Journal of Applied Botany and Food Quality 90, 165 - 173 (2017), DOI:10.5073/JABFQ.2017.090.021

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Seed inoculation with *Azospirillum lipoferum* alleviates the adverse effects of drought stress on wheat plants

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(Received November 12, 2016)

Summary

Drought is one of the major environmental stresses that adversely affects crop growth and productivity worldwide. The effect of inoculation with *Azospirillum lipoferum* on growth, yield, water status, osmoprotectant, antioxidant system and grain anatomy of wheat plants under drought stress conditions was investigated. The plants exposed to the drought stress exhibited a significant reduction in growth, grains yield, relative water content and leaf photosynthetic pigments, as well as alterations in grain anatomy. However, the treatment with *A. lipoferum* alleviated the stress generated by drought and improved the above-mentioned parameters. Drought stress increased proline, protein, soluble carbohydrates, relative membrane permeability and activities of antioxidant enzymes (SOD and POX). The antioxidant enzymes, phenols and grain anatomy exhibited changes in response to *A. lipoferum* inoculation in the absence or presence of drought stress. Our data suggest that inoculation with *A. lipoferum* could protect wheat plants from the harmful effects of drought stress through changes in the antioxidant defense system.

Keywords: *Triticum aestivum,* Drought stress, *Azospirillum lipoferum*, Antioxidant defense system, Anatomy, Compatible solutes

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops in the world, however this crop is exposed to a variety of abiotic stresses, such as drought, salt loading and freezing that influence its development, growth and productivity. Drought stress is among the most destructive abiotic stresses that increased in intensity over the past decades affecting world's food security. It is expected to cause serious plant growth problems for more than 50% of the arable lands by 2050 (KASIM et al., 2013). Drought affects plant water potential and turgor, enough to interfere with normal functions (HSIAO, 2000), and induces changes in physiological and morphological characters in the plants (RAHDARI and HOSEINI, 2012). Common plant symptoms after water stress are: stunting, limiting in CO_2 diffusion to chloroplasts by stomatal closure, reduction in photosynthesis rate, and acceleration of leaf senescence. Moreover, in wheat, a severe drought stress during the late growth stages (anthesis – post anthesis) induces chlorophyll degradation, cell solute leakage, and accelerated spike and grain maturation (BELTRANO et al., 1999). Drought stress also causes severe alterations in cell membrane selective permeability (leakage of cell solutes), fluidity and microviscosity (NAVARRI-IZZO et al., 1993; BELTRANO et al., 1999). It induces free radicals affecting antioxidant defenses and Reactive Oxygen Species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals resulting in oxidative stress. High concentrations of ROS can cause damage to various levels of organization (SMIRNOFF, 1993), like initiate lipid peroxidation, membrane deterioration and degrade proteins, lipids and nucleic acids in plants (HENDRY, 2005; SGHERRI et al., 2000; NAIR et al., 2008). Much of the injury in plants under abiotic stress is due to oxidative damage at the cellular level, which is the result of imbalance between the formation of reactive oxygen species (ROS) and their detoxification. Plant cells produce different antioxidant enzymes such as catalase (CAT), peroxides (POX), superoxide dismutase (SOD), glutathione peroxidase (GPX) and ascorbate peroxidase (APX) that scavenge the reactive free radicals (SIMOVA-STOILOVA et al., 2008). Generally, drought negatively affects quantity and quality of the plant growth. Therefore, to produce more food, the alleviation of drought stress is important to achieve the designated goals.

Globally, an extensive research is being carried out to develop strategies to cope with drought stress through development of drought tolerant varieties, shifting the crop calendars, resource management practices and others and other means (VENKATESWARLU and SHANKER, 2009) and most of these technologies are cost intensive. Recent studies indicate that microorganisms can also help plants to cope with drought stress. Bacteria of the genus *Azospirillum* are among the best investigated plant growth promoting rhizobacteria (PGPR) detected in the rhizosphere of many crop plants. They are able to produce plants hormones such as auxin, and proteins like polyamines, fix N_2, increase root growth and control pathogens. Such abilities collectively result in the enhanced growth of plants under stress (RAMOS et al., 2002; BHASKARA RAO and CHARYULU, 2005; RUSSO et al., 2008; CASSAN et al., 2009). Many researchers have indicated that *Azospirillum* spp. can mitigate the unfavorable effects of water stress on plant growth (ARZANESH et al., 2009; PEREYRA et al., 2009).

The present study was designed with the objective to examine changes in the antioxidant defense system of wheat plants under the effect of *Azospirillum lipoferum* applied by seed inoculation and exposed to drought stress. The tested hypothesis is that *A. lipoferum* will positively modify the level of antioxidant system that will mitigate the injuries generated by drought stress. Consequently, *A. lipoferum* will enhance the wheat performance under drought stress.

Materials and methods

Plant materials and bacterial strain

Seeds of wheat (*Triticum aestivum* L. cv. Giza 168) were obtained from the Crop Institute, Agricultural Research Center, Giza, Egypt. Bacterial strain *Azospirillum lipoferum* N040 was obtained from Agricultural microbiology department, Faculty of Agriculture, Cairo University for research purpose.

Inoculum preparation and bacterial growth

Bacterial culture was prepared by growing the *Azospirillum lipoferum* N040 in liquid nitrogen free biotin based (NFB) medium (PICCOLI et al., 1997) [5 g l^{-1} peptone and 3 g l^{-1} beef PH 7.0] at 25 ± 1 °C with shaking (150 rpm) for 48 h. The bacterial cells were

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pelletized by centrifugation (5000 rpm) for 10 min and re-suspended in sterilized tap water containing 0.025% (v/v) Tween-20 to the desired concentration (10^8 CFU ml^{-1}).

Experimental design and treatments

Two pot experiments were carried out at Demo Experimental Farm, Faculty of Agriculture, Fayoum University (Southeast Fayoum; 29° 17'N; 30° 53'E), during the two successive seasons of 2014 and 2015. Wheat seeds were surface-sterilized with 70% ethanol (3 min), treated with 2% sodium hypochlorite (NaClO) (5 min), and followed by repeated washing with sterile distilled water (3 times for 1 min). Then surface-disinfected seeds were incubated in sterilized liquid nitrogen free biotin based medium (as control) or in bacterial suspension (10^8 CFU ml^{-1}) for 2 h on a rotary shaker at 81 rpm. Ten inoculated seeds (10^7 bacteria per seed) or non-inculcated seeds (control) were sown in each plastic pot (32 cm in diameter, 25 cm in deep) containing 15 kg of soil and were thinned to five plants, one week after germination. Soil used in the pots had the following physic-chemical characteristics: sand, 2.7%; silt, 28.7%; clay, 68%; pH, 7.28 (1:2, w/v, soil and water solution); EC, 3.49 dS m^{-1} (1:2, w/v, soil and water solution); $CaCO_3$ 10.81% and organic matter 3.39%; total nitrogen, 39.6 (mg/kg dry soil); available phosphorus, 32.9 (mg/kg dry soil); extractable potassium, 8.33 (mg/kg dry soil). The first experiment was conducted on 15 November, 2014 and the second one was conducted on 19 November, 2015 in an open greenhouse. The average day and night temperatures were 22 ± 3 ºC and 11 ± 2 ºC, respectively. The relative humidity ranged from 38.1 to 69.8%, and day-length from 10 to 11 h. Pots were arranged in the greenhouse in a complete randomized block design with four replications for each treatment. Recommended doses of N, P, and K fertilizers (150-100-60 kg ha^{-1}) were applied to each pot and equal amounts of tap water was added to the pots to maintain the optimal soil moisture depending on plant and soil conditions (up to 1000 ml). Drought stress was applied after 30 days of planting to grain ripening. The two soil water conditions were either well-watered (100% of crop evapotranspiration (ETc)) or dried up (60% of crop evapotranspiration). Crop evapotranspiration was determined using gravimetrical method described by MAOA et al. (2014). Irrigation was applied twice a week during the experimental period.

Samples of wheat plants (36 per each treatment) were collected after 90 days from sowing to assess morphological data. Length of shoots and spikes (cm) was measured by using a meter scale. Numbers of fertile tillers per plant and numbers of spikelets per spike were counted. Flag leaf area (cm^2) was measured using a digital leaf meter (LI-3000 Portable Area meter Produced by LI-COR Lincoln, NE, USA). Samples of flag leaves were collected to estimate the concentration of total chlorophylls and total carotenoids, proline, total soluble protein, total soluble carbohydrates and total soluble phenols, relative membrane permeability, relative water content and activities of antioxidant enzymes. The experiment was terminated after 130 days from sowing after exposing the plants to water stress for 100 days. The 130-day-old plants from each treatment were collected for various measurements. The 130-day-old wheat plants were removed from the pots and moved smoothly to remove the adhering soil particles by dipped them in a bucket filled with water. Roots and straw were weighed to record their fresh weight, then were placed in an oven at 70 °C to reach a constant dry weight (DW). Grain yield per plant and 1000-kernel weight was also estimated. The powder of dried grains was used to determine the concentration of total soluble protein and total soluble carbohydrates.

Anatomical study

For observation of grain anatomy, samples were taken from the main spike at the age of 110 days and fixed in FAA solution (containing 50 cm^3 of 95% (v/v) ethanol + 10 cm^3 of formaldehyde + 5 cm^3 of glacial acetic acid + 35 cm^3 of distilled water) for 48 h. Thereafter, the samples were washed in 50% ethanol, dehydrated and cleared in tertiary butanol series, and embedded in paraffin wax. Cross sections, 25 µm thick, were cut by a rotary microtome (Leitz, Wetzlar, Germany), adhered by a Haupt's adhesive, stained with a crystal violet-erythrosin combination (SASS, 1961), cleared in carbol xylene, and mounted in Canada balsam. The sections were observed and documented using an upright light microscope (AxioPlan, Zeiss, Jena, Germany). Measurements were done using a micrometer eyepiece and average of five readings was calculated.

Photosynthetic pigments determination

Total chlorophyll and carotenoids concentration (mg g^{-1} FW) were estimated according to the procedure given by ARNON (1949). Flag leaves discs (0.2 g) of 90-day-old plants were homogenized with 50 ml 80% acetone. The slurry was strained through a cheese cloth and the extract was centrifuged at 15,000 × g for 10 min. the optical density of the acetone extract was measured at 663, 645 and 470 nm using a UV-160A UV Visible Recording Spectrometer, Shimadzu, Japan.

Free proline determination

Proline concentration in wheat flag leaves was measured following the rapid colorimetric method of BATES et al. (1973). Proline was extracted from 0.5 g of dry leaf samples by grinding in 10 ml of 3% sulpho-salicylic acid. The mixture was then centrifuged at 10,000 × g for 10 min. Two ml of the supernatant was added into test tubes and 2 ml of freshly prepared acid-ninhydrin solution was added. Tubes were incubated in a water bath at 90 °C for 30 min. The reaction was terminated in ice-bath. The reaction mixture was extracted with 5 ml of toluene and the vortex process was done for 15 s. The tubes were allowed to stand at least for 20 min in the dark at room temperature to allow the toluene and aqueous phases to be separated. The toluene phase was then carefully collected into test tubes and toluene fraction was read at 520 nm using a UV-160A UV Visible Recording Spectrometer, Shimadzu, Japan. The proline concentration in the sample was determined from a standard curve using analytical grade proline.

Total soluble proteins determination

The total soluble proteins concentration of the dry flag leaves and grains was determined according to the method described by BRADFORD (1976) with bovine serum albumin as a standard. An amount of 0.2 g of samples was ground in a mortar with 5 ml of phosphate buffer (pH 7.6) and was then transformed to the centrifuge tubes. The homogenate was centrifuged at 8000 rpm for 20 min. The supernatant of different samples was put in separate tubes. The volume of the samples in tubes was then made equal by adding a phosphate buffer solution and the extraction were stored in the refrigerator at 4 °C for further analysis. After extraction, 30 µl of different samples were taken out in separate tubes and were mixed with 70 µl of distilled water. Then, 2.9 ml of the Coosmassic Brilliant Blue solution was added to each sample tube and mixed thoroughly. The total volume was 3 ml in each tube. All tubes were incubated for 5 min at room temperature and then, the absorbance was recorded at 600 nm against the Blank. A standard curve of absorbance (600 nm) versus concentration (µg) of total soluble proteins was calculated.

Total soluble carbohydrates determination

Leaf and grains total soluble carbohydrates concentration were assessed by the method recommended by the ASSOCIATION OF OF-

FICIAL AGRICULTURAL CHEMISTS (1990) using phenol sulphuric acid reagent method.

Total soluble phenols determination

The soluble phenol concentration in wheat flag leaves was extracted as described by HSU et al. (2003). 0.2 g of dry leaves were homogenized in 80 ml methanol and kept overnight. The filtrates were diluted to 100 ml, and served as a stock solution. According to SLINKARD and SINGLETON (1997), 200 µl of the stock solution was added to 1.4 ml distilled water, and 0.1 ml of 50% (1N) Folin-Ciocalteu phenol reagent. After three min., 0.3 ml of 20% (w/v) sodium carbonate was added. The mixture was allowed to stand for 2 h. After gentle vortex, the absorbance was determined at 765 nm. Total soluble phenol concentration was standardized against tannic acid.

Relative water content determination

Relative water content (RWC) was determined in midrib excluding-fresh flag leaf discs of 2 cm^2 area. Discs were weighed quickly and immediately floated on double distilled water in Petri dishes to saturate them with water for the next 24 h, in dark. The adhering water of the discs was blotted and turgor mass was noted. Dry mass of the discs was recorded after dehydrating them at 70 °C for 48 h. By placing the values in the following formula (HAYAT et al., 2007), RWC was calculated:

$$RWC\ (\%) = (Fresh\ mass - dry\ mass) / (Turgid\ mass - dry\ mass) \times 100$$

Relative membrane permeability determination

For the RMP measurement, the flag leaves were cut into equal pieces and transferred to test tubes containing 20 ml of deionized distilled water. The test tubes were vortexed for 10 s and the solution was assayed for initial electrical conductivity (EC0). These tubes were kept at 4 °C for 24 h and then assayed for EC1. The same samples were autoclaved at 121 °C for 20 min to determine EC2. Percent RMP was calculated as following the formula described by YANG et al. (1996).

$$RMP\ (\%) = (EC1 - EC0) / (EC2 - EC0) \times 100$$

Enzyme assays

Flag leaves were excised from wheat plants and rapidly weighed. Each 1.0 g sample was ground with a pestle in an ice-cold mortar containing 10 ml of 50 mM phosphate buffer, pH 7.0. The homogenate was centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant was then filtered through two layers of cheese-cloth and used to measure various anti-oxidant enzyme activities.

Superoxide dismutase (SOD) activity was determined according to the method of FRIDOVICH (1975). One Unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of oxidation of nitroblue tetrazolium (NBT) at 560 nm. Each 3 ml reaction mixture contained 50 mM phosphate buffer pH 7.0, 200 mM methionine, 1.125 mM NBT, 1.5 mM EDTA, 75 mM riboflavin, and 10-40 µl of crude enzyme extract. The riboflavin was added as the last component. The tubes were shaken and placed 30 cm below two 15 W fluorescent lights. The reaction was started by switching on the light, and allowed to run for 10 min. Switching-off the light stopped the reaction. The tubes were then covered immediately with a black cloth and the absorbance was measured spectrophotometrically at 560 nm. A non-irradiated reaction mixture was set to zero absorbance as the blank. The volume of enzyme extracts that produced a 50% inhibition of the oxidation (color reaction) was read from the resulting graph.

Peroxidase (POX) activity in flag wheat leaves was measured using the method of THOMAS et al. (1981). POX was assayed using guaiacol as the substrate. The crude enzyme extract was prepared in a similar way to that used for SOD. Each reaction mixture consisted of 3 ml of 0.1 M phosphate buffer pH 7.0, 30 µl of 20 mM H_2O_2, 50 µl of crude enzyme extract, and 50 µl of 20 mM guaiacol. The reaction mixture was incubated for 10 min at room temperature in a cuvette. The absorbance was then measured at 436 nm. POX activity was expressed in A 436 Units g^{-1}FW leaf min^{-1}.

Statistical analysis

All the pots for two experiments (288) were arranged in a complete randomized design with nine pots per replicate and four replicates per treatment. Analysis of variance was performed using the SPSS software package to determine the least significant difference (LSD) among treatments at $P \leq 0.05$, and the Duncan's multiple range test was applied for comparing the means.

Results

Growth traits

Azospirillum lipoferum inoculation significantly improved the plant growth, straw and the grain yields of wheat in presence or absence of drought stress under open greenhouse conditions. However, drought stress significantly decreased the length of shoot, number of fertile tillers per plant, flag leaf area, length of spike, number of spiklets per spike, root fresh and dry weights (Tab. 1). Comparing to the control (100% ETc), the above-mentioned parameters were decreased under 60% of ETc by 27.17, 63.76, 64.85, 28.08, 19.95, 64.56 and 72.52%, respectively. After the inoculation of the wheat grains with

Tab. 1: Effect of *Azospirillum lipoferum* inoculation on the growth traits [length of shoot (cm), No of fertile tillers per plant, flag leaf area (cm^2), length of spike (cm), No of spikelets per spike, root fresh mass (g) and root dry mass (g)] of wheat (*Triticum aestivum* L., cv. Giza-168) plants grown under non-stressed and drought-stressed condition.

Treatments		Parameters						
Irrigation levels	Inoculation	Length of shoot	No of fertile tillers per plant	Flag leaf area	Length of spike	No of spikelets per spike	Root fresh weight	Root dry weight
100% ETc	No-inoculated	44.17±1.80b	3.67±0.58a	16.30±1.21b	9.83±0.20a	13.33±0.58b	0.790±0.06b	0.473±0.03b
	Inoculated	50.00±0.58a	4.67±0.58a	27.60±2.52a	9.87±0.35a	15.33±0.58a	1.600±0.48a	1.090±0.06a
60% ETc	No-inoculated	32.17±0.58d	1.33±0.58c	5.73±0.66c	7.07±0.12c	10.67±0.58c	0.280±0.02c	0.130±0.02c
	Inoculated	39.17±0.76c	1.67±0.58c	8.44±0.65c	8.20±0.75b	11.67±0.58c	0.280±0.03c	0.160±0.01c

Values are means ± SD (n=9) and differences between means were compared by the Duncan's multiple range test (LSD; $P \leq 0.05$). Mean pairs followed by different letters are significantly different.

A. lipoferum N040 and in absence of the water stress, the growth in most cases was significantly higher than that of the control ($P \leq 0.05$). Inoculation with the bacteria also improved the growth of the plants grown under the water stress and the values in some cases were significantly higher than those of the plants grown under the stress alone.

Yield components

Data in Tab. 2 show that wheat plants growing in the presence of water stress significantly decreased the yield components. However, the reductions were 52.45, 45.28, 71.97, 73.12, 38.49 and 19.27% for straw fresh weight per plant, straw dry weight per plant, spike fresh weight per plant, spike dry weight per plant, grain yield per plant and 1000-kernel weight respectively, compared to non-stressed and non-inoculated plants. Inoculation of grains of *Triticum aestivum* cv. Giza 168 with *A. lipoferum* N040 alleviated these deleterious effects of drought stress. In the presence of water stress *A. lipoferum* treatment showed 24.79 and 11.31%, 54.33 and 58.67%, 79.35 and 3.32%, increases in straw fresh and dry weights per plant, spike fresh and dry weights per plant, grain yield per plant and 1000-kernel weight respectively, compared to non-inoculated control. However, incubation the wheat grains in *A. lipoferum* suspensions significantly increased the above-mentioned parameters especially in the absence of the water stress (100% ETc) compared to the non-inoculated plants.

Anatomy of grain

As concerns the grain anatomical structure, drought stress decreased height and width of the grain by 9.09 and 9.63%, height and width of the endosperm by 13.78 and 10.82% as well as pericarp and aleurone layer by 23.08, and 17.22%, respectively, in comparison to the control (Tab. 3 and Fig. 1). However, incubation the seeds in *A. lipoferum* suspension caused positive changes in the above-mentioned characteristics in absence or presence of the water stress. For example, the maximum increase was achieved in *A. lipoferum* pretreated plants in absence of the water stress which recorded increments of 16.49 and 14.66, 41.67, 17.66 and 15.53 and 24.49% for length and width of grain, pericarp, length and width of endosperm and aleurone layer, as compared to non-inoculated and water-stressed plants.

Photosynthetic pigments

The water deficiency stress significantly decreased the concentration of total chlorophyll and carotenoids (Tab. 4). Comparing to non-inoculated control, the decrease reached 46.09 and 22.58% for total chlorophyll and carotenoids, respectively. After inoculation combined with drought stress the total chlorophyll and carotenoids content of wheat significantly increased compared to the non-inoculated plants (60% ETc). In absence of the drought stress, *A. lipoferum* inoculation significantly promoted the increases in total chlorophyll and carotenoids by 23.48 and 16.13% for total chlorophyll and carotenoids, respectively compared to non-inoculated plants (100% ETc).

Compatible solutes

Wheat plants subjected to drought stress exhibited a significant increase in the content of free proline, total soluble protein in leaves, total soluble carbohydrates in leaves and grains but showed a slight

Tab. 2: Effect of *Azospirillum lipoferum* inoculation on the yield component [straw fresh weight per plant (g), straw dry weight per plant (g), spike fresh weight per plant (g), spike dry weight per plant (g), grain yield per plant (g) and 1000-kernel weight (g)] of wheat (*Triticum aestivum* L., cv. Giza-168) plants grown under non-stressed and drought-stressed condition.

Treatments		Parameters					
Irrigation levels	Inoculation	Straw fresh weight per plant	Straw dry weight per plant	Spike fresh weight per plant	Spike dry weight per plant	Grain yield per plant	1000-kernel weight
100% ETc	Non-inoculated	9.60±0.40b	3.07±0.21b	11.95±1.00b	5.58±0.55b	2.52±0.03c	38.40±0.61b
	Inoculated	18.32±0.21a	6.03±0.64a	15.95±0.80a	7.07±0.64a	6.51±0.11a	40.80±0.26a
60% ETc	Non-inoculated	4.67±0.15d	1.68±0.03c	3.35±0.09d	1.50±0.10c	1.55±0.06d	31.00±2.00c
	Inoculated	5.85±0.35c	1.87±0.06c	5.17±0.40c	2.38±0.18c	2.78±0.03b	32.03±0.84c

Values are means ± SD (n=9) and differences between means were compared by the Duncan's multiple range test (LSD; $P \leq 0.05$). Mean pairs followed by different letters are significantly different.

Tab. 3: Effect of *Azospirillum lipoferum* inoculation on the height and width of grain and endosperm, thickness of percarp and aleurone layer [μm] of wheat (*Triticum aestivum* L., cv. Giza-168) plants grown under non-stressed and drought-stressed condition.

Treatments		Parameters					
Irrigation levels	Inoculation	Dimensions of grain		Dimensions of endosperm		Percarp thickness	Aleurone layer thickness
		Height	Width	Height	Width		
100% ETc	Non-inoculated	2062.4±11.6b	3873±12.6b	1884.8±6.9b	3646.3±18.7b	52.0±2.0a	60.0±1.0a
	Inoculated	2184.3±3.9a	4013±15.3a	1912.0±11.8a	3756.7±7.6a	56.7±3.1a	61.0±1.0a
60% ETc	Non-inoculated	1875.0±10.0c	3500±20.0d	1625.0±7.0d	3251.7±12.6c	40.00±2.0c	49.7±1.5b
	Inoculated	1875.2±13.9c	3752±17.6c	1711.8±11.5c	3619.0±16.8b	46.00±1.0b	57. 7±2.5a

Values are means ± SD (n=9) and differences between means were compared by the Duncan's multiple range test (LSD; $P \leq 0.05$). Mean pairs followed by different letters are significantly different.

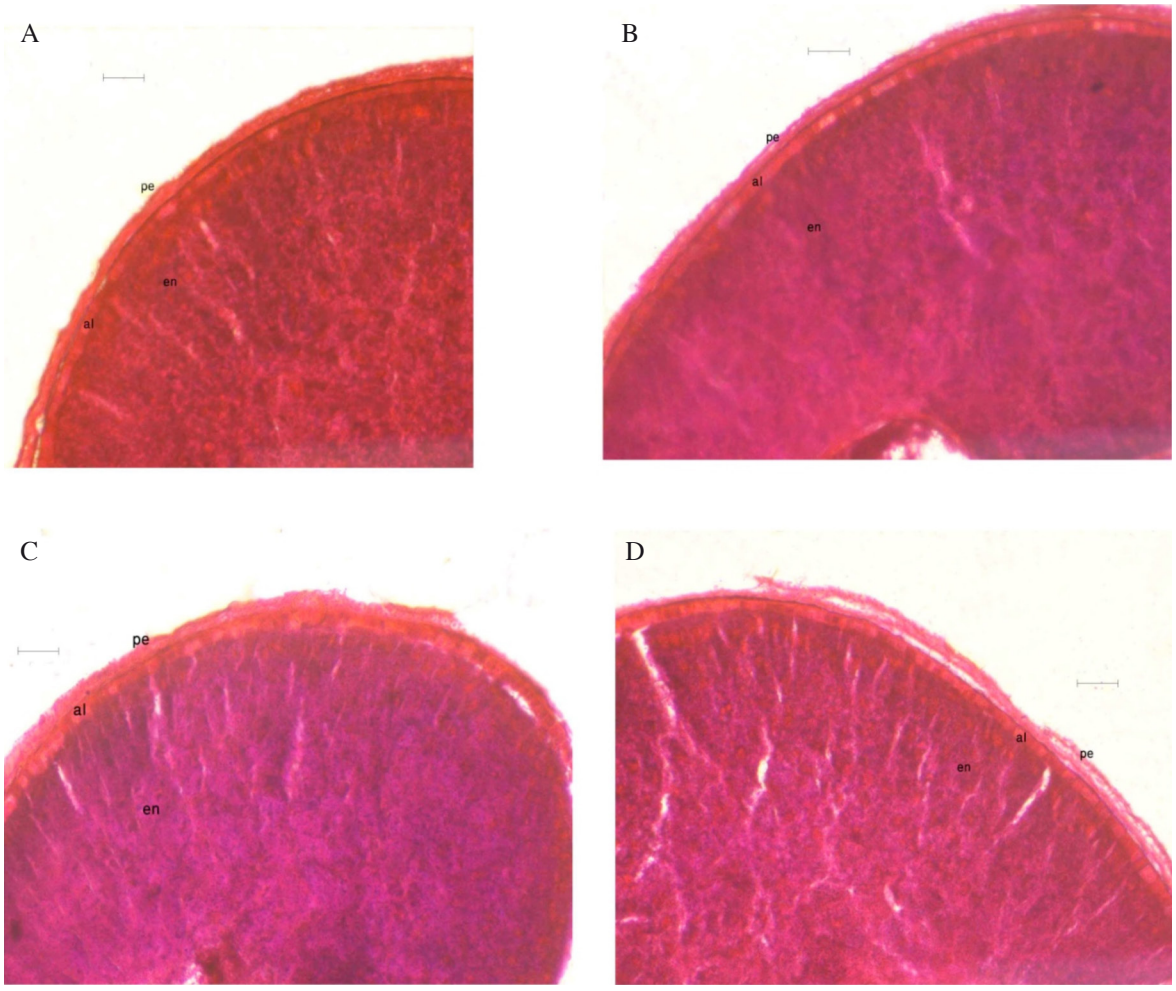

Fig. 1: Photographs of grain section of *Azospirillum lipoferum* N040 inoculated *Triticum aestivum* L. plants grown under water stress. A) Non-inoculated + 100% ETc; B) Inoculated + 100% ETc; C) Non-inoculated + 60% ETc; D) Inoculated + 60% ETc; al, aleurone layer; en, endosperm and pe, percarp, bars = 200 µm.

decrease in total soluble protein in grains compared to the non-inoculated and non-stressed plants (Tab. 4). The increases were 31.86, 97.22, 35.82 and 18.61%, respectively. However, in presence of drought stress inoculation of grains with *A. lipoferum* alleviated these effects of drought stress on the previous compatible solutes and significantly decreased these parameters compared to non-inoculated and water-stressed plants. The reductions were 12.08, 16.20, 7.31 and 11.35%, for leaf free proline, leaf total soluble protein, total soluble carbohydrates in leaves and grains, respectively.

Total soluble phenols

The stress generated by water deficit resulted in a slight decrease in total soluble phenols compared to non-water stressed plants (Tab. 5).

Tab. 4: Effect of *Azospirillum lipoferum* inoculation on total chlorophylls (mg g^{-1} FW), total carotenoids (mg g^{-1} FW), proline (mg g^{-1} DW), protein in leaves and grains (mg g^{-1}DW) and total soluble carbohydrates in leaves and grains (mg g^{-1} DW) of wheat (*Triticum aestivum* L., cv. Giza-168) plants grown under non-stressed and drought-stressed condition.

Treatments		Parameters						
Irrigation levels	Inoculation	Total chlorophylls	Total carotenoids	Proline	Protein in leaves	Protein in grains	Total soluble carbohydrates in leaves	Total soluble carbohydrates in grains
100% ETc	Non-inoculated	1.15±0.03	0.31±0.02b	1.13±0.07c	2.16±0.05d	18.75±0.48b	112.8±0.67d	623.23±0.96d
	Inoculated	1.42±0.10	0.36±0.01a	1.19±0.03c	3.05±0.13c	20.34±0.17a	131.7±0.47c	651.97±0.95c
60% ETc	Non-inoculated	0.62±0.03	0.24±0.01d	1.49±0.07a	4.26±0.04a	17.85±0.87b	153.2±1.48a	739.20±0.61a
	Inoculated	1.03±0.06	0.28±0.01c	1.31±0.03b	3.57±0.04b	18.10±0.21b	142.0±10.37b	655.27±1.01b

Values are means ± SD (n=6) and differences between means were compared by the Duncan's multiple range test (LSD; $P \leq 0.05$). Mean pairs followed by different letters are significantly different.

However, this attribute was significantly improved by *A. lipoferum* inoculation in presence or absence of the drought stress. In presence of the water deficiency stress, *A. lipoferum*-inoculated grains had a 36.37% increase in the total soluble phenols over its non-inoculated control.

Relative water content (RWC %) and relative membrane permeability (RMP %)

Data shown in Tab. 5 reveal that the RWC% of wheat plants was significantly reduced by 29.20% in presence of the water deficiency stress, but RMP% was significantly increased by 97.47% compared to the non-inoculated control (100% ETc). In presence of the drought stress, *A. lipoferum* inoculation reduced the injurious effects of drought stress on wheat plants, and maintained their RMP% and RWC% values at the near levels as in control plants.

Antioxidant enzyme activities

The activities of superoxide dismutase (SOD) and peroxidase (POX) are shown in Tab. 5. Growing wheat plants in presence of the water deficiency stress significantly increased SOD and POX activities by 19.22% and 51.58%, respectively, compared to the non-inoculated control (100% ETc). In addition, *A. lipoferum* inoculation of grains further increased these enzyme activities in presence of the drought stress by 8.82% and 31.25%, respectively compared to the control (i.e. non-inoculated and drought-stressed plants). Even in the absence of water deficiency stress *A. lipoferum* inoculation also significantly increased the activity of the two enzymes compared to the control (100% ETc).

Discussion

Water stress is one of the most adverse factors affecting plants growth and productivity. In addition, water stress causes over-production of reactive oxygen species (ROS) that can pose a threat to cells by causing oxidization of lipids, DNA, RNA and proteins, leading ultimately to cell death (Smirnoff, 1995; Mittler, 2002; Cruz de Carvalho, 2008; Kar, 2011; Sharma et al., 2012). A balance between the generation and degradation of ROS is required to avoid oxidative injury and to maintain metabolic functions under stress conditions. In plant tissues, the level of ROS is controlled by an antioxidant system that consists of antioxidant enzymes and non-enzymatic low molecular weight antioxidant molecules, including proline, ascorbic acid and carotenoids (Schutzendubel and Polle, 2002; Semida and Rady, 2014; Agami, 2016). In this study, water deficiency stress significantly reduced the growth of wheat plants, in terms of reduced length of shoot, number of fertile tillers per plant, flag leaf area, length of spike, number of spikelets per spike, root fresh and dry weights per plant, straw fresh and dry weights per plant, spike fresh and dry weights per plant, 1000-kernel weight and grain yield per plant. The observed reduction in the above-mentioned growth parameters under drought stress condition in this study may be due to the disturbance in metabolic process of the plant including chlorophyll destruction and the cell division (Tab. 3 and 4). Water deficiency stress causes losses in tissue water content, which reduce turgor pressure in the cell, thereby inhibiting enlargement and division of the cells causing a reduction in plant growth (Shao et al., 2007). Moreover, water stress decreased the growth rate, stem elongation and leaf expansion (Hale and Orcutt, 1987). The decline in fresh weight may be due to the decrease in water content of the stressed plant cells and tissues which lose their turgor and thus shrink (Boyer, 1988; Soha E. Khalil and El-Noemani, 2012). The decrease in dry weights of the stressed plants could be attributed to the disturbances in metabolic processes, which lead to decreases in meristematic activity, thereby inhibiting division of cells causing a reduction in dry mass production. Drought stress impairs mitosis, cell elongation and expansion result in reduced plant height, leaf area and crop growth (Nonami, 1998; Kaya et al., 2006; Hussain et al., 2008). The deleterious effects of drought stress on growth were reported by several researcher i.e. Beltrano and Ronco (2008) they found that, dry weight per plant was decreased in wheat plants subjected to severe drought stress, Arzanesh et al. (2011) they found that, straw yield and grain weight per ear were decreased under drought stress, Agami (2013) who reported that, drought stressed plants showed a significant reduction in growth traits and yield of lettuce comparison to non-water- deficiency stressed plants, Naveeda et al. (2014) they found that, drought stress had drastic effects on growth of maize plants; Wang et al. (2016) they found that severe stress had a negative impact on growth of *Heteropogon contortus* plants.

It has been shown from the results of this study that *A. lipoferum* inoculated grains in presence or absence of water deficit stress significantly improved plant growth characteristics and productivity of wheat plants. We have found that our bacterial strain has ameliorative effects on wheat growth grown in presence of water stress. This indicates that *A. lipoferum* is able to tolerate the drought stress and become active after the stress. This is very interesting regarding this strain, because there are so many situations in which the agricultural soils are subjected to moisture fluctuations. With such kind of ability of *Azospirillum* strain can alleviate the drought stress on plant growth and yield through stabilizing the plant growth conditions including plant water characters. These observations are in accordance with previous reports on the potential of endophytic bacteria having multiple beneficial traits in improving plant productivity and to enhance drought tolerance in plants (Sandhya et al., 2010; Vardharajula

Tab. 5: Effect of *Azospirillum lipoferum* inoculation on relative water content (RWC %), relative membrane permeability (RMP %), total soluble phenols (mg g^{-1} DW) and the activities of superoxide dismutase (Units g^{-1} FW leaf min^{-1}) and peroxidase (Units g^{-1} FW leaf min^{-1}) of wheat (*Triticum aestivum* L., cv. Giza-168) plants grown under non-stressed and drought-stressed condition.

Treatments		Parameters				
Irrigation levels	Inoculation	Relative water content	Relative membrane permeability	Total soluble phenols	SOD activity	POD activity
100% ETc	Non-inoculated	71.23±1.50a	6.33±0.25c	3.40±0.10b	14.93±0.61d	0.95±0.03d
	Inoculated	73.50±0.61a	6.57±0.31c	4.57±0.21a	16.07±0.38c	1.61±0.03b
60% ETc	Non-inoculated	50.43±1.80c	12.50±0.78a	3.30±0.10b	17.80±0.40b	1.44±0.10c
	Inoculated	63.00±5.52b	8.03±0.32b	4.50±0.20a	19.37±0.42a	1.89±0.03a

Values are means ± SD (n=9) and differences between means were compared by the Duncan's multiple range test (LSD; $P \leq 0.05$). Mean pairs followed by different letters are significantly different.

et al., 2011). PGPR including *Azospirillum* spp. affect plant growth through different activities including production of plant hormones such as IAA, N_2-fixation and controlling pathogens (Spaepen et al., 2008; Jalili et al., 2009). Production of plant hormones by *Azospirillum brasilense* increased root growth through enhancing nutrient uptake (Pereyra et al., 2009).

In this study, the drought stress markedly reduced dimension of wheat grain. This was mainly due to the reduction in height and width of endosperm, pericarp and aleurone layer thickness (Tab. 3 and Fig. 1). Tissues exposed to environments with low water availability have generally shown reduction in cell size, and increase in vascular tissue and cell wall thickness (Guerfel et al., 2009). In contrast, *A. lipoferum* was found to be more efficient in mitigating the adverse effects of stress by inducing positive changes the grain anatomy. The beneficial effect of *A. lipoferum* on wheat grain structure may be due to the crucial role of *A. lipoferum* in improving N_2-fixing potential, and plant growth regulators such as auxins, gibberellins and cytokinins which play a role in cell division and expansion.

Drought stress caused a significant reduction in the total chlorophylls and carotenoids concentration (Tab. 4). This reduction may be attributed to the increase in activity of chlorophyll-degrading enzyme chlorophyllase under stress conditions (Reddy and Vora, 1986). *A. lipoferum* inoculation could alleviate the reduction in total chlorophylls and carotenoids concentration under water deficiency stress. A similar result was reported by Heidari et al. (2011), who stated that inoculation of bacterial strain like *Pseudomonas* sp., *Bacillus lentus*, *Azospirillum brasilens*, increased chlorophyll content in basil (*Ociumum basilicm* L.) under drought stress.

Proline concentration in leaves of wheat plants were significantly increased under water deficiency stress (Tab. 4) which may be due to up regulation of proline biosynthesis pathway to keep proline in high levels, which helps in maintaining cell water status, protects membranes and proteins from stress (Yoshiba et al., 1997). The *A. lipoferum* inoculation in presence of drought stress showed lower values for the proline concentration than those in the water stress alone, suggesting that if proline is a stress indicator, wheat plants treated with *A. lipoferum* should have better drought tolerance. Because of *A. lipoferum* in presence of drought stress supported the antioxidant system in wheat plants to enable them to tolerate drought stress. Therefore, it could be expected that, the level of proline declined as result of recovery from stress. The reduced accumulation of proline may result in mitigating the stress effects of drought on wheat plants. This could be because proline is essential in proteins biosynthesis that necessary for cell division. Soluble sugars are key osmolytes contributing towards osmotic adjustment. In our study, the concentration of total soluble carbohydrates significantly increased in leaves of wheat plants subjected to drought stress. The increase in sugar concentration may be a result from starch degradation (Enebak et al., 1997). The increment in sugar concentration may be also a result of an interrupted starch metabolism to gain osmolytes for coping with the osmotic stress arising from water deficit. *A. lipoferum* inoculation decreased total soluble carbohydrates concentration in leaves of wheat plants under drought stress. This reduction in soluble carbohydrates concentration may be resulted from mitigating the stress generated by water stress. In this study inoculation also decreased total soluble proteins concentration in drought stress plants over than non-inoculated plants indicating *A. lipoferum* helps in alleviating the stress induced by water deficit.

The relative water content is a good indicator of drought stress (Fisher, 2000) and in this study, we noticed that drought stress caused a decrease in relative water content in both inoculated and non-inoculated plants compared to well watered plants, however, the inoculation of wheat stressed plants with the bacterium *A. lipoferum* N040 significantly increased the relative water content compared to the non-inoculated controls. This may be due to a reduction in the inhibitory effect of drought on roots and the development of a more effective root system in the inoculated plants (Dodd et al., 2010). Drought stress accelerated relative membrane permeability (RMP) in the inoculated and non-inoculated plants compared to well-watered plants (100% ETc). However, bacterial inoculation helped wheat plants to maintain the RMP and reduced leaf damage compared to non-inoculated plants under drought stress. A positive correlation between drought stress sensitivity and membrane damage were observed by Vardharajula et al. (2011) and Sandhya et al. (2010), and the bacterial inoculation reduced the membrane damage in plants stressed by drought stress.

Antioxidant enzymes are very good biochemical markers of stress and increasing their activities could be a potential to alleviate the oxidative stress-induced by water stress. In our study, drought induced activities of the two enzymes and this may be attributed to the generation of ROS. Consequently, the plant tries to force this by stimulation of antioxidants defense system (Li et al., 2011). The inoculation showed further induction in activities of both SOD and POD (Tab. 5), increasing the antioxidant defense system efficiency against superoxide (O_2^-) radicals produced under water stress. It is most probable that *A. lipoferum* improved plant defense enzymes such as superoxide dismutase, peroxidase or phenolic compounds, to mitigate the oxidative damage elicited by drought stress.

More work is necessary to find exactly the protecting roles of *Azospirillum* sp. on antioxidant enzymes system under drought stress, to provide potential new mechanisms of a plant's tolerance to drought stress, and to define the physiological roles of *Azospirillum* sp. in relation to environmental stresses, including drought stress.

Conclusions

The inoculation of wheat plants with the bacterium *A. lipoferum* N040 increased the activities of some key anti-oxidative enzymes (super-oxide dismutase and peroxidase) and the concentrations of non-enzymatic antioxidants such as total carotenoids and total phenols as well as the concentration of compatible solute such as proline, soluble carbohydrates and soluble proteins in wheat plants grown under drought stress. However, the antioxidant system exhibited considerable changes in response drought stress, suggesting that this increased antioxidant activity may be responsible, at least in part, for the greater tolerance of *A. lipoferum* treated wheat plants to drought stress, leading to improved plant growth, grain anatomy and productivity.

Acknowledgements

This study was founded by the Agricultural Botany Department, Faculty of Agriculture, Fayoum University and Research Center for Advanced Materials Science (RCAMS), Saudi Arabia.

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