method of Bates et al. (1973).

The activity of antioxidant enzymes.

Superoxide dismutase (SOD): Yu and Rengel (1999) at 560 nm were used to measure the total SOD.

Catalase (CAT): Velikova et al. (2000) method was used to obtain CAT enzyme activity at a wavelength of 240 nm.

Peroxidase (POD): The amount of POD at 470 nm was obtained by the method of Srinivas et al. (1999).

Polyphenol oxidase (PPO): Polyphenol oxidase was measured at 420 nm according to Kar and Mishra (1976) method.

2.4. Data analysis

All data were analyzed using SAS 9.4 software. Excel software was used to draw the graphs. Bartlett test was performed for the studied characteristics in each year, and because the differences were not significant, composite analysis was not performed.

3. Results

The Lowest temperature during the growing season 2019–2020 was recorded on 12 January (−9.5 °C), and during the growing season 2020–2021 on 6 January (−9 °C) (Fig. 1). While saffron grows in arid and semi-arid regions, the overground parts of the plant are exposed to winter freezes during the cold half of the year. Therefore, low winter

Table 2
Analysis of variance of studied characteristics in saffron under the influence of nutrition and planting depth in 2020.

S. O. V	df	Mean of squares											
		Relative water content	Membrane stability index	Leaf Proline	Mother corm Proline	Leaf Catalase	Mother corm Catalase	Leaf Peroxidase	Mother corm Peroxidase	Leaf Polyphenol oxidase	Mother corm Polyphenol oxidase	Leaf Superoxide dismutase	Mother corm Superoxide dismutase
Block	2	16.0 ^{ns}	1.06 ^{ns}	0.072 ^{ns}	0.004 ^{ns}	5886 ^{ns}	118 ^{ns}	0.0007 ^{ns}	0.0033 ^{ns}	273*	48.4 ^{ns}	0.0002 ^{ns}	0.00006 ^{ns}
Fertilizer (F)	3	70.6*	22.0**	9.007**	0.337**	25008**	22.2 ^{ns}	0.0107**	0.0316**	1588**	56470**	0.3621**	0.0351**
Error a	6	36.9	1.67	0.292	0.008	3417	197	0.0020	0.0020	115	180	0.0003	0.0012
Planting depth (P)	1	496 ^{ns}	8.05 ^{ns}	13.2**	0.086*	3927 ^{ns}	100 ^{ns}	0.0026 ^{ns}	0.0229*	3020**	83627**	0.0009 ^{ns}	0.0190*
F*P	3	4.93 ^{ns}	62.8**	11.2**	0.169**	4129 ^{ns}	1375**	0.0274**	0.0080 ^{ns}	2748**	48016**	0.0011 ^{ns}	0.0017 ^{ns}
Error	8	11.2	2.67	0.295	0.010	2918	130	0.001	0.0021	52.7	113	0.0009	0.0018
C.V%		4.33	2.76	25.2	13.0	24.8	9.98	22.0	26.2	10.0	12.0	6.26	14.8

** and * and ^{ns}: respectively indicate significance at the probability levels of one and five percent and the lack of significant differences. C.V: Coefficient of variation.

Table 3
Analysis of variance of studied characteristics in saffron under the influence of nutrition and planting depth in 2021.

S. O. V	df	Mean of squares											
		Relative water content	Membrane stability index	Leaf Proline	Mother corm Proline	Leaf Catalase	Mother corm Catalase	Leaf Peroxidase	Mother corm Peroxidase	Leaf Polyphenol oxidase	Mother corm Polyphenol oxidase	Leaf Superoxide dismutase	Mother corm Superoxide dismutase
Block	2	14.8 ^{ns}	16.1 ^{ns}	0.019 ^{ns}	0.027 ^{ns}	2130 ^{ns}	271 ^{ns}	0.034 ^{ns}	0.00003 ^{ns}	71521*	120420 ^{ns}	0.0001**	0.000009 ^{ns}
Fertilizer (F)	3	37.8 ^{ns}	126**	0.240**	0.655**	7433**	2824*	0.618**	0.0849**	598681**	10246365**	0.0003**	0.0010**
Error a	6	18.3	4.10	0.015	0.018	357	719	0.068	0.0007	7913	25485	0.0001	0.00006
Planting depth (P)	1	169**	275**	0.596**	0.711**	3408 ^{ns}	40838**	0.202*	0.0317 ^{ns}	1129268**	15683283**	0.0071**	0.0031**
F*P	3	61.4*	117**	0.076*	0.054 ^{ns}	7741**	10831**	0.119*	0.0221 ^{ns}	1307043**	7974188**	0.0073**	0.0008**
Error	8	13.5	10.6	0.102	0.0178	878	577	0.024	0.0064	10862	130643	0.000005	0.00003
C.V%		4.22	4.95	14.2	6.23	18.0	8.88	15.3	17.0	12.8	16.0	1.00	2.30

** and * and ^{ns}: respectively indicate significance at the probability levels of one and five percent and the lack of significant differences. C.V: Coefficient of variation.

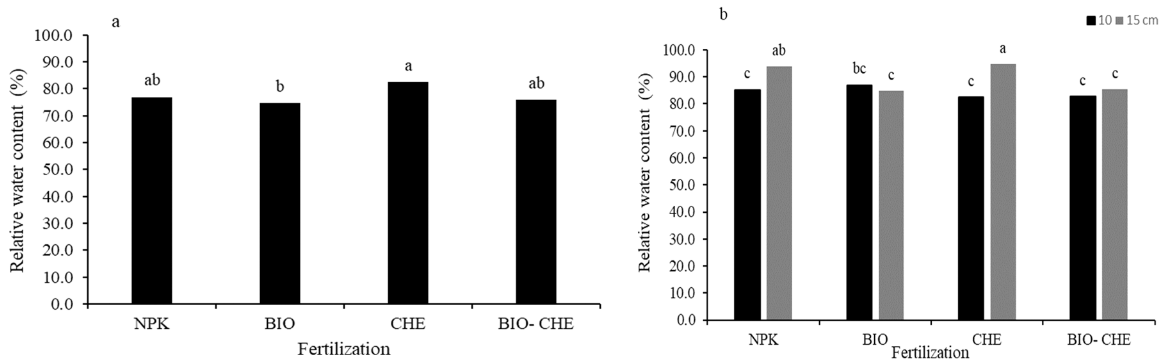

Fig. 2. The effect of fertilization on the relative water content of leaf in 2020 (a) and interaction fertilization and planting depth (10 and 15 cm) on the relative water content of leaf in 2021 (b) in saffron.

temperatures have been mentioned as one of the limiting factors of saffron production (Kafi et al., 2002). Therefore, when extreme cold occurs in certain years, the growth and development of saffron are negatively affected, and the yield reduces. The maximum tolerable cold of saffron has been reported to be − 18 °C (Jvanmard et al., 2002).

3.1. Relative water content (RWC)

In 2020, only fertilization significantly affected the RWC of the leaf (Table 2). Yet, in 2021, the interaction between fertilization and planting depth and planting depth significantly impacted yields (Table 3). In 2020, the CHE nutrition program yielded the highest RWC of the leaf but was not significantly different from the NPK and BIO-CHE nutrition programs (Fig. 2). Compared with NPK and BIO-CHE nutrition programs, the CHE nutrition program increased by 7.15% and 8.56% for the RWC of the leaf, respectively (Fig. 2a). In 2021, the leaf's highest RWC was obtained from the CHE nutrition program and planting depth of 15 cm, which was not significantly different from NPK treatment and planting depth of 15 cm but showed a 0.75% increase in the RWC of the leaf (Fig. 2b). The RWC of the leaf in 2021 has a significant and direct correlation with the MSI and leaf proline and an inverse correlation with the activity of the SOD enzyme (Table 5).

3.2. Membrane stability index (MSI)

In 2020, the effect of fertilization and interaction between fertilization and planting depth and in 2021, the impact of fertilization and planting depth and interaction between fertilization and planting depth on MSI were significant (Tables 2 and 3). The NPK nutrition program and planting depth of 15 cm brought the highest MSI in 2020 and 2021, but in 2021 it was similar to the CHE nutrition program and planting depth of 15 cm (Table 6). In both 2020 and 2021, with increasing planting depth from 10 to 15 cm in all nutrition programs, the MSI improved, except for the BIO-CHE nutrition program in 2020 and the BIO nutrition program in 2021, which was a decreasing trend (Table 6).

The membrane stability index in 2020 has a significant and direct correlation with the activity of the mother corm enzyme and an inverse correlation with the activity of the leaf enzyme (Table 4), and in 2021 a significant and direct correlation with the activity of the mother corms CAT, PPO enzymes and an inverse correlation with the activity of the mother corm SOD enzyme and the amount of mother corm proline (Table 5).

Means that have common letters are not significantly different at the 5% probability level based on the LSD test. Nitrogen, phosphate, and potassium fertilizers based on soil test (NPK), BIO (biofertilizers including nitrogen-fixing bacteria, phosphate, and potassium soluble bacteria), CHE (chemical fertilizers including calcium, potassium, microelements, and amino acid fertilizers) and BIO-CHE (combined method).

3.3. Proline

The effect of fertilization and planting depth and the interaction between fertilization and planting depth in both years (2020 and 2021) on leaf proline content was significant (Tables 2 and 3). The highest leaf proline content in 2020 was obtained from the BIO nutrition program at 10 cm planting depth, which showed an increase of 3.92 and 4.96 times compared with the NPK nutrition program for 10 and 15 cm planting depth, respectively (Table 7). The CHE nutrition program obtained the highest leaf proline content in 2021 at 15 cm planting depth, which was not significantly different from the BIO nutrition program at 15 cm planting depth (Table 7). The CHE nutrition program showed an 84.3% increase in leaf proline content at 15 cm planting depth compared with the NPK nutrition program (Table 7). In 2020, in all nutrition programs except the CHE nutrition program, the proline leaf content decreased by increasing the planting depth from 10 to 15 cm. Still, in 2021, in all nutrition programs, the leaf proline content increased by increasing the planting depth from 10 to 15 cm (Table 7).

As shown in Tables 2 and 3, the effects of fertilization and planting depth and the interaction of fertilization and planting depth on mother corm proline content became significant in 2020. Still, in 2021 only the effects of fertilization and planting depth on mother corm proline content became significant. The highest content of mother corm proline in 2020 was obtained from the BIO nutrition program at 10 cm planting depth, which was not significantly different from the NPK nutrition programs at 10 cm planting depth and the CHE at 15 cm planting depth (Table 7). The BIO nutrition program showed an increase of 9.41% for mother corm proline content compared with the NPK nutrition program at 10 cm planting depth (Table 7). By increasing the planting depth from 10 to 15 cm, the content of mother corm proline decreased in the NPK and BIO nutrition programs in 2020 but raised in the CHE and BIO-CHE nutrition programs (Table 7). A 10 cm planting depth and the BIO nutrition program induced the highest levels of mother corm proline in 2021 (Fig. 3). The BIO nutrition program, compared with the NPK nutrition program, showed an increase of 12.8% in the proline content of mother corm in 2021 (Fig. 3a). Planting depth of 10 cm compared with 15 cm of planting depth showed a 17.3% increase for mother corm proline in 2021 (Fig. 3b).

Leaf proline content in 2020 had a significant and direct correlation with mother corm proline content and mother corm PPO activity (Table 6), and in 2021, a significant and direct correlation with mother corm CAT and PPO activity and an inverse correlation with leaf and mother corm SOD enzyme activity (Table 7). Mother corm proline content in 2020 had a significant and direct correlation with the activity of leaf POD enzymes, leaf and mother corm PPO and mother corm SOD enzymes (Table 6), and in 2021 a significant and direct correlation with the activity of leaf POD and mother corm SOD enzymes and an inverse correlation with the activity of mother corm PPO enzyme (Table 7).

Table 4
Correlation coefficients of the number and dry weight of corm and antioxidants in saffron in 2020.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	Corms number	1													
2.	Corm dry weight	-0.08 ^{ns}	1												
3.	Relative water content	-0.07 ^{ns}	0.26 ^{ns}	1											
4.	Membrane stability index	0.17 ^{ns}	0.20 ^{ns}	0.020 ^{ns}	1										
5.	Leaf Proline	0.06 ^{ns}	-0.50*	-0.13 ^{ns}	0.22 ^{ns}	1									
6.	Mother corm Proline	-0.10 ^{ns}	0.27 ^{ns}	0.24 ^{ns}	0.26 ^{ns}	0.47*	1								
7.	Leaf Catalase	-0.42*	0.43*	0.18 ^{ns}	0.22 ^{ns}	-0.07 ^{ns}	0.44*	1							
8.	Mother corm Catalase	-0.20 ^{ns}	-0.36 ^{ns}	-0.09 ^{ns}	-0.20 ^{ns}	0.07 ^{ns}	-0.005 ^{ns}	-0.24 ^{ns}	1						
9.	Leaf Peroxidase	-0.46*	0.22 ^{ns}	-0.0002 ^{ns}	-0.58**	-0.17 ^{ns}	0.23 ^{ns}	0.42*	0.10 ^{ns}	1					
10.	Mother corm Peroxidase	-0.03 ^{ns}	0.22 ^{ns}	-0.29 ^{ns}	0.52**	-0.02 ^{ns}	0.11 ^{ns}	0.37 ^{ns}	-0.23 ^{ns}	-0.06 ^{ns}	1				
11.	Leaf Polyphenol oxidase	0.12 ^{ns}	0.45*	0.23 ^{ns}	0.07 ^{ns}	0.04 ^{ns}	0.46*	0.44*	-0.42*	0.19 ^{ns}	0.22 ^{ns}	1			
12	Mother corm Polyphenol oxidase	0.03 ^{ns}	-0.54**	-0.12 ^{ns}	0.15 ^{ns}	0.96**	0.41 *	-0.07 ^{ns}	0.002 ^{ns}	-0.12 ^{ns}	-0.03 ^{ns}	0.09 ^{ns}	1		
13	Leaf Superoxide dismutase	0.12 ^{ns}	0.21 ^{ns}	-0.003 ^{ns}	0.35 ^{ns}	-0.23 ^{ns}	0.17 ^{ns}	0.39 ^{ns}	0.04 ^{ns}	-0.05 ^{ns}	0.60**	0.30 ^{ns}	-0.29 ^{ns}	1	
14	Mother corm Superoxide dismutase	-0.36 ^{ns}	0.61**	0.27 ^{ns}	0.15 ^{ns}	-0.09 ^{ns}	0.52**	0.49*	-0.004 ^{ns}	0.22 ^{ns}	-0.06 ^{ns}	0.13 ^{ns}	-0.17 ^{ns}	-0.17 ^{ns}	1

^{ns}, * and ** are non-significant and significant at five and one percent probability levels, respectively.

3.4. Enzymatic activity

Catalase[1]: In 2020, the effect of fertilization on leaf CAT activity was significant (Table 2). In 2021, the effect of fertilization and interaction fertilization and planting depth on leaf CAT activity was significant (Table 3). The leaf CAT activity in 2020 was highest by NPK treatment (similar to the BIO and CHE nutrition programs) (Fig. 4a). While in 2021, the activity of CAT in leaf was highest by the BIO nutrition program at a planting depth of 15 cm (which was similar to NPK and BIO treatment at a planting depth of 10 cm, CHE at a planting depth of 10 and 15 cm and BIO-CHE at a planting depth of 15 cm) (Table 8). The BIO nutrition program at a 15 cm planting depth showed a 96.3% increase in leaf CAT enzyme activity compared with NPK treatment at a planting depth of 10 cm (Table 8). In all nutritional programs except NPK treatment, leaf CAT activity increased by increasing the planting depth from 10 to 15 cm (Table 8).

In 2020, the interaction between fertilization and planting depth on the activity of CAT enzyme in the mother corm was significant (Table 2). The highest activity of CAT in the mother corm in 2020 was obtained from the NPK treatment at a planting depth of 10 cm and CHE at a planting depth of 15 cm, which not was significantly different from the BIO nutrition programs at planting depths of 10 and 15 cm and BIO-CHE at planting depth of 10 cm (Table 8). In 2021, all the effects of fertilization and planting depth and the interaction between fertilization and planting depth on the activity of CAT in the mother corm was significant (Table 3). In 2021, the highest activity of CAT in the mother corm was obtained from the CHE nutrition program at a planting depth of 15 cm, (which was f similar to the BIO and BIO-CHE nutrition programs at planting depth of 15 cm) (Table 8). The CHE nutrition program, compared with NPK treatment at planting depth of 15 cm, showed a 26.6% increase in the activity of CAT in the mother corm (Table 8).

The leaf CAT activity in 2020 is directly related to leaf POD, PPO, and mother corm SOD enzyme activity (Table 4). The activity of the PPO enzyme in the mother corm and the PPO enzyme in the leaf correlated significantly and directly in 2021 (Table 5).

Peroxidase (POD): In 2020, the effect of fertilization and interaction fertilization and planting depth on leaf POD activity was significant (Table 2). In 2021, the effects of fertilization and planting depth and interaction fertilization and planting depth on leaf POD activity were significant (Table 3). In 2020, the BIO nutrition program had the highest leaf POD activity at a planting depth of 15 cm (Table 8). The BIO nutrition program at a planting depth of 15 cm showed a 36.8% increase in leaf POD activity compared with the NPK nutrition program at a planting depth of 10 cm (Table 8). In 2021, the NPK nutrition program at a planting depth of 15 cm had the highest leaf POD activity, which was not significantly different from the NPK, BIO and BIO-CHE nutrition programs at a planting depth of 10 cm (Table 8). In 2020, all nutrition programs except NPK and CHE showed an increase in leaf POD enzyme activity when planting depth was increased from 10 to 15 cm. Nonetheless, by 2021, planting depth of 10–15 cm decreased the activity of leaf POD enzyme in all programs except NPK. (Table 8).

It was found that fertilization, planting depth, and fertilization alone significantly influenced mother corm POD enzyme activity in 2020 and 2021, respectively (Tables 2 and 3). In 2020, the highest activity of mother corm POD was obtained from the NPK nutrition program, which was not significantly different from the BIO nutrition program (Fig. 4b). In 2020, the highest activity of mother corm POD was obtained from the NPK nutrition program, which was not significantly different from the BIO nutrition program (Fig. 4b).

In 2021, the highest activity of mother corm POD enzyme was obtained from the BIO nutrition program, which showed an increase of 88.7% compared with the NPK nutrition program (Fig. 4d).

A significant and direct correlation exists between leaf POD activity and mother corm SOD activity in 2021, and it is inversely correlated with the activity of the mother corm PPO enzyme (Table 5). The amount of POD activity enzyme in 2020 has a significant and direct correlation

Table 5
Correlation coefficients of the number and dry weight of corm and antioxidants in saffron in 2021.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	Corms number	1													
2.	Corm dry weight	0.73**	1												
3.	Relative water content	0.08 ^{ns}	0.19 ^{ns}	1											
4.	Membrane stability index	-0.12 ^{ns}	0.08 ^{ns}	0.47*	1										
5.	Leaf Proline	-0.17 ^{ns}	0.10 ^{ns}	0.52**	0.22 ^{ns}	1									
6.	Mother corm Proline	0.26 ^{ns}	-0.14 ^{ns}	-0.06 ^{ns}	-0.53**	-0.21 ^{ns}	1								
7.	Leaf Catalase	-0.26 ^{ns}	-0.20 ^{ns}	-0.27 ^{ns}	-0.35 ^{ns}	0.37 ^{ns}	-0.15 ^{ns}	1							
8.	Mother corm Catalase	-0.10 ^{ns}	-0.08 ^{ns}	0.68**	0.47*	0.60**	-0.36 ^{ns}	-0.16 ^{ns}	1						
9.	Leaf Peroxidase	0.10 ^{ns}	-0.06 ^{ns}	0.12 ^{ns}	0.04 ^{ns}	-0.18 ^{ns}	0.43*	-0.35 ^{ns}	-0.20 ^{ns}	1					
10.	Mother corm Peroxidase	0.10 ^{ns}	-0.16 ^{ns}	0.19 ^{ns}	-0.16 ^{ns}	0.35 ^{ns}	0.22 ^{ns}	0.20 ^{ns}	0.24 ^{ns}	-0.38 ^{ns}	1				
11.	Leaf Polyphenol oxidase	-0.07 ^{ns}	0.07 ^{ns}	-0.33 ^{ns}	-0.06 ^{ns}	-0.22 ^{ns}	-0.08 ^{ns}	0.17 ^{ns}	-0.64**	-0.10 ^{ns}	-0.004 ^{ns}	1			
12	Mother corm Polyphenol oxidase	0.08 ^{ns}	0.04 ^{ns}	0.32 ^{ns}	0.52**	0.44*	-0.62**	-0.04 ^{ns}	0.74**	-0.57**	0.30 ^{ns}	-0.27 ^{ns}	1		
13	Leaf Superoxide dismutase	-0.17 ^{ns}	-0.22 ^{ns}	-0.52**	-0.14 ^{ns}	-0.51**	-0.01 ^{ns}	0.06 ^{ns}	-0.73**	0.03 ^{ns}	-0.35 ^{ns}	0.74**	-0.38 ^{ns}	1	
14	Mother corm Superoxide dismutase	-0.05 ^{ns}	0.05 ^{ns}	-0.34 ^{ns}	-0.46*	-0.58**	0.59**	-0.06 ^{ns}	-0.78**	0.53**	-0.32 ^{ns}	0.38 ^{ns}	-0.92**	0.53**	1

^{ns}, * and ** are non-significant and significant at five and one percent probability levels, respectively.

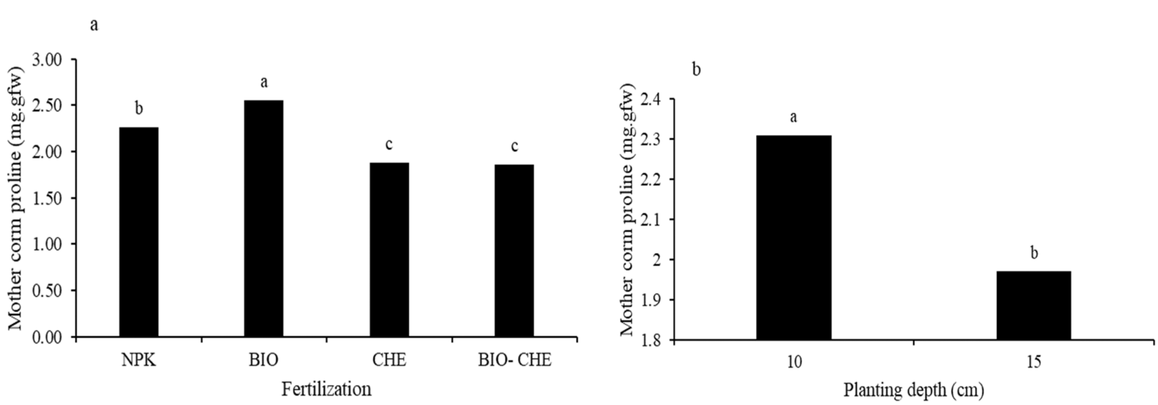

Fig. 3. The effect of fertilization on the content of mother corm proline in 2021 (a) and the effect of planting depth (10 and 15 cm) on the content of mother corm proline in 2021 (b) in saffron. Nitrogen, phosphate, and potassium fertilizers based on soil test (NPK), BIO (biofertilizers including nitrogen-fixing bacteria, phosphate, and potassium soluble bacteria), CHE (chemical fertilizers including calcium, potassium, microelements, and amino acid fertilizers) and BIO-CHE (combined method). The averages that have common letters are not significantly different at the level of one percent probability based on the LSD test.

with the activity of leaf SOD enzyme (Table 4).

Polyphenol oxidase (PPO): In 2020 and 2021, all the effects of fertilization and planting depth and the interaction of fertilization and planting depth on leaf PPO activity enzyme were significant (Tables 2 and 3). In 2020 and 2021, the CHE nutrition program obtained the highest leaf PPO activity enzyme at a planting depth of 10 cm (Table 9). Leaf PPO activity enzyme in the CHE nutrition program at a planting depth of 10 cm increased by 38.8% compared with the NPK nutrition program at a planting depth of 10 cm in 2020 and by 2.55 times in 2021 compared with the NPK nutrition program and planting depth of 15 cm (Table 9). In 2020, with increasing the planting depth from 10 to 15 cm in all nutrition programs except in the BIO-CHE nutrition program, and in 2021, except in the NPK nutrition program, the activity of leaf PPO activity enzyme decreased (Table 9).

In 2020, the highest activity of mother corm PPO activity enzyme was obtained from the BIO nutrition program at a planting depth of 10 cm, which showed an increase of 91.6% compared with the NPK nutrition program and planting depth of 10 cm (Table 9).

In 2021, the CHE nutrition program at a planting depth of 15 cm had the highest activity of mother corm PPO activity enzyme, which was not significantly different from the BIO- CHE nutrition program and planting depth of 15 cm (Table 9). The Chemical fertilizers nutrition program at a planting depth of 15 cm showed 3.47 times increase in mother corm PPO activity enzyme activity compared with the NPK nutrition program at a planting depth of 15 cm (Table 9). The activity of leaf PPO enzyme in 2021 correlates significantly with the leaf SOD activity enzyme (Table 5). The activity of the mother corm PPO enzyme in 2021 has a significant and inverse correlation with the activity of the mother corm SOD enzyme (Table 5).

Superoxide dismutase (SOD): In 2020, only the effect of fertilization, and in 2021 all the effects of fertilization and planting depth and the interaction between fertilization and planting depth became significant (Tables 2 and 3). The NPK nutrition program resulted in the highest leaf SOD enzyme activity in 2020 (Fig. 5a).

In 2021, the CHE nutrition program got the most increased activity of leaf SOD enzyme at a planting depth of 10 cm (Table 9). The CHE nutrition program at a planting depth of 10 cm showed a 27.9% increase in leaf SOD activity compared with the NPK nutrition program at

Table 6
Interaction fertilization and planting depth on membrane stability index in saffron in 2020 and 2021.

Fertilization	Planting depth (cm)	Membrane stability index (%)	
		Leaf	
		2020	2021
NPK	10	57.1^{de}	59.4^{d}
	15	65.8^{a}	77.6^{a}
BIO	10	61.0^{bc}	60.8^{cd}
	15	54.5^{e}	57.6^{d}
CHE	10	58.3^{cd}	66.7^{bc}
	15	61.9^{b}	71.9^{ab}
BIO- CHE	10	58.0^{cd}	62.2^{cd}
	15	56.9^{de}	69.1^{b}

Nitrogen, phosphate, and potassium fertilizers based on soil test (NPK), BIO (biofertilizers including nitrogen-fixing bacteria, phosphate, and potassium soluble bacteria), CHE (chemical fertilizers including calcium, potassium, microelements, and amino acid fertilizers) and BIO-CHE (combined method)) and planting depth (10 and 15 cm) in 2020 and 2021. In each column, the averages that have common letters, based on the LSD test, are not significantly different at the level of one percent probability.

Table 7
Proline content in saffron leaf and mother corm under the influence of different fertilization levels and planting depth.

Fertilization	Planting depth (cm)	Proline (mg gfw^{-1})		
		Leaf		Mother corm
		2020	2021	2020
NPK	10	1.72^{b}	0.566^{bc}	1.020^{ab}
	15	1.36^{b}	0.688^{bc}	0.687^{c}
BIO	10	6.75^{a}	0.565^{bc}	1.116^{a}
	15	1.23^{b}	0.935^{ab}	0.644^{c}
CHE	10	1.36^{b}	0.658^{bc}	0.919^{b}
	15	1.87^{b}	1.268^{a}	1.101^{ab}
BIO- CHE	10	1.76^{b}	0.413^{c}	0.317^{d}
	15	1.20^{b}	0.573^{bc}	0.552^{c}

Nitrogen, phosphate, and potassium fertilizers based on soil test (NPK), BIO (biofertilizers including nitrogen-fixing bacteria, phosphate, and potassium soluble bacteria), CHE (chemical fertilizers including calcium, potassium, microelements, and amino acid fertilizers) and BIO-CHE (combined method)) and planting depth (10 and 15 cm) in 2020 and 2021. In each column, the averages that have common letters, based on the LSD test, are not significantly different at the level of one percent probability.

planting depths of 10 and 15 cm (Table 9).

Fertilization and planting depth significantly affected the activity of the mother corm SOD enzyme in 2020 (Table 2). The CHE nutrition program obtained the highest activity of mother corm SOD enzyme in 2020, which showed an increase of 49.8% compared with the NPK nutrition program (Fig. 5b). Regarding planting depth, planting depth of 15 cm had the highest activity of mother corm SOD enzyme in 2020 (Fig. 5c).

In 2021, fertilization, planting depth, and interaction between fertilization and planting depth significantly affected mother corm SOD enzyme activity (Table 3). SOD enzyme activity was highest when mother corms were planted to a depth of 10 cm with BIO and BIO-CHE nutrition programs or at a deep 15 cm with NPK nutrition programs. The BIO nutrition program at a planting depth of 15 cm and the CHE nutrition program at a planting depth of 10 cm (Table 9). In 2021, in all nutritional programs, with increasing the planting depth from 10 to 15 cm, the activity of the mother corm SOD enzyme decreased (Table 9). Leaf SOD activity enzyme in 2021 has a significant and direct correlation with the activity of mother corm SOD enzyme (Table 5).

3.5. *Number and dry weight of corm*

In 2020, the effect of fertilization and interaction fertilization and planting depth on the number of corms per unit area was significant (Table 10). The highest number of corms in 2020 was obtained from the BIO-CHE nutrition program and planting depth of 15 cm, which was not significantly different from NPK and BIO-CHE nutrition programs and planting depth of 10 cm (Table 11). The number of corms decreased by increasing the planting depth from 10 to 15 cm in all nutrition programs (except in the BIO-CHE nutrition program) (Table 11). Fertilization and planting depth significantly influenced corm production in 2021 (Table 6). A BIO nutrition program and planting depth of 10 cm produced the most corms, which was not significantly different from an NPK nutrition program and planting depth of 15 cm. Compared to the NPK nutrition program, the BIO program produced 8.51% more corms (Table 11). The NPK and BIO-CHE nutrition programs showed increased corms after planting depths increased to 15 cm, but BIO and CHE nutrition programs decreased (Table 11). The number of corms in 2020 has a significant and inverse correlation with the activity of leaf CAT and POD enzymes (Table 4) and in 2021 has a significant and direct correlation with the dry weight of corms (Table 5).

Regarding the dry weight of corm per unit area in 2020, the effect of fertilization and planting depth and the interaction between fertilization and planting depth became significant (Table 10). The highest dry weight of corms in 2020 was obtained from the CHE nutrition program and planting depth of 10 cm, which showed an increase of 11.7% compared with the NPK nutrition program and planting depth of 15 cm (Table 11).

For the dry weight of corms in 2021, only the interaction between fertilization and planting depth became significant (Table 10). At a planting depth of 10 cm, the BIO-CHE nutrition program induced the highest dry weight of corms, which was not significantly different from the CHE nutrition program and 15 cm planting depth (Table 11). Comparing the BIO-CHE nutrition program with planting depths of 10 cm (which had the highest dry weight of corms in 2021) with the BIO nutrition program and planting depths of 10 cm (which had the greatest number of corms), dry corm weight increased by 69.9% but corm number decreased by 34.0% (Table 11). The BIO nutrition program apparently resulted in more corms but at a lower weight. While the BIO-CHE nutrition program was able to make less number of corms, but with more weight in saffron planting. Consider planting saffron with fewer corms but larger ones to ensure more flowers since larger corms produce more flowers.

The dry weight of corm in 2020 has a significant and direct correlation with the activity of leaf CAT and PPO enzymes and mother corm SOD enzymes and inversely correlated with leaf proline content and mother corm PPO activity enzymes (Table 4).

4. Discussion

4.1. *Leaf relative water content (RWC)*

Cold stress affects leaf tissue water, and RWC is an important indicator. It indicates how well a plant retains water in its tissues. RWC is higher in plants that are less stressed (Yildiztugay et al., 2017). Jia et al. (2017) used this indicator to measure the balance between leaf tissue water supply and transpiration rate. As a result of reduced water uptake due to cold (Iseri et al., 2013), plants under cold stress show similar signs to those under drought stress (Karimi and Ershadi, 2015).

As mentioned, in 2020, the CHE nutrition program and in 2021, the CHE nutrition program and planting depth of 15 cm had a higher RWC (Fig. 2). Factors such as plant access to water and the plant's ability to regulate stomatal and osmotic movements affect the amount of tissue water content. In addition to reducing ROS, proline also plays a role in osmotic regulation. It reduces the potential for water inside the plant, and the plant can absorb and retain more water. Soluble carbohydrates

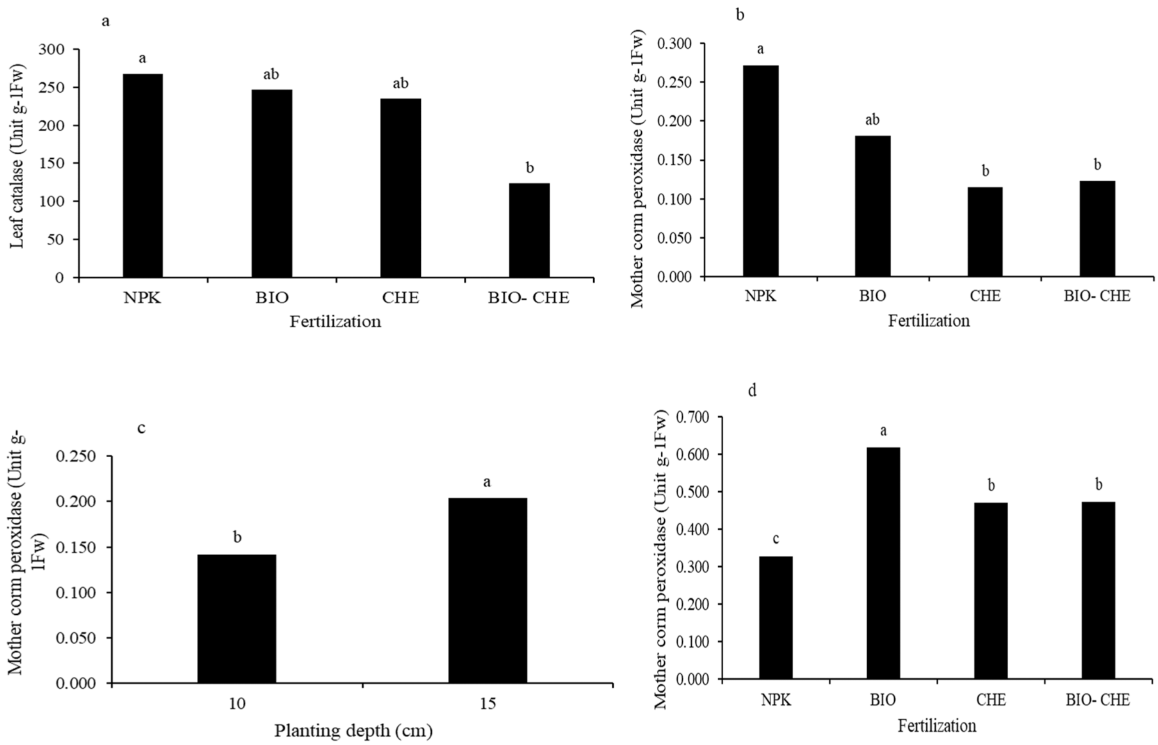

Fig. 4. Effect of fertilization on leaf catalase activity in 2020 (a), mother corm peroxidase activity in 2020 (b), the effect of planting depth (10 and 15 cm) on mother corm peroxidase activity in 2020 (c) and effect of fertilization on the activity of mother corm peroxidase in 2021 (d) in saffron. Nitrogen, phosphate, and potassium fertilizers based on soil test (NPK), BIO (biofertilizers including nitrogen-fixing bacteria, phosphate, and potassium soluble bacteria), CHE (chemical fertilizers including calcium, potassium, microelements, and amino acid) and BIO-CHE (combined method). The means that have common letters according to the LSD test are not significantly different in the level of probability of one and five percent.

Table 8
The activity of catalase and peroxidase enzymes in saffron leaf and mother corm under the influence of fertilization and planting depth treatments.

Fertilization	Planting depth (cm)	Catalase (Unit g^{-1}Fw)			Peroxidase (Unit g^{-1}Fw)	
		Leaf	Mother corm		Leaf	
		2021	2020	2021	2020	2021
NPK	10	134^{ab}	129^{a}	266^{b-d}	0.212^{b}	1.375^{ab}
	15	108^{b}	100^{bc}	301^{a-c}	0.078^{de}	1.520^{a}
BIO	10	132^{ab}	114^{a-c}	242^{b-d}	0.137^{cd}	1.147^{a-c}
	15	263^{a}	119^{ab}	237^{cd}	0.290^{a}	0.910^{b-d}
CHE	10	188^{ab}	94.3^{c}	195^{d}	0.179^{bc}	0.868^{b-d}
	15	189^{ab}	129^{a}	381^{a}	0.130^{cd}	0.758^{cd}
BIO- CHE	10	89.7^{b}	128^{a}	215^{cd}	0.056^{e}	0.996^{a-d}
	15	147^{ab}	101^{bc}	328^{ab}	0.170^{bc}	0.463^{d}

Nitrogen, phosphate, and potassium fertilizers based on soil test (NPK), BIO (biofertilizers including nitrogen-fixing bacteria, phosphate, and potassium soluble bacteria), CHE (chemical fertilizers including calcium, potassium, microelement, and amino acid) and BIO-CHE (combined method)) and planting depth (10 and 15 cm) in 2020 and 2021. In each column, the averages that have common letters, based on the LSD test are not significantly different at the level of one and five percent probability.

also play a role in osmotic regulation.

According to Table 5, in 2021, the CHE nutrition program and planting depth of 15 cm produced more leaf proline content. Many evidences indicate that mineral nutrients play an important role in winter freezing tolerance. Among all the minerals, potassium plays an essential role in plants' growth, metabolism, and survival under environmental stress. Potassium plays a vital role in enzyme activation, protein synthesis, photosynthesis, osmotic regulation, stomatal movements, energy transfer, material transport in the phloem, cation-anion balance, and stress resistance (Marschner, 2012). Higher RWC in the CHE nutrition program can be attributed to higher stomatal conductance, better osmotic regulation, or root ability to absorb water, in all of which potassium plays an important role. Potassium also plays an important role in improving leaf water content and plant water relationships (water potential, osmotic potential, and turpentine potential), increasing the RWC (Thalooth et al., 1990).

4.2. Membrane stability index (MSI)

Membrane stability index is closely related to its lipid composition (Wang et al., 2013). The Plasma membrane is the first place to perceive stress and induce oxidation and lipid peroxidation of the membrane (Huang et al., 2015). Consequently, electrolyte leakage increases as the material leave the cell, and the MSI decreases. The NPK nutrition program and planting depth of 15 cm in both years (2020 and 2021) had a higher MSI (Table 4). The reason for the decrease in leaf electrolyte leakage with increasing planting depth needs to be better understood. But it may be due to the transmission of resistance messages from the underground organs to the overground organs. Increasing planting depth lowers temperature fluctuations and reduces electrolyte leaks by increasing membrane stability and reducing freezing damage. Potassium is essential for high crop yield and can be a limiting factor for crops under certain environmental conditions such as drought, salinity, and high temperature (Mengel and Kirkby, 2001). Among mineral nutrients, potassium is the most abundant cation in plants. It plays an important role in regulating the reduction of damage of ROS due to stress to plasma membranes, maintaining cell membrane integrity and maintaining enzyme activity in cells (Raza et al., 2013). Improving the cell MSI can

Table 9
Polyphenol oxidase and superoxide dismutase activities in saffron leaf and mother corm under the influence of fertilization and planting depth.

Fertilization	Planting depth (cm)	Polyphenol oxidase (Unit g^{-1}Fw)				Superoxide dismutase (Unit g^{-1}Fw)	
		Leaf		Mother corm		Leaf	Mother corm
		2020	2021	2020	2021	2021	
NPK	10	90.8^{b}	61.7 ^{f}	35.9$^{c\text{-}e}$	1026^{bc}	0.212^{b}	0.251^{a}
	15	80.3^{b}	755$^{c\text{-}e}$	29.0^{de}	1524^{bc}	0.212^{b}	0.248^{a}
BIO	10	78.0^{b}	946^{bc}	426^{a}	1567^{bc}	0.212^{b}	0.252^{a}
	15	56.6 ^{cd}	808 ^{cd}	41.0 ^{cd}	496^{c}	0.212^{b}	0.250^{a}
CHE	10	126^{a}	1924^{a}	54.5^{bc}	2104^{b}	0.294^{a}	0.248^{a}
	15	45.3^{de}	428$^{d\text{-}f}$	20.7^{e}	5285^{a}	0.155^{c}	0.201^{b}
BIO- CHE	10	40.6^{e}	1204^{b}	73.8^{b}	1096^{bc}	0.210^{b}	0.252^{a}
	15	63.0^{c}	410^{ef}	27.6^{de}	4955^{a}	0.211^{b}	0.214^{b}

Nitrogen, phosphate, and potassium fertilizers based on soil test (NPK), BIO (biofertilizers including nitrogen-fixing bacteria, phosphate, and potassium soluble bacteria), CHE (chemical fertilizers including calcium, potassium, microelements, and amino acid) and BIO-CHE (combined method)) and planting depth (10 and 15 cm) in 2020 and 2021. In each column, the averages that have common letters based on the LSD test are not significantly different at the level of one percent probability.

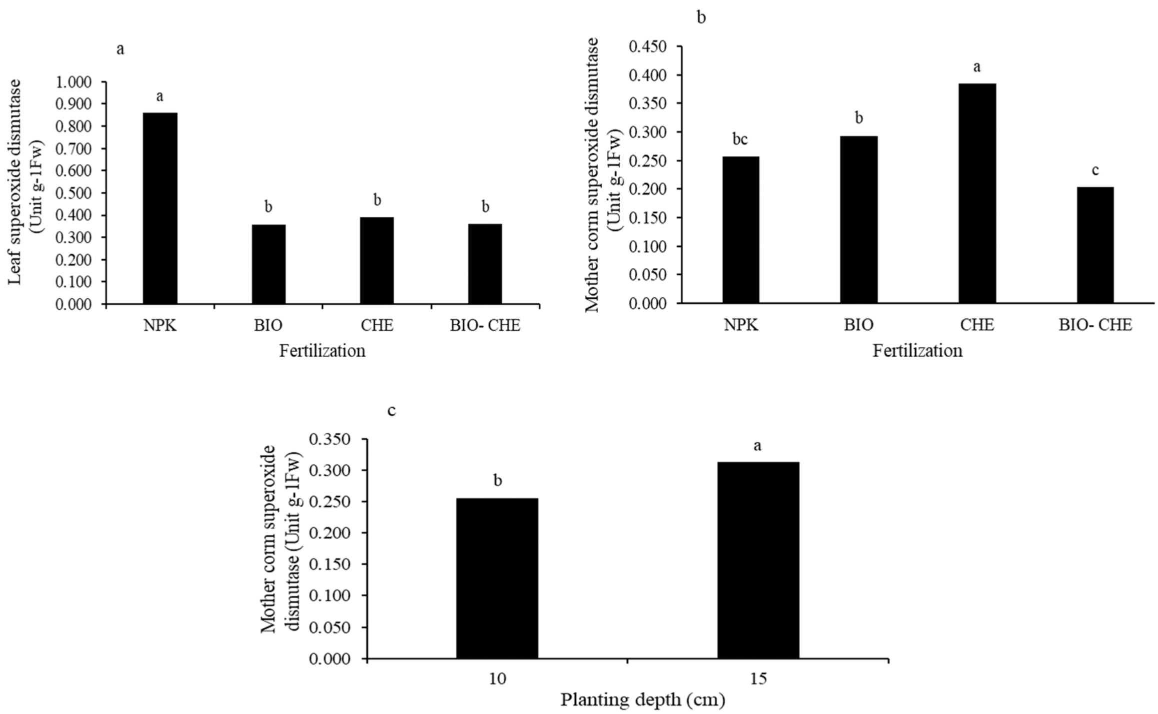

Fig. 5. Effect of fertilization on leaf superoxide dismutase activity enzyme in 2020 (a), the activity of mother corm superoxide dismutase enzyme in 2020 (b) and the effect of planting depth (10 and 15 cm) on the activity of mother corm superoxide dismutase enzyme in 2020 (c) in saffron. Nitrogen, phosphate, and potassium fertilizers based on soil test (NPK), BIO (biofertilizers including nitrogen-fixing bacteria, phosphate, and potassium soluble bacteria), CHE (Chemical Fertilizers including Calcium, Potassium, microelement, and amino acid) and BIO-CHE (combined methods). The means that have common letters are not significantly different at the level of one percent probability based on the LSD test.

be attributed to the role of nitrogen, potassium and phosphate elements in increasing the activities of antioxidant enzymes in reducing the damage of stress factors to plasma membranes (Tables 6 and 7 and Figs. 4 and 5).

4.3. Proline

Proline is a non-enzymatic antioxidant, and regulation of biosynthesis of proline concentration in tissue is a mechanism that occurs during cold stress (Ritonga and Chen, 2020). In addition to scavenging free radicals and stabilized membranes, proline served as a nitrogen and carbon source (Szabados and Savouré, 2010). The BIO nutrition programs, and a planting depth of 10 cm caused the highest levels of leaf and mother corm proline (CHE nutrition programs caused higher leaf proline in 2021, but it was similar to the effect of BIO nutrition programs) (Table 5 and Fig. 3). In addition to the nitrogen reservoir function of proline, bacteria acting as nitrogen stabilizers appear to be able to increase the production of proline. Also, potassium is involved in protein-degrading enzymes and protein conversion to the amino acid proline (Moeinian et al., 2011). It has been reported that with decreasing planting depth and temperature, the amount of proline in leaf, corm mass and roots has improved significantly (Koocheki and Seyyedi, 2019b).

4.4. Enzymatic activity

Under oxidative conditions, the amount of free radicals increases and plants reduce free radicals levels by producing a variety of antioxidants and regulating cellular homeostasis (Kamran et al., 2020). Plants have a variety of enzymatic and non-enzymatic antioxidants to protect against

Table 10
Analysis of variance (mean squares) effect of fertilization and planting depth on the number of corms and dry weight of saffron corms in 2020 and 2021.

S. O. V	df	Mean of squares			
		Corms number		Corm dry weight	
		2020	2021	2020	2021
Block	2	248 [ns]	38.8 [ns]	140 [ns]	13798 [ns]
Fertilizer (F)	3	2640**	3018 [ns]	27058**	17846 [ns]
Error a	6	226	1892	729	35440
Planting depth (P)	1	523 [ns]	1204 [ns]	27880**	6215 [ns]
F*P	3	1941*	50747**	8088**	30205*
Error	8	280	826	302	5004
C.V %		11.9	9.31	6.42	17.9

** and * and [ns]: respectively indicate significance at the level of probability of one and five percent and the lack of significant differences. C.V: Coefficient of variation.

Table 11
Interaction fertilization and planting depth on the number of corms and dry weight of saffron corm.

Fertilization	Planting depth (cm)	Corms number (no/ m^2)		Corm dry weight (g/ m^2)	
		2021	2020	2021	2020
NPK	10	213[e]	150[ab]	357[b]	257[c]
	15	388[ab]	144[b]	413[b]	333[b]
BIO	10	421[a]	144[b]	339[b]	173[d]
	15	159[e]	88[c]	315[b]	266[c]
CHE	10	352[bc]	138[b]	372[b]	372[a]
	15	331 [cd]	131[b]	446[ab]	337[b]
BIO- CHE	10	278[d]	150[ab]	576[a]	144[d]
	15	329 [cd]	181[a]	341[b]	283[c]

Nitrogen, phosphate, and potassium fertilizers based on soil test (NPK), BIO (biofertilizers including nitrogen-fixing bacteria, phosphate, and potassium soluble bacteria), CHE (chemical fertilizers including calcium, potassium, microelement, and amino acid) and BIO-CHE (combined methods)) and planting depth (10 and 15 cm) in 2020 and 2021. In each column, the averages with common letters based on the LSD test are not significantly different at the one and five percent probability levels.

oxidative stress. Catalase, glutathione peroxidase, ascorbate peroxidase, and superoxide dismutase are enzyme antioxidants. Catalase and glutathione peroxidase enzymes catalyze hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) (Öktem et al., 2008). In-plant cells, the first line of defence against ROS is SOD. Therefore, the increase in SOD activity indicates an increase in the production of ROS. The SOD can repair damaged plant cells by catalyzing the conversion of superoxide anion (O^{-2}) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). In general, high SOD activity leads to reduced membrane lipid peroxidation (Zhang et al., 2004).

Antioxidant enzyme activity reduces ROS and improves membrane integrity and stress resistance, including cold stress (Gill and Tuteja, 2010). Under environmental stress, plants' need for nutrients, especially potassium, increases. Potassium is needed to maintain photosynthetic carbon dioxide[1] stabilization (Jiang and Zhang, 2002). Reactive oxygen species removes potassium from cells by lipid peroxidation (Cuin and Shabala, 2007). Increasing access to nitrogen has improved plant resistance to oxidative stress (Ramalho et al., 1998). Nitrogen deficiency increases the activity of SOD, glutathione reductase and ascorbate peroxidase enzymes (Logan et al., 1999). In phosphorus shortage conditions, plants are more sensitive to cold stress (Starck et al., 2000). Phosphorus supply increases the activity of SOD, ascorbate peroxidase and guaiacol peroxidase enzymes (Ahn et al., 2002). Potassium plays an important role in reducing ROS by activating biosynthetic pathways of antioxidants and increasing antioxidant activity (Cakmak, 2005).

4.5. Number and dry weight of corm

The effect of all environmental and soil conditions and the plant's response to these conditions can ultimately be seen in plant yield. The highest number and dry weight of corms in 2020 were related to the BIO-CHE nutrition program and planting depth of 15 cm and CHE and planting depth of 10 cm, respectively (Table 9). In 2021, the highest number and dry weight of corm were obtained from the BIO nutrition program with a planting depth of 10 cm and the BIO-CHE with a planting depth of 10 cm, respectively. In 2020 and 2021, nutrition programs that induced lower antioxidant enzyme activities produced higher corm numbers and dry weights. This shows that these nutritional programs have made the plant less aware of freezing stress, and the plant spends all its energy on increasing the number and weight of corms (increasing the yield of the vegetative part).

5. Conclusion

One of the critical factors in saffron production is freezing stress. Different factors can help the plant to confront freezing stress by improving the growth characteristics of the plant. It is important to consider freezing stress when producing saffron. Many factors can improve the plant's growth characteristics and help it overcome freezing stress. Planting depth and fertilization are among these factors. The present experiment showed that fertilization and planting depth could reduce the effects of freezing stress and thus improve saffron yield. In the first year of saffron planting, the leaves are more sensitive to freezing stress than the mother corms and have higher antioxidant activity. Unlike in the first year, the mother corms are more susceptible to freezing stress in the second year. In this case, biofertilizer requires less planting depth (10 cm), which could be attributed to soil surface elements' richness and good ventilation. Among nutrition programs, the chemical fertilizers (CHE) nutrition program could have higher leaf relative water content, higher activity of antioxidant enzymes and higher dry weight of corm, which can be recommended as a superior nutrition program.

CRediT authorship contribution statement

Seyyed Jalal Azari carried out the experiments and wrote the manuscript with support from Ali Sorooshzadeha. Ali Sorooshzadeha supervised the project and the author contributed to the final version of the manuscript. Jafar Nabati contributed to the design and implementation of the research. Ehsan Oskoueian, developed the theoretical framework.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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