Moisture- and period-dependent interactive effects of plant growth-promoting rhizobacteria and AM fungus on water use and yield formation in dryland wheat

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Research Article

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Abstract

Purpose

In drought-prone soils, plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungus (AMF) might positively affect water uptake and crop yield via rhizosphere interactions.

Methods

Sole and combined additions of *Bacillus amyloliquefaciens* producing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and *Rhizophagus irregularis* into rhizospheric soils were performed under well-watered (WW; 80% field water capacity), moderate water stress (MWS; 50% FWC) and severe water stress (SWS; 35% FWC) in pot-cultured wheat (*Triticum aestivum* L.).

Results

In moderate and severe drought stress, water use efficiency (WUE_B) was increased by 27.9–34.3% in PGPR and 20-22.1% in AMF treatments, respectively, and grain yield was improved by 20.03–30.77% in PGPR and 12.13–34.34% in AMF treatments, respectively, compared with CK. Importantly, the co-inoculation of AMF and PGPR significantly promoted WUE_B by 57.46–98.49% and grain yield by 131.82–94.94% compared to the average value of two sole inoculations in MWS and SWS treatments, respectively. Biomass production followed a similar trend as yield. Particularly, the above parameters were significantly enhanced with the prolonged developmental stages ($p < 0.05$). ACC deaminase significantly reduced ACC accumulation in MWS and SWS, enhanced AMF root colonization, and promoted rhizosphere microbial biomass carbon and nitrogen levels across all three developing stages. Furthermore, AMF-PGPR co-inoculation enhanced chlorophyll and carotenoid contents during anthesis while reducing them during pre-harvesting. Enhanced water uptake and root activities upsurged photosynthetic attributes throughout the growing season.

Conclusion

AMF-PGPR co-inoculation acted as a promising solution to cope with the droughted environment via root activities for stronger water capture.

Introduction

Wheat (*Triticum aestivum* L.) is a crucial cereal crop and vital staple food in most parts of the world. It holds nutritional components (55% carbohydrates and 8–12% proteins), bringing down malnutrition worldwide (Sabença et al. 2021). The annual demand for wheat is growing at a rapid rate of 1.6%, while

its yield significantly declined due to water scarcity (Rady et al. 2021). The need of the hour is to restore yield production in arid and semi-arid areas.

The biotic (pathogens and pests) and abiotic (drought, waterlogging, salinity, poor nutrition, soil compaction, and heavy metals) stresses are the foremost reasons behind food scarcity around the globe. The irrigation water demand might increase up to 10% by 2050 due to fluctuation in temperature, thus affecting the atmosphere. Drought is critical stress for truncated crop productivity in arid and semi-arid areas. Drought stress stimulates ethylene production through enhanced levels of 1-aminocyclopropane-1-carboxylic acid [ACC] (Yuan et al. 2022) which acts as a precursor in the biosynthesis of ethylene. Ethylene lessens the roots elongation, triggers insufficient photosynthesis, less intake of nutrients, and reduces the water supply, resulting in stunted growth and low yields of crops (Brunetti et al. 2021).

Different traditional and advanced agricultural approaches enhance crop productivity by alleviating drought stress (Seleiman et al. 2021). However, 1-aminocyclopropane-1-carboxylate (ACC) deaminase-producing plant growth-promoting rhizobacteria (PGPR) is an effective alternative technique to cope with water scarcity. Plenty of ACC-deaminase-producing PGPR species has been reported to promote plant growth under stress conditions (Goswami and Deka 2020). Apart from this, ACC-deaminase producing PGPR promotes root colonization of arbuscular mycorrhizal fungi and helps plants enhance secretions of growth hormones (Moon and Ali 2022a), root development, and immobilized nutrients solubilization (Kumar et al. 2021).

Arbuscular mycorrhizal fungi (AMF) can establish symbiosis with up to 80% of terrestrial plants. AMF is ubiquitous and exists in all types of agricultural soil. The extra-radical hyphal network of AMF and plant roots allows the plants to cover more soil surface area (Vallejos-Torres et al. 2021). AMF can act as bio-regulators (promoting plant performance such as growth, flowering, and yield), bio-protectors (demoting the influence of biotic and abiotic stresses), and bio-fertilizers (stimulating plant nutrient uptake). AMF helps the host plants to overcome drought stress via mechanisms such as increasing root areas (Benaffari et al. 2022), improving rhizosphere soil (Samiappan et al. 2021), providing defense against oxidative damages (Pasbani et al., 2020), and enhancing chlorophyll contents, photosynthetic rate, and gaseous exchange attributes and boosting water use efficiency (Ilyas et al. 2020).

The co-inoculation of AMF and PGPR has synergetic and snowballing effects. The combination of AMF and PGPR has been reported to increase the synthesis of organic acids, soluble sugars, substances for ROS scavenging, antioxidant enzymes, lower Na^+ levels in plants under water stress, prevent phytopathogens, improve phytoremediation (Pasbani et al., 2020), fruit quality (Sani and Yong 2021), yields of crops (Wilkes et al. 2021), and condense the use of chemical fertilizers (El-Sawah et al. 2021). Some *Bacillus* species help in mycorrhizal development, known as mycorrhization helper bacteria (MHB) (Sangwan and Prasanna 2021).

The present study aimed to examine AMF-PGPR's co-inoculation to lessen the drought stress during different growth stages of wheat and improve the yield by using unsterilized soil (providing natural

competition for microbes) in a pot experiment. We hypothesized that co-inoculation of AMF and ACC deaminase producing PGPR (a) would reduce ACC concentration, enhance AMF root colonization and microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN), (b) improve chlorophyll contents, carotenoids, and gaseous exchange properties of wheat under water scarcity conditions during the different growth period, and (c) would have a better impact on water use efficiency, root activities, physiological properties and yield of wheat in a water stress environment.

Materials And Methods

Site description

The pot experiment was performed in the rain-protected shelter house located at Sub-campus, Lanzhou University, Yuzhoung County, Lanzhou, China (latitude: 35°50' N, longitude: 104°06' E, the elevation of 1620 m) from March to August 2019. During the growing interval, the mean relative humidity was 60–85%, while day temperature was ranged between 25 ℃ to 30 ℃, and night temperature was 10 ℃ to 15 ℃ inside the shelter house.

Inoculants, ACC deaminase assay, and inoculation procedure

Bacterium *Bacillus amyloliquefaciens* was used as a bacterial treatment, supplied by the Institute of Biology, Gansu Academy of Sciences, Lanzhou, China. This PGPR strain was screened (Fig. 1b) for the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme (Murali et al. 2021) prior to use. Bacterial isolate was grown in 50ml tryptic soybean broth (TSB) medium for 24 hours at 30 ℃ at 120 rpm. Cells were harvested by centrifugation at 3000g for 5 minutes and washed twice in 1 ml 0.5 M Tris-HCl. Spot inoculation was performed on petri dishes containing Dworkin and Foster (DF) minimal salt medium with ACC as the sole nitrogen source, without ACC as negative control and $(NH_4)_2SO_4$ as a positive control. Bacterial isolate was grown in 200 ml Lauria-Bertani broth (LB) media for 24 hours at 30 ℃ at 200 rpm. Bacterial cells were harvested by centrifuging 50 ml inoculated LB media at 3000 rpm for 10 minutes. The supernatant was discarded, and the pellet was washed twice with normal saline and suspended in autoclaved distilled water. The cell density of the bacterial strain was adjusted to be 1×10^8 CFU mL^{-1} and stored at 4 ℃ for seed treatments. The AMF used in this study was *Rhizophagus irregularis*, supplied by the Institute of Biology, Gansu Academy of Sciences, Lanzhou, China. The mycorrhizal inoculum was prepared by growing maize as a host plant, with spores of *Rhizophagus irregularis* as trap culture, three months before the experiment. The trap culture medium was composed of sand and pre-autoclaved soil (1:1 w/w). The final mycorrhizal inoculum was composed of mycorrhizal roots, mycelia, and soil with fungal spores (968 spores/100 g of soil) and stored in polyethylene bags at 4 ℃ before use.

Crop material

The wheat (*Triticum aestivum* L.) cultivar Longchun 29 was used as a plant material. Seeds were treated with 10% (v/v) hydrogen peroxide for 10 minutes for sterilization. Sterilized seeds were further washed

with deionized water.

Experimental design and treatments

The experiment had three factors, PGPR inoculation, AMF inoculation, and water treatment. Microbial inoculation was further divided into four levels, inoculated with a combination of AMF and PGPR, AMF, PGPR, and non-inoculated or control (CK), while water treatment had three levels, well-watered (WW), moderate water stress (MWS), and severe water stress (SWS).

Each treatment had 12 replicates resulting in 144 pots in total. Each plastic pot had a 9 kg vermiculite and soil mixture (1:2 w/w). Sterilized wheat seeds were immersed in fresh bacterial inoculum in an autoclaved petri-plate for 48 hours and let dry before sowing. Six seeds were sown in each pot at a uniform depth. After one week of germinations, thinning was performed to get three plants per pot, showing consistent growth. For AMF treatment, 100 grams of *Rhizophagus irregularis* inoculum was mixed with 6 kg of each pot soil. For non-AMF treatments, 100 grams of sterilized soil and 10 mL of unsterilized inoculum aqueous filtrate maintain soil properties (nutrients, organic matter, and microbial diversity).

Drought treatment started after four weeks of sowing, letting the plants grow properly and allowing AMF to colonize in wheat roots. One-third of pots were watered to maintain 80% field water capacity (WW), one-third were watered to maintain 50% field water capacity (MWS), while the rest of the pots were watered to maintain 35% field water capacity (SWS) (Boutraa et al. 2010). These treatments were carried out one month before harvesting.

Assessments

The growth and yield attributes were measured at the harvesting stage. Three plants per treatment were selected for plant height (cm), root and shoot dry weight (g/plants), thousand kernel weight (TKW), and grain yield (g/plant). Water use efficiency (WUE_B) was measured by the ratio of dry shoot weight to total water used during the growing season (Batool et al. 2019).

$$\mathrm{WUE}_B(\mathrm{g\,L^{-1}H_2O}) = \frac{\text{Shoot dry weight}}{\text{Quantity of water used}}$$

Triplicates of pots from each treatment were picked at three stages (tillering, anthesis and pre-harvesting). The ACC levels in plant root samples were measured with the method provided by Wang and Woodson (1989). Liquid nitrogen was crushed roots and then homogenized in 80% ethanol for 10–15 minutes at 55 ℃. Followed by centrifugation for 10 minutes at 10000 × g, the supernatant of samples was evaporated for dryness at 55 ℃ under vacuum. In distilled water, the final products were suspended. In addition, the levels of isolated ACC were indirectly measured by converting ACC to ethylene. Gas chromatography was used to determine the amount of evolved ethylene.

Fresh roots were properly washed using running tap water and cut into 1-cm pieces. 10% KOH solution was used to clean roots for 1 hour at 90 ℃ and again, washed with running tap water and treated with H_2O_2 for 10 minutes. Roots were washed adequately with running tap water again and dipped in 1% HCL solution for acidification for 2 minutes. Roots were stained with 0.05% trypan blue and dye for 20 minutes at 90 ℃. Stained roots were kept in lactoglycerol overnight for color separation (Caruso et al. 2021; Tajuddin and Salleh 2022). AMF root colonization rate was observed and recorded with an optical microscope (Zeiss Axio Lab.A1 having 10 Mp camera) using the gridline intersect method. The following equation was used for calculating the percentage of mycorrhizal colonization:

$$\text{Mycorrhizal colonization (\%)} = \frac{\text{No. of root segments colonized with AMF}}{\text{No. of root segments observed}} \times 100$$

The chloroform fumigation-extraction method analyzed the soil MBC and MBN (Sapkota et al. 2021).

The top fully expanded, fresh wheat leaves were collected at tillering, anthesis, and pre-harvesting for chlorophyll a, chlorophyll b, and total chlorophyll contents determination (Danish and Zafar-ul-Hye 2019). Fresh leaves extract was collected by using 80 % acetone solution. The chloophyll a and chlorophyll b estimations were measured at 663 and 645nm wavelength, respectively, using a spectrophotometer. Following equations were used for calculation.

$$\text{Chlorophyll a (mg/g)} = 12.7\ (\text{OD } 663) - 2.69\ (\text{OD } 645)\ \text{V}/1000\ (\text{W})$$

$$\text{Chlorophyll b (mg/g)} = 22.9\ (\text{OD } 645) - 4.68\ (\text{OD } 663)\ \text{V}/1000\ (\text{W})$$

$$\text{Total chlorophyll (mg/g)} = \text{Chlorophyll a} + \text{Chlorophyll b}$$

where OD = Optical density (wavelength), V = Final volume made, W = Fresh leaf weight (g)

Total carotenoid contents were calculated using the following formula (Lichtenthaler and Wellburn 1983):

$$\text{Total carotenoid contents} = (1000 \times \text{A470} - 3.27 \times \text{Ca} - 104 \times \text{Cb})/229$$

Ca and Cb are the contents of chlorophyll a and b in mg/g, and A470 is the absorbance (TU-1950 UV-VIS spectrophotometer) at 470 nm wavelengths.

Gas exchange attributes were measured for fully-grown stressed and unstressed flag leaves on the main tiller of three plants per treatment with the help of a portable infrared gas exchange analyzer (IRGA; Li-Cor 6400, Lincoln, NE, USA). Gas exchange attributes performed at tillering, anthesis, and pre-harvesting included photosynthetic rate (P_n), stomatal conductance (g_s), transpiration rate (E), and internal carbon dioxide concentration (Ci). The portable photosynthesis measuring system had a 6400-02B LED source providing a photosynthetic photon flux density PPFD of 1300 µmol m^{-2} s^{-1}. CO_2 concentration was

maintained at 350 g m^{-3}, relative humidity at 70%, and temperature at 25 °C (Subrahmanyam et al. 2006).

Statistical analysis

Data are depicted as mean ± SE (standard error) of three independent biological replicates. The normality of residuals was tested using the Shapiro-Wilk test. The significance of differences among treatments was scrutinized using analysis of variance (ANOVA) methods fitting for the factorial design according to a complete randomized design (CRD). The effect of microbial inoculation, water treatments, and developing stages, and the significance of differences among treatment means (Tukey's test) were ascertained by using the "ggpubr" package in R. The principal component analysis (PCA) method was used to reduce the dimensions between yield and root colonization, chlorophyll content, carotenoids, photosynthesis, and gas exchange attributes. PCA results were envisioned using a Biplot constructed between the first two PCA (PC1 and PC2) using the "ggbiplot" R package.

Results

WUE_B, plant growth, and yield attributes

Microbial inoculations and water treatments significantly influenced WUE_B, plant height, 1000-kernal weight, and grain yield. Root and shoot dry weight were significantly affected by microbial inoculations, water treatments, and their interactions.

WUE_B, plant height (Fig. 1), root and shoot dry weight, 1000-kernal weight, and grain yield were significantly reduced with increased water stress (WW > MWS > SWS). In addition, microbial inoculations significantly enhanced WUE_B, plant height, root and shoot dry weight, 1000-kernal weight, and grain yield compared to their CK.

WUE_B for AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF were significantly enhanced by 17.7%, 7%, and 2.3% in well water treatment (Table 1), 37.8%, 27.9%, and 20% in moderate water (Table 1), and 56%, 34.3%, and 22.1% severe water stress (Table 1) as compared to their controls. AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF significantly enhanced root dry weight by 16%, 8%, and 4% in well water treatment, 21.1%, 15.8%, and 10.5% in moderate water treatment, and 42.9%, 28.26% and 21.4% in severe water stress, while upsurged grain yield by 21.1%, 5.4% and 9.7% in well water treatment, 37.3%, 20.2% and 12.3% in moderate water stress, and 63.6% 31.4% and 34.7% in severe water stress respectively as compared to control plants.

Irrespective of water treatment, AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF significantly improved plant height by 12.74%, 11.03%, and 9.55%, root dry weight by 21.18%, 14.75%, and 11.04%, shoot dry weight by 16.93%, 10.88%, and 6.82%, 1000-kernal

weight by 16.73%, 11.75% and 6.03% and grain yield by 19.83%, 16.15% and 10.05% as compared to non-inoculation plants.

Table 1
Effect of microbial inoculation on plant height, root and shoot dry weight, 1000-kernal weight, grain yield, and water use efficiency for biological yield (WUE_B) at the harvesting stage of wheat under different water regimes

Water treatments	Microbial inoculation	Plant height (cm)	Root dry weight (g/plant)	Shoot dry weight (g/plant)	1000-kernal weight (g)	Grain yield/plant	WUE_B (g/L H_2O)
WW	**AMF-PRPR**	86.40 ab	0.29 a	3.26 a	42.21 a	4.01 a	2.01 a
	PGPR	93.50 a	0.27 b	3.19 ab	39.27 b	3.49 bc	1.82 b
	AMF	88.33 ab	0.26 bc	3.11 b	37.00 c	3.63 b	1.75 b
	No inoculation	82.57 b	0.25 c	3.09 b	35.06 c	3.31 c	1.71 b
MWS	**AMF-PRPR**	75.53 a	0.23 a	2.89 a	35.99 a	3.13 a	1.96 a
	PGPR	72.83 ab	0.22 b	2.61 b	35.45 a	2.74 b	1.82 ab
	AMF	71.67 ab	0.21 b	2.52 b	33.17 b	2.56 b	1.70 b
	No inoculation	65.33 b	0.19 c	2.24 c	31.58 c	2.28 c	1.42 c
SWS	**AMF-PRPR**	60.17 a	0.20 a	2.08 a	33.30 a	1.98 a	1.80 a
	PGPR	51.50 bc	0.18 b	1.88 b	30.48 b	1.59 b	1.55 b
	AMF	54.27 ab	0.17 c	1.71 c	28.64 c	1.63 b	1.41 b
	No inoculation	45.90 c	0.14 d	1.51 d	26.21 d	1.21 c	1.16 c
ANOVA	**WT**	***	***	***	***	***	***
	MI	***	***	***	***	***	***
	WT × MI	ns	**	**	ns	ns	ns

Mean (n = 3) with the same letter in a column within the same water regime are statistically similar at $p < 0.05$ according to Duncan's multiple range test. ns indicates non-significant while **, *** indicate significance levels at 0.01 and 0.001, respectively. AMF = arbuscular mycorrhizal fungus, PGPR = Plant growth-promoting rhizobacteria, WT = Water treatments, MI = Microbial inoculation, WW = Well watered (80% FWC), MWS = Moderate water stress (50% FWC), SWS = Severe water stress (35% FWC)

ACC accumulation

A significant microbial inoculation, water treatment, developmental stages, microbial inoculation with water treatment and developmental stages; the interaction between water treatment and developmental stages; and interaction among microbial inoculation, water treatment, and developmental stages effect observed for ACC accumulation ($p < 0.001$). ACC accumulation significantly increased during anthesis while significantly reduced during the pre-harvesting stage (Fig. 2a). ACC accumulation was significantly enhanced with an increase in drought stress. Microbial inoculation did not significantly influence ACC accumulation during tillering and anthesis for well-watered treatments; however, microbial treatments (AMF and PGPR co-inoculation as well as alone) significantly reduced the ACC accumulation during tillering, anthesis, and pre-harvesting stages compared to control for moderate and severe water stresses.

Mycorrhizal colonization

Mycorrhizal colonization was significantly affected by microbial inoculation, water treatment, developmental stages, microbial inoculation with water treatment and developmental stages; the interaction between water treatment and developmental stages; and interaction among microbial inoculation, water treatment, and developmental stages. Regardless of water treatments, AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF averagely increased mycorrhizal colonization by 98.8%, 29.2%, and 66.5% at tillering (Fig. 2b), 112.61%, 29.06%, and 71.09% at anthesis (Fig. 2b), and 107.1%, 27.3%, and 62.2% at pre-harvesting (Fig. 2b) respectively, comparing with CK. Irrespective of developmental stages, mycorrhizal colonization in AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF was 102.87%, 67.65%, and 25.49% higher in well-watered 101.46%, 56.85%, and 31.57% higher in moderate water stress, 114.33%, 73.66%, and 27.71% higher in severe water stress than that of their respective controls. Average mycorrhizal colonization was also significantly higher in severe water stress and moderate water stress compared to well-watered treatment (SWS > MWS > WW) across three growing stages.

Microbial biomass carbon and microbial biomass nitrogen

Microbial inoculation, water treatment, developmental stages, the interaction between microbial inoculation and developmental stages significantly influenced MBC ($p < 0.001$), while the interaction between microbial inoculation and developmental stages, water treatment and developmental stages, and water treatment and microbial inoculations also significantly influenced MBN ($p < 0.001$). MBC and MBN significantly upsurged during anthesis and reduced during pre-harvesting stages. MBC significantly increased with an increase in water stress (WW > MWS > SWS) (Fig. 2c), while MBN decreased in moderate water stress and enhanced in severe water stress (Fig. 2d). Microbial inoculations significantly increased MBC and MBN during three water treatments across all growth stages.

Chlorophylls and carotenoid contents

Microbial inoculation, water treatment, developmental stages, and interaction between water treatment and developmental stages significantly affected chlorophyll a content. Usually, AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF had significantly higher chlorophyll a content than their respective CK in severe water stress during tillering and anthesis, while the trend was changed during pre-harvesting where chlorophyll a content was higher in well-watered treatment. In general, chlorophyll a content was increased by 9.23%, 5.87%, and 4.53% by AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF, respectively, compared to their CK. Chlorophyll a content in AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF, on average, was increased by 10.6%, 7.6%, and 6.7% at tillering (Fig. 3a), 8.33%, 4.95%, and 3.16% at anthesis (Fig. 3a), and 8.9%, 5.2% and 4% at pre-harvesting (Fig. 3a) in comparison with CK, respectively.

Chlorophyll b content was significantly affected by microbial inoculation, water treatment and developmental stages, and interaction between water treatment and developmental stages. Across all three developing stages, AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF had significantly higher chlorophyll b content than their respective CK in severe water stress. Averagely, chlorophyll b content in AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF was 26.68%, 14.66%, and 8.53% higher in well water treatment (Fig. 3b), 38.59%, 23.31%, and 15.38% higher in moderate water stress (Fig. 3b) and 56.98%, 39.19%, and 23.03% higher in severe water stress (Fig. 3b), as compared to their relative CK across three developing stages. Chlorophyll b content was increased in AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF 36.4%, 23.6%, and 14.3% at tillering, 35.31%, 19.69%, and 11.57% at anthesis and 40%, 26%, and 16.5% at pre-harvesting, as compared to their corresponding CK.

Similarly, total chlorophyll was significantly affected by microbial inoculation, water treatment and developmental stages, and interaction between water treatment and developmental stages. Generally, total chlorophyll increased during anthesis, while it again reduced during the pre-harvesting stage. Total chlorophyll continued to lessen with increased drought stress across tillering and anthesis, while it again upsurges in severe stress during pre-harvesting. Total chlorophyll in AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF were enhanced by 13.25%, 7.87%, and 4.90% in well water treatment (Fig. 3c), 12.81%, 8.12%, and 5.51% in moderate water stress (Fig. 3c) and 15.56%, 10.18%, and 8.12% in severe water stress (Fig. 3c), as compared to their CK respectively. During developmental stages, AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF was 14.9%, 10.3%, and 7.9% higher at tillering, 13.09%, 7.55%, and 4.65% higher at anthesis and 13.5%, 8.3%, and 5.8% higher at pre-harvesting, than their corresponding CK.

Carotenoid content concentration was also significantly affected by microbial inoculation, water treatment and developmental stages, and interaction between water treatment and developmental stages. In general, carotenoids increased during anthesis and were reduced for well-watered and moderate water stress at pre-harvesting. In case of severe water stress, carotenoids continued to increase through whole developmental stages. Carotenoid content in AMF-ACC deaminase producing PGPR co-

inoculation, ACC deaminase producing PGPR, and AMF were increased by 23.33%, 15.94%, and 10.73% in well-water treatment (Fig. 3d), 26.91%, 17.74%, and 11.09% in moderate water stress (Fig. 3d), and 39.39%, 27.07%, and 16.25% in severe water stress (Fig. 3d), as compared to their corresponding CK. Carotenoid content in AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF was 28.1%, 18.3%, and 11.9% higher at tillering, 23.96%, 17.02%, and 10.41% increased at anthesis, and 36.7%, 24.7%, and 15.6% increased at pre-harvesting than their comparative CK.

Photosynthesis and gas exchange attributes

Microbial inoculation, water treatments, developmental stages, and interaction significantly ($p < 0.05$) influenced the photosynthetic rate. The photosynthetic rate increased from tillering to anthesis and decreased during pre-harvesting for well water and moderate water treatments, while there was a significant upsurge observed for severe water stress during the pre-harvesting stage. As compared to no inoculation, the photosynthetic rate of AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF treatments were improved by 30.77%, 20.39%, and 14.41% in well water treatment (Fig. 4a), 36.63%, 25.97%, and 16.04% in moderate water stress (Fig. 4a) and 48.85%, 29.82% and 14.84% in severe water stress (Fig. 4a). When averaging the water treatments, AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF significantly increased photosynthetic rate by 34.8%, 21.3%, and 17.6% at tillering, 43.99%, 31.27%, and 19.57% at anthesis and 35.4%, 22.4%, and 9.9% at the pre-harvesting stage, compared to that observed in non-inoculation conditions, respectively.

Microbial inoculation, water treatments, developmental stages, and their interactions significantly impacted the stomatal conductance. In general, stomatal conductance reduced with an increase in water stress (WW > MWS > SWS), while it increased with each growing stage (tillering < anthesis < pre-harvesting). Averagely, stomatal conductance was significantly upsurged for AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF by 19.15%, 12.55%, and 8.19% during well water treatment (Fig. 4b), 24.59%, 15.81%, and 10.27% during moderate water stress (Fig. 4b), and 24.06%, 15.69% and 10.87% during severe water stress (Fig. 4b) than their respective controls. While stomatal conductance for AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF was significantly Improved by 13.8%, 8.6%, and 5.6% at tillering, 22.86%, 14.87%, and 9.47% at anthesis and 27.9%, 18.4% and 12.7% at pre-harvesting, as compared to non-inoculated conditions.

The intracellular CO_2 was significantly influenced by microbial inoculation, water treatments, developmental stages, and interactions. Intracellular CO_2 reduced from tillering to anthesis, while upsurge during pre-harvesting for well water treatment, while it was increased from tillering to anthesis and reduced during pre-harvesting for both moderate and severe water stress. The intracellular CO_2 for AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF significantly reduced 10.5%, 7.1%, and 4.3% at tillering (Fig. 4c), 15.69%, 11.06%, and 7.14% at anthesis (Fig. 4c) and 15.4%, 11.2%, and 7.3% during pre-harvesting (Fig. 4c), relative to their respective controls.

Similarly, Microbial inoculation, water treatments, developmental stages, and their interactions significantly influenced the transpiration rate. The general trend was similar to stomatal conductance, where it lessened with an escalation in water stress (WW > MWS > SWS), while it amplified with each growing stage (tillering < anthesis < pre-harvesting). The transpiration rate, averagely, for AMF-ACC deaminase producing PGPR co-inoculation, ACC deaminase producing PGPR, and AMF was increased by 20.75%, 13.74%, and 9.73% during well water treatment (Fig. 4d), 21.26%, 14.55%, and 9.23% during moderate water stress (Fig. 4d), and 46.41%, 35.84%, and 27.5% during severe water stress (Fig. 4d) as compared to their relative non-inoculations.

Principal component analysis (PCA)

Dim 1 explained 60.4%, 59.1%, and 45.9%, and dim 2 explained 28.2%, 23.0%, and 31.1% of the total variance in well-watered, moderate water stress, and severe water stress. Figure 5 showed that intracellular carbon dioxide was significantly away from photosynthesis, transpiration, and stomatal conductance with increased water stress. ACC accumulation was significantly away from root colonization, transpiration, and stomatal conductance during well-watered treatment (Fig. 5a), root colonization, chlorophyll contents, transpiration, stomatal conductance, and photosynthesis during moderate water stress (Fig. 5b), and significantly away from root colonization, microbial biomass carbon and nitrogen, photosynthesis, transpiration, stomatal conductance, chlorophyll content, and carotenoids during severe water stress (Fig. 5c).

Discussion

In the present study, the application of exotic AMF and ACC deaminase producing PGPR strain inoculations either as single or co-inoculation treatments were very effective in helping wheat plants to lessen the detrimental effects of drought stress on mycorrhizal colonization, MBC and MBN, chlorophyll, and carotenoids contents, gas exchange attributes, WUE_B, root and shoot dry weight, growth, and yield attributes. The treated plants displayed a high level of drought stress tolerance under water scarcity.

WUE_B, root attributes, microbial activities, and plant growth are important aspects to enhance plants' yield attributes. These attributes were significantly high in AMF and ACC deaminase-producing PGPR treated plants, while these attributes were reduced with an upsurge in water scarcity. AMF and ACC deaminase-producing PGPR co-inoculation significantly enhanced MBC and MBN in severe water treatments, indicating the upsurge of microbial activities in rhizospheric soil. AMF and ACC deaminase producing PGPR enhanced the root dry weight, which enhanced the WUE_B and uptake of nutrients, thus escalating the plant height, shoot dry weight, 1000-kernal weight, and grain yield under drought stress. The significant improvement in these parameters is due to the upsurge of ACC deaminase activity, microbial biomass, root colonization, chlorophyll contents, total carotenoids contents, stomatal conductance, transpiration rate, photosynthetic rate, and reduction in intracellular carbon dioxide due to AMF-ACC deaminase producing PGPR co-inoculation under water deficit conditions. These findings were in line with the previous investigations (Adesemoye et al. 2008; Xun et al. 2015; Calvo-Polanco et al. 2016).

The environmental stresses usually damage plants due to the upsurge level of ethylene. Drought stress is linked with ACC production, a precursor of ethylene (Moon and Ali 2022b). *Bacillus amyloliquefaciens* produces ACC deaminase enzyme and has developed a defensive mechanism for host plants to cope with ethylene stress under drought conditions (Zafar-Ul-Hye et al. 2019). In our study, ACC concentration rises with an increase in drought stress, while ACC deaminase producing PGPR alone and combined with AMF significantly reduced the ACC concentration. The upsurge in ethylene levels significantly retards legume plant nodulation (Kumari et al. 2021) and inhibits AMF species (Jajoo and Mathur 2021).

Our findings showed that the exogenous AMF successfully infected the plants inoculated with AMF or ACC deaminase producing PGPR or a combination of AMF and ACC deaminase producing PGPR under well-watered and drought circumstances. In the presence of increased drought stress, mycorrhizal colonization upsurge significantly with co-inoculation of AMF and ACC deaminase producing PGPR. Our verdicts are in line with numerous studies exhibiting that mycorrhizal infection increased when the host plants were subjected to water stress (Chareesri et al. 2020). Ben Laouane (2019) and Raklami (2019) reported a positive effect of ACC deaminase producing PGPR on augmenting root colonization by AMF. Water scarcity improved soil pores and soil aeration by reducing soil compaction, allowing AMF to be well established in roots to perform the plant's normal functioning. However, our results illustrated the germination and colonization of AMF in the growing roots at the tillering stage, which had further improved at the anthesis stage and finally attained the maximum when wheat plants reached the pre-harvesting stage. The alterations in root colonization at differing developing stages of wheat had been stated in earlier studies (Wahdan et al. 2021). The improvement in root colonization with developing stages may be due to the time required for germination of spores, growth of germ tube, and penetration to the host plant's roots.

Soil MBC and MBN are soil microbial activity indicators associated with fertility and soil health (Song et al. 2019; Rodgers et al. 2021). An increase in microbial biomass is due to enzymatic activities of AMF and PGPR, indicating the continuous growth of microorganisms (Zhou et al. 2015). Our results align with these studies showing the significant increment in MBC and MBN under drought stress in AMF, and ACC deaminase produces PGPR treatments compared to control. The rise in MBC and MBN contents under drought stress is due to the upsurge enzymatic activity of ACC deaminase producing PGPR and root colonization of exotic AMF.

Photosynthetic traits establish an imperative tool to study the impact of water stress on plants. Our finding showed increased photosynthetic pigment synthesis (chlorophyll a, b, total chlorophyll, and carotenoids) in wheat plants inoculated with AMF, ACC deaminase producing PGPR, or AMF and ACC deaminase producing PGPR combination. Photosynthetic pigments are enhanced from tillering to anthesis, followed by a reduction during the pre-harvesting stage. Similar trends were observed in previous studies (Hashem et al. 2019; Li et al. 2019; Anli et al. 2020), which indicated the increase in photosynthetic pigments with AMF and ACC deaminase producing PGPR inoculation while a reduction in photosynthetic pigments with the increase in water stress. Drought stress damaged the plant machinery, resulting in reactive oxygen species (ROS) synthesis, spoiling the photosynthetic pigments. AMF and ACC

deaminase-producing PGPR co-inoculated plants could absorb more nutrients and water, reducing the ROS damage and alleviating the drought stress.

Our data indicated an increase in photosynthesis rate, transpiration rate, and stomatal conductance while a decrease in intracellular CO_2 in leaves during water stress in treated wheat plants compared to microbes' accessible controls. These outcomes agree with previous reports using PGPR or AMF (Begum et al. 2019; Hashem et al. 2019; Zai et al. 2021; Talaat 2021). AMF and ACC deaminase producing PGPR co-inoculation increased the chlorophyll content, which helps to enhance the photosynthetic rate. An upsurge in stomatal conductance in AMF and ACC deaminase-producing PGPR infected plants during water stress enhanced the CO_2 assimilation in leaves due to stomatal opening for a longer time. AMF and ACC deaminase producing PGPR symbiosis with plant roots influenced the stomatal functioning of leaves. Decreased levels of intracellular CO_2 in AMF and ACC deaminase-producing PGPR co-inoculation treated plants enhanced photosynthesis because these plants utilized more CO_2 than non-treated controls. These photosynthetic and gaseous exchange trends indicate AMF and PGPR treated plants' survival in drought stress environments.

Conclusions

In this research, it has been spotlighted that AMF and ACC deaminase producing PGPR generate a positive impact on ACC deaminase activity, microbial biomass, root colonization, root dry weight, WUE_B, growth, and yield attributes, mainly due to the numerous nutrition-related assistances that this class of beneficial soil microorganisms is proficient at delivering to the host plants. Moreover, our data propose that AMF-ACC deaminase-producing PGPR co-inoculation builds up drought stress-adaptive maneuvers by stimulating microbial mechanisms such as ACC deaminase activity and plant mechanisms, such as chlorophyll carotenoid contents, photosynthetic machinery, and gas exchange attributes which helps in enhancing root activities and WUE_B. This work makes the AMF-ACC deaminase-producing PGPR co-inoculation technique more likely to be reasonable for producers in harsh regions and farmers in developing countries to attain a sustainable crop growing approach. However, further studies need to be performed to understand better the potential of AMF-ACC deaminase-producing PGPR co-inoculation in field trials under drought conditions.

Declarations

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Conflicts of interest/Competing Interest

The authors declared that there are no conflicts of interest or competing interests.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

The R scripts/codes used during the current study are available from the corresponding author on reasonable request.

Author Contribution

You-Cai Xiong, Muhammad Maqsood Ur Rehman, and Ying Zhu contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Muhammad Maqsood Ur Rehman, Ying Zhu, Wasim Khan, Muhammad Abrar, Wei Wang, Awais Iqbal, Yuan Chen, and Muhammad Aammar Tufail. The first draft of the manuscript was written by Muhammad Maqsood Ur Rehman, and You-Cai Xiong, Jian-Sheng Ye, Anum Khan, and Muhammad Rafiq reviewed, edited, and commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

Not applicable.

Consent to participate

The authors agreed to participate.

Consent for publication

All authors consent to the publication of the manuscript.

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Figures

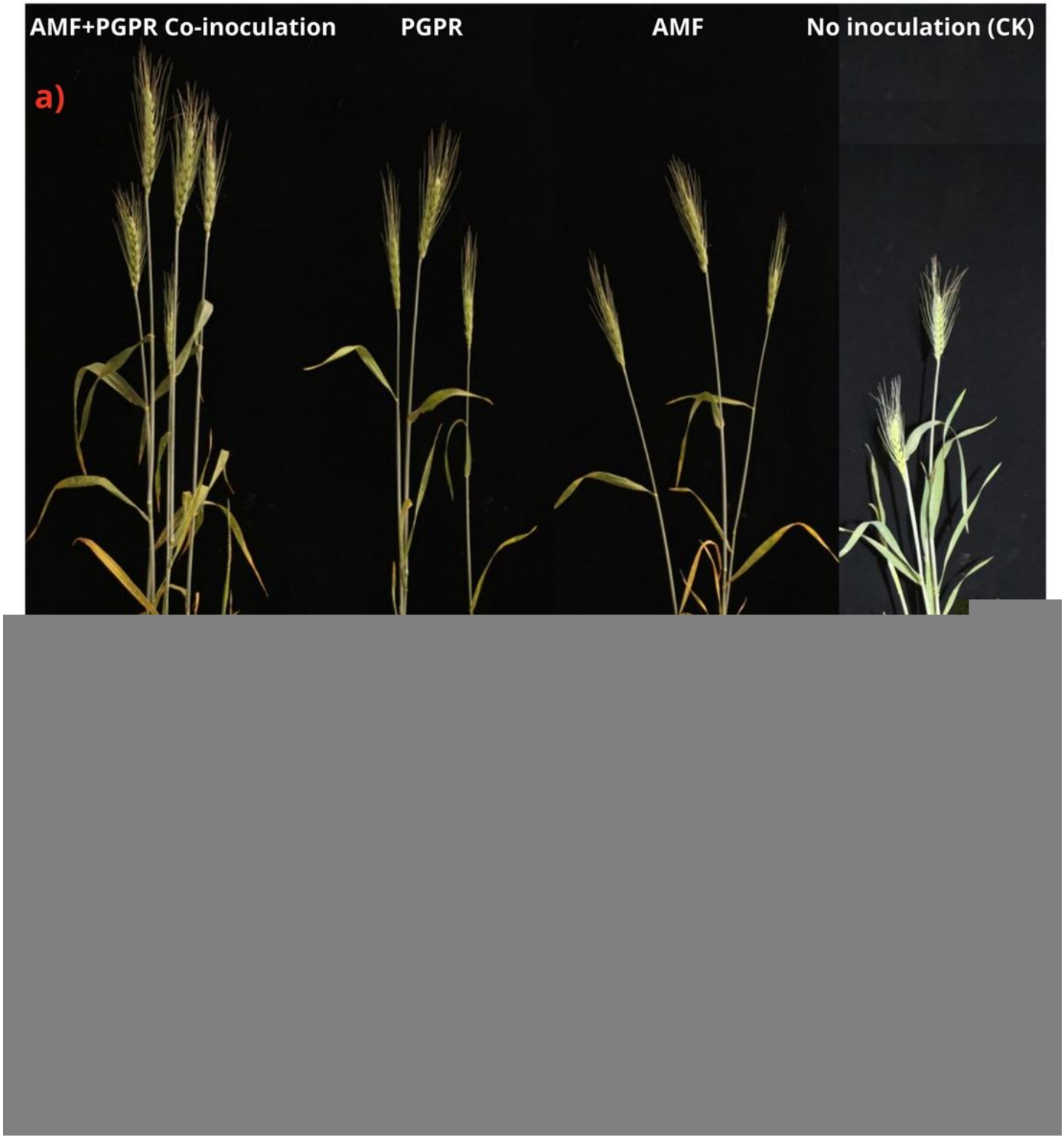

Figure 1

(a) Impact of arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) co-inoculation (AMF-PGPR), only PGPR or AMF inoculation and of the tested control (non-inoculated) on wheat plant growth **(b)** Screening of PGPR's isolates for 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme production; **1.** *Bacillus amyloliquefaciens* **2.** *Bacillus megaterium* **3.** *Bacillus*

licheniformis **4.** *Corynebacterium glutamicum* **(c)** Light micrographs of mycorrhizal colonization of wheat roots

Figure 2

Influence of different water regimes (WW = Well-watered; 80% field water capacity, MSW = Moderate water stress; 55% field water capacity, and SWS = Severe water stress; 35% field water capacity) on **(a)** ACC accumulation **(b)** Root colonization (%) **(c)** Microbial biomass carbon and **(d)** Microbial biomass nitrogen in wheat plants inoculated with arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) co-inoculation (AMF-PGPR), only PGPR or AMF and in control (non-inoculated) across three developing stages (Tillering, Anthesis, and Pre-harvesting). Data are the mean ± SE of three biological replicates, while the same letter within the same water regime is statistically similar at $p< 0.05$ according to Tukey's multiple range test

Figure 3

(a) Chlorophyll a **(b)** Chlorophyll b **(c)** Total chlorophyll content and **(d)** Carotenoid content in wheat plant leaves under three water regimes (WW = Well-watered; 80% field water capacity, MSW = Moderate water stress; 55% field water capacity and SWS = Severe water stress; 35% field water capacity) of the arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) co-inoculated (AMF-PGPR), only PGPR or AMF inoculated and of the tested control (non-inoculated) plants across three developing stages (Tillering, Anthesis, and Pre-harvesting). Data are mean ± SE of three biological replicates, while NS indicates non-significant, **, ***, **** indicate significance level at 0.01, 0.001, and 0.0001, respectively

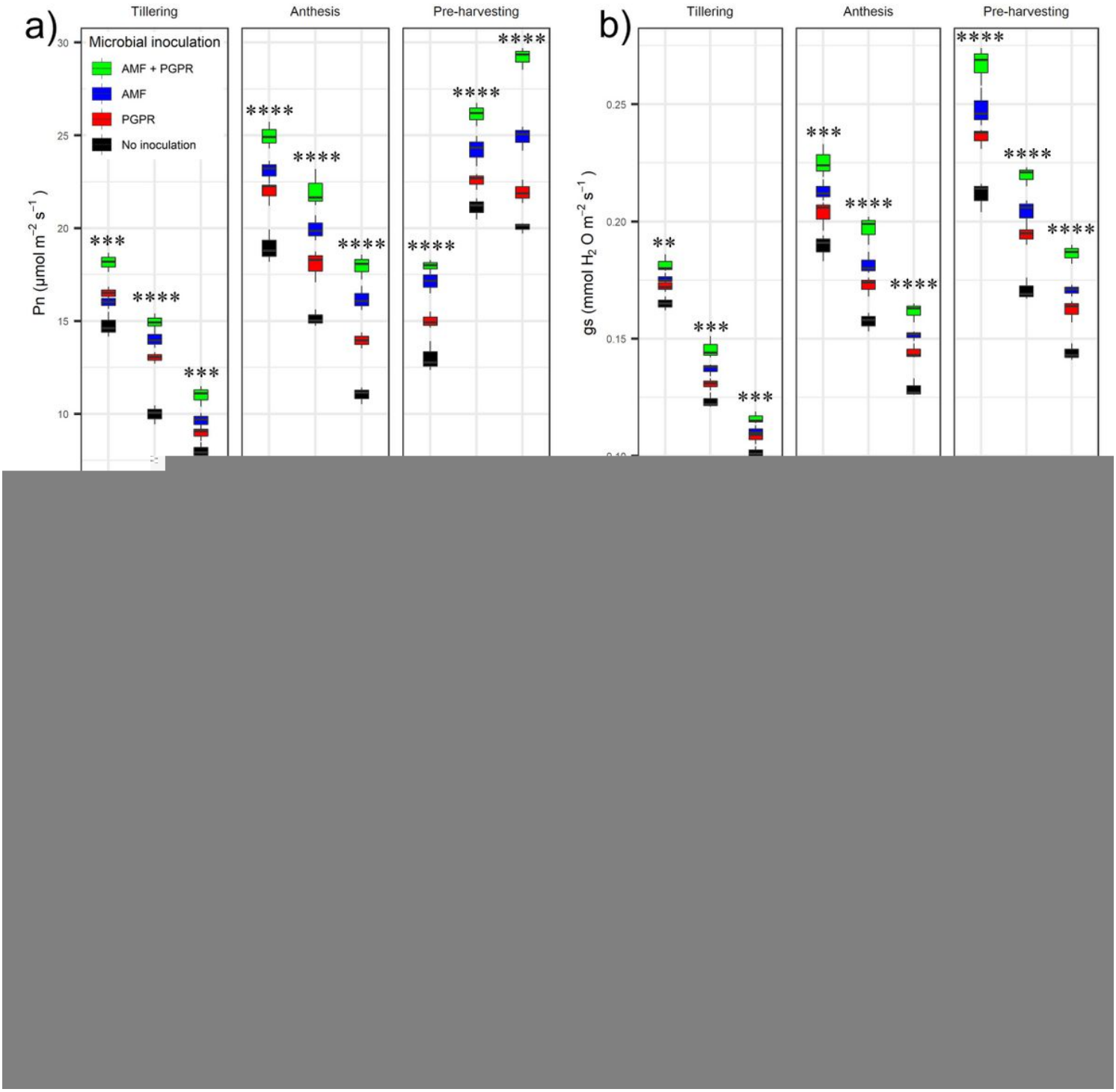

Figure 4

(a) Photosynthetic rate (Pn) **(b)** Stomatal conductance (gs) **(c)** Intracellular carbon dioxide (Ci) and **(d)** Transpiration rate (E) in wheat plant leaves under three water regimes (WW = Well-watered; 80% field water capacity, MSW = Moderate water stress; 55% field water capacity and SWS = Severe water stress; 35% field water capacity) of the arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) co-inoculated (AMF-PGPR), only AMF or PGPR inoculated and of the tested control (non-inoculated) plants across three developing stages (Tillering, Anthesis, and Pre-harvesting). Data are

mean ± SE of three biological replicates, while **, ***, **** indicate significance levels at 0.01, 0.001, and 0.0001, respectively

Figure 5

Principal component analysis (PCA) of the different studied traits and treatments under three water treatments [**(a):** (WW = Well-watered; 80% field water capacity **(b):** MSW = Moderate water stress; 55% field water capacity **(c):** SWS = Severe water stress; 35% field water capacity)] across three developing stages (Tillering, Anthesis, and Pre-harvesting). Chla, chlorophyll a; Chlb, chlorophyll b; TChl, total chlorophyll; IntraC, intracellular carbon dioxide; TransR, transpiration rate; StomCon, stomatal conductance; PhotoR, photosynthesis; RC, Root colonization; ACC; 1-aminocyclopropane-1-carboxylate, MBC; Microbial biomass carbon, MBN; Microbial biomass nitrogen