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The effects of bio-fertilization and two levels of chemical fertilization on wheat (*Triticum aestivum* L.) under drought conditions

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Abstract

A filed experiment was conducted to consider the impacts of bio-fertilizers (*Azotobacter chroococcum* and *Glomus mosseae*) and two levels of chemical fertilization (50% and 100% of recommended dose) on proline content and activities of antioxidant enzymes (catalase and peroxidase) in wheat *Triticum aestivum* L. cultivar IPA 99 under drought conditions (50% and 20% of soil field capacity). Bio-fertilization involved treatment with *A. chroococcum* (Azoto) and *G. mosseae* (AMF), singly or in combination. The experiment was conducted by applying a Randomized Complete Block Design (RCBD) with three replications. The results of this study showed that the treatment utilizing Azoto+AMF fertilizer with concentrated chemical fertilizer (100%) significantly decreased proline content and the activities of antioxidant enzymes under drought conditions. In addition, the percentage of mycorrihzal root infection was increased. The lowest values of proline content and catalase and peroxidase activities (3.35 µmole/g, 84.52 unit/ml, 90.90 unit/ml, respectively) were achieved by the application of combined bio-fertilizers with 100% of chemical fertilizer and 50% water deficit. The combined bio-fertilizer application with 50% of chemical fertilizer and 20% water deficit achieved the maximum mycorrihzal infection percentage (90%).

Keywords: Antioxidant enzymes, *Azotobacter* sp., Bio-fertilizers, *Glomus mosseae*, Wheat, drought conditions.

تأثير الاسمدة الحيوية ومستويين من الاسمدة الكيميائية على نبات الحنطة *Triticum aestivum* L تحت ظروف الجفاف

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الخلاصة:

نفذت التجربة الحقلية لدراسة تأثير الأسمدة الحيوية (*Azotobacter chroococcum* and *Glomus mosseae*) ومستويان من الاسمدة الكيميائية (50% و 100% من الجرع الموصى بها) على محتوى البرولين والانزيمات المضادة للأكسدة (فعالية الكاتليز والبيروكسيديز) لنبات الحنطة *Triticum aestivum*

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.L صنف أباء 99 تحت ظروف الجفاف (50% و20% من السعة الحقلية). باستخدام الجنس *A. chroococum* (Azoto) وفطريات المايكورايزا *G. mosseae* (AMF) بصورة منفردة أو متحدة باستخدام تصميم القطاعات الكاملة العشوائية بترتيب الألواح المنشقة (CRBD) مع ثلاث مكررات. اظهرت نتيجة هذه الدراسة ان معاملة التسميد Azoto+AMF بمستوى سماد كيميائي 100% ادى الى خفض معنوي في محتوى البرولين والانزيمات المضادة للأكسدة تحت ظروف الجفاف, بالإضافة الى زيادة في النسبة المئوية للجذور المصابة بالمايكورايزا . تم تحقيق اقل قيمة لمحتوى البرولين, الكاتليز , البيروكسيديز باستخدام اسمدة حيوية مزدوجة بمستوى (100%) من الاسمدة الكيميائية مع عجز مائي بنسبة 50% (3.35 ميكرو مول / جم ، 84.52 وحدة / مل ، 90.90 وحدة / مل) على التوالي. حقق التسميد المزدوج أقصى نسبة إصابة بالمايكورايزا (90٪) بمستوى (50٪) من الأسمدة الكيمائية و 20٪ عجز مائي.

Introduction

Crop plants are vulnerable to various natural stresses, all of which influence plant growth and development, thus hampering crop efficiency [1]. Drought is considered to be the most destroying single natural stress, which, more than any other environmental stress, reduces crop productivity [2]. Drought influences morphological, physiological, biological, and molecular processes in plants, resulting in development restraint. Plants generally accumulate few types of compatible solutes such as proline, betaine, and polyols in the cytosol to elevate the osmotic pressure, thus maintaining both turgor and gradient-driven water absorption, and protect membranes and proteins [3]. In plant cells, the activity of antioxidant enzymes increases as a response to natural stresses. The development of Reactive Oxygen Species (ROS**s**) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and singlet oxygen ($^1O_2$) can result from environmental stresses [4]. These ROS**s** can initiate destructive oxidative processes, including lipid oxidation, oxidation of proteins, and nucleic acids damage [5]. Plants exposed to stress from drought can overcome oxidative stress via the activation of some or all antioxidant enzymes [6,7].

Mycorrhizae is a mutualistic symbiosis between fungi and plant roots. The mycorrhizal fungi could increase plant growth by increasing the nutrient adsorption, particularly phosphorus [8]. They also have the effects of increasing plant resistance to drought, controlling root infection by pathogens, producing the growth stimulating compounds, and stimulating the activities of some advantageous organisms to improve soil structure and aggregation as well as mineral nutrient distribution [9].

Nitrogen -fixing bacteria (*Azotobacter*) are known to supply additional nitrogen in an eco-friendly manner. *Azotobacter* has been found to synthesize plant growth-promoting substances, such as auxins, gibberellins, cytokinins and some antibiotic metabolites. This bacteria can impact plant growth indirectly by expanding the population of beneficial microorganisms in the rhizosphere [10].

This study aimed to assess the impacts of the inoculation of bio-fertilizers (*A. chroococcum*, *G. mosseae*, and *A. chroococcum* + *G. mosseae*) and chemical fertilizer (50% and 100% of recommended dose) under 50% and 20% of soil field capacity on proline content and the activities of antioxidant enzymes (catalase and peroxidase) in wheat *Triticum aestivum* L.

Materials and methods

Soil collection

Four soil samples were collected from wheat and barley fields suffering from drought in Al Ramadi city, West Iraq, during March 2018. Samples were collected in pored polyethylene bags from a depth of 0 – 30 cm below the soil surface, i.e. from the Rhizosphere of roots, and stored at room temperature to be used for the isolation of arbuscular mycorrhizal fungi (AMF) and *A. chroococcum*.

Isolation and identification of AM fungi from root -soil texture

The AM fungi spores were isolated by utilizing the wet sieving and decanting procedure [11]. The procedure used was as in the following:

- The root-soil blend was thoroughly mixed with a 30 sec glass bar.
- The mixture was left for 10 sec to settle heavier particles and organic material. The remaining soil-root-hyphae-spores suspension was slowly poured through a set of three sieves (pores of 85, 65, and 25 µ, respectively).
- The extract was washed away from the sieves to petri dishes of 10 cm diameter.
- A dissecting microscope was used to visualize the spores, aggregates, and sporocarps which were collected by a pipette.

The fresh spores were used for the identification, based on morphology of spores, spore – bearing structures, and sporocarps [12].

Isolation of *Azotobacter chroococcum* from Soil

Azotobacter chroococcum was isolated from soil samples following a previously described method [13].

- Gradient dilutions of soil solution (3-10, 5-10) were prepared for each sample.
- One ml from each dilution was set in 250 ml flask containing 50 ml of N- free Jensen's broth and incubated at 30º C for 2-5days.
- The flasks were examined for a film of surface growth formation. A wet mount, preferably of the surface film, was prepared and observed with compound microscope.
- Plates of N – free Jensen's agar were streaked and incubated at 30 º C for 1-2 days.
- The plates were examined for the presence of colonies, which were wet mounted and examined for Gram staining.
- The pure colonies were examined and used as inoculums for a slant of N - free Jensen's agar medium.
- All the isolates of *Azotobacter* sp. were subjected to biochemical characterizations, which included Gram stain reaction and growth on N- free medium containing 1 % sucrose, manifold, and rhamnose as a sole carbon source.

Field experiment

The experiment was conducted during 2018-2019 in the Biology Department's research field, College of Science, University of Baghdad, Baghdad, Iraq. The chemical and physical characteristics of field soil were measured in the laboratory of Soil Department, College of Agriculture, University of Baghdad (Table 1). Field plots (48 plots, 1×2 m) were prepared in the field that was equipped with a greenhouse. To prevent possible horizontal movement of irrigated water and inoculants, the plots were separated from each other by a plastic sheet inserted vertically into the soil to a depth of 35 cm. Cultivar grains of wheat (IPA 99) were manually sown in their respective plots in rows of two meters each with a distance of 20 cm between them (3 rows per plot) and a seeding rate of 10 g per row (150 kg/ha^{-1}). The plots were treated with bio-fertilizers consisted of *G. mosseae*, *A. chroococcum*, separately or in combination. Chemical fertilizers (Chf) used were urea at 140 kg/ ha^{-1} and super phosphate (P_2O_5) at 250 kg/ha^{-1}. Before seed planting, the whole amount of phosphorus fertilizer was added, while urea was split into two equal quantities. The first amount (50%) was added before planting, i.e. during the preparation of the soil, whereas the second one (100%) was added 40 days after sowing (during the early tillering stage). The seeds of *Triticum aestivum* L. cultivar (IPA 99) were sowed in 28 November 2018. Water stress was applied to the soil-field capacity by irrigating the plots and then withholding the next irrigation until soil moisture reached 50% and 20 % of soil field capacity. Over the course of the analysis, all weeds were uprooted by hand-weeding. The soil humidity of the plots was measured using a weight basis method [14].

Results

Physical and chemical properties of soil

The results of soil analysis revealed that the soil texture was loam with EC of 1.1 and pH of 7.4. Concentrations of available N, P, and K were 14.58, 24.36, and 375.16 mg. kg^{-1} , respectively (Table 1).

Table 1- Some physical and chemical properties of soil used in the experiment.

Sand (g/ kg soil)	Silt (g /kg soil)	Clay (g/kg soil)	Soil texture	Field capacity	pH	EC ds/ m	Available nutrients mg. kg^{-1}		
							N	P	K
320	430	250	Loam	31	7.4	1.1	14. 58	24.36	375.16

Proline content in wheat treated with bio- and chemical fertilizers

The results presented in table 2 reveal that proline content was essentially increased by the stress of water deficit. The highest mean level of proline (4.62 µmole/g) was recorded at 20% water deficit, while the lowest (3.56 µmole/g) was at 50% water deficit.

Besides, according to bio-fertilizer treatment, the high mean level of proline (4.78 µmole/g) was recorded in the control group, while the lowest (3.78 µmole/g) was recorded by the treatment with Azoto +AMF. According to chemical fertilizer treatment, the highest mean (4.18 µmole/g) was at 50% chemical fertilization, while the lowest was (4.00 µmole/g) at 100 % chemical fertilization.

In addition, the interaction between chemical fertilization and water deficit significantly affected proline content in the plant; the highest mean was 4.72 µmole/g at 50% chemical fertilization with 20% water deficit, while the lowest was 3.49 µmole/g at 100% chemical fertilization with 50% water deficit.

Also, the interaction between chemical and bio-fertilization significantly affected proline content; the highest value was 4.86 µmole/g at 50% chemical fertilization, while the lowest was 3.70 µmole/g at Azoto +AMF with 100% chemical fertilization. Moreover, the relationship between water deficit and bio-fertilizers significantly affected the results; the highest proline level was 5.72 µmole/g at 20% water deficit, but the lowest was 3.40 µmole/g at Azoto +AMF with 50% water deficit.

Moreover, the triple interaction among chemical fertilizers, water deficit, and bio-fertilizers significantly affected proline content. Maximum proline content (5.84 µmole/g) was recorded at 50% chemical fertilizers with 20% water deficit, while minimum proline content (3.35 µmole/g) was recorded by Azoto+AMF with 100% chemical fertilizers and 50% water deficit.

Table 2-Effects of chemical fertilizers, water availability, and AMF and AZOTO inoculation on proline content (µmole /g) in wheat

Chf %	(H): Water %	Biological treatments				(chf x H)	
		(AMF)	(Azoto)	(Azoto + AMF)	Control		
50	20	4.44	4.34	4.28	5.84	4.72	
	50	3.66	3.57	3.45	3.88	3.64	
100	20	4.24	4.17	4.05	5.60	4.51	
	50	3.42	3.41	3.35	3.79	3.49	
LSD	--	LSD (chfHT) = 0.492				LSD (chfH)= 0.451	
(chf x T)		LSD (chfT) = 0.801				Mean of (chf)	
(chf): 50		4.05	3.95	3.86	4.86	4.18	LSD (chf)= 0.173
(chf): 100		3.83	3.79	3.70	4.69	4.00	

(H x T)					Mean of (H)
(H): 20	4.34	4.25	4.16	5.72	4.62
(H): 50	3.54	3.49	3.40	3.84	3.56
LSD	LSD (HT) = 0.330				LSD (H)= 0.173
Mean of (T)	3.94	3.87	3.78	4.78	LSD (T)= 0.246
(P<0.05).					

Chf: chemical fertilizer, H: water deficit , T: treatment

Impacts of bio- and chemical fertilizers on catalase and peroxidase activities on leaves of wheat under drought conditions

a- Catalase enzyme activity

The results introduced in Table 3 demonstrate that the mean value of catalase activity was greatly increased by the stress of water deficit . The highest catalase activity (93.69 unit/ml) was recorded at 20% water deficit, while the lowest (87.64 unit/ml) was at 50% water deficit.

Besides, based on bio-fertilizer treatment, the high mean catalase activity (92.99 unit/ml) was recorded in the control, while the lowest (89.18 unit/ml) was recorded at Azoto +AMF. Based on chemical fertilizer treatment, the highest mean was 91.56 unit/ml at 50% chemical fertilization, while the lowest was 89.76 unit/ml at 100% chemical fertilization.

Furthermore, the interaction between chemical fertilization and water deficit significantly affected the results; the highest mean catalase activity was 94.51 unit/ml at 50% chemical fertilization with 20% water deficit, while the lowest was 86.65 unit/ml at 100% chemical fertilization with 50% water deficit.

Also, the interaction between chemical and bio-fertilization significantly affected the results; the highest value was 93.36 unit/ml at 50% chemical fertilization, while the lowest was 88.06 unit/ml at Azoto +AMF with 100% chemical fertilization. Moreover, the relationship of water deficit with bio-fertilizers significantly affected the results; the highest value was 95.47 unit/ml at 20% water deficit, but the lowest was 85.89 unit/ml at Azoto +AMF with 50% water deficit.

However, the triple interaction among chemical fertilizers, water deficit, and bio-fertilizers significantly affected catalase activity. Maximum catalase activity (95.93unit/ml) was recorded by treatment with 50% chemical fertilizers and 20% water deficit, while minimum catalase activity (84.52 unit/ml) was recorded by Azoto+AMF with 100% chemical fertilizers and 50% water deficit.

Table 3- Effects of chemical fertilizers, water availability, AMF and *A. chroococcum* inoculation on catalase enzyme activity (unit / ml) in the leaves of wheat.

Chf %	(H): Water %	Biological treatments				(chf x H)	
		(AMF)	(Azoto)	(Azoto + AMF)	Control		
50	20	94.83	93.95	93.35	95.93	94.51	
	50	88.65	87.77	87.27	90.80	88.62	
100	20	92.69	92.20	91.60	95.01	92.87	
	50	86.51	85.38	84.52	90.22	86.65	
LSD	--	LSD (chfHT) = 4.607				LSD (chfH)= 1.32	
(chf x T)		LSD(chfT)= 3.91				Mean of (chf)	
(chf): 50		91.74	90.86	90.31	93.36	91.56	LSD (chf)= 0.214
(chf): 100		89.60	88.79	88.06	92.61	89.76	
(H x T)						Mean of (H)	
(H): 20		93.76	93.07	92.47	95.47	93.69	
(H): 50		87.58	86.57	85.89	90.51	87.64	
LSD		LSD (HT) = 1.291				LSD (H)= 0.214	
Mean of (T)		90.67	89.82	89.18	92.99	LSD (T)= 0.303	
(P<0.05).							

Chf: chemical fertilizer, H: water deficit , T: treatment

b- Peroxidase enzyme activity

Peroxidase activity in the leaves of wheat was significantly increased with the progression of water stress period in all treatments under study. The greatest peroxidase activity (118.81 unit/ml) was recorded at 20% water deficit, while the lowest (99.79 unit/ml) was at 50% water deficit (Table 4).

Besides, according to bio-fertilization treatment, the highest mean peroxidase activity (116.92 unit/ml) was recorded by the treatment of control, while the lowest (104.43 unit/ml) was at Azoto +AMF. Also, according to chemical fertilization, the highest mean (112.35 unit/ml) was at 50% chemical fertilization, while the lowest (106.24 unit/ml) was at 100% chemical fertilization.

Moreover, the interaction between chemical fertilization and water deficit significantly affected peroxidase activity; the highest mean (121.47 unit/ml) was at 50% chemical fertilization with 20% water deficit, while the lowest (96.33 unit/ml) was at 100% chemical fertilization with 50% water deficit.

Also, the interaction between chemical and bio-fertilization significantly affected the results; the highest value (118.14 unit/ml) was at 50% chemical fertilization, while the lowest (101.32 unit/ml) was at Azoto+AMF with 100% chemical fertilization. Moreover, the interaction of water deficit and bio-fertilizers significantly affected the results; the highest value (126.20 unit/ml) was at 20% water deficit, but the lowest (95.11 unit/ml) was at Azoto +AMF with 50% water deficit.

Additionally, the triple interaction between chemical fertilizers, water deficit and bio-fertilizers significantly affected peroxidase activity. Maximum peroxidase activity (126.80 unit/ml) was recorded at 50% chemical fertilizers with 20% water deficit, while minimum peroxidase activity (90.90 unit/ml) was recorded by Azoto+AMF treatment with 100% chemical fertilizers and 50% water deficit.

Table 4-Effect of chemical fertilizers levels, water availability, AMF and *A. chroococcum* inoculation in peroxidase enzyme activity (unit/ ml)

Chf %	(H): Water %	Biological treatments				(chf x H)	
		(AMF)	(Azoto)	(Azoto + AMF)	Control		
50	20	123.53	119.81	115.76	126.80	121.47	
	50	103.81	100.38	99.31	109.48	103.24	
100	20	114.66	112.61	111.74	125.61	116.16	
	50	95.78	92.83	90.90	105.80	96.33	
LSD	--	LSD (chfHT) = 6.599				LSD (chfH)= 4.27	
(chf x T)		LSD (chfT) = 5.23				Mean of(chf)	
(chf): 50		113.67	110.09	107.53	118.14	112.35	LSD (chf)= 0.565
(chf): 100		105.22	102.72	101.32	115.70	106.24	
(H x T)						Mean of H	
(H): 20		119.09	116.21	113.75	126.20	118.81	
(H): 50		99.79	96.61	95.11	107.64	99.79	
LSD		LSD (HT) = 5.36				LSD (H)= 0.565	
Mean of(T)		109.44	106.41	104.43	116.92	LSD (T)= 0.799	
(P<0.05).							

Chf: chemical fertilizer, H: water deficit , T: treatment

Mycorrhizal colonization in roots

The results in Table 5 show that the percentage of mycorrhiza colonization decreased significantly when the level of chemical fertilizer was increased from 50% to 100% for all treatments. The highest value was recorded upon adding 50% of the chemical fertilizer and all treatments. The treatments of *G. mosseae* and *G. mosseae + Azotobacter* with 20% water deficit and 50% chemical fertilizer gave the highest value (90%) as shown in Figure 1.

Table 5-Effects of bio-fertilizers and chemical fertilizers on the percentage of mycorrhizal colonization in wheat roots

Treatments	Chemical fertilizer	Mycorrhizal infection
G. + Azoto+ 50% water stress	100%	20%
G. mosseae + 20% water stress	100%	25%
G. + Azoto+ 20% water stress	100%	25%
G. mosseae + 50 % water stress	100%	20%
G. mosseae + 20 % water stress	50%	90%
G. mosseae +50% water stress	50%	70%
G. + Azoto+ 50% water stress	50%	80%
G. + Azoto+ 20% water stress	50%	90%

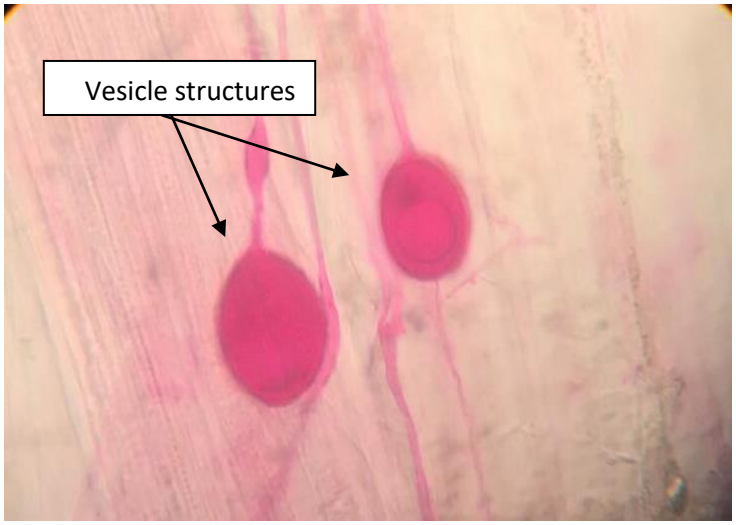

Figure 1-Mycorrhiza vesicles at treatment with *G. mosseae* + 20 % water stress at 50% chemical fertilizer (40X)

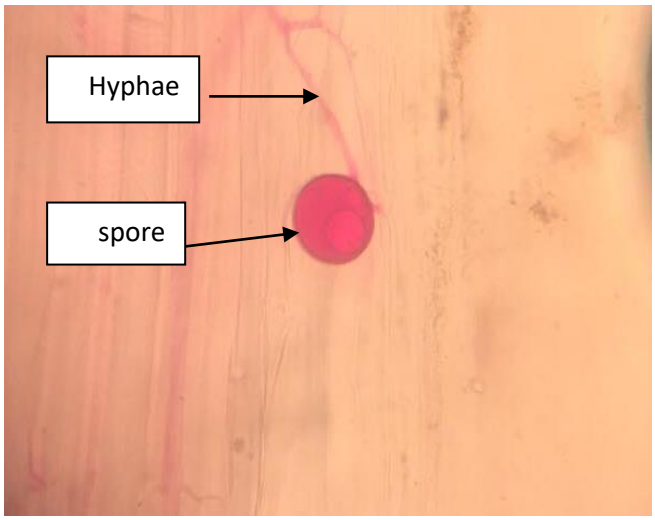

Figure 2-Mycorrhiza vesicles at treatment with *G.mosseae+A.chroococcum*+ 50% water stress at 50% chemical fertilizer (40X)

Discussion

Rhizosphere is a wealthy habitat of micro-organisms that was explored in order to obtain the potential plant growth- promoting rhizo-bacteria (PGPR), that can be useful in the development of bio-inoculants to enhance crop growth and yield. Bio-fertilizer inoculation improved yield and yield component of wheat plant. In this study, one genus of each of free living bacteria and fungi were isolated, purified, and identified to study their effects synergistically with chemical fertilizers on wheat plant.

The results of the field experiment showed that the lowest values of proline content and antioxidant enzymes' activities in leaves of wheat were recorded in response to the treatment with Azoto+AMF with 100% chemical fertilization and 50% water deficit. These decreases indicate the potential action of bio-fertilizers. The results illustrated the accumulation of proline under drought conditions. It is believed that proline plays a major role in maintaining membrane stability and subsequently reducing the leakage of nutrients and the loss of water in cells grown under drought stress. These results agree with those of [15] who reported the decrease of proline content in leaves of wheat inoculated with bio-fertilizers under drought conditions.

The effects of *A. chroococcum* were described in relation to nitrogen fixation and uptake from the soil after converting it into amino acids and then into protein compounds derived from the plant. This improves the growth of the plant and increases its weight as a result of encouraging the absorption of phosphorus to form a dense root mass that help absorb water and nutrients [16] .In addition, symbiosis of AMF with plant roots was reported to be accommodating in tolerating and overcoming episodes of water stress in various plant species [17], including wheat [18].

Antioxidant activity upon stress conditions could prevent oxidative damage caused by reactive oxygen species. Also, the generation of catalase increases at water stress conditions. These results agree with those of [19] who revealed that, under drought stress, the expression levels of CAT in seedlings of the bio-fertilizers were lower than those of control treatment.

The results also showed superior peroxidase activity under control conditions as compared with bio-fertilizer treatment which decreased the peroxidase activity. These results agree with those of [15,20] who mentioned lower peroxidase and catalase activities in wheat plant inoculated with a bio-fertilizer under drought condition.

The reason that the percentage of mycorrhizal infection increased significantly is that, under phosphorus deficiency conditions, the amount of phospholipids in the root cell

membranes decreases, leading to increased permeability of these membranes. This leads to increased root secretion of reduced sugars and amino acids, thus increasing the proportion of infected roots. Under phosphorus availability conditions, the permeability of the membranes of the root cells is reduced due to the increase in their phospholipids and the decreases in the amounts of sugars and amino acids, leading to a decrease of infected roots [21]. This negative effect of high nitrogen and phosphate-content fertilizers on the percentage of mycorrhizal infection is in agreement with that described by many researchers [22,23].

In conclusion, the use of bio-fertilizers with chemical fertilizers improves the yield and yield components and decreases proline content and antioxidant enzymes' activities of wheat plant under drought conditions.

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