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Effect of Bio-chemical Fertilizer on Proline Accumulation, Catalase and Peroxidase Enzymes Activity in Leaves of Two Wheat Cultivars (Ipa99 and Rabyaa) Under Water Deficit Stress.

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Abstract

Field experiment was carried out during 2013- 2014 at the research field of the Department of Biology, College of Science, Baghdad University, to study the effect of bio-chemical fertilizers and chemical fertilizers on some agronomic traits and yield components of two wheat cultivars (IPA99 and Rabyaa) under drought stress conditions. The bio-chemical fertilizers were mixture of (*Azotobacter chroococcum. Azospirillum brasilense.* and *Pseudomonas fluorescens.*) with chemical fertilizers (only 50% of the recommended dosage of N and P), while the chemical fertilizers was 100% of recommended dosage of N and P. Field plots (1×1 m) were randomly made in the field equipped with rainfall shelter to avoid rains. Grains of wheat cultivars were sown manually in their respective plots in rows with a distance of 20 cm between rows and at seed rate of 3 g per row (150 kg/ha). The experiment was conducted in randomized complete block design (RCBD) under split plot arrangement with five replications. Adding fertilizer were kept in the sub plot, while water stress treatment was assigned as main plot. The data were analyzed by analysis of variance (ANOVA). Water stress was applied by irrigated the plots to the soil field capacity then withheld next irrigation until the soil moisture of the respective plots depleted to 80 (control), 30 % of soil field capacity. The results also showed significant decrease antioxidant enzymes activity with the decreased water stress. Also, the antioxidant enzymes activity (CAT and POD) were more activity after 107 days than 65 days because of no irrigation for harvesting preparation. Besides, at high drought level, the response of the antioxidant enzymes activity (CAT and POD) and proline accumulation were similar to that observed in control treatment, while with bio-chemical fertilizers the significant decreased respectively. These bio-chemical fertilizers would play key role in productivity and sustainability of protect the environment as eco-friendly fertilizers and cost effective inputs for the farmers. With using bio-chemical fertilizers that contain different microbial strains has led to decrease in the use of chemical fertilizers, which can be help achieving sustainability of farms and with no harmful agrochemicals for human safety.

Keywords: Biofertilizer, chemical fertilizers, water deficit, wheat, proline, catalase, peroxidase

اثر الاسمدة الحيوية والكيمياوية على كمية البرولين و فعالية الانزيمات الكتليز والبيروكسيديز في اوراق صنفين لمحصول الحنطة اباء 99 وربيعة تحت ظروف الجفاف

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الخلاصـــــــة:

نفذت تجربة حقلية خلال الموسم الزراعي 2013 –2014 في حقل الأبحاث التابع لقسم علوم الحياة في كلية العلوم، جامعة بغداد لدراسة تأثيرات الاسمدة العضوية و الكيمائية على بعض الصفات الخضرية والحاصل لصنفين من الحنطة (اباء وربيعة) تحت تأثير شدود مختلفة لماء التربة . تضمن توليفة السماد العضوي ثلاث انواع من البكتريا وهي *Azospirillum brasilense., Azotobacter chroococcum.)* *Pseudomonas fluorescens*.) مع 50 % من سمادي النيتروجين والفسفور وحسب الجرع الموصى بها للحنطة ، في حين تضمن معاملة التسميد الاخرى استخدام 100 % من الاسمدة النيتروجينية والفوسفاتية . حرثت وسويت تربة الحقل وقسمت إلى ألواح بإبعاد 1x1 م، ثم بذرت حبوب الأصناف يدويا في المكان الخاص بها في الألواح على شكل خطوط بطول متر وبمسافة 20 سم بين خط وآخر وبنسبة بذار3غم لكل خط (150 كغم/هكتار). فصلت الألواح فيما بينها بطبقة من البلاستك أنزلت في التربة بعمق 35 سم لمنع الحركة الأفقية للماء بين ألواح التربة. أجريت التجربة باستخدام تصميم الألواح المنشقة بترتيب القطاعات العشوائية الكاملة وبخمسة مكررات لكل معاملة. احتلت معاملة التسميد الألواح الثانوية بينما احتلت معاملات الشد المائي الألواح الرئيسية. طبق الشد الرطوبي بسقي الألواح وصولا إلى السعة الحقلية للتربة، وبعد ذلك حجبت السقية التالية إلى حين استنزاف رطوبة التربة لألواح المقارنة إلى 80 % وألواح الشد الجفاف30 % من السعة الحقلية للتربة على التوالي .أظهرت النتائج انخفاض ملحوظ لنشاط الانزيمات المضادة للأكسدة مع زيادة الإجهاد المائي. كما أن مضادات الأكسدة الانزيمات النشاط (الكتليز و البيروكسيديز) كانت أكثر نشاطا وربما يعود ذلك لعدم ري النباتات قبل الحصاد. الى جانب ذلك، ومع زيادة الجفاف، زادة فعالية الانزيمات المضادة للأكسدة (الكتليز و البيروكسيديز) كما زادة كمية البرولين في الاوراق لكلى الصنفين ولجميع المعاملات بما فيها معاملة السيطرة، بينما و مع اضافة الأسمدة الحيوية و الكيميائية انخفضت فعالية الانزيمات بشكل كبير على التوالي. اظهرت الدراسة امكانية ان تلعب الاسمدة الحيوية الكيمياوية دورا فعالا في ادامة خصوبة التربة وان تكون صديقة البيئة لاحتوائها على ميكروبية مختلفة تساعد النباتات للحصول على العناصر الغذائية وزيادة تحملها لبعض الاجهادات البيئة للتقليل من خطر تلوث البيئة بالأسمدة الكيميائية لضمان سلامة الانسان .

Introduction

Wheat (*Triticum* ssp.) is one of the most important food crops in the world in terms of the area harvested, production and nutrition; as it supplies about 19% of the calories and 21%of the protein to the world's population [1]. Due rapidly increasing population and changing dietary patterns ,the demand for wheat by 2050 is expected to increase by 31%over the 683 million tons consumed in 2008[1,2]. At present the productivity of wheat is limited due to several environmental stresses including high temperature and drought [3-5].

Drought and high temperature are two important environmental factors that adversely affect performance and yield of wheat crop [6,7]. Drought increases senescences by enhancing chlorophyll degradation ,nitrogen loss ,and lipid peroxidation [8].Very high accumulation of cellular proline (up to 80% of the amino acid pool under stress and 5%under normal condition)due to increased synthesis and decreased degradation under a variety of conditions such as salt and drought has been documented in many plant species[9], proline, which is an amino acid especially known for its sensitivity to stress, is synthesized under stress conditions [10]. In plants proline accumulation has been well correlated with tolerance to salinity drought [11].Drought tolerance in plants various physiological responses ,including antioxidant defenses and osmolytes [12]. Drought stress causes imbalance between the generation and quenching of reactive oxygen species (ROS). ROS, such as superoxide radicals(OH),which are highly reactive in the absence of effective protective mechanism .Drought can seriously damage plants by lipid peroxidation ,protein degradation, breakage of DNA and cell death[13,14].

To minimize and eliminate oxidative damage, plants have evolved on antioxidant system composed of both non-enzymatic and enzymatic constituents present in plant cells, such as ascor bate peroxidase (APX). Catalase (CAT), guaiacol peroxidase (POD), superoxide dismutase (SOD) [15].The antioxidants could remove, neutralize and scarenge the ROS at different cellular locations, helping to reduce lipid peroxidation and maintaining cell membrane stability [16,17]. Previous studies generally agreed that maintaining active antioxidant enzymes is important for drought tolerance in various plant species by increasing the protection capacity against oxidative [18,19]. Furthermore general idea is that morphological for long-term accumulation, of these compatible solutes, such as soluble sugar, betaine and free proline, is one of the most common response of plants to water deficit [20].

Biofertilizer is a substance, which contains living microorganisms applied to seed, plant surfaces or soil. Biofertilizers add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus and stimulating plant growth through the natural processes of nitrogen fixation, solubilizing phosphorus and stimulating plant growth through the synthesis of growth promoting substances. Bio-fertilizers can be expected to reduce the use of chemical fertilizer and pesticides. The microorganisms in bio-fertilizers restore the soil's natural nutrient cycle and build soil organic matter. One of the biochemical reactions to drought stress is the antioxidant defense system, including enzymatic and non- enzymatic compounds. Also enzymatic systemic includes superoxide dismutase (SOD, catalase (CAT), ascorbate peroxidase (APX), glutathione(GR) and polyphenol oxidase(POD) [21,22]. Antioxidant enzymes can eliminate the active radical oxygen (ROS) which generate in abiotic stress condition .The enzymes protect membranes from destructive effect of ROS and make plants to resist against stress conditions [23]. Difference of stress stimulation on antioxidant enzymes also species and age of plant [24]. The present investigations were aimed to study the role of bio-chemical fertilizers effects on proline and antioxidant enzymes of wheat cultivars under drought stress.

Materials and methods

Two bread wheat cultivars namely Rabyaa and IPA 99 were used in this study. State Board redistricted the cultivars for Seed Testing and Certification, Ministry of Agriculture, Iraq. The cultivars were kindly provided by Seed Technology Center, Ministry of Science and Technology and by State Board for Seed Testing and Certification, Ministry of Agriculture. Field experiment was conducted during 2013- 2014 in silt loam soil at the research field of the Department of Biology, College of Science, Baghdad University, Baghdad, Iraq. The chemical and physical characteristics of field soil were measured in laboratory of soil department, college of agriculture, Baghdad University. The field was equipped with transparent cover to avoid the effect of the rain during the course of study. Field plots (1×1 m) were randomly made in the field. The plots were separated from each other by a plastic sheet inserted vertically in the soil to 35 cm depth in order to prevent the possible horizontal movement of irrigated water. Grains of wheat cultivars were sown manually in their respective plots in rows of one meter each with a distance of 20 cm between rows (10 rows per plot) and at seed rate of 3g per row (150 kg/ha). Chemical fertilizers were used for the half of the plot, while another half were treated bio-fertilizer, which was a mixture of *Azotobacter chroococcum*, *Azospirillum liboferum* and *Bacillus megatherium* applied with chemical fertilizers (only 50% of the recommended dosage of NP). The bio-fertilizer brought from the experimental station of crops department, Abu-Ghraib, State Board of Agricultural Researches in Baghdad, Iraq. Chemical fertilizers used were urea (46% N) at 200 kg ha^{-1} and triple super phosphate (46% P2O5) at 100 kg ha^{-1}. All phosphorus fertilizer was applied at planting during seedbed preparation, while urea was divided into two equal amounts. The first amount was added during the land preparation prior to planting, the second was added 30 days after sowing (during the early tillering stage) and the final amount was added at panicle initiation.

The bio-fertilizer inoculums preparation was completed by growing the bio-fertilizer in 250 ml conical flask having 100 ml nutrient broth by incubating at 28±1ºC in the orbital shaking incubator with 100 rpm for three days to attain uniform cell density (108-109 CFU.ml^{-1}). The seeds of wheat were inoculated by mixing with sterilized peat containing sugar solution 10% and Arabic gum with three-day-old inoculums of respective strain, while seed for control and other treatments were mixed with sterilized peat containing sterilized broth and solution of sugar and Arabic gum. Inoculated seed were air dried under shade for 6-8 hr before sowing [19]. Seed of wheat *Triticum aestivum* L. cultivar Rabyaa and IPA 99 after inoculation according to the treatment plan were introduced (on 1 December

2013). Weeds were eliminated by hand. Growth parameters taken after 120 days from sowing at flowering stage while yield parameters were taken at harvest 8 may 2014.

Water stress was applied by irrigated the plots to the soil filed capacity then withheld next irrigation until the soil moisture in the respective plots depleted to 80 (control), 25 % of soil field capacity. Soil moisture of the plots was recorded by weight basis method (Standards Association of Australia, 1977)

OW = (WS1 - WS2)/WS2 x 100

Where :

OW = Soil moisture percentage on dry weight basis

WS1 = Soil sample weight before oven drying

WS2 = Soil sample weight after oven drying

Proline determination

Free proline content of wheat leaves was determined following the method of [25]. Samples of 0.5 g of fresh weight of leaves from each treatment were homogenized in sulphosalicylic (3% w/v H2O), then centrifuged at 3000 rpm for 5 minutes. Samples of 2 ml from the supernatant were added to 2 ml of each of ninhydrin and glacial acetic acid and incubated at 100ºC for 1 hour in water bath. The reaction was arrested in an iced bath and the chromophore was extracted with 4 ml toluene and its absorbance at 520 nm was determined in spectrophotometer (Varian Australia PTY LTD). Proline concentration was determined using a calibration standard curve prepared with authentic proline and calculated its amount on fresh weight basis using the following formula.

µmoles of proline/g of fresh material = [(µg proline / ml × ml toluene) / 115.5 µg /µmole] / [(g sample (0.5)/5]

The results were analyzed using ANOVA.

Determination catalase activity enzyme

The activity of catalase was determined according to [26]in 3ml, of reaction solution , which contained 2ml of phosphate buffer pH 7.0 and 0.3ml of hydrogen peroxide solution (3%) in a test tube , then 0.2ml of extract containing enzyme (supernatant) was added .The blank was composed from : 2.3 ml of phosphate buffer pH 7.0 and 0.2 ml of extract containing enzyme (supernatant).After 1 min the absorbance was measured at wave length of 240 nm using UV-Vis spectrophotometer for activity test and blank tubes .The activity of catalase was calculated as shown below :

$$\text{Enzyme activity (unit/ml)} = \frac{\Delta Ab(Abt-Abb)x\ dilution\ factor\ (DF)X\ reaction\ volume(V)}{Extinction\ coefficient(EC)Xvolume\ of\ enzyme\ (EV)}$$

Abt =absorbance of test tube/min

Abb = absorbance of blank tube/min

DF=dilution of supernatant (15)

V= Volume of reaction (3)

EC=40

EV=Volume of enzyme (0.2 ml).

Determination of peroxidase activity enzyme

1.5 ml of hydrogen peroxide solution (1.7mM) was mixed with 1.5ml of 4-Aminoantipyrine reagent (2.5mM),then 0.1ml of extract containing enzyme(supernatant) was added to the absorbance were measured at wave length of 510 nm using UV-Vis spectrophotometer .The activity of peroxidase was calculated as shown in below equation :

$$\text{Enzyme activity (unit/ml)} = \frac{\Delta A/\min x\ RVXD}{6.58XEV}$$

Where:

ΔA/min=The change in absorbance at 510nm/minute

RV=Total volume of reaction mixture (3ml)

D=Enzyme dilution factor (30)

6.58=Extinction coefficient

EV=Volume of enzyme sample (0.1ml).

[27,28].

The experiment was conducted in randomized complete block design (RCBD) under split plot arrangement with five replications. The fertilizer kinds were kept in the sub plot, while water stress treatment was assigned as main plot. The data were analyzed using analysis of variance (ANOVA). The least significant differences test was used to compare the averages of treatments [29].

Results and discussion

Effect of bio-chemical and chemical fertilizer on proline content of leaves of two wheat cultivars under water deficit stress.

Results presented in figures 1 and 2 exhibited that average of proline content was significantly increased by water deficit stress. Exposure of plants to 30 % water stress led to increased proline content of leaves by 13.87 and 10.18 % after 65 and 107 days after sowing, respectively.

Average of proline content was significantly different among the fertilizer treatment. The highest proline content (5.86 and 4.61 µmole/g) were recorded by control treatment after 65 and 107 days respectively, while bio-chemical and chemical treatments recorded the lowest proline content (3.99 and 4.19 µmole/g),after 65 and 107 days, respectively.

The interaction between water deficit stress and wheat cultivars with fertilizer treatments significantly affected proline content of leaves. At abnormal irrigation (30% of field capacity) and without fertilizer, Rabyaa cultivar has statistically higher leaves proline content (9.28 µmole/g) than the other treatment after 56 days. However, normal irrigation (80% field capacity) and with adding bio-chemical fertilizers, differential response in terms of leaves proline content has been observed. Minimum proline content (3.35µmole/g) was found in IPA99 after 107 days.

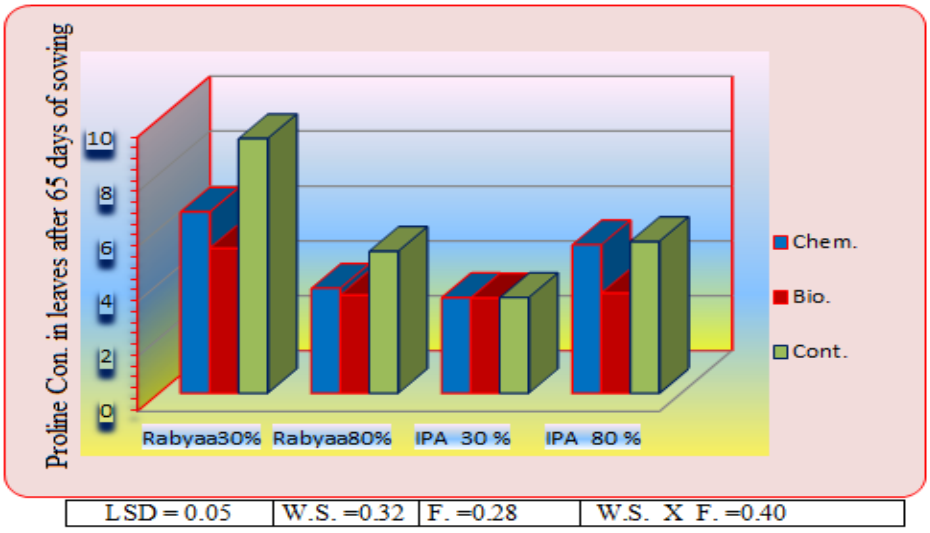


Figure 1-Proline concentration. (µmole/g) in leaves tissue after 65 days under field conditions**.**

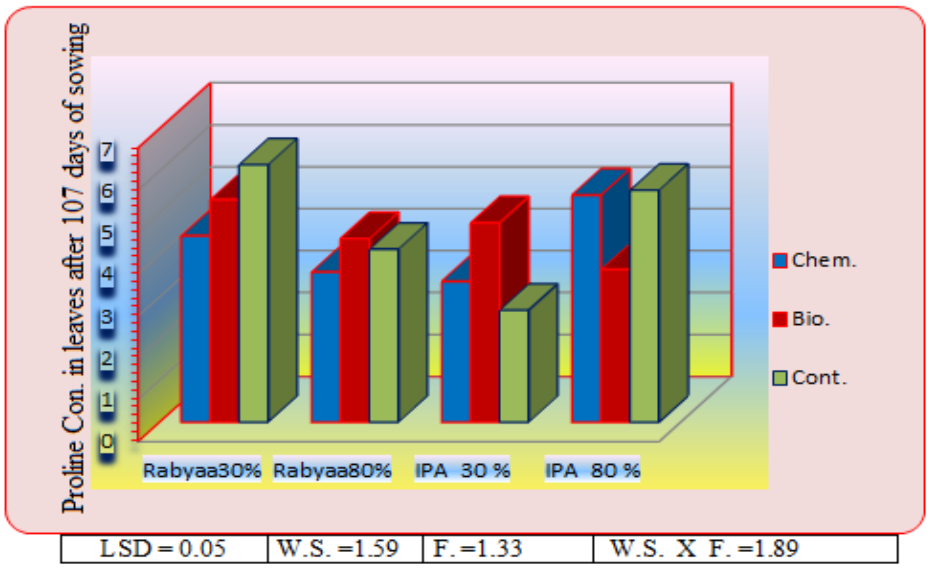
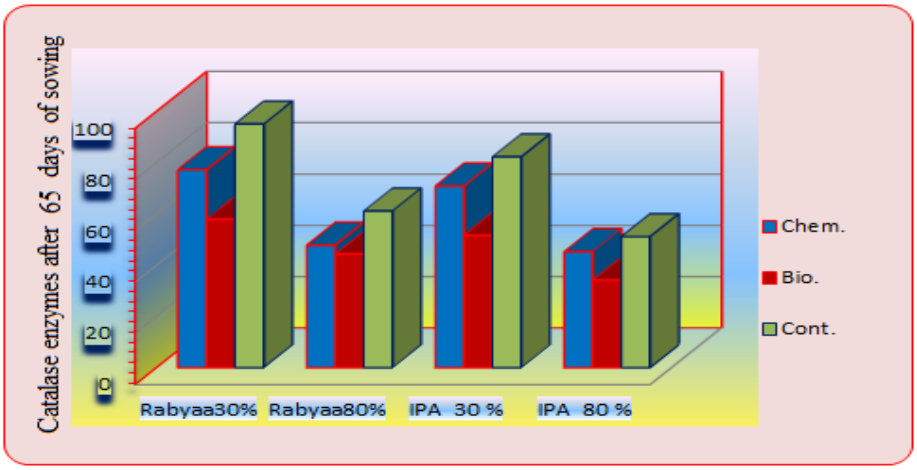


Figure 2-Proline concentration. (µmole/g) in leaves tissue after 107 days under field conditions**.**

Effect of bio-chemical fertilizer on Catalase enzymes activity of leaves.

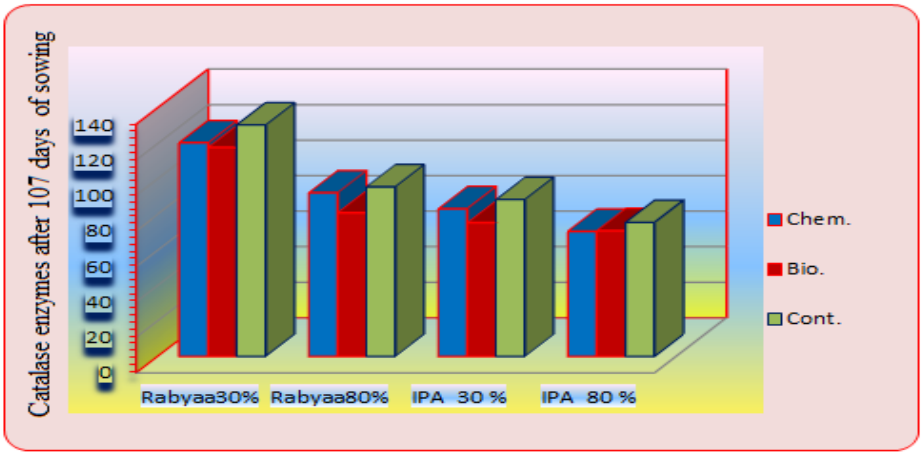
Data presented in figures 3 and 4 show the effect of fertilizers and water deficit stress on Catalase activity after 65 and 107 days of wheat plants grown either in 30% or 80% field capacity. Results presented exhibited that average of catalase activity was significantly increased by water deficit stress. Exposure of plants to 30% water stress led to increased

Catalase activity in leaves by (53.23 and 26.49 %) after 65 and 107 days after sowing, respectively.



LSD ≤ 0.05	W.S. =2.34	F. =1.5	W.S. X F. =2.12

Figure 3-Catalase enzymes activity (unit/ml) in leaves tissue after 65 days under field conditions.



LSD ≤ 0.05	W.S. =3.63	F. =3.48	W.S. X F. =4.9

Figure 4-Catalase enzymes activity (unit/ml) in leaves tissue after 107 days under field conditions**.**

Figure 4 and 5 showed that, plants with bio-chemical fertilizer treatments showed a decrease in the catalase production under control (47.01 and 86.18 unit/ml) after 65 and 107 days, respectively. Maximum results were found in control treatments (72.38 and 97.42 unit/ml) after 65 and 107 days, respectively.

Also, there were significantly increases the catalase content in control treatment (without fertilizer) interaction with water shortage especially after 107 days. cultivating Rabyaa cultivar under 30% field capacity caused an increment of up to 130.36 unit/ml after 107 and in the control treatments. Although this increment was much less than that (34.18 unit/ml) when IPA99 cultivar treated with bio-chemical fertilizers under 80% field capacity after 65 days, respectively.

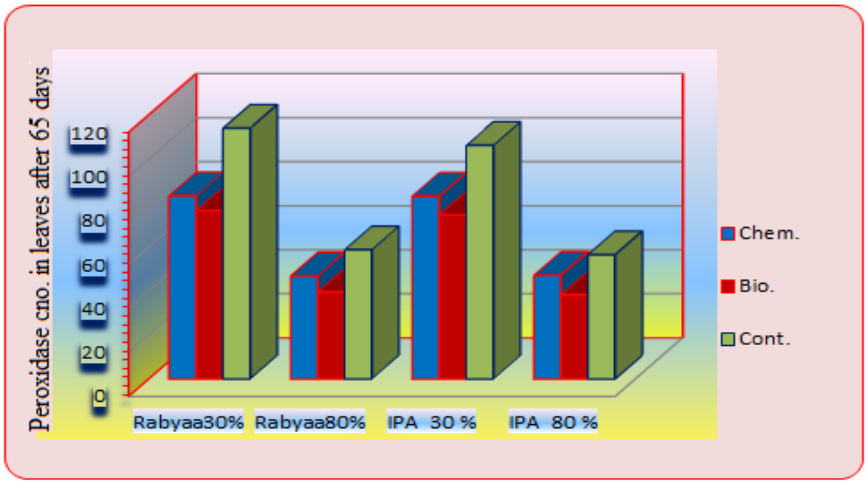
Effect of bio-chemical fertilizer on peroxidase enzymes activity of leaves.

The peroxidase activity (figures 5,6) was significantly ($P \geq 0.05$) increased with the progression of water stress period in all treatments under study. The peroxidase activity under control treatment condition at 107 days for all wheat cultivars caused an increase than at peroxidase activity after 65 days. At 30% field capacity maximum average increase by (87.41% and 40.78%) in peroxidase

activity after 65 and 107 days, respectively .while least increase in activity was found in 80% field capacity.

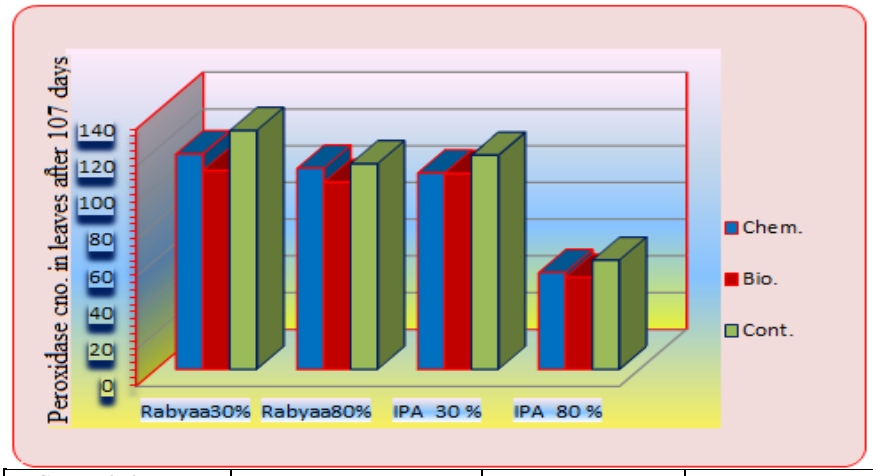
The activity of this antioxidant enzyme again decreased within bio-chemical and chemical fertilizers treatments. This decrease was less after 65days treatments. The maximum average increase in peroxidase activity (83.97 and 104.51 unit/ml) were though control level treatment , while minimum (57.19 and 91.71 unit/ml) were recorded in bio-chemical treatments after 65 and 107 days, respectively.

The highest increase activity(130.06 unit/ml) was found in Rabyaa control treatment at 30% field capacity after 107 days compared to lowest values of peroxidase activity (38.30 unit/ml) which found in IPA99 at 80 % field capacity bio-chemical treatment after 65 days from the sowing



LSD ≤ 0.05	W.S. =1.23	F. =1.34	W.S. X F. =1.90

Figure 5-Peroxidase enzymes activity (unit/ml) in leaves tissue after 65days under field conditions**.**



LSD ≤ 0.05	W.S. =3.63	F. =6.40	W.S. X F. =9.05

Figure 6-. Peroxidase enzymes activity (unit/ml) in leaves tissue after 107 days under field conditions**.**

Environmental stresses are becoming a major problem and productivity is declining at an unprecedented rate. Water deficit is considered as a major environmental factor affecting many aspects of plant physiology and biochemistry [30]. Because morphological and physiological mechanisms are involved in a plant's response to drought stress, It is a fact that yield and yield components of the plant in drying soil are reduced even in tolerant genotypes. The grain yield depends on many characters such as proline accumulation, CAT and POD antioxidant enzymes activity.

The present study demonstrated that Rabyaa are the most tolerant cultivars since it had the highest values of grain and biological yield at high water deficit stress, while IPA 99 was the most drought sensitive cultivars since lower grain and biological yield were obtained when grown at high level of water deficit stress. Subsequent data analyses revealed that the drought tolerant cultivars appeared

superior in yielding, number of tillers, spikes, grains per spikes, 1000-grain weight, grain yield, biological yield ,harvest index ,leaf area , plant height, chlorophyll , biomass fresh weight and dry weight, proline , catalase and peroxidase enzymes content compared to the drought sensitive cultivar (IPA 99).

The test cultivars also varied significantly for leaf area where the tolerant cultivars Rabyaa, had higher leaf area than drought sensitive cultivars IPA 99 under field condition although both cultivars showed significant reduction with increased water deficit stress. The reduction of leaf area could probably be one of the drought mechanisms adapted by drought tolerant plant grown under the limited soil moisture content [31]. With a small leaf area a crop is able to limit water loss because the size of the evaporating surface is small. In present study, the results were not coincided with the results of other studies. The ability of tolerant wheat cultivars to maintain higher leaf area under drought condition may be attributed to the ability of these cultivars to grow efficient root system that can absorb water enough to maintain normal higher leaf area.

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