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ORIGINAL ARTICLE

Differential response of single and co-inoculation of *Rhizobium leguminosarum* and *Mesorhizobium ciceri* for inducing water deficit stress tolerance in wheat

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Abstract Limited soil water availability is a major threat to agricultural productivity because it inhibits plant growth and yields. Various strategies have been adopted to mitigate water deficit stress in plants; however, using extremophilic microbes with plant growth promoting traits could be an environmentally friendly and cost-effective approach to improve crop stress resilience. Rhizobia are well known for their symbiotic association with legumes, but they can also improve the fitness of non-legumes under stressed conditions. Thus, different rhizobial strains were isolated from nodules of two legumes (lentil and chickpea) and tested for osmoadaptation at four different polyethylene glycol (PEG-6000) levels, i.e., − 0.05, − 0.65, − 1.57, and − 2.17 MPa. Two stress-tolerant rhizobial strains, SRL5 and SRC8, were selected to evaluate their potential to induce tolerance against water deficits in wheat grown at four different percentages of field capacity (FC; 40, 60, 80, and 100%). Rhizobial inoculation improved physiological parameters and growth of wheat under water deficit; however, co-inoculation of selected rhizobia was better than sole application. Grain yield was most limited at the highest level of water deficit but sole inoculation with SRC8 and SRL5 improved yield by 24% and 19%, respectively. Combined inoculation increased grain yield by up to 48% compared to the uninoculated control. Thus, rhizobia from different legumes possess enormous potential for improving the resilience of cereals (non-legumes) to water deficit stress. Moreover, co-inoculation of rhizobia could be more beneficial than their sole application.

Keywords Chickpea · Drought · Lentil · Osmoadaptation · Photosynthesis

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Introduction

Climate change has become a big challenge for modern agriculture and is a serious threat to global food security. Agricultural production of many crops is declining due to rapid changes in rainfall and temperature patterns. Increasing scarcity of good quality water is becoming a limiting factor for sustainable agriculture. Besides, an increasing population is consuming more water for domestic and industrial use, which is ultimately threatening the sustainable production of crops like wheat (Raza et al. 2012).

In arid and semi-arid regions of the world, water deficit is a major threat to agricultural productivity. Water deficit stress causes hormonal imbalance, reduces photosynthesis, and induces nutrient deficiencies, which ultimately leads to reduced plant vigor and yield (Asghar et al. 2015). *Triticum aestivum* L. (wheat) is globally significant but 70% of its cultivated area is located in semi-arid and arid regions (Zhang et al. 2011b). Soil salinity and drought are the most critical abiotic stresses which reduce the productivity of staple food crops all over the world (Munns 2011). Drought adversely affects the development and growth of wheat such as flowering and physiological maturity (Farooq et al. 2012), inhibits the growth of primary and secondary roots (Zhang et al. 2011a), and decreases fresh and dry weight of wheat seedlings (Li and Ma 2013; Yan

and Shi 2013). Moreover, water deficit stress inhibits plant growth by disturbing various biochemical and physiological processes, including nutrient metabolism, uptake of essential ions, respiration, translocation of carbohydrates, and photosynthesis (Farooq et al. 2008). Water deficit stress retards wheat growth by lowering transpiration rate, photosynthetic rate, and stomatal conductance (Zhan et al. 2011).

However, beneficial plant interactions with microbes can improve the fitness of crop plants under various environmental stresses (Nadeem et al. 2016; Khan et al. 2017; Ali et al. 2017), including water deficit stress (Nadeem et al. 2014). Rhizobia are known for their beneficial symbiotic association with legumes and fix nitrogen by forming nodules on the roots but also have the potential to promote cereals (non-legumes) growth via indirect mechanisms and direct mechanisms or strategies (Yanni et al. 1997; Antoun et al. 1998; Mehboob et al. 2009; Ahmad et al. 2015). Rhizobia can act as plant growth promoting bacteria (PGPB) for non-legumes, such as sunflower (Alami et al. 2000; Ullah et al. 2017), wheat (Asghar et al. 2015; Yanni et al. 2016), rice (Yanni et al. 1997, 2001; Biswas et al. 2000), and maize (Hussain et al. 2016). In the absence of the host (legume) plant, rhizobia adopt different strategies for their normal survival, such as formation of biofilms on both biotic and abiotic surfaces, allowing nutrient dispersion and liquid flow (An and Friedman 2000; Rinaudi et al. 2006). Rhizobia ameliorate adverse impacts of stresses and induce tolerance in plants by adopting different mechanisms, including induction of systemic tolerance by certain chemical or physical changes (Yang et al. 2009). Rhizobia can alleviate environmental stresses and promote plant growth by producing exopolysaccharides (EPS) and catalase (Hussain et al. 2014), chaperons and sugars (Berjak 2006), organic compounds like trehalose (Zahran 1999), phytohormones (Khalid et al. 2006), siderophores (Arora et al. 2001), and enhancing the availability of essential nutrients (Hussain et al. 2009) by mechanisms such as phosphate solubilization (Zaidi et al. 2009). This study aimed to investigate the effect of rhizobia from different legumes on the growth, physiology, and yield of wheat under water deficit conditions. Furthermore, the comparative efficacy of single and co-inoculation of *Rhizobium leguminosarum* and *Mesorhizobium ciceri* for inducing water deficit stress tolerance in wheat was investigated. Although the potential of rhizobia from different legumes to ameliorate water deficit stress in cereals has been previously addressed (Alami et al. 2000; Hussain et al. 2014; Asghar et al. 2015; Yanni et al. 2016), co-inoculation of different water deficit stress tolerant rhizobia isolated from nodules of lentil and chickpea for mitigating water deficit in wheat has rarely been studied. This is surprising since lentil and chickpea are often grown in rotation with wheat (Gan et al. 2015); thus, there is considerable potential for residual rhizobia to colonize the root system of germinating wheat seedlings.

Materials and methods

Isolation of rhizobia from nodules of legumes

Lentil and chickpea were uprooted at the flowering stage and plant roots were washed with tap water to remove adhering soil. Nodules were cut from the roots, dipped in 95% ethanol solution for 10 s, then in 3% (v/v) sodium hypochlorite solution for 4 min, and, finally, nodules were rinsed with sterilized water (Somasegaran and Hoben 2012). These nodules were crushed to make a suspension, which was streaked on Petri plates containing autoclaved yeast extract mannitol (YEM) agar medium supplemented with Congo red dye (2.5 mL/1000 mL) and incubated at 28 ± 1 °C for 72 h. Only white, elevated, and translucent bacterial colonies were purified by repeated streaking (three to four times) using YEM agar medium supplemented with Congo red. For authentication of isolates as rhizobia, surface-sterilized seeds of respective legume (lentil and chickpea) were inoculated with these isolates and sown in jars containing twice autoclaved (at 121 °C temperature and 15 psi pressure for 20 min) sand (500 g/jar) as growth medium. Nitrogen-free Hoagland's nutrient solution was used as a source of irrigation and nutrients. The experiment was performed under controlled gnotobiotic conditions and four replications of each isolate were maintained. Effective nodulation was observed 30 days after sowing in jars, with isolates that formed pink nodules putatively considered as rhizobia. Further confirmation of rhizobia was done by BIOLOG® (MicroLog System Release 4.2; Biolog Inc., Hayward, CA, USA) using the GN database release 6.01.

Osmoadaptation assay

Rhizobial isolates, ten from each legume (lentil and chickpea), were tested for their osmoadaptation potential by using different levels (0, 10, 20, and 30%) of polyethylene glycol (PEG-6000) in yeast extract mannitol broth (YMB) media (Busse and Bottomley 1989). The osmotic potential of broth media was measured by an osmometer (OSMOMAT 030-D, Gonotec, Germany). One milliliter of rhizobial suspension (≈10^8 CFU mL^{-1}) was used to inoculate each test tube containing 20 mL of YMB media. Three replications were maintained at each PEG-6000 level and placed in a mechanical shaking incubator for 4 days at a temperature of 28 ± 1 °C at 100 rpm, then the optical density was determined at a wavelength of 550 nm by a densitometer (Den-1 Densitometer, McFarland, UK). Moreover, population counts (CFU mL^{-1}) were determined by dilution plating.

Characterization of rhizobia for plant growth promoting traits

Selected rhizobial isolates were tested for their plant growth promoting traits using standard procedures, such as indole

acetic acid (IAA) production (Sarwar et al. 1992; MacFaddin 1980), organic acid production (Vincent 1970), phosphate (P) solubilization (Mehta and Nautiyal 2001), siderophores production (Schwyn and Neilands 1987), oxidase activity (Kovacs 1956), catalase activity, Gram staining (Holt et al. 1994), and EPS production (Nicolaus et al. 1999). Their ability to colonize wheat roots was investigated by adopting the procedure explained by Simon et al. 1996. Aggregation abilities of selected rhizobial isolates were determined by following the procedure described by Madi and Henis (1989) and Burdman et al. (1998). Selected rhizobial isolates were tested for synergism/antagonism between them by following the procedure adopted by Naveed et al. (2014a, b) and both rhizobia (SRL5 and SRC8) were found to be synergistic with each other.

Pot experiment

Two selected efficient water deficit stress-tolerant rhizobial strains were evaluated in a pot experiment for inducing water deficit stress tolerance in wheat. For this, soil was air dried, passed through a 2-mm sieve, and then analyzed for physical and chemical properties. Soil was classified as sandy clay loam with saturation percentage 33.5, CEC 5.01 Cmolc kg^{-1}, pH 7.4, EC 1.41 dS m^{-1} organic matter 0.67%, and total nitrogen 0.05%. The field capacity (FC) of soil was measured by using pressure membrane apparatus. Before filling the pots, the soil water retention curve was measured using suction plates at 0.3, 0.6, 1.0, 3.0, and 4.5 bar pressure, and a linear regression equation determined by plotting ln (h) versus ln θ/θs to obtain the water contents at the permanent wilting point (θWP) and field capacity (θFC) of soil. The following linear regression equation was developed by taking ln θ/θs versus ln (h) to obtain θWP, θFC, θAWC etc.:

$$\ln P = \ln Pe + b \ln \theta/\theta s$$

where P is the matric potential (kPa), Pe (intercept) is the air entry value/bubbling pressure, which is inversely related to α, and b is the slope of ln P vs. ln θ/θs of the water retention curve (Imran et al. 2014).

Rhizobial cells were harvested by centrifugation at 1300 × g for 20 min after culturing in YMB. Then, bacterial cells (pellets) were washed and suspended in sterilized saline solution (0.9% NaCl) and uniform bacterial cell density (10^7–10^8 CFU mL^{-1}) was achieved by maintaining the optical density at a wavelength of 550 nm by a densitometer (Den-1 Densitometer, McFarland, UK). This suspension of rhizobial cells in 0.9% NaCl solution was used as a inoculum. Wheat seeds were inoculated by soaking (for 15 min) in inocula of selected rhizobial strains (10^7–10^8 cfu mL^{-1}) having 1% carboxymethyl cellulose (CMC) as an adhesive agent. Uninoculated (control) treatments were also maintained to segregate the effect of inoculations. Both inoculated as well as uninoculated (control) wheat seeds were soaked in the same way. In the case of the uninoculated control, the wheat seeds were soaked with the same 1% CMC as an adhesive agent but sterilized saline solution (0.9% NaCl) was used instead of living bacterial cells. In the case of co-inoculation, inocula of desired plant growth promoting rhizobial strains were mixed in equal proportion and vortexed for 5 min to ensure homogenized cell density of different rhizobial strains before seed soaking. The inoculated and uninoculated (control) wheat seeds (five seeds per pot) were sown at four different FC levels (40, 60, 80, and 100%), following a completely randomized design with factorial arrangement comprising three replicates. The gravimetric method was used for maintaining the FC on a daily basis under ambient light and temperature conditions in the warehouse. Plant growth and yield were measured 130 days after sowing, at maturity.

Assessment of physiological and chemical parameters

At booting stage, leaf gas exchange parameters, i.e., photosynthetic rate, stomatal conductance, substomatal CO_2 concentration, photosynthetic water use efficiency of plants, and vapor pressure deficit were measured using a CIRAS-3 Portable Photosynthesis System (Amesbury, MA, USA). The free proline content was determined according to the method described by Bates et al. (1973). The SPAD value of the chlorophyll content was measured using SPAD-502 meter (Konica Minolta, Japan). For chlorophyll contents (a and b), 0.5 g of leaf samples from each treatment was homogenized with 80% acetone (v/v) and then the homogenate was filtered through filter paper. The absorbance of the resulting solution was read by a spectrophotometer at 663 and 645,480 nm for chlorophylls a and b, respectively (Arnon 1949). Crude protein was determined by multiplying the grain nitrogen content by a factor of 6.25 (Thimmaiah 2004). The relative water content (RWC) was determined by using the following formula, as described by Mayak et al. (2004):

$$\mathrm{RWC}\ (\%) = [(\mathrm{FW}-\mathrm{DW})/(\mathrm{FTW}-\mathrm{DW})] \times 100$$

where FW is the fresh weight, DW the dry weight, and FTW is the fully turgid weight.

The membrane stability index (MSI) of wheat was determined by the method given by Talaat and Shawky (2014). For this, 200 mg of leaf sample was taken in 10 mL of double distilled water in two sets. One set was heated at 40 °C for 30 min in a water bath and then the electrical conductivity bridge (C_1) was measured by a conductivity meter. The second set was boiled at 100 °C for 10 min and its conductivity

was also measured (C_2). The membrane stability index was measured by the following formula:

$$\text{MSI}\ (\%) = \left[\left(1-(C1/C2)\right]\right. \times 100$$

Nutrients analysis

Nitrogen, phosphorus, and potassium were determined after digestion. The plant samples were digested according to the method of Wolf (1982). K contents were determined with a flame photometer, P contents were determined with a spectrophotometer, and the Kjeldahl method was used to determine the N in the plant samples.

Statistical analysis

Data were statistically analyzed by using statistical software (Statistix 8.1®; Analytical Software, Tallahassee, FL, USA). The two-way analysis of variance (2-way ANOVA) technique was used by following a completely randomized factorial design and Tukey's honest significant difference (HSD) test ($P < 0.05$) was applied to compare the means.

Results

In total, 20 rhizobial strains (ten from chickpea and ten from lentil) were isolated. All 20 strains were assayed further for their drought tolerance at four PEG-6000 levels (0, 10, 20, and 30%). Increasing levels of PEG-6000 decreased the cell numbers of rhizobial strains, as well as optical density (OD) values; however, strain SRC8 (chickpea) and SRL5 (lentil) showed the best resistance, with more colony units (CFU $mL^{-1} \times 10^3$) and greater OD values as compared to others against all PEG levels, which relates to rhizobia survival under harsh conditions (Table 1). In the case of lentil rhizobial isolates, SRL5 showed population counts (CFU $mL^{-1} \times 10^3$) of 31.52, 27.83, 18.81, and 11.7 at 0, 10, 20, and 30% of PEG-6000, which were greater than the other lentil isolates, while among chickpea rhizobial isolates, SRC8 had maximum survival at 0, 10, 20, and 30% PEG-6000 levels, with population counts (CFU $mL^{-1} \times 10^3$) of 48.5, 45.4, 34.2, and 19.9, respectively. Similarly, both rhizobial isolates (SRC8 and SRL5) showed higher OD values compared with the other isolates.

A pot study was carried out to test the combined and separate effects of two different rhizobial strains (SRC8 and SRL5) to ameliorate water deficit stress in wheat. The highest water deficit stress tolerant rhizobial isolates (SRL5 and SRC8) showed various plant growth promoting characteristics (Table 2). Selected rhizobia were positive for P-solubilization, organic acid production, and siderophore production. Rhizobial isolates, SRL5 and SRC8, had the ability to produce IAA of about 1.86 ± 0.18 and 5.16 ± 0.11 mg L^{-1}, respectively. SRL5 showed less root colonization of wheat as compared to SRC8. Rhizobial isolate SRC8 (9.83 ± 0.55) showed more aggregation as compared to SRL5 (5.60 ± 0.33). Both rhizobial isolates were positive for oxidase as well as catalase activities.

The data in Table 3 reveal a significant increase in the growth and yield of wheat by the co-inoculation of strains (SRL5 and SRC8) at various water deficit stress levels as compared to the control (no inoculation). Co-inoculation (SRC8 + SRL5) performed the best at all water deficit levels as compared to sole inoculation. Co-inoculation enhanced the number of tillers per plant, straw yield, and grain yield by 166, 32, and 48%, respectively, at the highest water deficit condition (40% FC), while hundred-seed weight and number of spikes per plant increased by 30 and 67%, respectively, at 40% FC compared with the uninoculated control. The data regarding gaseous exchange parameters presented in Table 4 depict that the photosynthetic rate, transpiration rate, photosynthetic water use efficiency, stomatal conductance, and vapor pressure deficit were reduced at different FC levels and decreases were maximal at higher levels of water deficit stress (40% FC). But inoculation of rhizobial strains improved these gaseous exchange parameters over the uninoculated control, while the most appreciable improvement was denoted by combined inoculation of SRL5 and SRC8 as compared to their sole application as well as the uninoculated control at various levels of water deficit stress. Combined application of SRL5 and SRC8 improved the photosynthetic rate (106%), transpiration rate (52%), water use efficiency (36%), stomatal conductance (91%), and vapor pressure deficit (20%) at the 40% FC water deficit stress level. In the case of substomatal CO_2 concentration, the maximum reduction (20%) was noticed by combined application of rhizobial strains at the 40% water deficit stress level. Single inoculation of the rhizobial strain SRC8 was comparatively more effective versus the rhizobial strain SRL5. The data in Table 5 show that chlorophyll a, chlorophyll b, relative water contents, and crude proteins were decreased with increasing water deficit stress levels. However, rhizobial inoculation significantly enhanced these parameters in comparison to uninoculated control treatment. Single application of rhizobial strains showed good results; however, the best results were found with combined seed inoculation of strains SRL5 and SRC8 as compared to treatments where no inoculation was done. Co-inoculation improved the SPAD value, chlorophylls a and b, relative water contents, and crude proteins by 63, 168, 126, 32, and 55%, respectively, at 40% FC over the uninoculated control, followed by sole inoculation of SRC8 (46, 112, 95, and 24%, respectively, over uninoculated control at 40% FC). Nutrients concentrations in grains and straw of wheat were significantly decreased at various levels of water deficit (Table 6), such as 80, 60, and 40% FC, as compared to plants grown at normal or 100% FC levels. Increasing water deficit stress level significantly reduced the NPK contents of wheat plant; however,

Table 1 Osmoadaptation of rhizobial strains at four different PEG-6000 levels

Isolates	Optical density				Rhizobial population counts (CFU $mL^{-1} \times 10^3$)			
	0% PEG (− 0.05 MPa)	10% PEG (− 0.65 MPa)	20% PEG (− 1.57 MPa)	30% PEG (− 2.17 MPa)	0% PEG (− 0.05 MPa)	10% PEG (− 0.65 M Pa)	20% PEG (− 1.57 MPa)	30% PEG (− 2.17 MPa)
Lentil rhizobia								
SRL1	4.4 ± 0.15	3.7 ± 0.15	3.1 ± 0.09	2.6 ± 0.12	27.27 ± 0.35	22.86 ± 0.60	16.14 ± 0.27	8.9 ± 0.47
SRL2	5.6 ± 0.09	4.6 ± 0.09	4.1 ± 0.21	3.1 ± 0.20	30.49 ± 0.75	25.71 ± 0.55	18.31 ± 0.16	10.3 ± 0.09
SRL3	4.3 ± 0.12	3.7 ± 0.12	2.9 ± 0.03	2.5 ± 0.15	25.55 ± 0.70	20.24 ± 0.73	12.76 ± 0.35	6.6 ± 0.54
SRL4	4.5 ± 0.09	3.9 ± 0.19	3.4 ± 0.18	2.6 ± 0.18	28.18 ± 0.32	23.32 ± 0.20	16.29 ± 0.20	8.7 ± 0.43
SRL5	6.2 ± 0.03	5.2 ± 0.03	4.6 ± 0.10	3.3 ± 0.06	31.52 ± 0.45	27.83 ± 0.43	18.81 ± 0.55	11.7 ± 0.31
SRL6	3.2 ± 0.05	2.6 ± 0.05	2.0 ± 0.05	1.4 ± 0.02	21.40 ± 0.34	20.56 ± 0.39	13.71 ± 0.02	5.8 ± 0.26
SRL7	3.5 ± 0.12	2.7 ± 0.12	2.1 ± 0.23	1.6 ± 0.09	22.57 ± 0.34	17.89 ± 2.11	11.89 ± 2.02	5.6 ± 1.28
SRL8	3.6 ± 0.07	3.0 ± 0.07	2.3 ± 0.12	1.8 ± 0.03	23.11 ± 0.40	21.54 ± 0.24	13.86 ± 0.12	8.8 ± 0.27
SRL9	4.5 ± 0.06	4.2 ± 0.06	3.6 ± 0.15	2.7 ± 0.20	28.62 ± 0.34	21.98 ± 0.13	15.38 ± 0.31	8.7 ± 0.34
SRL10	5.0 ± 0.03	4.3 ± 0.03	3.8 ± 0.26	2.9 ± 0.19	28.95 ± 0.09	24.59 ± 0.25	17.29 ± 0.25	10.1 ± 0.26
Chickpea rhizobia								
SRC1	6.7 ± 0.15	5.5 ± 0.15	4.3 ± 0.15	3.7 ± 0.12	38.28 ± 0.53	36.1 ± 0.27	25.3 ± 0.20	11.9 ± 1.07
SRC2	7.1 ± 0.19	5.7 ± 0.19	5.0 ± 0.12	4.0 ± 0.10	41.35 ± 0.53	38.7 ± 0.25	28.0 ± 0.44	13.8 ± 0.37
SRC3	7.4 ± 0.18	6.2 ± 0.18	5.1 ± 0.03	4.3 ± 0.15	42.75 ± 0.36	40.4 ± 0.33	30.8 ± 0.45	15.4 ± 0.62
SRC4	8.1 ± 0.12	7.1 ± 0.12	6.4 ± 0.15	5.1 ± 0.12	43.62 ± 0.28	40.8 ± 0.67	30.6 ± 0.70	15.7 ± 0.76
SRC5	6.2 ± 0.15	5.1 ± 0.15	3.8 ± 0.32	3.2 ± 0.18	37.82 ± 0.51	34.8 ± 0.65	24.7 ± 0.66	10.1 ± 0.41
SRC6	6.5 ± 0.20	5.3 ± 0.20	3.9 ± 0.29	3.4 ± 0.07	38.78 ± 0.37	36.3 ± 0.48	26.7 ± 0.30	15.3 ± 2.42
SRC7	6.9 ± 0.09	5.6 ± 0.21	4.7 ± 0.12	3.8 ± 0.03	40.90 ± 0.29	37.9 ± 0.27	28.3 ± 0.29	13.4 ± 0.22
SRC8	8.9 ± 0.09	7.9 ± 0.09	7.0 ± 0.38	6.5 ± 0.12	48.50 ± 0.31	45.4 ± 0.30	34.2 ± 0.74	19.9 ± 0.48
SRC9	7.8 ± 0.15	7.0 ± 0.15	5.6 ± 0.18	5.4 ± 0.15	43.41 ± 0.54	40.6 ± 0.25	31.0 ± 0.24	15.6 ± 0.47
SRC10	8.5 ± 0.23	7.5 ± 0.23	6.4 ± 0.24	5.9 ± 0.19	44.52 ± 0.30	40.8 ± 0.19	30.8 ± 0.39	15.5 ± 0.63

Data are the averages of three replications ± standard error

rhizobial inoculation enhanced the NPK contents as compared to the uninoculated control. Rhizobial strains performed best when used as co-inoculation as compared to their single inoculation at all FC levels. At 40% FC, prominent increases in the NPK contents of grains by co-inoculation of SRC8 and SRL5 strains were recorded up to 44, 40, and 47% as compared

Table 2 Characterization and identification of selected rhizobial isolates

Characteristic	SRL5	SRC8
[b]Gram staining	–	–
[b]Oxidase activity	+	+
[b]Catalase activity	+	+
[b]Exopolysaccharide production	++	+++
[b]Organic acid production	+	+
[b]P-solubilization	++	+++
[b]Siderophores production	+	+
[b]IAA production (mg L^{-1})[a]	1.86 ± 0.18	5.16 ± 0.11
[b]Root colonization of wheat (10^5 CFU g^{-1})[a]	1.98 ± 0.21	2.73 ± 0.16
[b]Aggregation (%)[a]	5.60 ± 0.33	9.83 ± 0.55
[c]BIOLOG® similarity	*Rhizobium leguminosarum*	*Mesorhizobium ciceri*
[c]Similarity value (%)	93	96

[a] Data are the averages of three replications ± standard error

[b] Specific functional characteristics

[c] Taxonomic significant characters

Table 3 Effect of rhizobial strains on growth and yield parameters of wheat at various levels of water deficit

Treatments	Plant height (cm)	No. of spikes ($plant^{-1}$)	No. of tillers ($plant^{-1}$)	Straw yield ($g\ pot^{-1}$)	Grain yield ($g\ pot^{-1}$)	Hundred-grain weight (g)
100% FC						
No inoculation	81.16 ± 0.76^{c}	3.33 ± 0.58^{de}	4.33 ± 0.58^{bc}	20.50 ± 0.50^{c}	11.06 ± 0.40^{d}	3.12 ± 0.03^{c}
SRL5	83.00 ± 1.00^{c}	4.33 ± 0.58^{bc}	5.00 ± 1.00^{b}	20.96 ± 0.55^{bc}	11.90 ± 0.40^{bc}	3.17 ± 0.02^{c}
SRC8	86.33 ± 2.52^{b}	4.66 ± 0.58^{ab}	6.33 ± 0.58^{a}	21.70 ± 0.36^{b}	12.56 ± 0.45^{ab}	3.54 ± 0.02^{b}
SRL5 + SRC8	90.66 ± 1.53^{a}	5.33 ± 1.15^{a}	6.66 ± 1.53^{a}	22.73 ± 0.55^{a}	13.26 ± 0.40^{a}	3.78 ± 0.02^{a}
80% FC						
No inoculation	72.00 ± 2.00^{e}	2.33 ± 0.58^{fg}	3.00 ± 1.00^{de}	15.26 ± 0.60^{e}	9.56 ± 0.40^{ef}	2.89 ± 0.02^{d}
SRL5	78.33 ± 2.08^{d}	3.33 ± 0.58^{de}	3.66 ± 0.58^{cd}	15.56 ± 0.55^{e}	10.00 ± 0.40^{e}	3.15 ± 0.02^{c}
SRC8	82.33 ± 1.53^{c}	3.66 ± 0.58^{cd}	4.33 ± 0.58^{bc}	16.56 ± 0.40^{d}	11.00 ± 0.30^{d}	3.50 ± 0.03^{b}
SRL5 + SRC8	86.66 ± 2.08^{b}	4.66 ± 0.58^{ab}	5.00 ± 1.00^{b}	17.36 ± 0.55^{d}	11.23 ± 0.35^{cd}	3.77 ± 0.03^{a}
60% FC						
No inoculation	52.00 ± 1.00^{h}	1.33 ± 0.58^{h}	2.33 ± 0.58^{ef}	8.60 ± 0.40^{h}	6.40 ± 0.50^{h}	2.45 ± 0.15^{f}
SRL5	63.66 ± 1.53^{f}	2.33 ± 0.58^{fg}	2.66 ± 0.58^{de}	9.10 ± 0.70^{gh}	7.63 ± 0.45^{g}	2.91 ± 0.14^{d}
SRC8	69.33 ± 1.53^{e}	2.66 ± 0.58^{ef}	3.00 ± 1.00^{de}	9.90 ± 0.50^{g}	7.66 ± 0.40^{g}	3.12 ± 0.09^{c}
SRL5 + SRC8	76.66 ± 2.08^{d}	3.00 ± 1.00^{df}	4.33 ± 0.58^{bc}	10.80 ± 0.60^{f}	8.93 ± 0.31^{f}	3.43 ± 0.13^{b}
40% FC						
No inoculation	39.33 ± 1.15^{j}	1.00 ± 0.00^{h}	1.00 ± 0.00^{g}	3.76 ± 0.25^{k}	3.70 ± 0.46^{k}	2.12 ± 0.06^{g}
SRL5	48.33 ± 1.53^{i}	1.00 ± 0.00^{h}	1.33 ± 0.58^{fg}	4.03 ± 0.31^{jk}	4.40 ± 0.50^{j}	2.46 ± 0.10^{f}
SRC8	54.66 ± 1.53^{gh}	1.33 ± 0.58^{h}	2.00 ± 0.00^{efg}	4.70 ± 0.40^{ij}	4.60 ± 0.82^{i}	2.57 ± 0.08^{f}
SRL5 + SRC8	57.33 ± 1.53^{g}	1.66 ± 0.58^{gh}	2.66 ± 0.58^{de}	5.00 ± 0.20^{i}	5.50 ± 0.20^{i}	2.76 ± 0.03^{e}

Data are the averages of three replications ± standard deviations. Means sharing the same letter(s) in a column do not differ significantly according to Tukey's HSD test ($P < 0.05$)

to the uninoculated control, while increases in NPK by single inoculation of SRC8 were up to 27, 23, and 13%, respectively, and single inoculation of SRL5 increased the NPK contents up to 20, 10, and 12%, respectively, over uninoculated control treatment. Moreover, co-inoculation resulted in high nitrogen and phosphorous concentrations in wheat straw, with increments of 43.92 and 53.33%, respectively, at the maximum stress level of 40% FC. Increasing levels of water deficit increased the production of proline concentration but seed inoculation of strains SRL5 and SRC8 minimized the proline contents at all water deficit levels. Co-inoculation of rhizobial strains SRC8 and SRL5 decreased the proline contents compared to sole seed inoculation over the control treatment; however, the maximum reduction in proline concentration (21.56%) was found at the 40% FC level with their combined application, while sole inoculation of rhizobial strains decreased the proline contents by 20.57% (SRC8) and 7.09% (SRL5) over the uninoculated control.

Discussion

Certain microbes such as bacteria possess enormous mechanisms which help them to withstand harsh environments and enhance crop growth and development via direct and indirect relations with plants (Khan et al. 2017). Our study focused on the evaluation of rhizobia having multiple mechanisms to ameliorate water deficit stress on wheat. Twenty different strains of rhizobia were isolated from nodules of legumes collected from different regions of Punjab province, Pakistan. These strains were assayed for their osmoadaptation potential at different PEG-6000 levels. All strains showed variable tolerance at different water deficit (PEG) levels. Rhizobial strains SRC8 and SRL5 performed better regarding their survivability and were selected for further study. It was hypothesized that such enormous water deficit stress tolerance by these rhizobial isolates could be due to the production of organic solutes such as glycerol, proline, betaines, glutamine, sugars, and sugar alcohols, which maintained cell turgor and cell volume (Le Rudulier et al. 1984; Smith and Smith 1989). Moreover, the production of oxidases and catalases to avoid the disruption of nucleic acid and cellular membrane under stress (Goyal et al. 1986; Boumahdi et al. 1999), release, and accumulation of stress proteins, osmolytes, EPS, and antioxidants (Goyal et al. 1986; Vanderlinde et al. 2010) might have contributed to inducing water deficit stress tolerance in bacteria. Abolhasani et al. (2010) found that rhizobia can survive up to − 3.5 MPa under water deficit stress. Furthermore,

Table 4 Effect of rhizobial strains on different gas exchange parameters of wheat at various levels of water deficit

Treatments	A (μmol CO_2 m^{-2} s^{-1})	*E* (mmol m^{-2} s^{-1})	WUE	Ci (mmol m^{-2} s^{-1})	Gs (mmol m^{-2} s^{-1})	VPD (kPa)
100% FC						
No inoculation	11.48 ± 0.77^{c}	3.39 ± 0.13^{d}	3.39 ± 0.32^{bcd}	140.58 ± 9.17^{ij}	259.3 ± 7.13^{d}	2.57 ± 0.04^{cde}
SRL5	13.09 ± 0.20^{b}	3.63 ± 0.27^{bc}	3.61 ± 0.31^{abc}	130.93 ± 4.16^{j}	287.08 ± 5.40^{c}	2.65 ± 0.04^{ab}
SRC8	13.32 ± 0.60^{b}	3.66 ± 0.28^{c}	3.65 ± 0.32^{ab}	129.39 ± 1.42^{j}	321.56 ± 19.36^{b}	2.66 ± 0.04^{ab}
SRL5 + SRC8	17.14 ± 0.44^{a}	4.33 ± 0.06^{a}	3.86 ± 0.08^{a}	111.07 ± 4.42^{k}	352.31 ± 12.12^{a}	2.68 ± 0.02^{a}
80% FC						
No inoculation	09.20 ± 0.08^{fg}	3.08 ± 0.10^{ef}	2.98 ± 0.08^{efg}	158.86 ± 8.99^{g}	202.83 ± 7.05^{fg}	2.52 ± 0.01^{e}
SRL5	10.38 ± 0.62^{de}	3.25 ± 0.06^{de}	3.18 ± 0.14^{def}	155.63 ± 7.34^{gh}	238.85 ± 6.15^{e}	2.61 ± 0.02^{bcd}
SRC8	11.13 ± 0.91^{cd}	3.30 ± 0.04^{d}	3.36 ± 0.24^{bcd}	153.74 ± 7.75^{gh}	256.67 ± 6.97^{d}	2.63 ± 0.04^{abc}
SRL5 + SRC8	13.66 ± 0.41^{b}	3.85 ± 0.11^{b}	$3.55 \pm 0.13^{a–d}$	145.73 ± 11.80^{hi}	289.52 ± 9.25^{c}	2.66 ± 0.05^{ab}
60% FC						
No inoculation	06.52 ± 0.63^{j}	2.45 ± 0.05^{i}	2.66 ± 0.30^{gh}	222.15 ± 5.53^{de}	158.5 ± 4.55^{h}	2.21 ± 0.04^{h}
SRL5	08.35 ± 0.66^{gh}	2.90 ± 0.03^{fgh}	2.88 ± 0.25^{fg}	213.40 ± 7.45^{ef}	196.07 ± 12.22^{g}	2.37 ± 0.05^{f}
SRC8	09.75 ± 0.08^{ef}	2.99 ± 0.07^{fg}	3.26 ± 0.06^{cde}	207.36 ± 7.10^{f}	217.30 ± 6.88^{f}	2.39 ± 0.05^{f}
SRL5 + SRC8	10.70 ± 0.25^{cd}	3.20 ± 0.08^{de}	$3.34 \pm 0.09^{b–e}$	163.82 ± 7.04^{g}	247.95 ± 6.86^{de}	2.57 ± 0.04^{cde}
40% FC						
No inoculation	04.44 ± 0.08 k	1.86 ± 0.06^{j}	2.38 ± 0.03^{h}	345.07 ± 6.04^{a}	82.64 ± 5.15^{j}	2.12 ± 0.05^{h}
SRL5	06.89 ± 0.24^{ij}	2.51 ± 0.04^{i}	2.73 ± 0.08^{gh}	323.10 ± 6.91^{b}	125.45 ± 11.24^{i}	2.38 ± 0.04^{f}
SRC8	07.48 ± 0.91^{hi}	2.72 ± 0.1^{1h}	2.76 ± 0.44^{g}	304.14 ± 8.42^{c}	143.08 ± 13.62^{h}	2.42 ± 0.04^{f}
SRL5 + SRC8	09.19 ± 0.44^{cd}	2.84 ± 0.08^{gh}	3.23 ± 0.16^{df}	224.74 ± 6.51^{d}	157.81 ± 6.59^{h}	2.55 ± 0.07^{de}

Gas exchange parameters include photosynthetic rate (*A*), transpiration rate (*E*), photosynthetic water use efficiency (*WUE*), substomatal CO_2 concentration (*Ci*), stomatal conductance (*Gs*), and vapor pressure deficit (*VPD*)

Data are the averages of three replications ± standard deviations. Means sharing the same letter(s) in a column do not differ significantly according to Tukey's HSD test ($P < 0.05$)

Hussain et al. (2014) also demonstrated that rhizobia have survivability up to − 2.18 MPa (25% of PEG-6000), and increasing the PEG-6000 concentration decreased the number of rhizobial cells.

These water deficit stress tolerance rhizobia improved the growth, physiology, and yield contributing parameters of wheat under varying levels of field capacity. Rhizobial inoculation not only improved the wheat growth when used as a single inoculation but, also, co-inoculation of these rhizobia resulted in further improvement of growth and physiology of wheat. Photosynthesis and chlorophyll contents of wheat were improved with the combined application of rhizobia at all levels of field capacity, which might be due to the production of siderophores, which improved iron (part of chlorophyll) availability (Arora et al. 2001) and improved the uptake of macro- as well as micronutrients like Mo, Fe, and Mg (Yanni et al. 2001). Uptake of these nutrients might result in enhanced photosynthesis being an integral part (co-factor) of the photosynthetic enzyme. Rhizobia benefit non-legumes directly through the production of phytohormones, oligosaccharides, and lumichromes, which reduce water loss via transpiration and decrease stomatal conductance (Mehboob et al. 2009), hence, improved photosynthesis under water deficit stress might lead to improved plant growth. Our findings are in line with the results of Hussain et al. (2016), who found a significant increase in the photosynthesis of maize plant as a result of inoculation with *Rhizobium phaseoli* and *Mesorhizobium ciceri* under drought stress. In our experiment, under water deficit stress, co-inoculation of rhizobial isolates augmented the hundred-seed weight, NPK contents, crude proteins, and grain and straw yields. This might be due to the production of EPS in the rhizosphere, which aids nutrient supply by improving the physical and chemical properties of soil (Kaci et al. 2005; Hussain et al. 2014). Moreover, acquisition of soil mineral nutrients through the efficient modulation of the roots architecture aids directly in the uptake of NPK, Ca, Mg, Zn, Mo, and Na and resultantly increases the vegetative and reproductive biomass of cereals (Yanni et al. 2001). Rhizobia mineralize organic P and improve nutrient uptake in cereals through the modification of root morphology (Mehboob et al. 2009). These mechanisms of nutrients availability might remain very effective under water deficit stress by assisting the plant in coping with stress. Our results are in line with the experiment of Asghar et al. (2015), who observed a significant increase in the fresh and dry weights of roots and shoots of wheat plant via multi-strain inoculation of rhizobia under water deficit conditions. Similarly, Singh et al. (2006) noticed a significant increase in N and P concentration, dry weight, and height of rice plant through the inoculation of three different rhizobial strains. These possible mechanisms

Table 5 Effect of rhizobial strains on different physiological parameters of wheat at various levels of water deficit

Treatments	SPAD	Ch "a" (mg g^{-1} fresh weight)	Ch "b" (mg g^{-1} fresh weight)	RWC (%)	MSI (%)	Crude proteins (%)
100% FC						
No inoculation	45.82 ± 2.5^{cde}	2.54 ± 0.1^{c}	1.29 ± 0.04^{c}	73.43 ± 1.5^{de}	55.00 ± 2.0^{c}	13.15 ± 0.37^{de}
SRL5	48.23 ± 2.0^{bc}	2.77 ± 0.02^{b}	1.39 ± 0.01^{b}	77.93 ± 6.13^{c}	56.66 ± 2.08^{bc}	13.86 ± 0.29^{bc}
SRC8	50.21 ± 2.53^{b}	2.82 ± 0.06^{b}	1.41 ± 0.01^{b}	82.06 ± 1.48^{b}	58.00 ± 2.00^{ab}	14.08 ± 0.35^{b}
SRL5 + SRC8	55.62 ± 2.54^{bcd}	3.03 ± 0.10^{a}	1.54 ± 0.11^{a}	84.76 ± 1.76^{a}	60.00 ± 2.00^{a}	14.75 ± 0.30^{a}
80% FC						
No inoculation	40.83 ± 2.08^{fg}	2.11 ± 0.18^{f}	1.06 ± 0.05^{e}	64.76 ± 1.66^{f}	41.00 ± 2.00^{ef}	11.95 ± 0.27^{h}
SRL5	44.32 ± 1.28^{def}	2.31 ± 0.02^{de}	1.15 ± 0.02^{d}	71.33 ± 2.08^{e}	42.66 ± 1.53^{ef}	12.60 ± 0.29^{fg}
SRC8	48.65 ± 3.73^{bc}	2.35 ± 0.01^{de}	1.17 ± 0.01^{d}	74.33 ± 1.53^{d}	43.66 ± 1.53^{e}	12.84 ± 0.19^{ef}
SRL5 + SRC8	49.34 ± 2.62^{a}	2.87 ± 0.06^{b}	1.44 ± 0.03^{b}	78.00 ± 1.73^{c}	49.33 ± 1.53^{d}	13.46 ± 0.30^{cd}
60% FC						
No inoculation	34.04 ± 2.55^{h}	1.87 ± 0.02^{g}	0.91 ± 0.02^{g}	53.66 ± 1.53^{i}	28.33 ± 1.53^{h}	8.29 ± 0.31^{l}
SRL5	38.07 ± 2.30^{g}	2.13 ± 0.02^{f}	1.04 ± 0.02^{e}	61.33 ± 1.53^{g}	33.33 ± 1.53^{g}	10.23 ± 0.35^{j}
SRC8	43.58 ± 2.27^{ef}	2.23 ± 0.02^{ef}	1.09 ± 0.02^{e}	66.66 ± 1.53^{f}	35.00 ± 1.00^{g}	11.06 ± 0.32^{i}
SRL5 + SRC8	47.44 ± 2.93^{a}	2.43 ± 0.02^{cd}	1.19 ± 0.02^{d}	71.00 ± 2.00^{e}	40.00 ± 2.00^{f}	12.30 ± 0.40^{gh}
40% FC						
No inoculation	26.00 ± 2.00^{i}	0.73 ± 0.06^{j}	0.43 ± 0.02^{j}	39.66 ± 1.53^{k}	18.33 ± 1.15^{j}	5.97 ± 0.46^{n}
SRL5	33.73 ± 1.33^{h}	1.32 ± 0.21^{i}	0.73 ± 0.02^{i}	48.33 ± 1.53^{j}	21.66 ± 1.15^{i}	7.44 ± 0.32^{m}
SRC8	37.86 ± 2.45^{g}	1.56 ± 0.02^{h}	0.84 ± 0.03^{h}	52.33 ± 1.15^{i}	24.33 ± 1.53^{i}	8.14 l ± 0.30
SRL5 + SRC8	42.34 ± 2.13^{b}	1.97 ± 0.03^{g}	0.98 ± 0.02^{f}	58.33 ± 1.15^{h}	30.33 ± 1.53^{h}	9.22 ± 0.39^{k}

Data are the averages of three replications ± standard deviations. Means sharing the same letter(s) in a column do not differ significantly according to Tukey's HSD test ($P < 0.05$)

advocate the efficacy of rhizobia to enhance crop yield in stress environments. Plant height, number of tillers, and number of spikes per plant increased due to the synergistic influence of SRC8 and SRL5, which might be due to the production of phytohormones, such as auxins and gibberellins (Yanni et al. 2001), cytokinins (Noel et al. 1996), and/or phytoeffective metabolites, which might have improved nutrient uptake (Höflich et al. 1994). Our results are in line with the findings of Höflich (1999), who inoculated different cereals with *Rhizobium leguminosarum* R39 and observed a 19–33% increase in the shoot growth of these plants. Similarly, Shakir et al. (2012) found a significant increase in the shoot length and number of tillers per plant of wheat through the inoculation of ACC deaminase containing rhizobacteria under water deficit. In our study, inoculation improved the membrane stability index, relative water contents, and reduced the proline concentration of wheat plant at normal as well as severe levels of FC (40%), which may be due to the production of trehalose (Cytryn et al. 2007), lumichrome (Mehboob et al. 2009), and abscisic acid (Minamisawa et al. 1996) by bacteria, which help in sustaining cell turgidity and avoiding extra water loss from plant cells under water deficit conditions. The production of catalase (Hussain et al. 2014), antibiotics (Labuschagne et al. 2010), and phenolic acids (Mishra et al. 2006) might also play a role in improving membrane stability by avoiding plant cell injury under water deficit conditions, while improvement in the stress resilience of wheat in response to rhizobial inoculation might be due to the induction of systemic resistance (Hussain et al. 2014; Yanni et al. 2016) and nutrients and water availability to wheat in extreme environments (Vanderlinde et al. 2010). Moreover, the production of EPS and siderophores (Hossain and Mårtensson 2008) by rhizobia in the plant rhizosphere may also enhance the survivability and tolerance of plants under water deficit stress conditions. The study of Ortiz et al. (2015) revealed that inoculation enhanced relative water contents and reduced proline accumulation and electrolyte leakage as compared to uninoculated control plants. Moreover, our study revealed that combined/ consortium application was better than sole inoculation of rhizobium.

Conclusion

Our study revealed that Rhizobia can be used as plant growth promoting bacteria (PGPB) to induce systemic tolerance in wheat under limited water availability. Moreover, our study also advocated that co-inoculation of different rhizobia (isolated from different legumes) could be a better option than single strain inoculation for improving plant growth under limited availability of water.

Table 6 Effect of rhizobial strains on nutrients concentrations and proline contents of wheat at various water deficit levels

Treatments	N in grain (%)	N in straw (%)	P in grain (%)	P in straw (%)	K in grain (%)	Proline (μg g^{-1} DW)
100% FC						
No inoculation	2.03 ± 0.06^{b}	1.60 ± 0.27^{bc}	0.64 ± 0.03^{a}	0.26 ± 0.02^{c}	1.55 ± 0.03^{b}	1.64 ± 0.03^{ghi}
SRL5	2.20 ± 0.03^{a}	1.64 ± 0.19^{abc}	0.65 ± 0.02^{a}	0.28 ± 0.01^{bc}	1.62 ± 0.01^{a}	1.61 ± 0.01^{hi}
SRC8	2.24 ± 0.01^{a}	1.66 ± 0.34^{ab}	0.66 ± 0.02^{a}	0.29 ± 0.02^{b}	1.63 ± 0.02^{a}	1.59 ± 0.01^{hi}
SRL5 + SRC8	2.25 ± 0.02^{a}	1.69 ± 0.28^{a}	0.68 ± 0.02^{a}	0.32 ± 0.01^{a}	1.65 ± 0.02^{a}	1.57 ± 0.02i
80% FC						
No inoculation	1.52 ± 0.05^{g}	1.46 ± 0.25^{f}	0.47 ± 0.02^{c}	0.19 ± 0.02^{f}	1.33 ± 0.03^{f}	1.72 ± 0.02^{fgh}
SRL5	1.68 ± 0.03^{d}	1.50 ± 0.28^{ef}	0.48 ± 0.03^{c}	0.23 ± 0.01^{d}	1.46 ± 0.02^{d}	1.68 ± 0.01^{ghi}
SRC8	1.74 ± 0.02^{c}	1.53 ± 0.30^{de}	0.50 ± 0.02^{bc}	0.24 ± 0.01^{d}	1.49 ± 0.02^{cd}	1.65 ± 0.01^{ghi}
SRL5 + SRC8	1.79 ± 0.03^{c}	1.57 ± 0.29^{cd}	0.52 ± 0.02^{b}	0.26 ± 0.01^{c}	1.52 ± 0.02^{bc}	1.62 ± 0.01^{hi}
60% FC						
No inoculation	1.30 ± 0.03^{hi}	1.03 ± 0.03^{j}	0.33 ± 0.02^{e}	0.15 ± 0.01^{h}	1.19 ± 0.03^{g}	2.20 ± 0.20^{b}
SRL5	1.54 ± 0.04^{fg}	1.33 ± 0.02^{gh}	0.34 ± 0.01^{de}	0.18 ± 0.02^{f}	1.33 ± 0.02^{f}	1.90 ± 0.10^{c}
SRC8	1.59 ± 0.02^{ef}	1.34 ± 0.01^{gh}	0.35 ± 0.02^{de}	0.2 ± 0.02^{ef}	1.34 ± 0.02^{ef}	1.83 ± 0.01^{def}
SRL5 + SRC8	1.62 ± 0.02^{e}	1.37 ± 0.02^{g}	0.37 ± 0.01^{d}	0.22 ± 0.01^{de}	1.39 ± 0.02^{e}	1.78 ± 0.02^{fg}
40% FC						
No inoculation	1.08 ± 0.03^{k}	0.93 ± 0.03^{k}	0.18 ± 0.03^{h}	0.10 ± 0.01^{i}	1.07 ± 0.09^{h}	2.50 ± 0.22^{a}
SRL5	1.31 ± 0.02^{j}	1.23 ± 0.04^{i}	0.20 ± 0.02^{gh}	0.13 ± 0.01^{h}	1.21 ± 0.02^{g}	2.30 ± 0.12^{b}
SRC8	1.38 ± 0.03^{i}	1.29 ± 0.04^{hi}	0.22 ± 0.02^{fg}	0.14 ± 0.01^{gh}	1.22 ± 0.01^{g}	1.99 ± 0.01^{c}
SRL5 + SRC8	1.45 ± 0.03^{h}	1.34 ± 0.04^{gh}	0.25 ± 0.04^{f}	0.15 ± 0.01^{g}	1.39 ± 0.03^{f}	1.90 ± 0.01^{cde}

Data are the averages of three replications ± standard deviations. Means sharing the same letter(s) in a column do not differ significantly according to Tukey's HSD test ($P < 0.05$)

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