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Research Article

BIOREMEDIATION OF DROUGHT STRESSED WHEAT (*TRITICUM AESTIVUM* L.) SEEDLINGS

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ABSTRACT
A study was conducted to evaluate the effects of microbin biofertilizer, a mixture of nitrogen-fixing and phosphate-dissolving bacteria (*Azotobacter* sp. and *Azospirillium* sp. and *Bacillus megaterium*) for ameliorating negative effects of drought stress on growth and growth indices, photosynthetic efficiency, antioxidant enzymes, and phytohormones of wheat (*Triticum aestivum* L.). Two levels of irrigation (90% and 50% of field capacity) were employed in presence and absence of biofertilizer. Wheat seedling exhibited a retardation of growth, a reduction of the photosynthetic efficiency, relative water content, and membrane stability index, as well as the increase of the antioxidant enzymes like superoxide dismutase, peroxidase, ascorbate peroxidase and catalase, the production of malondialdehyde, hydrogen peroxide, and the extensive impairments in cellular ultra-structure in response to drought stress. Phytohormones indole-3 acetic acid (IAA), gibberellic acid (GA) contents were decreased as a result of water deficit; conversely, absicic acid (ABA) showed a marked increase. The interaction of microbin biofertilizer and water stress showed a remarkable improving in growth characteristics and in some physiological parameters. The overall results suggest that application of microbin biofertilizer inoculation alleviates to some extent the deleterious effects of drought stress on wheat seedlings.

Keywords: *Antioxidant Enzymes, Biofertilizer, Photosynthetic Efficiency, Phytohormones, Water Stress*

INTRODUCTION
Drought is a worldwide problem, constraining global crop production seriously and has detrimental effects on most of plant growth stages such as germination, seedling growth, physiological activities and ultimately the plant yield (Azimi *et al.,* 2013). Wheat (*Triticum aestivum* L.) is considered the most strategic crop for Egypt and many other developing countries. Wheat should be irrigated when 50–55% of the available soil water is depleted in the root zone (Doorenbos and Pruitt, 1992). Limitation of irrigation water resources is a widespread growing problem for cultivation of agricultural crops. When plants are exposed to environmental stresses such as drought, reactive oxygen species (ROS) like, superoxide ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen (1O) are produced. The balance between the production of ROS and the quenching activity of the antioxidants is upset and this often results in oxidative damage (Jung, 2004). To reduce the toxicity of ROS, plant cells have developed an anti oxidative system, consisting of protective enzymes. Superoxide radicals are scavenged by superoxide dismutase, while the resulting H_2O_2 is reduced to H_2O by catalase, peroxidase and ascorbate peroxidase (Apel and Hirt, 2004). Plants respond and adapt to drought stress through various biochemical changes including changes of the endogenous hormone levels (Monneveux and Belhassen, 1996). Plants can recruit abscisic acid as an endogenous, signal to initiate adaptive responses (Wang *et al.,* 2008). However, the variation of IAA and GA contents under drought stress are contradictory. It was reported that drought resulted in a decrease of IAA content in the leaves of wheat (Xie *et al.,* 2003). However, Pustovoitova *et al.,* (2004) reported that adaptation to drought was accompanied with an increase in the IAA content. Current soil management strategies are mainly dependent on inorganic chemical-based fertilizers, which caused a serious threat to human health and environment. The exploitation of beneficial microbes as a biofertilizer has become paramount importance in agriculture sector for their potential role in food safety and sustainable crop production (Bhardwaj *et al.,* 2014). Biofertilizers containing microorganism, which have an ability to convert nutritionally important elements to available form through biological processes

(Vessey, 2003). Water and nutrients are the most important factors during plant growth and development. Deficit irrigation and use of biological fertilizers are the critical components to crop production in sustainable farming systems (Canbolat *et al.,* 2006). Microorganisms could play a significant role in stress management, also provide excellent models for understanding stress tolerance mechanisms that can be subsequently engineered into crop plants. The main objective of this study was to investigate the effect of microbin biofertilizer on wheat (*Triticum aestivum* L.) growth, photosynthetic pigments, photochemical activity of photo system II (PSII), ultra-structure, antioxidant defense system, in addition to monitor the relative water content, lipid peroxidation, and membrane stability index, as well as the changes in the endogenous level of phytohormones under normal irrigation and drought stress.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* L.) cv. Sakha 93 was used in this study, purchased from the International Research Center, El-Dokki, Giza, Egypt. Grains were subjected to surface sterilization with 0.1% sodium hypochlorite solution for 5min and then rinsed several times with distilled water. Grain inoculation was performed by mixing wheat grains with the microbin using Arabic gum as adhesive material. Microbin, a commercial multi-strains biofertilizer is produced by biofertilizers unit, General Organization of Agriculture Equalizaton Fund, Agriculture Research Centre, Ministry of Agriculture, Giza, Egypt. It is constituted of a mixture of N_2-fixing and P-dissolving bacteria, the treated biofertilizer containing N-free living bacteria (*Azotobacter sp.* and *Azospirillium sp*.) and a phosphate dissolving bacteria (*Bacillus megaterium*). The coated grains were then air dried in shade for 30 minutes and inoculated and uninoculated grains were sown immediately in pots containing soil and sand mixture (1:2). The pots (in triplicates) were placed in growth chamber under 14h light/10h dark cycle at 23/18±2°C for 20 days. Pots were divided into two sets (inoculated and uninoculated) both irrigated with half strength Hoagland´s nutrient solution (Hoagland and Arnon, 1950) till water field capacity. Drought stress treatment was started after 5 days from sowing, two treatments were imposed viz, 90%, and 50% of the field capacity, these treatments reflecting conditions achieved as optimum level of water supply (control plants) and drought stress, respectively.

Plant Growth Parameters

At harvest (20 days) plant samples were collected, in order to avoid damaging the roots when they pull out, all of the pots were temporarily waterlogged for 1h. After carefully uprooting, the plant samples were divided into shoots and roots. The samples were washed several times with distilled water, and the plant shoot height and root length (cm) were measured. To determine the dry weight, the shoots and roots were oven-dried separately at 70°C for 72h. Fresh leaves were used for different biochemical analysis.

Photosynthetic Pigment Contents

The pigment fractions (chlorophyll a, chlorophyll b and carotenoids) were estimated by the method described by Lichtenthaler (1987). Leaf tissues (50mg) were homogenized in 10ml chilled acetone (80%). The homogenate was centrifuged at 4,000g for 12min. Absorbance of the supernatant was recorded at 663, 647 and 470nm (using a T80 UV–Vis spectrophotometer - double beam).

Chlorophyll Fluorescence

Measurements of chlorophyll fluorescence (Fv/Fm) were performed by OS-30P pulse modulated chlorophyll fluorimeter (Opti-sciences, Hudson, USA). Before every measurement, leaves were dark-adapted for 30 min with leaf-clips (Branquinho *et al.,* 1997).

Estimation of Photosynthetic Activity (PS II)

Chloroplast thylakoid membranes (PS II membranes) were isolated from wheat leaves according to Arnon (1949) method. Photo system II activity, as indicated by the rate of 2,6-dichlorophenol indophenol (DCPIP) photo reduction, was monitored at 600nm using a spectrophotometer as described by Trebest (1972).

Ultra-Structural Analysis

Fresh tissues (1–2mm^2) taken from last fully expanded leaves were prepared according to method of Loreto *et al.,* (2001) for electron microscopy processing. The ultra-structure visualization and

photographing were carried out using the Transmission Electron Microscope (JEOL-TEM 100 CX) at the Electron Microscopic Unit, Faculty of science, Alexandria University.

Relative Water Content

The relative water content was estimated according to Turner (1981) and was evaluated from the equation: RWC (%) = (FW – DW) / (TW – DW) x100, where FW is the fresh weight of the leaves, TW is the weight at full turgor, measured after floating the leaves for 24 hours in water at room temperature and DW is the weight estimated after drying the leaves for 4 hours at 80°C or until a constant weight is achieved.

Membrane Stability Index

Membrane stability index (MSI) was determined by recording the electrical conductivity of leaf leachates in double distilled water at 40 and 100°C. Leaf samples (0.1g) were cut into discs of uniform size and taken in test tubes containing 10 ml of double distilled water in two sets. One set was kept at 40°C for 30 minutes and another set at 100°C in boiling water bath for 15 minutes and their respective electric conductivities C_1 and C_2 were measured by conductivity meter (Deshmukh *et al.,* 1991).

Membrane stability index = $[1- (C_1/C_2)] \times 100$

Determination of Malondialdehyde and Hydrogen Peroxide Content

The level of lipid peroxidation products was estimated and expressed as malondialdehyde (MDA) according to Buege and Aust (1978). The MDA concentrations were expressed as µmol g^{-1} DW. Hydrogen peroxide levels were determined according to Sergiev *et al.,* (1997) and the concentrations were expressed as mmol g^{-1} DW.

Extraction and Determination of Antioxidant Enzymes Activities

Fresh wheat leaves (1g) were homogenized in ice cold 50mM phosphate buffer (pH 7.5) containing 0.5mM EDTA with prechilled pestle and mortar.

The homogenate was centrifuged at 10,000g for 10 min at 4°C and the supernatants were collected and used for the determination of different enzyme activities. The protein content in the enzyme extract was determined according to Hartree (1972).

Superoxide dismutase (SOD) activity was determined following the method used by Giannopolitis and Ries (1977) where its ability to inhibit the photo reduction of nitroblue tetrazolium (NBT) was measured. Catalase (CAT) activity was assayed with the method of Zhang *et al.,* (2005).

One unit of enzyme determined the amount necessary to decompose 1µmol of H_2O_2 per min at 25°C. Peroxidase (POD) activity was determined according to Noreen and Ashraf (2009). Changes in absorbance of the reaction solution at 470nm were determined after every 20s.

Ascorbate peroxidase (APX) activity was assayed by the following reduction in absorbance at 290nm as ascorbate was oxidized according to the method of Boominathan and Doran (2002). The mixture contained 50mM phosphate buffer (pH 7), 0.2mM EDTA, 0.5mM ascorbic acid and 50µg bovine serum albumin.

Determination of Plant Phytohormons

Hormones were extracted and purified following Kettner and Doerffling (1995) procedure. The plant leaves (1g) were ground in 80% methanol at 4°C for 72 hr with an antioxidant, butylated hydroxyl toluene. The extracted sample was centrifuged and the supernatant was reduced to aqueous phase using rotary evaporator.

The pH of aqueous phase was adjusted to 2.5-3.0 and extracted four times with half volume of ethyl acetate. The ethyl acetate was dried completely using rotary evaporator and the dried sample was redissolved in 1ml of methanol (100%) and was analyzed on HPLC (Agilent technologies 1200 series) Exclipse XDB C18 (5 µm, 4.6 X 150 mm) column and using UV detector. Samples were injected into HPLC column for identification and quantification.

Isocratically eluted at 2ml min^{-1} with methanol, 2% acetic acid and H_2O (40:20:20) as mobile phases, detection was performed with an absorbance monitor operating at 254 nm. Identification and peak assignment of the compound was based on comparison of its retention time with corresponding standard and by spiking of sample with the standard.

Statistical Analysis

Statistical analysis of the results was carried out according to Duncan's multiple range tests using SPSS-20. Data were subjected to one-way ANOVA following the method of Sokal and Rohlf (1995). Differences between treatment-means were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Results

Data represented in Figure 1 showed that the wheat plants (*Triticum aestivum* L.) inoculated with microbin biofertilizer, recorded a significant ($P<0.05$) increase in all plant growth parameters (shoot and root lengths and dry weights) and photosynthetic pigments content comparing with uninoculated plants in the normal irrigation (control plants) or drought treatment. In general, plants were negatively affected by water deficit treatment as compared with normal irrigation treatment. Water stress could restrict dry weight and tissue water content, also shoot and root lengths. The reduction in shoot and root lengths and dry weights was 35%, 42% and 39%, 60% respectively, compared to the control. There was a reduction in RWC, the maximum reduction in RWC reached about 52% compared to the control. Microbin inoculated plants showed significantly increase in dry matter, longer shoot and root length in comparison with the drought stressed plants (Figure 1B).

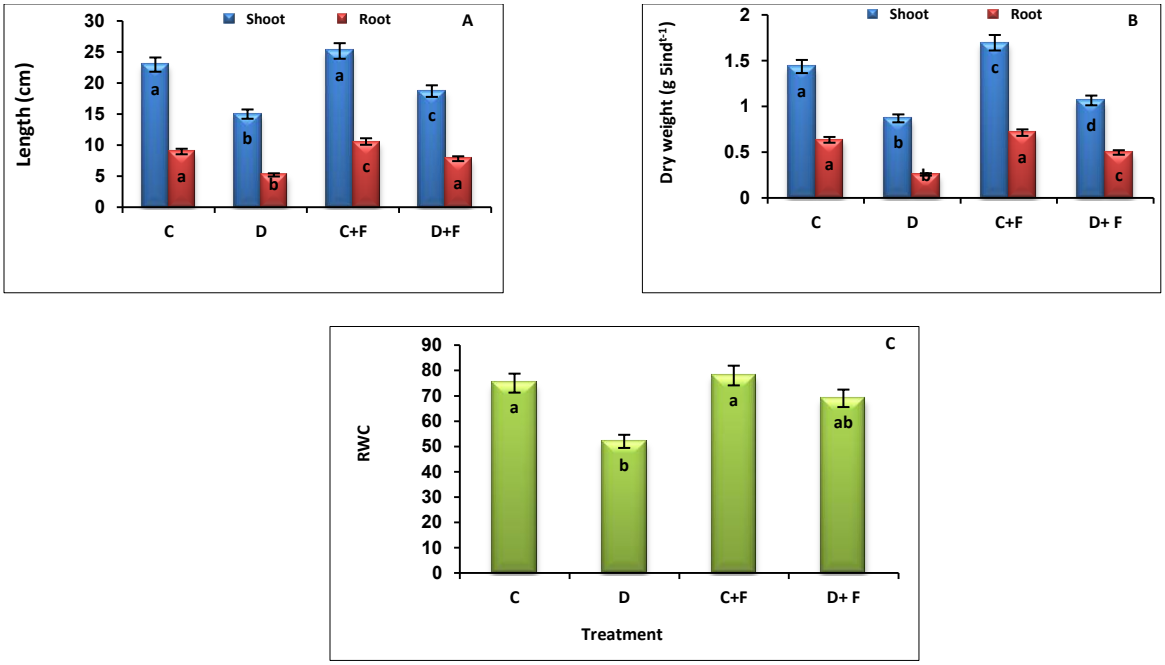

Figure 1: Effect of Inoculation by Bacterial Biofertilizer on Shoot and Root, (A) Lengths, (B) Dry Weights and (C) Leaf RWC at Normal and Drought Treatment of Wheat Plants. Values are Means ±SD Based on Triplicate Independent Determinations, and Different Letters Means Significant Difference as Evaluated by Duncan´s Multiple Comparison Test; C: Normal Irrigation (Control), D: Drought, C+F: Control+ Biofertilizer, D+F: Drought + Biofertilizer

Chlorophylls and carotenoids content were also affected by drought stress; control plants exhibited higher total chlorophyll content, the percent inhibition reached 53% in drought stressed plants. Application of biofertilizer increases to some extent the total chlorophyll content, the increase value reached nearly about 1.2-fold compared to the control. Carotenoids were insignificantly affected by application of biofertilizer, conversely, there was a significant increase in response to drought, and the increase value was about 1.3-fold compared to the control (Figure 2A). In addition, the ratio of Car/Chl significantly increased under drought with respect to the control and the biofertilizer treated plants (Figure 2B). The study of PSII

photochemistry showed that there was a marked decrease in the PSII and in maximal quantum yield Fv/Fm of water-stressed plants compared to the control, the reduction percentage was about 40% and 31% respectively. Application of biofertilizer alleviated the adverse effect of drought stress in wheat leaves (Figure 2C and D).

Compared with the control, water deficit treatment led to considerable ultra structural alterations in organelles. Focusing on the chloroplast, control wheat leaves exhibited a typical chloroplast structure, ellipsoidal shape, with well displayed thylakoid membranes organized in granal and stromal membranes (Figure 3). In the corresponding tissues of the plants submitted to drought stress, the chloroplasts suffered severe damage. Two types of changes could be distinguished, the first one was the chloroplasts became more rounded instead of an ellipsoidal shape; and the second, there was disruption of the membrane structure and the integrity of the outer membrane and the internal network of thylakoid membranes was almost completely lost. During drought and subsequent biofertilizer recovery in the chloroplasts, slight changes of the stromal and grana lamellae region could be observed.

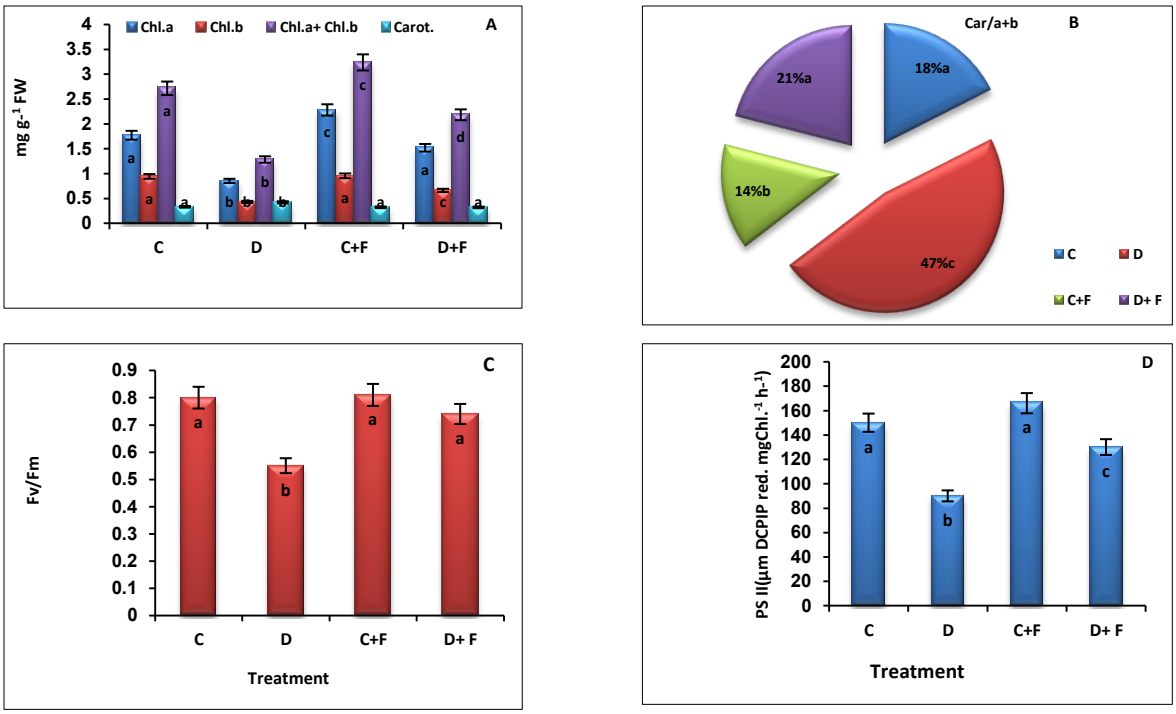

Figure 2: Effect of Inoculation by Bacterial Biofertilizer on Photosynthetic Pigments (A), Ratio between Carotenoids and Chlorophylls and Photosynthesis Activity {Fv/Fm (C) and PSII (D)} at Normal Irrigation and Drought Treatment of Wheat Plants. Values are Means ±SD Based on Triplicate Independent Determinations and Different Letters Means Significant Difference as Evaluated by Duncan´s Multiple Comparison Test; C: Normal Irrigation (Control), D: Drought, C+F: Control+ Biofertilizer, D+F: Drought+ Biofertilizer

Data in Figure 4(A and B) revealed that the concentration of MDA, and H_2O_2 were significantly increased in the wheat leaves due to drought stress, values reached about 2.5- and 1.7- fold higher than the control. Treatment of biofertilizer accompanied with water deficit significantly decreased the MDA and H_2O_2 contents compared to the control. Data of MSI were represented in Figure 4C, compared to the control plants; the MSI was markedly decreased under drought condition, while plants previously inoculated with biofertilizer seemed to be ameliorated. Simple linear regression was obtained in the present study (Figure 5A and B) showed that the coefficient of determination (R^2) between H_2O_2 , MDA content in treated and control leaves and MSI were about 0.908 and 0.881, respectively.

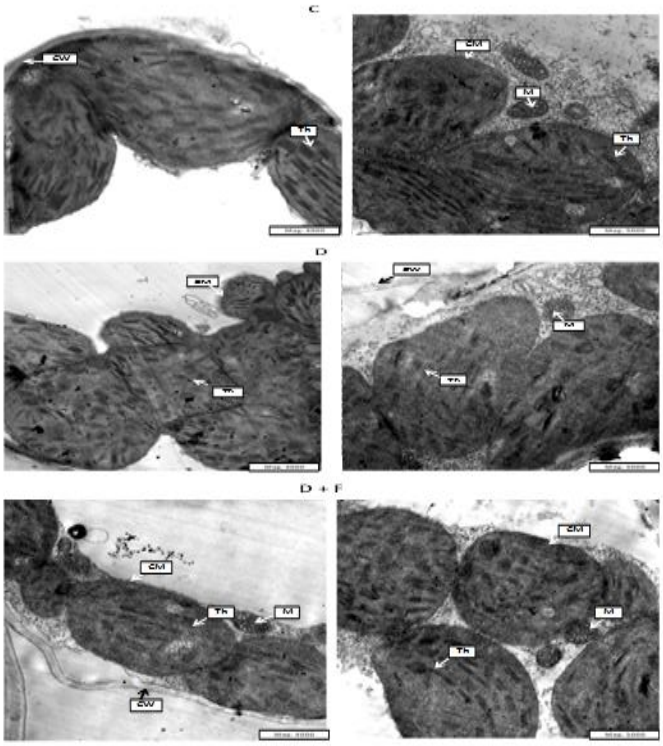

Figure 3: Transmission Electron Micrographs of Leaf Mesophyll Cells from Normal Irrigation and Drought Treatment of Wheat Plants Inoculated by Bacterial Biofertilizer and Uninoculated; C: Normal Irrigation (Control), D: Drought, D+F: Drought + Biofertilizer, (Th) Thylakoid, (CM) Chloroplast Membrane, (M) Mitochondria, (CW) Cell Wall

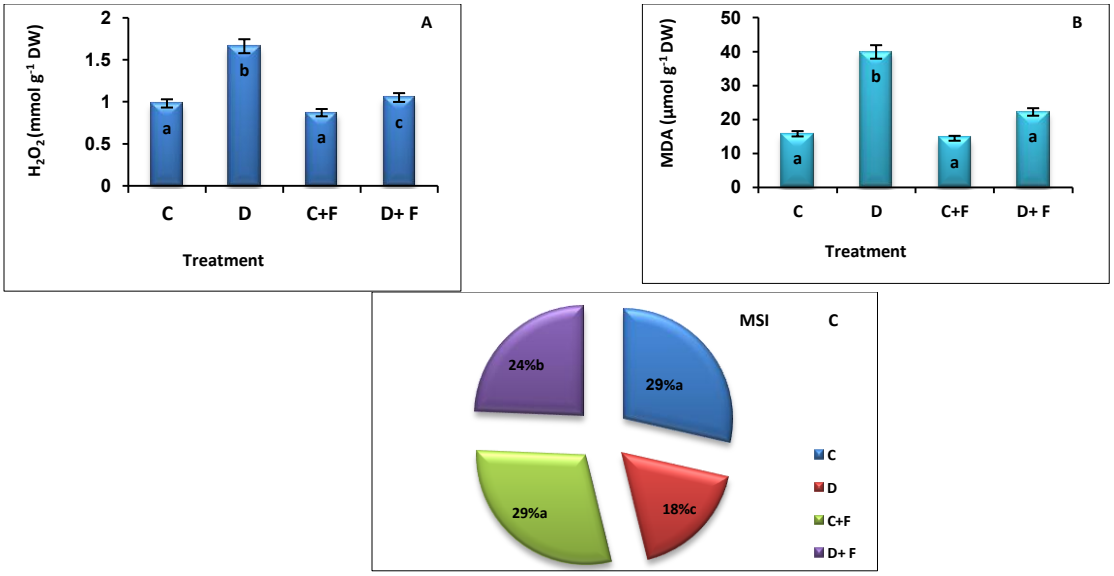

Figure 4: Effect of Inoculation by Bacterial Biofertilizer on (A) H_2O_2), (B) MDA Content and (C) MSI at Normal Irrigation and Drought Treatment of Wheat Plants. Values are Means ±SD Based on Triplicate Independent Determinations, and Different Letters Means Significant Difference as Evaluated by Duncan´s Multiple Comparison Test; C: Normal Irrigation (Control), D: Drought, C+F: Control+ Biofertilizer, D+F: Drought + Biofertilizer

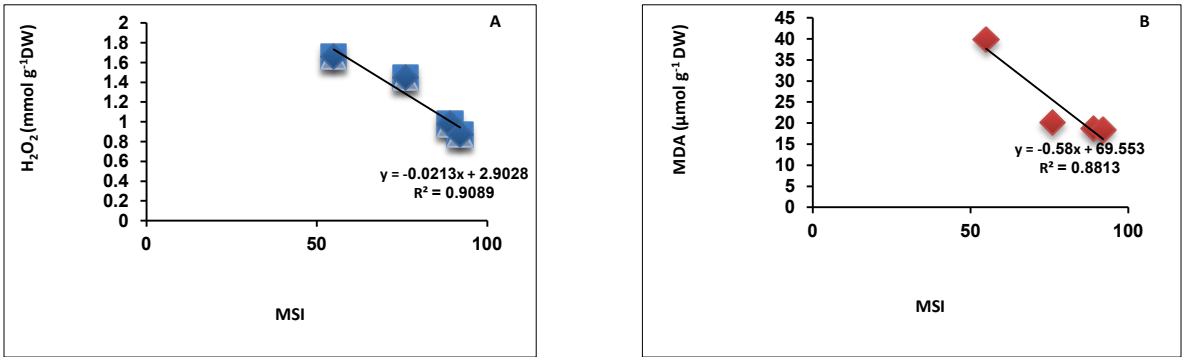

Figure 5: Regression Analysis between the H2O2, (A) MDA (B) and MSI

Results in Figure 6 illustrated that drought stress caused a significant increase of SOD, POD CAT, and APX specific activities, the values were 2-, 1.7-, 1.6-, and 1.6-fold higher than that of the control. Data of plants under well watered conditions, inoculated with biofertilizer showed activities nearly similar to that of the control. On the other hand plants inoculated with biofertilizer and stressed showed reduction in the specific activities of all tested antioxidant enzymes compared to the drought stressed ones.

Except for abscisic acid, the IAA and GA contents of wheat leaves were inhibited by ratio of 28% and 46% respectively due to water deficit compared to the control. Application of biofertilizer in well watered plants enhanced both IAA and GA, the amount was increased about 1.8- and 1.2-fold, respectively compared with uninoculated plants. Absicic acid was markedly increased under drought stress; the amount was 5-fold greater than control. In leaves treated with biofertilizer and grown under drought condition the ABA content was reached 35% less compared with drought stressed leaves (Figure 7).

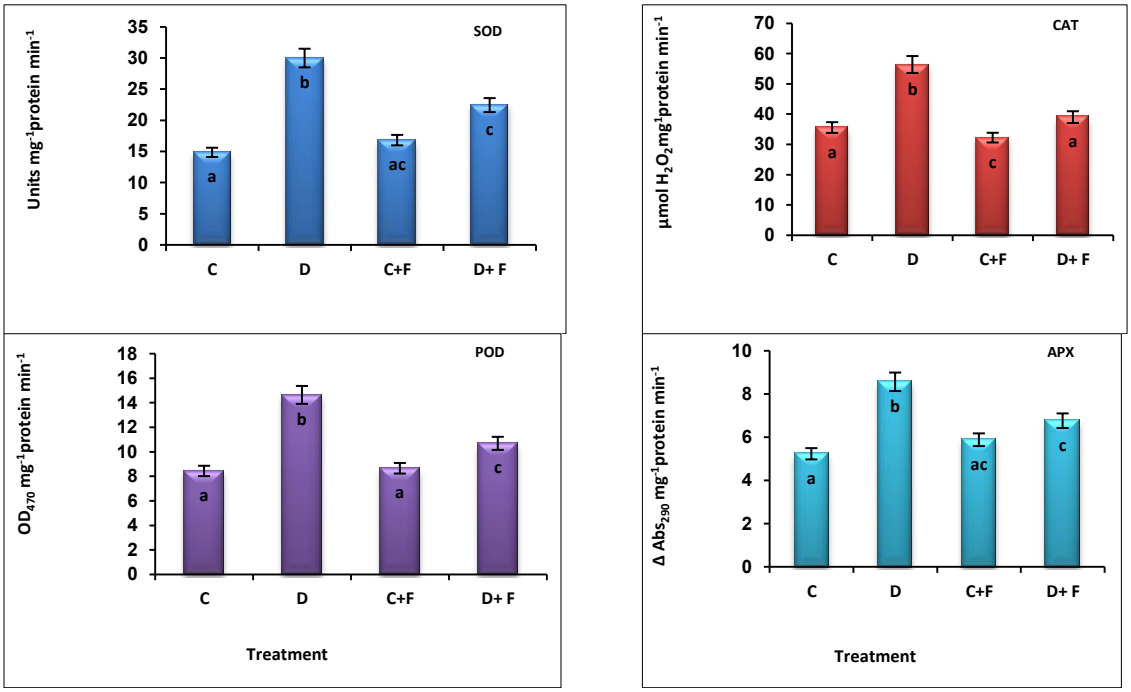

Figure 6: Effect of Inoculation by Bacterial Biofertilizer on Antioxidant Enzymes Activities (SOD, CAT, POD, and APX) at Normal Irrigation and Drought Treatment of Wheat Plants. Values are Means ±SD Based on Triplicate Independent Determinations, and Different Letters Means Significant Difference as Evaluated by Duncan´s Multiple Comparison Test; C: Normal Irrigation (Control), D: Drought, C+F: Control + Biofertilizer, D+F: Drought + Biofertilizer

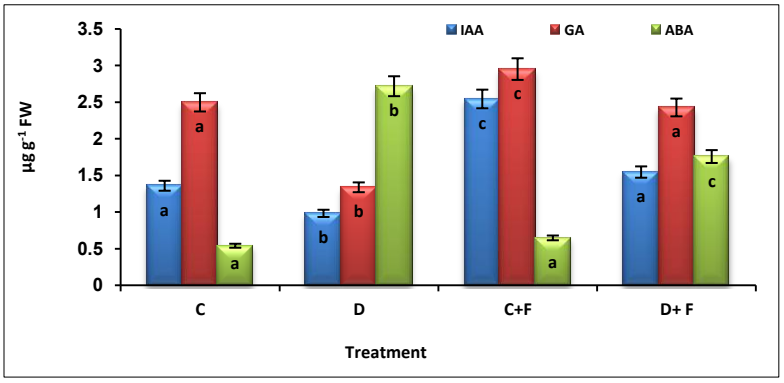

Figure 7: Effect of Inoculation by Bacterial Biofertilizer on Phytohormons (IAA, GA, and ABA) at Normal Irrigation and Drought Treatment of Wheat Plants. Values are Means ±SD Based on Triplicate Independent Determinations, and Different Letters Means Significant Difference as Evaluated by Duncan´s Multiple Comparison Test; C: Normal Irrigation (Control), D: Drought, C+F: Control + Biofertilizer, D+F: Drought + Biofertilizer

Discussion

Different bacterial strains are being used in bioremediation processes, in this study, microbin a mixed biofertilizer was used to determine its effect to improve wheat plant tolerance for drought stress. Among studied treatments the microbial inoculations was significant and the highest promoting effect observed in well irrigated treatment compared with the uninoculated stressed ones. According to the results, there was a marked decrease in growth parameters, shoot and root lengths, and dry weight, RWC and the total chlorophyll content under drought stress condition compared with normal water supply treatment (control plants). In consensus, Khalil and Yousef (2014) reported that, the reduction in plant growth in response to water stress may be due to blocking up of xylem and phloem vessels thus, hindering any translocation through them. Also, drought causes losses in tissue water content which reduce turgor pressure in cell, thereby inhibiting enlargement and division of cell causing of reduce of plant growth and dry mass accumulation (Delfine *et al.*, 2002). In this investigation, the maximum photochemical efficiency of PSII and the ratio Fv/Fm were estimated, their amounts were decreased under drought stress. The cellular ultra-structural analysis illustrated that the chloroplasts became rounded, with a disintegrating envelope and disrupted and irregularly shaped grana lamellae (Figure 3), this indicates serious damage to chloroplast structure, consistent with Batra *et al.*, (2014) who reported that drought stress causes not only a substantial damage to photosynthetic pigments, but it also leads to deterioration of thylakoid membranes. Under prevailing study, the carotenoids (Car) content and Car/Chl ratio were increased in response to drought stress, this may referred to protective role of carotenoids in photosynthesis; photo protection and their antioxidant activity. Drought caused considerable oxidative stress by accumulation of ROS (Mittler, 2002). In present study the activities of SOD, POD, CAT, and APX enzymes in wheat leaves were increased as a result of water stress. These results are in a good accord with the study of Rohman *et al.*, (2016) who found that enzymes like SOD, POD and APX were involved in scavenging ROS in maize in breds under drought stress. Data showed that the MDA content was significantly higher in stressed leaves this may be referred to the induction of membrane lipid per oxidation by means of ROS our results were in agreement with Moussa and Aziz (2008). The lower membrane stability index reflects the extent of lipid peroxidation, which in turn is a consequence of higher oxidative stress due to water stress conditions, our data on H_2O_2 content also support these findings (Figure 4). In accordance with our results Buchanan *et al.*, (2000) confirmed that water stress caused water loss from plant tissues which seriously impair both membrane structure and function. The results of this study also indicated a significant decrease of IAA and GA production and increase in ABA in the leaves due to drought stress that might be responsible, at least partly, for reduction of the root and shoot dry weight. Saleem *et al.*, (2007) declared that, the over production of ABA in response to abiotic and biotic stresses leads to

inhibition of root growth and, consequently, inhibition of the plant growth under stress conditions. Hu *et al.,* (2010) reported that ABA may induce antioxidant defense systems and suppress toxicity of ROS under drought stress. Results showed that the effect of interaction between water stress and biofertilizer (microbin) treatment was evaluated positively. The present results are in agreement with those obtained by Heidari and Golpayegani (2012) who found that inoculation with rhizobacteria could be efficiently used to improve growth, antioxidant status and photosynthetic pigments under water stress. They also reported that a significant increase was found in the inoculation with growth promoting rhizobacteria, especially combination of the bacterial species. Based on the results, the lower values of MDA and H_2O_2 in response to application of microbin combined with water deficit indicate that at a cellular level wheat plants are equipped with an efficient free radical quenching system that offers protection against oxidative stress. Moreover, microbial inoculation affect positively toward IAA and GA accumulation and reduction in ABA content compared to the stressed leaves. The improve of the photosynthetic capacity of wheat treated with microbin biofertilizer might be due to the increase of the availability of phosphorus, nitrogen and other nutrients, this appeared to coincide with the positive effects on the dry mass accumulation. In agreement with our results García-Fraile *et al.,* (2015) reported that, bacterial biofertilizers can improve plant growth through the synthesis of plant nutrients or phytohormones, which can be absorbed by plants. Likewise, Selvakumar *et al.,* (2012) reported that, biofertilizer significantly improved chlorophyll concentration, this is because, nitrogen is the chief constituent of protein, essential for the formation of protoplasm, which leads to cell enlargement, cell division and ultimately resulting in increased plant growth. Furthermore, Abou-Zeid (2014) reported that, the photosynthetic capacity of maize treated with biofertilizer increased due to the increase of intracellular macronutrients such as K, Ca, Mg, and P that might enhance chlorophyll biosynthesis and protect the photosynthetic systems. Microbin contains *Azotobacter* sp. and *Azospirillium* sp. and *Bacillus megaterium.* Cohen *et al.,* (2015) declared that as compensation to root exudates secreted by plants, the microorganism plant association may improve plant growth by synthesis of vitamins, antibiotics, enzymes and phytohormones, *Azospirillum sp* not only generate plant growth promoting hormones, but also sprinkle hormones such as ABA in stress conditions. (Marulanda-Aguirre *et al.,* (2008), observed an increased level of chlorophyll content in *Lactuca sativa* inoculated with *Bacillus megaterium,* this could be indicating the positive interaction of *B. megaterium this* interaction might trigger the chlorophyll related enzymes for chlorophyll synthesis and leads to increase the photosynthesis; also, plant-bacterial interaction alters the carbohydrate metabolisms of plants. Recently, Esmaeili *et al.,* (2016) stated that the adequate supplies of the micronutrients and biofertilizer containing *Azotobacter* will have favorable effects on soybean quality and growth.

Conclusion

From the obtained results, it can be concluded that the dual application of phosphorus and nitrogen biofertilizer resulted in improve and overcome the depressive effect of water stress on wheat plant. Moreover, biofertilizers are important component in integrated nutrients managements, they are cost effective, eco-friendly and renewable source of plant nutrients. From the present work the data showed that the microbin biofertilizer improved some physiological parameters of the wheat plant. Biofertilizer could play an important role in adaptation strategies and increase of tolerance to abiotic stresses such as drought in agricultural plants. They might replace part of the use of chemical fertilizers, reduces amount and cost of chemical and thus prevents the environment pollution from extensive application of chemical fertilizers.

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