Deciphering the Mechanisms of Microbe Mediated Drought Stress Alleviation in Wheat

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Abstract

Drought stress adversely influences the crop plants. Herein, present research was designed to elucidate the role of plant growth promoting microbes for amelioration of water stress in wheat. A pot experiment was conducted for screening the microorganisms on the basis of plant growth, chlorophyll and proline content under water stress. *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 were found more promising strains that positively influenced the plant growth, chlorophyll and proline status of seedlings under water stress condition. Further, *Bacillus* sp. BT-3 and *Klebsiella* sp. HA9 along with check strain (BioNPK) were used for elucidating their detailed effect on morphological, biochemical, physiological and molecular traits to mitigate drought stress in wheat. Microbial inoculation significantly enhanced plant growth, biomass, relative water content, chlorophyll content and root morphological parameters over the uninoculated water stressed (30% FC) control. Likewise, sugar content, protein content and antioxidant enzymes were also significantly enhanced due to microbial inoculation under water stress (30% FC). Microbial inoculation significantly decreased proline, glycine betaine, lipid peroxidation, peroxide and superoxide radicals in wheat over the uninoculated water stressed (30%FC) control. Quantitative real-time (qRT)- PCR analysis revealed that *Bacillus* sp. BT-3, *Klebsiella* sp. HA9 and BioNPK inoculation significantly upregulated stress responsive genes (*DHN*, *DREB*, *L15* and *TaABA-8OH*) over the uninoculated water stressed (30% F.C.) control. The study reports the potential of *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 along with BioNPK in water stress alleviation in wheat which could be recommended as effective biofertilizers.

Introduction

Drought stress is considered as one of the major agricultural impediment creating changes in the morphological, physiological, biochemical and molecular attributes of crop plants. Drought causes the production of free radicals and reactive oxygen species (ROS) in plant cells. Higher amount of ROS may cause oxidative damage and reduce chlorophyll content (Anjum et al. 2011; Hasanuzzaman et al. 2018; Ngumbi and Kloepper 2016; Rahdari et al. 2012). ROS also initiate lipid peroxidation, protein denaturation, membrane leakage and oxidative damage of nucleic acids (Hasanuzzaman et al. 2020; Impa et al. 2012). Water scarcity causes diminished turgor pressure and water potential resulting in stomata closure leading to reduced rate of photosynthesis (Martin-StPaul et al. 2017; Rodriguez-Dominguez and Brodribb 2020). Water scarcity also influenced nutrients absorption and translocation, as nutrients move to the roots through water (Bista et al. 2018; Rouphael et al. 2012). Under drought stress, biosynthesis of ethylene gas is a major physiological manifestation leading to plant growth retardation. Thus, drought stress negatively influences the plant growth, biomass and yield (Ullah et al. 2017, 2018a; Kumar and Verma 2018).

In order to over-come deleterious effects of drought, plant develops different adaptive mechanisms such as production and accumulation of osmoprotectants and antioxidants (Anjum et al. 2017; Hasanuzzaman et al. 2018; Kaushal and Wani 2016; Kim et al. 2017). Phytohormones also enhance the survivability of plants under abiotic stresses such as salinity stress, drought stress or micronutrients

deficiency (Ullah et al. 2018b). According to literature, levels of abscisic acid (ABA) is increased in response to water stress and trigger drought- responsible signalling pathways which subsequently regulate morphological, physiological and biochemical responses accordingly (Egamberdieva et al. 2017; Khan et al. 2018; Wilkinson et al. 2012; Zong et al. 2020). Plant responses to drought stress is a multistep process that include the modulation of stress related genes such as *DHN, DREB, NAC, Hsp, EREBPs, LOXs, L15, WRKY* etc. (Gontia-Mishra et al. 2016; Li et al. 2012; Sallam et al. 2019; Zhu et al. 2016). Drought tolerance genes are expressed concurrently under drought and produce products that respond to signal transduction, stress response, and assist plants in coping with drought stress (Zhou et al. 2010). Two different types of protein are mostly involved during stress management; functional protein, like LEA protein, chaperones, and osmotic regulators, and regulatory proteins, which are part of signalling pathways and gene transcription (Takahashi and Shinozaki 2019).

Wheat (*Triticum aestivum* L.) is the world's most important food grain crop, followed by rice and maize. Different abiotic factors such as soil salinity, water scarcity and micronutrients deficiency etc. pose a threat to wheat productivity (Kumar and Verma 2018). Daryanto et al. (2016) reported that wheat productivity was declined by 21% with a 40% reduction in water. Improving wheat's drought tolerance ability is one of the important researchable concerns around the world in order to supply with the world's expanding population. In this regards, numerous plant breeding and biotechnological approaches are used to combat drought stress. Agronomic strategies such as water saving irrigation and mulching are used to raise the capacity of plants in order to bear the water stress. These technologies are highly technical, expensive, labour intensive and non-renewable. Therefore, sustainable alternatives are required. Plant growth promoting microorganisms can be employed as an alternative, sustainable synergistic biological and environment friendly strategy to manage drought stress.

Plant growth-promoting microorganisms can help plants cope with drought stress by increasing plant growth and improving nutrient uptake and translocation. These microbes can mitigate the drought response of crop plants by improving water potential of cells through modulating accumulation of osmoprotectants, antioxidants, up-regulation or down regulation of stress responsive genes (Singh et al. 2020; Ullah et al. 2019). Plant growth-promoting microorganisms also help crop plants to endure drought by accelerating biosynthesis and metabolism of ACC (1-aminocyclopropane- 1-carboxylate) deaminase enzymes, phytohormones and volatile organic compounds (Danish et al. 2020; Gontia-Mishra et al. 2016; Ngumbi and Kloepper 2016; Vurukonda et al. 2016). ACC deaminase enzymes reduce the ethylene concentration in plant body and help the plant against the environmental stress (Ali and Kim 2018). A large number of reports have demonstrated the role of microbes in alleviating drought stress in crops like, mungbean, pea, maize, wheat and chickpea etc. (Kasim et al. 2013; Mayak et al. 2004; Naveed et al. 2014; Sandhya et al. 2010; Sarma and Saikia 2014; Tiwari et al. 2016; Zahir et al. 2008). However, there are few claims illustrating the key role of PGPRs in ameliorating water stress in wheat crops. In order to improve drought tolerance, the interaction of plant growth promoting microorganisms (PGPMs) with wheat crop was explored in this study. Various morphological, physiological, and biochemical features, as well as the expression levels of stress-related genes (*DHN, DREB, L15*, and *TaABA-8OH*) were examined under drought stress to get a clear picture of plant-microbe interactions.

Materials And Methods

Microorganisms

Ten bacterial isolates procured from National Agriculturally Important Microbial Culture Collection, ICAR-NBAIM, India, five bacterial isolates obtained from Microbial Technology Unit II, ICAR-NBAIM, India, along with one BioNPK formulation were used in this study (Table 1). In this study, BioNPK was utilised as a check which has found effective in stimulating plant growth under drought stress (Saxena et al. 2020).

Growth conditions of microorganisms

All the microorganisms were grown in Nutrient Agar (Himedia Pvt. Limited) except *Nesterenkonia* which was grown in nutrient broth supplemented with 4% NaCl. Microorganisms were incubated at 30°C for 48 h at 150 rpm in a shaking incubator. Four isolates viz. *Bacillus* sp. RPB03, *Pseudomonas* sp. RPB22, *Bacillus* sp. RPB602 and *Pseudomonas* sp. RPB609 were incubated at 50°C for 48 h at 150 rpm.

Pot experiment for screening of microorganisms for drought stress alleviation

An initial screening was conducted with all the microorganisms used in the study on the basis of chlorophyll content, proline content and various plant growth parameters. The best isolates obtained from the study would be used later for carrying out detailed investigation. Therefore, one month pot trial was conducted in a glass house at ICAR-National Bureau of Agriculturally Important Microorganisms, Mau, Uttarpradesh, India. Each pot (4” diameter) was filled with 0.5 kg sterilized sand: soil mixture (1:3) which was sterilized in autoclave by tyndallisation technique. The pots were weighed and 100% saturated with water to calculate the field capacity (FC). Thereafter, the weight of each pot (sand: soil and water) was calculated for 50% and 30% FC which was then used to maintain the water levels at FC according to the treatments. Drought stress levels were assessed using 30% (Stressed Control) and 50% (Un-stressed Control) FC. Log phase broth cultures (containing10^9 CFU/ mL) were mixed with 0.2% carboxymethyl cellulose (CMC) carrier and coated on wheat (HD2967) seeds. Seeds treated with only nutrient broth and CMC were used as uninoculated control. Prior to seed treatment, the wheat seeds were surface sterilised for 1 minute with 70% ethanol and then for 5 minutes with a 1.5% sodium hypochlorite solution (Rudolph et al. 2015). Eight seeds were sown in each pot which were trimmed to four plants after germination. All treatments were taken in triplicates and randomised. Recommended dose of NPK (60:30:20) mg kg^{-1} of soil was applied in all the treatments. The pots were weighed everyday and water was simply supplied to maintain field capacity (FC) according to drought stress levels of treatments. The experiment was set up using the following treatments –(i) 30% FC- Uninoculated stressed control; (ii) 50% FC- Uninoculated non-stressed control; (iii) 30% FC + microbial inoculation. In our study, a total of 16 microorganisms were used making the total number of treatments as eighteen (Tables 3 and 4).

Similar setup was used for elucidating the impact of selected/efficient bioinoculants on morphological, physiological, biochemical and molecular traits of wheat under drought stress. *Bacillus* sp. BT-3,

Klebsiella sp. HA9 and BioNPK (consortium of *Azotobacter* sp. , *Paenibacilus* sp. and *Bacillus* sp.) were selected for further study. The experiment was designed using the following treatments –

(i) 30% FC- Uninoculated stressed control; (ii) 50% FC- Uninoculated un-stressed control; (iii) 30% FC + . *Bacillus* sp. BT-3; (iv) 30% FC + *Klebsiella* sp. HA9; (v) 30%FC + BioNPK (consortium of *Azotobacter chroococum, Paenibacilus tylopili* and *Bacillus decolorationis*). The total treatments maintained were five and are presented in Table 5.

Analyses of plant growth and biomass

After 30 days of sowing, three plants were uprooted from each treatment replicate. Root and shoot length were measured by using inch tap. Fresh weight of root and shoot was measured by using weighing balance. To determine the dry weight, wheat roots and shoots were incubated in hot air oven at 80°C for three days.

Analysis of root morphology

Root studies were carried out by collecting plants from three replicates after 30 days of sowing. The adherence of the soil to the roots was detached by the method of Costa et al. (2000). The LA2400 (3rd Gen.) scanner was used to measure root length, surface area, projected area, volume, average diameter, number of root tips, number of forks and number of links. Thereafter, WIN RHIZO Programme V. 2017a software (Regent Instruments Inc. Ltd., Quebec, Canada) was used to analysed actual values of each root parameters.

Relative water content

Weatherly's method was used to analyse the relative water content (RWC) (Weatherly 1950). Leaves were harvested from the plants and weighed. Thereafter, leaves were transferred in distilled water for 24 hrs. Afterthis the leaves were fully turgid and were weighed again. After weighing, the leaves were put in oven at 80°C for 72 h and dry weight was recorded. RWC was calculated using the following formula.

$$RWC = [\frac{fresh\ weight - dry\ weight}{turgid\ weight - dry\ weight}] \times 100$$

Chlorophyll and carotenoid content

Chlorophyll a, b, total chlorophyll and carotenoid were analysed following the method described by Arnon (1949). One gram fresh leaves were grounded in liquid nitrogen followed by homogenization in 80% acetone. A spectrophotometer was used to measure the absorbance of the supernatant at 663, 645, and 480 nm (Analytik Jena).

Quantitative determination of osmoprotectants

Proline content

The acid-ninhydrin approach established by Bates et al. (1973) was used to determine the proline content of root and leaf tissues using spectrophotometry. 0.5 g plant sample was crushed with liquid nitrogen and homogenized in10 ml of 3.0% sulfosalicylic acid. Then, it was centrifuged at 10,000 rpm for 20 min. 2.0 ml of supernatant was mixed with equal amounts of acid ninhydrin and glacial acetic acid followed by heating at 100°C for 1 hr. Reaction was stopped by putting the tubes in ice. 4.0 ml of toluene was added and mixed thoroughly by vortexing for 15 to 20 min. Absorbance of the upper layer was recorded at 520 nm by using a spectrophotometer (Analytik Jena). A standard curve was prepared using L-proline (10-100 µg/ml) as a standard.

Sugar content

Dubois et al (1951) method was used to calculate sugar content. 200 mg plant samples (root and leaves) were cooked for one hour with 10 ml of 80% ethanol. The extract was filtered through Whatman no. 1 filter paper after cooling. One ml of filtrate was added with 1.0 ml of 5% phenol and 5 ml concentrated H_2SO_4 and vortexed well. Absorbance was recorded at 490 nm and the concentration of sugar was calculated with reference to standard curve made from glucose (0-100 µg/ml).

Protein content

The Bradford assay was used to analyse the protein content of plant roots and leaves (Bradford 1976). Plant materials weighing 0.5 g were crushed in liquid nitrogen and homogenised in 5 mL sodium phosphate buffer (pH 7.0). The suspension was then centrifuged for 20 minutes at 12000 rpm, and 0.5 mL of clear supernatant was combined with 3 mL of Bradford reagent. A spectrophotometer was used to measure the absorbance at 595 nm (Analytik Jena). The concentration of protein in unknown sample was calculated with reference to standard curve made from Bovine serum albumin (100-1000 µg ml^{-1}).

Glycine betaine

Grieve and Grattan (1983) method was applied to analyse the glycine betaine in plant tissues.

Lipid peroxidation

Lipid peroxidation was assayed following the methods described by Heath and Packer (1968). Plant samples were finely ground in liquid nitrogen and homogenised in 10.0 ml of 0.1% trichloro-acetic acid. The homogenate was centrifuged at 1500 g for 15 min. 1.0 ml of supernatant was added with 4.0 ml of 0.5% thiobarbituric acid in 20% trichloro-acetic acid. The mixture was then heated for 30 minutes at 95°C before being chilled in an ice bath. The mixture was centrifuged for 10 minutes at 10,000 g after cooling. At 532 nm and 600 nm, the absorbance of the supernatant was measured. The extinction coefficient for thiobarbituric acid reactive substance is 155 mM^{-1} cm^{-1}. The results were represented as nmol malondialdehyde (MDA) equivalents per gram of fresh weight.

Quantitative determination of Antioxidant enzymes

Superoxide dismutase (SOD)

The methodology reported by Dhindsa et al. (1981) was used to determine SOD activity (1981). Plant samples (100 mg) were crushed and centrifuged (15000 g, 20 min.) in 0.1 M phosphate buffer (pH 7.5). 0.2 ml 200 mM methionine, 0.1 ml 2.25 mM nitroblue tetrazolium chloride (NBT), 0.1 ml 3 mM EDTA, 1.5 ml 100 mM phosphate buffer (pH 7.8), 0.1 ml 1.5 M sodium carbonate, and 0.1 ml enzyme extract were used to make 3 ml reaction mixtures. Water was used to make up the final volume (3 ml). Thereafter, 0.4 mL of 2µmol l^{-1} riboflavin was added and exposed to light (15 W fluorescent lamp, 15 min). After deactivating the enzyme activity in the dark, the absorbance was measured at 560 nm. One unit of SOD was represented by a 50% decrease in absorbance when compared to the control, which lacked enzyme extract.

Peroxidase (POD)

POD activity was determined by using the method reported by Castillo et al. (1984). Plant samples (100 mg) were ground, homogenised, and centrifuged (15000 g, 20 min.) in 0.1 M phosphate buffer (pH 7.5). 0.5 ml of 96 mM guaiacol, 1.0 ml of 100 mM phosphate buffer (pH 6.1), 0.5 ml of H2O2 (12 mM), and 0.1 ml enzyme extract were combined to make a 3.0 ml reaction mixture. The change at 470 nm was recorded at every 30s interval and the enzyme activity was calculated as Units (U) (tetra guaiacol) min^{-1} g^{-1}fresh weight. Tetra guaiacol has an extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Ascorbate peroxidase (APX)

APX was analysed by using Nakano and Asada method (Nakano and Asada 1981). Plant samples (100 mg) were ground, homogenised, and centrifuged (15000 g, 20 min.) in 0.1 M phosphate buffer (pH 7.5) containing 1mM ascorbic acid and 0.5 mM EDTA. 0.1 ml of EDTA (3 mM), 1.5 ml of 100 mM phosphate buffer (pH 7.0), 0.1 ml of 0.1 mM H_2O_2, 0.5 ml of 3.0 mM ascorbic acid, and 0.1 ml enzyme extract were used to make a 3.0 ml reaction mixture. After 60 seconds, absorbance was taken at 290 nm, and activity was represented as U min^{-1} g^{-1}fresh weight.

Catalase (CAT)

CAT was assayed by Aebi method (Aebi 1983). Plant samples (100 mg) were ground in 0.1 M phosphate buffer (pH 7.5), homogenized and centrifuged (15000 g, 20 min.). 1.5 ml of 100 mM (pH 7.0) buffer, 0.5 ml of 75 mM H2O2, and 50 l enzyme extract were combined to make a 3.0 ml reaction mixture. The final volume of the reaction mixture was made up by adding water. At 30s intervals, the change at 240 nm was measured, and the enzyme activity was expressed as Umin^{-1} g^{-1}fresh weight.

Glutathione reductase (GR)

Smith et al. (1988) method was used for measuring glutathione reductase activity. Plant samples (100 mg) were ground, homogenised, and centrifuged (15000 g, 20 min.) in 10 ml of 0.1 M phosphate buffer (pH 7.5). 1.0 ml of 0.2 M phosphate buffer containing 1 mM EDTA, 0.5 ml of 3.0 mM 5, 5-dithiobis [2-nitrobenzoic acid] (DTNB), 0.1 ml of 2.00 mM NADPH, 0.1 ml of 20 mM glutathione disulphide (GSSG), and 0.1 ml of enzyme extract were used to make a 3.0 ml reaction mixture. Spectrophotometric measurements were taken to determine the increase in absorbance at 412 nm. The extinction coefficient of NADPH is 6.22 mM^{-1} cm^{-1}. The activity was expressed as U min^{-1} g^{-1}fresh weight.

Histo-chemical detection of peroxide and superoxide radicals

Method described by Fryer et al. (2002) was followed for staining of superoxide and peroxide radicals in plant leaves. Plant leaves were placed in tubes and immersed in nitroblue tetrazolium (NBT) (0.2%) and 3, 3'-Diaminobenzidine (DAB) (1.0 mg/ ml, pH 3.8) staining solution for staining of superoxide and peroxide radicals respectively. The tubes were placed in the desiccator and attached to a vacuum pump for increasing the infiltration of staining solution. Tubes were rolled up in aluminium foil and left overnight at room temperature. After incubation period, staining solution was drained off. Stained leaves were thoroughly washed for 5 minutes in an acetic acid-glycerol-ethanol (1:1:3) solution at 100°C. Leaves were transferred onto a paper towel saturated with 60% glycerol. Superoxide radicals were visualised as a dark blue due to NBT precipitation and peroxide radicals were visualised as reddish brown due to DAB polymerization.

Analyses of expression of genes associated with drought response in root and shoot of wheat

Thirty-day-old root and shoot samples were collected, and total RNA was extracted using the Trizol method (Rio et al. 2010). qPCR was used to validate the expression of genes (*DHN, DREB, L15*, and *TaABA-8OH*) with potential roles in drought stress response. Three independent samples of each were used. TOPscript^{TM} cDNA synthesis kit (Enzynomics, Republic of Korea) was used synthesize cDNA from 2 g of total RNA, according to the manufacturer's protocol. Table 2 lists the gene-specific primers used for qPCR. The Agilent Mx3000P™ PCR platform and Maxima SYBR Green qPCR kit Master Mix (2X) Universal (Thermo Fisher Scientific Baltics, UAB) were used for the qPCR, which was carried out according to the manufacturer's instructions. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of the selected genes normalised to the expression level of actin from cycle threshold values. Three independent biological replicates with three technical replicates were used in the experiment.

Statistical Analyses

The results of the experiment were provided as the average of three replications. The results of each experiment were statistically analysed using MiniTab 17's one-way analysis of variance (ANOVA). Tukey's test was used to compare mean values of acquired data between treatments ($P \leq 0.05$).

Results

Screening of microorganisms

Microorganisms were screened for drought stress alleviation on the basis of plant growth, chlorophyll content and proline content in wheat leaves. Plant growth parameters such as root length, shoot length (plant height), fresh and dry weight significantly decreased in un-inoculated stressed (30% FC) plants as compared to their counterparts at 50% FC. Likewise, chlorophyll content was also decreased (Table 3). Almost, all microbial inoculations significantly improved the root length of wheat as compared to un-inoculated control at 30% FC. Inoculation of *Serratia* sp. HA5, *Klebsiella* sp. HA9, *Bacillus* sp. BT3, *Bacillus* sp. NKC35, *Bacillus subtilis* DS178, *Nesterenkonia* sp. Nest, *Pseudomonas* sp. RPB609, *Pseudomonas* sp. RPB22, *Bacillus* sp. RPB03 and BioNPK (consortium of *Azotobacter chroococum, Paenibacilus tylopili* and *Bacillus decolorationis*) resulted in significantly better root fresh weight as compared to uninoculated control plants growing at 30% F.C. In case of root dry weight, HA9, SWC20, and BT3 significantly outperformed other inoculants and uninoculated control at 30% FC (Table 3). In case of shoot length, only *Klebsiella* sp. HA9 and *Bacillus* sp. BT3 showed significant increase over the water stressed controls (30% FC). Moreover, they also significantly improved the shoot fresh and dry weight at 30% F.C. Highest dry weight of root was recorded with *Klebsiella* sp. HA9 (71.00 mg plant^{-1}) followed by *Leucobacter* sp. SWC-20 (63.75 mg plant^{-1}) and *Bacillus* sp. BT-3 (63.58 mg plant^{-1}). Likewise, the highest dry weight of shoot was recorded with *Klebsiella* sp. HA9 (67.50 mg plant^{-1}) followed by *Bacillus* sp. BT3 (59.58 mg plant^{-1}) (Table 3).

Almost all bioinoculants significantly enhanced the chlorophyll content over the uninoculated stressed control (30% FC). Highest chlorophyll content was recorded with *Bacillus* sp. BT-3 (12.35 mg g^{-1} FW) followed by *Klebsiella* sp. HA9 (10.86 mg g^{-1} FW). Highest proline content in wheat leaves (8.50 mg g^{-1} FW) and root (3.67 mg g^{-1} FW) was recorded for uninoculated water stressed plants. Whereas, lowest proline content in leaves (2.87 mg g^{-1} FW) and root (1.20 mg g^{-1} FW) was found with uninoculated non-stressed plants (50% FC). Proline content was higher in the leaves as compared to roots. Proline accumulation significantly decreased due to microbial inoculation as compared to uninoculated stressed control (30% FC). Highest percentage reduction in proline accumulation was recorded with *Klebsiella* sp. HA9 followed by *Bacillus* sp. BT3 under 30% FC (Table 4). Thus, *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 were found to be more promising bioagent to enhance the plant growth and were further selected for detailed investigation.

Response of selected microbial inoculants on plant growth

In the next set of experiments, we further evaluated *Bacillus* sp. BT3, *Klebsiella* sp. HA9 and BioNPK for their performance to alleviate water stress with detailed morpho-physiological and biochemical analyses. During this experiment again, shoot length (plant height) and fresh weight of uninoculated stressed (30% FC) control plants significantly decreased over the uninoculated non-stressed (50% FC) plants. In case of shoot length, all the microbial inoculations were statistically at par with each other and significantly better than the uninoculated stressed (30% FC) control. Inoculation of *Bacillus* sp. BT3 recorded the highest shoot length (19.33 cm). Microbial application significantly enhanced the fresh weight and dry

weight of shoot over uninoculated stressed (30% FC) control. Inoculation of *Klebsiella* sp. HA9 recorded maximum shoot fresh weight (317.33 mg). Inoculation of *Klebsiella* sp. HA9 (57.33 mg) recorded significantly higher root dry weight. In case of fresh and dry weight of root, there was no significant difference between the uninoculated stressed (30% FC) and uninoculated non-stressed (50% FC) treatments (Table 5).

Response of selected microbial inoculants on root architecture

Under water stressed (30% FC) conditions, total root length, number of root tips and number of forks in wheat plants decreased significantly as compared to the non-stressed uninoculated treatment (Fig. 1A and Fig. 1B). Almost all microbial inoculations significantly increased the total root length, number of root tips, number of links (Fig. 1A and Fig. 1B) and surface area (Fig. 2A) over the uninoculated stressed (30% FC) control. There was no significant effect of microbial inoculation on the projected area (Fig. 2A), root volume and root diameter (Fig. 2B) under 30% FC. Highest number of root tips, numbers of forks and number of links was recorded with inoculation of BioNPK followed by *Klebsiella* sp. HA9 under water stressed (30% FC) (Fig. 1A and Fig. 1B, Supplementary Figure 1).

Response of selected microbial inoculants on relative water content (RWC)

Relative water content (RWC) significantly reduced in uninoculated water stressed (30% FC) control as compared to the uninoculated non-stressed control (50% FC). Microbial inoculation significantly increased the RWC over uninoculated water stressed (30% FC) control (Table 6). Inoculation of *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 resulted RWC statistically at par with uninoculated non-stressed control.

Response of selected microbial inoculants on chlorophyll and carotenoids

Chlorophyll a, Chlorophyll b, carotenoids and total chlorophyll content significantly decreased in uninoculated water stressed (30% FC) control as compared to the uninoculated non-stressed control. All microbial inoculations significantly increased the Chlorophyll a, total Chlorophyll and carotenoids over the uninoculated water stressed (30% FC) control (Table 6).

Response of selected microbial inoculants on osmoprotectant levels

Organic osmoprotectants such as total soluble sugar, protein, glycine betaine and proline significantly increased in both leaves and roots of the uninoculated wheat plants growing at 30% F.C. as compared to uninoculated non-stressed (50% F.C.) control (Fig. 3A, Fig. 3B, Fig. 4A , Fig. 4B and Fig. 5). Osmoprotectant accumulation was higher in leaves as compared to roots. Significantly higher total soluble sugar and protein content in leaves were recorded in the plants receiving microbial inoculations over the uninoculated water stressed (30% F.C.) control. Inoculation of *Bacillus* sp. BT3 significantly increased the protein content of leaves over the other treatments (Fig. 3A). In case of roots, only *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 significantly enhanced the sugar content over the uninoculated water stressed (30% F.C.) control (Fig. 4A).

Glycine betaine and proline content in both root and leaves significantly decreased due to microbial inoculation over the uninoculated water stressed (30% F.C.) control (Fig. 3B, Fig. 4B and Fig. 5). There were no significant differences among the microbial inoculants with respect to glycine betaine content in both leaves and root. However, significantly higher reduction of glycine betaine in leaves and roots were recorded with inoculation of *Klebsiella* sp. HA9 and *Bacillus* sp. BT3 respectively, over uninoculated water stressed (30% FC) control (Fig. 3B and Fig. 4B). There were no significant differences among the microbial inoculations with respect to proline content in leaves. Both in roots and leaves, inoculation of *Bacillus* sp. BT3 significantly decreased the proline content over uninoculated water stressed (30% F.C.) control (Fig. 5).

Lipid peroxidation (MDA content)

Lipid peroxidation in both leaves and roots significantly increased in uninoculated water stressed (30% F.C.) control over the uninoculated non-stressed (50% F.C.) control. Microbial inoculation significantly reduced the lipid peroxidation in leaves. Lowest lipid peroxidation in both leaves (29.94 mg g^{-1} F.W.) and roots (6.33 mg g^{-1} F.W.) was recorded with *Klebsiella* sp. HA9 followed by *Bacillus* sp. BT3 (Fig. 3B and Fig. 4B). Both in leaves and roots, inoculation of *Klebsiella* sp. HA9 reduced the MDA content by two fold over the uninoculated water stressed (30% F.C.) control. Whereas, inoculation of *Bacillus* sp. BT3 reduced the MDA content by 1.7 fold in both roots and leaves over the uninoculated water stressed (30% F.C.) control.

Response of selected microbial inoculants on antioxidants enzymes

In case of leaves, antioxidant enzymes such as super oxide dismutase (SOD), Ascorbate peroxidase (APX), peroxidase (POD) and catalase (CAT) significantly increased in uninoculated water stressed (30% F.C.) control over the uninoculated non-stressed (50% F.C.) control. Almost, microbial inoculation significantly increased the SOD, APX, CAT, POD and Glutathione reductase (GR) over the uninoculated water stressed (30% F.C.) control. There was no significant difference among the microbial inoculation with respect to SOD activity. However, highest APX activity (7.80 U $min.^{-1}$ g^{-1} F.W.) and catalase activity (12.17 U $min.^{-1}$ g^{-1} F.W.)were recorded with inoculation of *Klebsiella* sp. HA9. Besides, *Bacillus* sp. BT3 showed highest peroxidase (59.87 U $min.^{-1}$ g^{-1} F.W.) and glutathione reductase (4.27 U $min.^{-1}$ g^{-1} F.W.) activities.

In case of roots, only SOD and POD significantly increased in uninoculated water stressed (30% F.C.) control over the uninoculated non-stressed (50% F.C.) control. Highest SOD (8.47 U g^{-1} F.W.), POD (33.67 U $min.^{-1}$ g^{-1} F.W.) and APX (2.23 U $min.^{-1}$ g^{-1} F.W.) activities were recorded with *Klebsiella* sp. HA9 inoculation while highest CAT (3.40 U $min.^{-1}$ g^{-1} F.W.) and GR (1.80 U $min.^{-1}$ g^{-1} F.W.) activities were found in the plants inoculated with *Bacillus* sp. BT3 (Table 7).

Histochemical staining

Histological staining results showed maximum superoxide radicals (as a dark blue spots) accumulation in uninoculated water stressed (30% F.C.) control plants (Supplementary Figure 2A). Likewise, peroxide radicals as reddish brown spots were also visualised more in uninoculated water stressed (30% F.C.) control (Supplementary figure 2B). Inoculation with *Bacillus* sp. BT3, *Klebsiella* sp. HA9 and BioNPK reduced the intensity of dark blue spots and reddish brown spots over the uninoculated water stressed (30% F.C.) control. The intensity of dark blue spots and reddish brown spots was very low for the uninoculated non-stressed (50% F.C.) control plants.

Gene expression

Four drought responsive genes *DHN, L15, DREB* (*P18-R & P25-F*) and *TaABA-8OH* were targeted for expression studies using gene specific primers. All genes were expressed in wheat root and shoot under drought. All the genes were up-regulated at variable extent both in roots and leaves due to microbial inoculations. In case of roots, highest up-regulation (2.05 folds) of *DHN* was recorded due to inoculation of BioNPK. While, 1.70 folds and 1.42 folds up-regulation of L15 and *TaABA-8OH* genes were recorded in case of plants inoculated with *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 respectively (Fig. 6A). In case of leaves, highest up-regulation (10.35 folds) of *DHN* was recorded due to inoculation of *Klebsiella* sp. HA9 while ~5 folds up-regulation of both L15 and *TaABA-8OH* genes were observed in case of plants inoculated with *Bacillus* sp. BT3. In leaves, highest up-regulation (1.50 folds) of *DREB* gene was observed in plants treated with BT3 (Fig. 6B).

Discussion

Global climate change has brought about an elevation in the temperature along with decline in rainfall resulting in drought stress adversely affecting crop productivity. Drought stress disrupts normal plant function (Hsiao 2000) causing serious manifestations in the physiological and morphological traits of the plant. Moreover, the induction of free radicals and reactive oxygen species (ROS) hampers the various levels of organization mainly by membrane degradation, lipid peroxidation and disruption of various biomolecules in the plant (Bartels and Sunkar 2005; Hasanuzzaman et al. 2018; Meena et al. 2017; Ngumbi and Kloepper 2016). The application of microbes that promote plant growth is seen as a possible strategy to mitigate the harmful effects of water stress in a faster, more sustainable and cost-effective way. Agriculturally important microorganisms can boost the plant growth through nutrient mobilization and solubilisation, growth hormones secretion, disease suppression along with strengthening the induce systemic resistance (ISR) thereby improving its yield and productivity. In the present study, two bacterial cultures viz. *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 were found to be the best among the screened microorganisms which positively modulated plant growth parameters, chlorophyll content and proline status that enhanced the ability of wheat plant to tolerate the imposed water stress.

Further, promising strains (*Bacillus* sp. BT3 and *Klebsiella* sp. HA9) along with check strain (BioNPK biofertilizer) were used for elucidating their detailed impact on plant morphological, physiological, biochemical and molecular characters to alleviate the water stress in wheat. Again, the higher plant

growth was also recorded with *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 strains than BioNPK under water stress (30% FC). Similar findings were also acquired in wheat plants by Chakraborty et al. (2013) who reported that inoculation of *Bacillus safensis* and *Ochrobactrum pseudogregnonense* increased the plant biomass, plant height as well as photosynthetic pigments under water stress. Different strains of PGPRs like *Azospirillum* sp., *Azotobacter* sp., and *Pseudomonas fluorescens* are distinguished for their beneficial role on plants in water scarcity (Zhu et al. 2020). Singh et al. (2020) also reported that drought decreased the rice growth but inoculation of rice plants with *Trichoderma* and *Pseudomonas* minimised the negative impact of drought. Khan et al. (2019) found that *Azospirillum, Pseudomonas*, *Bacillus* and *Azotobacter* inoculation improved the plant growth and biomass of field crops under water deficit condition. In second pot experiment, equal enhancement of chlorophyll content was recorded with *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 (88%) over the uninoculated water stressed.

Chakraborty et al. (2013) and Naveed et al. (2014) also investigated that inoculation of microbes enhanced the chlorophyll content in wheat and boost the plant growth in drought. Increased pigment content in the leaves because of inoculation with PGPM could be due to increased availability of nutrients and photosynthesis. Relative water content (RWC) is an indicator of the plant's water balance and could be seen as a strategy to improve drought tolerance (Nounjan et al. 2018). In our present study, *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 inoculation significantly enhanced the RWC in wheat leaves by 14.33% and 17.67% respectively over the uninoculated water stressed (30% FC) control. The contribution of beneficial microbes in maintaining the RWC for alleviating drought stress has been reported earlier as well (Ngumbi and Kloepper 2016; Naseem and Bano 2014). Abdela et al. (2020) reported that co-inoculation of *Mesorhizobium ciceri* and *Pseudomonas fluorescens* increased the RWC in chickpea by 22.2% over the uninoculated control under drought stress.

Root system architecture is most important traits of plants among the many adaptive traits for enduring drought stress (Huang and Gao 2000; Huang et al. 2014). In this study, inoculation of *Bacillus* sp. BT3, *Klebsiella* sp. HA9 and BioNPK significantly altered almost all root morphological parameters. Individual inoculation of *Bacillus* sp. BT3 or *Klebsiella* sp. HA9increased the root length by 2.0 folds over the uninoculated stressed (30% FC) control. *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 inoculation enhanced the root surface area by 48% and 70% respectively over the uninoculated water stressed (30% FC). An increase in the number of lateral roots and root hairs under drought stress not only increases the surface area for uptake, but also improves the hydraulic conductivity of the root (Miyahara et al. 2011). Likewise, number of root tips and number of forks were also enhanced by more than 100%. Earlier reports suggested that microbial inoculation positively altered root architecture and enhanced the absorption sites for water and nutrients (Gouda et al. 2017; Hosseini et al. 2017; Khan et al. 2020). Jochum et al. (2019) found that inoculation of maize and wheat with *Bacillus* and *Enterobacter* significantly enhanced the survivability of seedlings through modification in root architecture under moisture deficit conditions.

MDA, a lipid peroxidation byproduct, represents the level of oxidative stress to the cell membrane caused by stress (Gontia Mishra et al. 2016). In our present investigation, *Bacillus* sp. BT3, *Klebsiella* sp. Wheat seedlings inoculated with HA9 and BioNPK under water stress had remarkably low MDA content in roots

and shoots, suggesting that inoculation of beneficial microbes shielded plant cellular homeostasis against the deleterious impacts of stress. Our results are in agreement with previous studies denoting that microbial inoculation encounters oxidative damage caused by water deficit (Gontia-Mishra et al. 2016; Tiwari et al. 2016). Osmoprotective substances are marked biochemical signals of plant stress tolerance. Osmoprotectants adjust the osmotic potential inside the plant cell, maintain the cell's turgidity under the drought stress and protect the plant from oxidative damage (Ullah et al. 2017; Wang et al. 2019). The results of our study revealed that microbial inoculation significantly influenced the amount of osmoprotectants. For example- inoculation of *Bacillus* sp. BT3 and *Klebsiella* sp. HA9decreased the glycine betaine and proline content in leaves by over 40% over the uninoculated water stressed (30% FC) control. It was obvious that PGPM treated plants were not subjected to as much drought stress, consequently, less proline and glycine betaine were accumulated in wheat leaves and roots in the presence of beneficial bacteria. Inoculation with PGPM can stimulate root exudation, biofilm formation and soil moisture conservation thereby improving root growth and nutrient uptake thus ameliorating stress conditions (Tiwari et al. 2016). Gontia-Mishra et al. (2016) investigated that levels of proline and sugars were enhanced in wheat plants under drought stress condition. However, microbial inoculation significantly decreased the proline content in wheat plants over the uninoculated wheat plants in drought stress.

Similar results were made in previous studies by Grover et al. (2014) and Tiwari et al. (2016). Many researchers believe that inoculating plants with PGPMs can improve water potential of plant cells by increasing total soluble sugar, protein, glycine betaine, and proline content (Asghari et al. 2020). This however can be due to different plant genotypes and microbe types.

Our results revealed that antioxidant enzymes such as POD, SOD, APX, CAT and GR were found to be enhanced in uninoculated water stressed (30% FC) control wheat plants as compare to uninoculated un-stressed (50% FC) control. Similar results were also found by Kaushal and Wani (2016), Mishra et al. (2020) and Tiwari et al. (2016). Inoculation of *Bacillus* sp. BT3 and *Klebsiella* sp. HA9 significantly enhanced the concentration of antioxidant enzymes (SOD, APX, POD, GR and CAT) in wheat leaves under water stress (30% FC) over the uninoculated water stress (30% FC). Similarly, Yaseen et al. (2020) investigated that inoculation of *Pseudomonas moraviensis* significantly increased the APX and CAT accumulation in wheat leaves over the uninoculated wheat plants under drought stress. Batool et al. (2020) reported that inoculation of *Bacillus subtilis* HAS31 significantly enhanced the SOD, POD and CAT accumulation in potato plant over the uninoculated control plants under water stress condition. Our findings are also consistent with previous research on the role of plant growth promoting rhizobacteria in modulating antioxidant enzymes in rice, maize, and tomato and improving crop plant drought tolerance (Haddidi et al. 2020; Narayanasamy et al. 2020; Sood et al. 2020; Tsai et al. 2020). Histo-chemical staining results of the present study revealed that microbial inoculation decreased the accumulation of peroxide and superoxide radicals in wheat leaves under water stress which further supports the increased activity of antioxidant enzymes like SOD and POD. Our results supported by Gontia-Mishra et al. (2016) who found that microbial inoculation significantly decreased H_2O_2 accumulation in wheat seedlings

under drought stress. It might be possible due to less encounter of inoculated plants to water stress or more accumulation of antioxidant enzymes by inoculated plants.

Plant growth promoting microbes (PGPM) have recently been identified in many crop plants as mediating drought tolerance via the induction of various genes linked to abiotic stresses (Kasim et al. 2013; Nautiyal et al. 2013; Naveed et al. 2014; Sarma and Saikia 2014; Saakre et al. 2017). Hence, qRT-PCR was used to analyse the expression of drought-responsive genes (DHN, DREB/P18-R-P25F, L15, and TaABA-8OH) in wheat seedlings under water stress with or without microbial inoculation. Dehydrin (*DHNs*) play the key role in plant protective reactions under drought stress such as-holding the water molecules, scavenging the reactive oxygen species and binding with DNA, RNA, protein and lipid to maintain their biological activity (Liu et al. 2017). The dehydration- responsive element binding proteins (*DREB*) impart drought stress tolerance to plants through the induction of water stress responsive genes (Agarwal et al. 2006). The *TaABA-8OH* gene is implicated in abscisic acid catabolism, which converts ABA to 8'-hydroxy-ABA. Subsequently, the 8'-hydroxy ABA is transformed to phaseic acid (Kushiro et al. 2004). This particular gene significantly contributes towards ABA catabolism and especially to reduce the endogenous levels of ABA promptly after the dehydration stress is removed (Umezawa et al. 2006)

Our results revealed that microbial inoculation enhanced the expression levels of *DHN, DREB, L15* and *TaABA-8OH* genes over the uninoculated water stressed (30% FC) control. Microbial mediated upregulation of stress responsive genes (*DHN, DREB, L15* and *TaABA-8OH*) might have helped wheat plants in tolerating the water stress. Our findings are accordance with Wu et al. (2020) who investigated that inoculation of *Azospirillium brasilense* and *Bacillus amyloliquefaciens* alleviated the drought stress in wheat crop by up-regulating the drought stress responsive genes such as APX1, HSP17.8 and SAMS1 over the uninoculated control under drought stress. Ahmad et al. (2019) investigated that *Pseudomonas fluorescens* inoculation enhance the expression level of *DHN1* gene of maize over the uninoculated control during prolonged stress at 6 days after sowing. Singh et al (2020) also found that inoculating rice plants with *Pseudomonas fluorescens* OKC and *Trichoderma asperellum* T42 enhanced the expression levels of stress responsive genes such as- OSPiP1, *DHN* and *DREB* over the uninoculated control under drought stress.

Conclusion

Drought stress causes serious ramifications for the growth and development of a plant system. It severely affects the membrane integrity, root growth, osmotic potential and overall health of wheat. Inoculation of PGPMs helped wheat plants to improve their growth through multifarious beneficial effects on root morphology, relative water content, chlorophyll content, osmoprotectant and antioxidant enzymes in water stress (30% FC). Moreover, they also increased the expression levels of *DHN, DREB, L15* and *TaABA-8OH* which contributed towards activation stress responsive mechanisms and ABA homeostasis. Our results provided an overall comprehensive overview on the microbe mediated drought stress alleviation of wheat. Thus, the use of these microorganisms can be recommended as efficient bioinoculants against water stress in wheat plants.

Declarations

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Figures

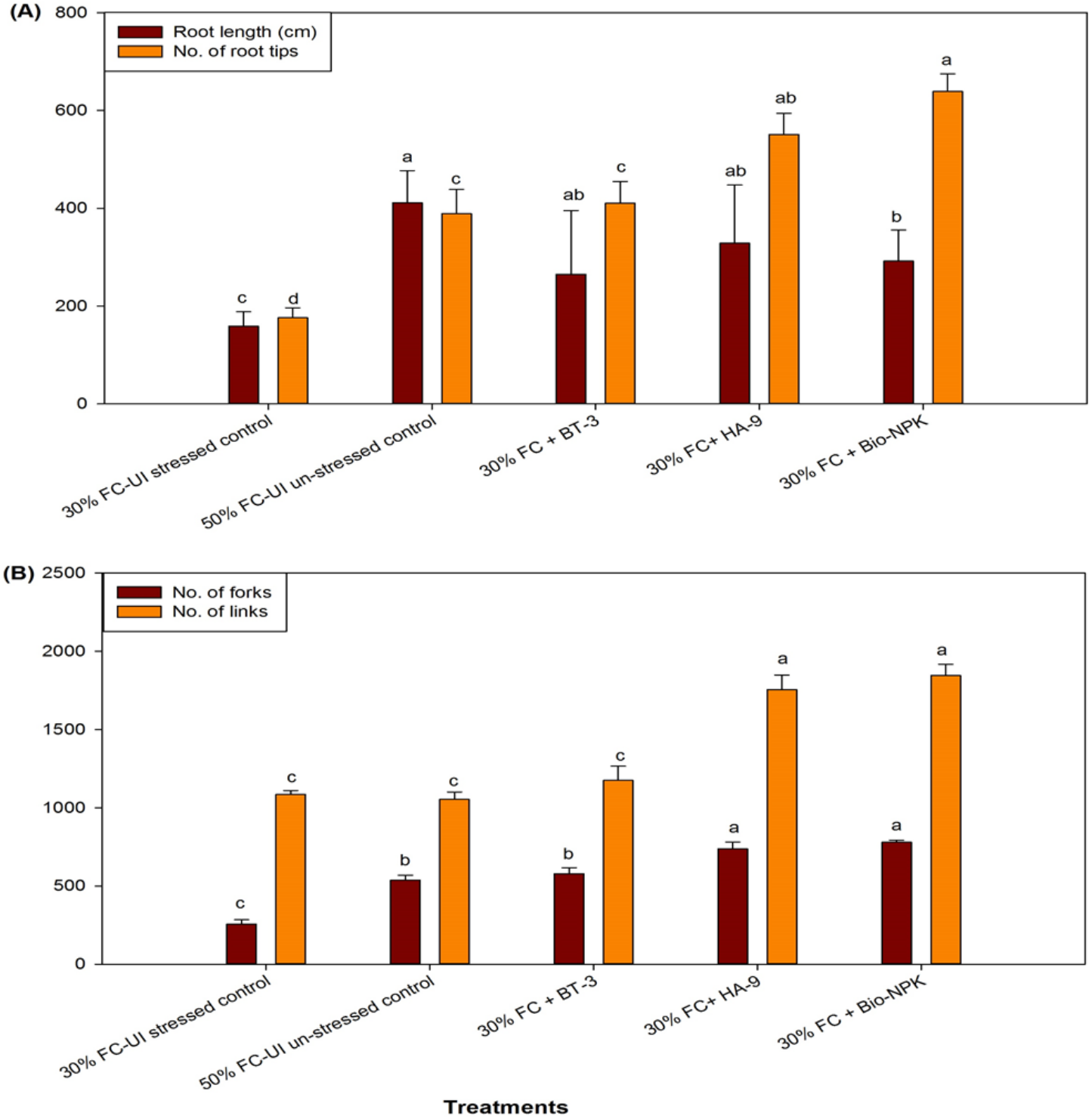

Figure 1

Response of microbial inoculation in relation to wheat root morphology under drought stress. (A) root length, number of root tips; (B) number of forks and number of links. Data are the average of three replicates ± SD; Grouping information between mean values of obtained data was carried out by Tukey's test and 95% confidence ($P \leq 0.05$).

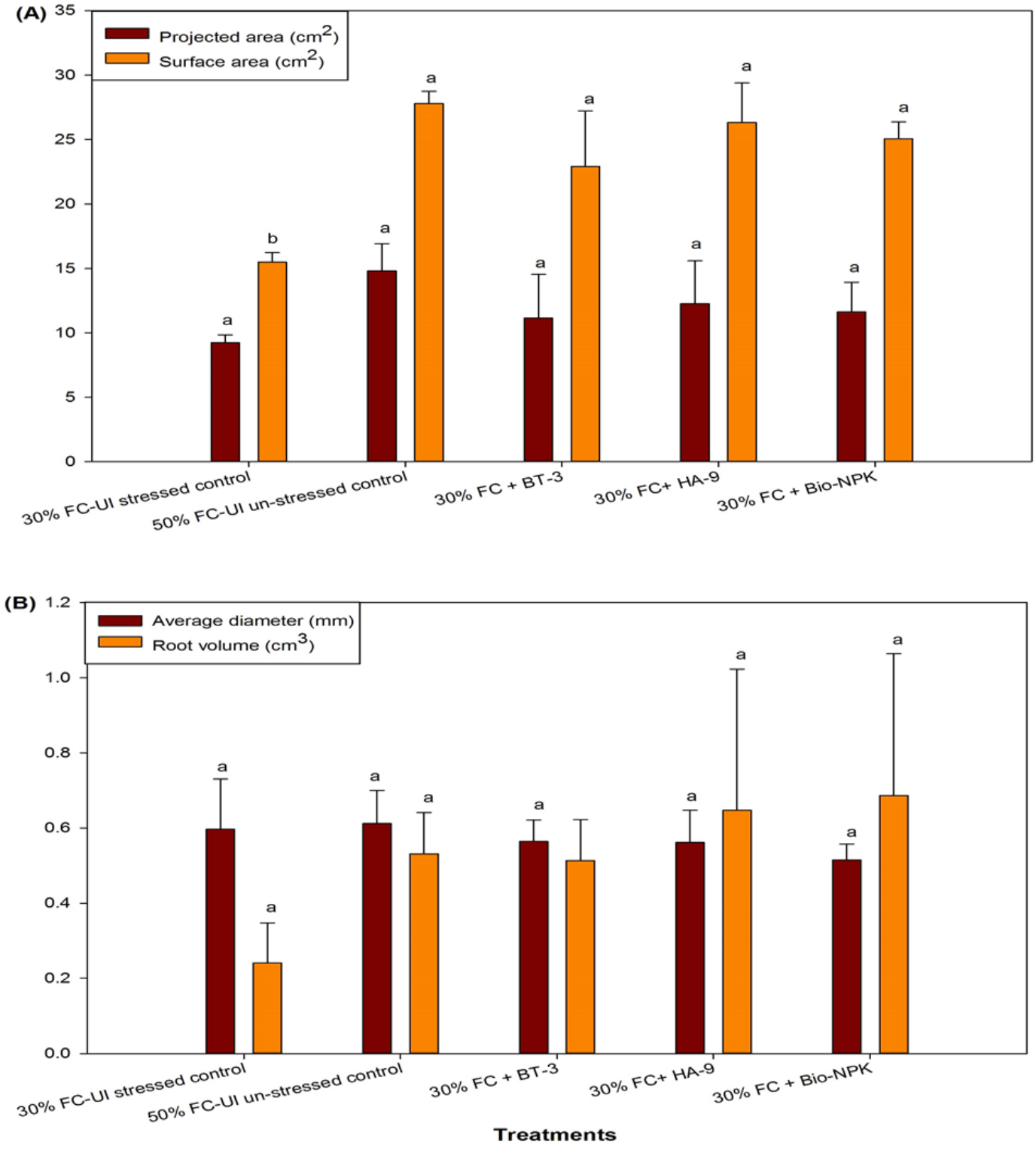

Figure 2

Response of microbial inoculation in relation to wheat root morphology under drought stress. (A) projected area and surface area; (B) average diameter and root volume. Data are the average of three replicates ± SD; Grouping information between mean values of obtained data was carried out by Tukey's test and 95% confidence ($P \leq 0.05$).

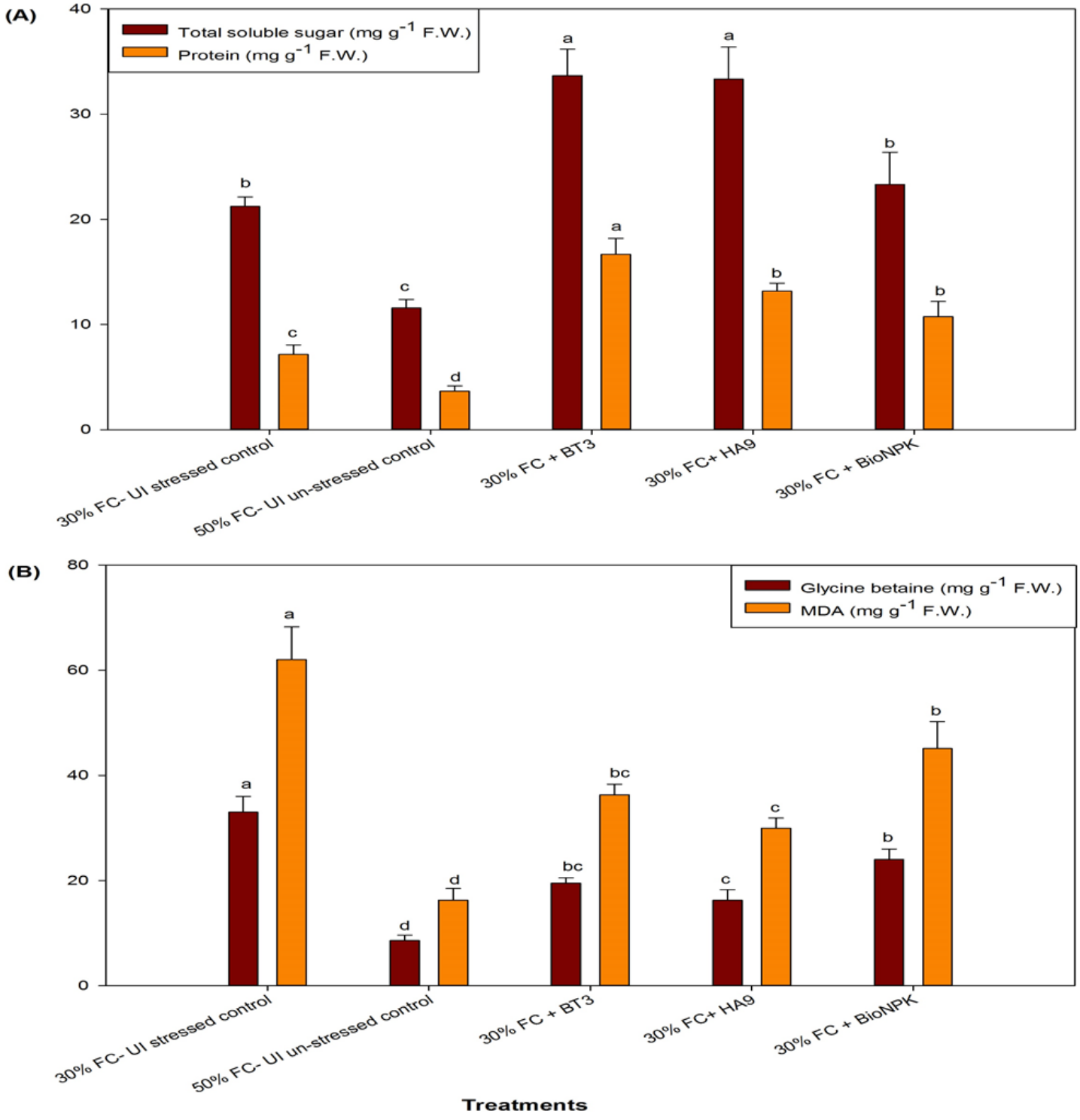

Figure 3

Response of microbial inoculation in relation to osmoprotectant (protein, sugar and glycine betaine) and MDA content in wheat leaf under drought stress. (A) total soluble sugar and protein; (B) glycine betaine content and lipid peroxidation or malondialdehyde (MDA) in leaves. Data are the average of three replicates ± SD; Grouping information between mean values of obtained data was carried out by Tukey's test and 95% confidence (P≤ 0.05).

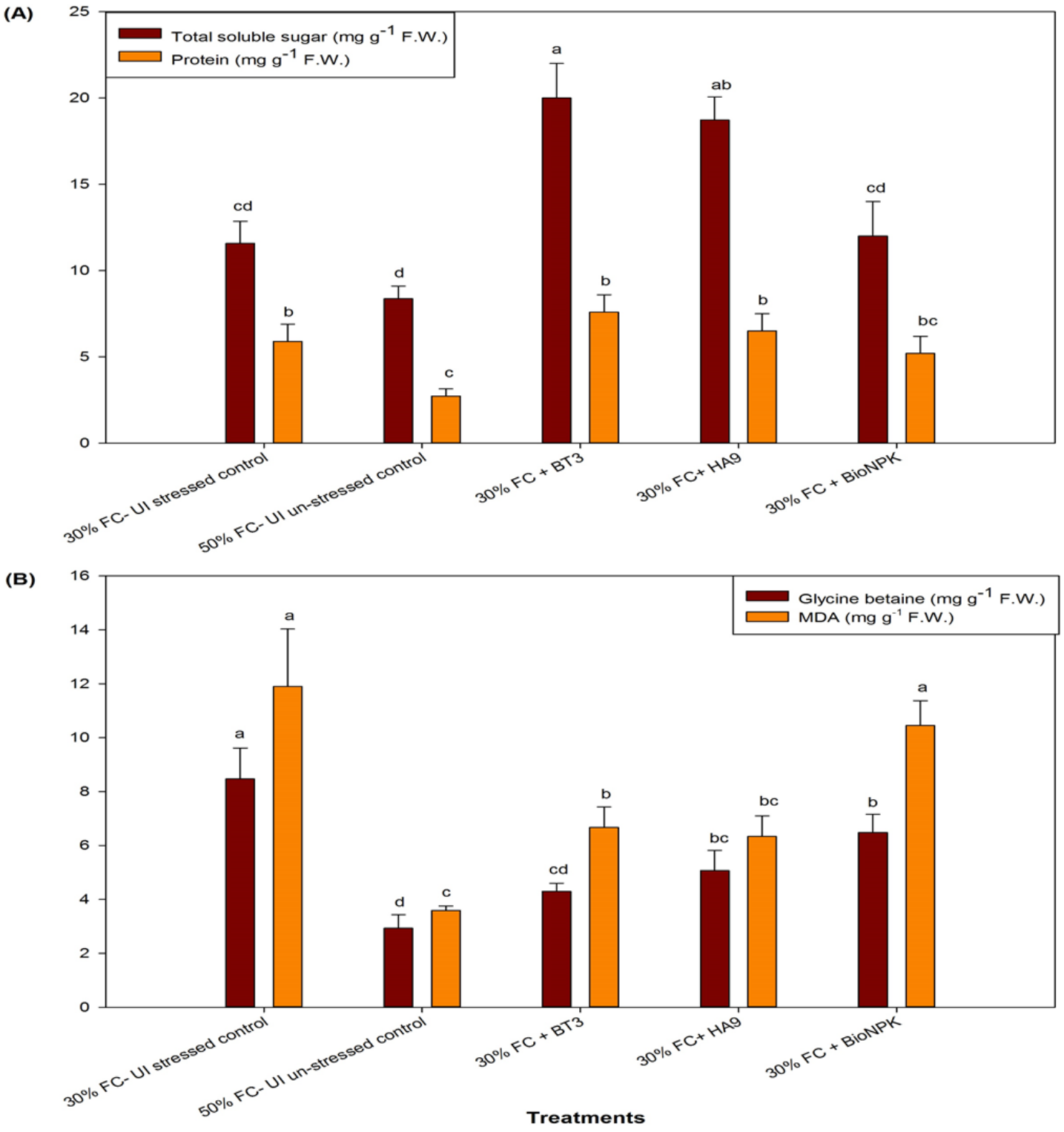

Figure 4

Response of microbial inoculation in relation to osmoprotectant (protein, sugar and glycine betaine) and MDA content in wheat roots under drought stress. (A) total soluble sugar and protein; (B) glycine betaine content and lipid peroxidation or malondialdehyde (MDA) in roots. Data are the average of three replicates ± SD; Grouping information between mean values of obtained data was carried out by Tukey's test and 95% confidence (P≤ 0.05).

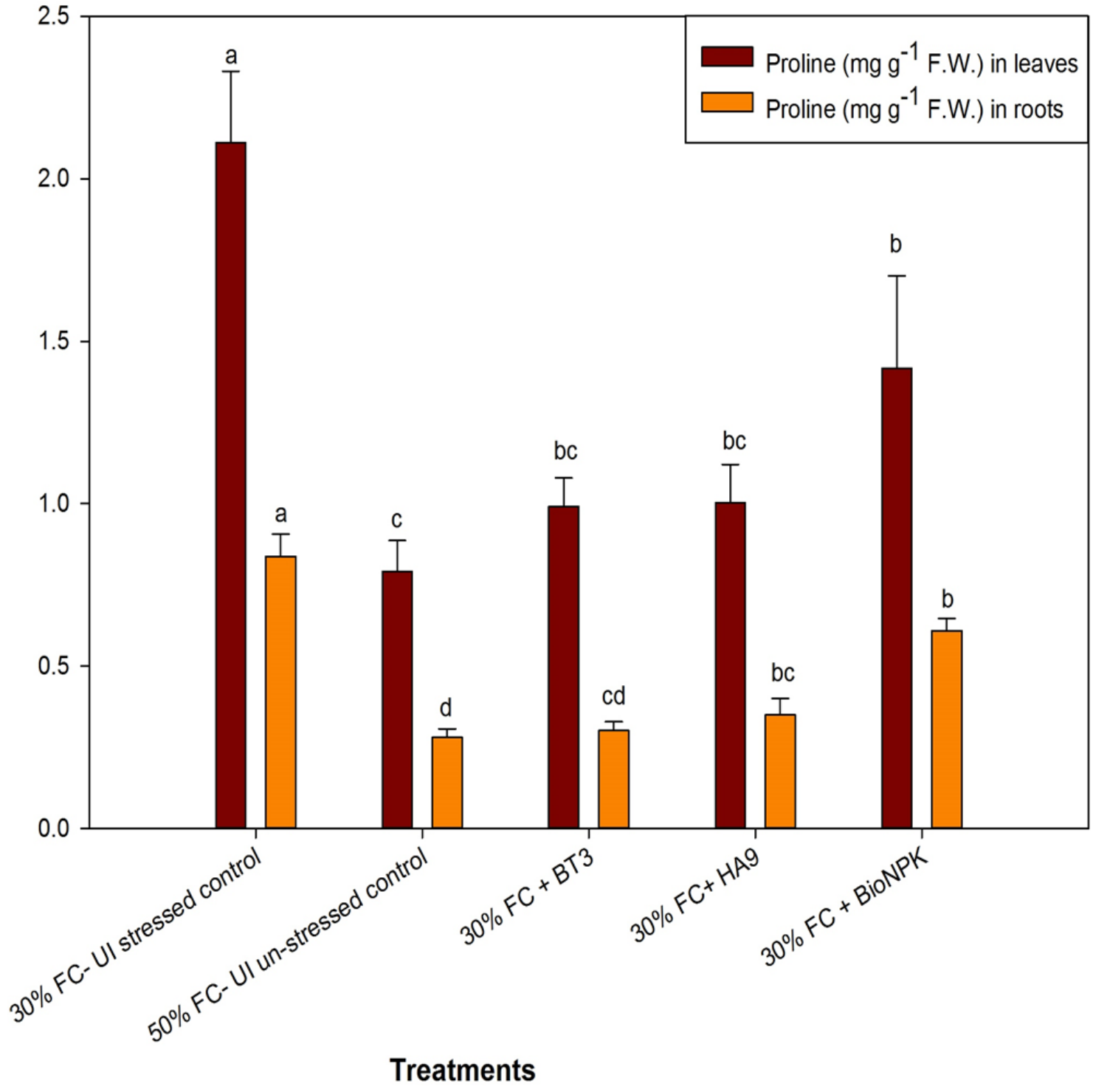

Figure 5

Response of microbial inoculation in relation to proline content in wheat roots and leaves under drought stress. Data are the average of three replicates ± SD; Grouping information between mean values of obtained data was carried out by Tukey's test and 95% confidence ($P \leq 0.05$).

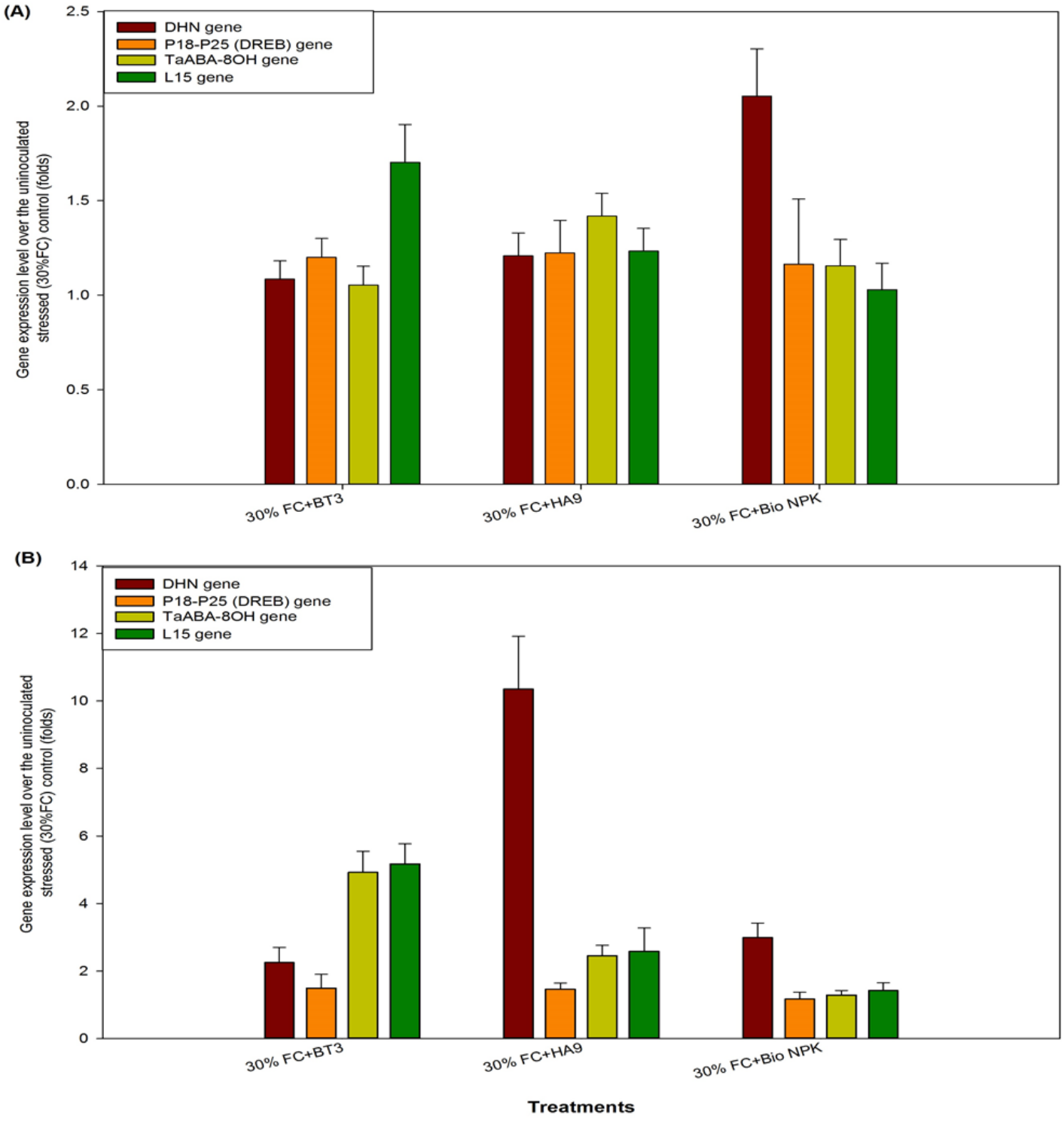

Figure 6

Modulation of gene expression levels (expressed as fold increase) of DHN, L15, P18 and ABA-8OH genes of wheat due to inoculation of plant growth promoting bacteria under drought stress. (A) expression of DHN, L15, P18 and ABA-8OH genes in roots; (B) expression of DHN, L15, P18 and ABA-8OH genes in leaves. Data are the average of three replicates ± SD.

Supplementary Files

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- SupplementaryFig.1.tif
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