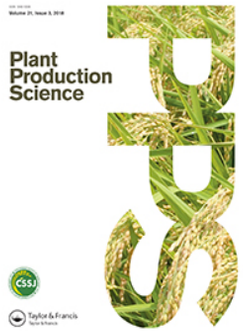

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Effect of carbonic anhydrase-containing endophytic bacteria on growth and physiological attributes of wheat under water-deficit conditions

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ABSTRACT

Drought is one of the major limitations to agricultural productivity, suppressing plant growth and yield of food crops throughout the world particularly in arid and semiarid regions. Drought-tolerant carbonic anhydrase (CA; EC 4.2.1.1)-containing endophytic bacteria may improve plant growth under stressed conditions. In the present study, effect of drought-tolerant CA-containing endophytic bacteria on growth and physiology of wheat under water-deficit conditions was studied. One hundred and fifty isolates were isolated from wheat plants and screened for their ability to tolerate polyethylene glycol (PEG) 6000-induced water-deficit stress (−0.31 to −3.20 MPa). Fifty isolates exhibiting intrinsic ability to tolerate stress were further screened for CA activity. Ten drought-tolerant isolates with higher CA activity were evaluated for improving wheat growth under water-deficit conditions (−0.04, −1.09, −1.23 MPa). Results showed that PEG-mediated water-deficit stress significantly reduced growth of wheat. However, inoculation with isolates WR2, WS11 and WL19 significantly enhanced seedling growth by improving maximum root length, shoot length, root and shoot dry weight under non-stressed as well as stressed conditions. These isolates were identified by 16S rRNA as *Bacillus marisflavi* (WR2) *Bacillus thuringiensis* (WS11) and *Bacillus subtilis* (WL19). Isolate WL19 also improved chlorophyll content, photosynthetic rate, CA activity and relative water content compared to uninoculated control plants. Overall, our findings suggest that endophytic bacterial isolates WR2, WS11 and WL19 with CA activity can enhance photosynthesis and biomass of wheat seedlings under water-deficit conditions.

Abbreviations: CA: Carbonic anhydrase; PEG: Polyethylene glycol; CO_2: Carbon dioxide; HCO^{3-}: Bicarbonate; TSA: Tryptic Soy Agar; LB: Luria Bertani; A: CO_2 assimilation rate; E: Transpiration rate; g_s: Stomatal conductance; C_i: Substomatal CO_2 concentration; RWC: Relative water content; EL: Electrolyte leakage

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Bacterial endophytes; carbonic anhydrase activity; drought; photosynthetic rate; wheat

Introduction

Climate change is a serious threat to productivity of food crops throughout the world due to rise in temperature and erratic change in rainfall pattern. The global temperature is predicted to increase drastically from 1.8 to 3.6°C by the year 2100 (Sharma, Bhattacharyyal, Rajkhowa, & Jha, 2014). Such climatic variables may project to increasingly hotter summer and severe drought stress in arid and semiarid regions. The drought area is predicted to increase 2-fold while water resources will decline up to 30% by the year 2050 (Falkenmark, 2013). Plants growing under water-deficit conditions face limited water supply and nutrient deficiencies. In agricultural regions, up to 50% or more yield loss can be experienced by drought (Wood, 2005). Drought impairs plant growth and development by reducing leaf size, stem thickness, root proliferation and disturbing plant water relations. It induces a range of changes in physiological and cellular functions of plants such as reduced carbon dioxide assimilation, membrane damage and disturbed enzymatic activity (Farooq, Wahid, Kobayashi, Fujita, & Basra, 2009). Reduced stomatal aperture and photosynthetic rate under severe water-deficit conditions thereby decrease crop yields (Anjum et al., 2011). Therefore, increasing productivity per drop of water is becoming important for many regions.

Several measures have been adopted to mitigate the drought-induced yield reduction and produce high and satisfactory yield under stress environments. Molecular breeding programs and transgenic approaches have been used to develop drought-tolerant plants but these have certain limitations (Cominelli, Sala, Calvi,

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Supplementary data for this article can be accessed here.

Gusmaroli, & Tonelli, 2008; Cominelli, Conti, Tonelli, & Galbiati, 2013). Plant growth-promoting rhizobacteria (PGPR) have also been reported to mitigate the adverse effects of drought (Vardharajula, Ali, Grover, Reddy, & Bandi, 2011). However, survival of PGPR may not be sustainable for longer run due to rhizospheric competition and various environmental injuries like drought, salinity, etc. Recently, inoculation of plants with endophytic bacteria to enhance the drought tolerance (Akbari, Akbari, & Golakiya, 2016; Khan et al., 2016) has gained interest among the scientific community. Compared to other bacteria which colonize the plant epiphytically, endophytes are better protected from the environmental stresses like temperature, osmotic potential and ultraviolet radiation. Endophytic bacteria colonize the internal plant tissues without causing any negative effect and sign of infection to the host plant (Schulz & Boyle, 2006). These bacteria are almost present in all plants. They reside inside the plant in intercellular, in vascular bundle and within the cell (Ulrich, Ulrich, & Ewald, 2008). They can promote plant growth and development by producing variety of products that could be beneficial to the host plant (Ryan, Germaine, Franks, Ryan, & Dowling, 2008). Among the traits of plant growth-promoting endophytes are: ability to fix atmospheric nitrogen, solubilize phosphorus and produce siderophores, certain organic acid and phytohormones like auxins, gibberellins and cytokinins. These plant growth-promoting bacteria (PGPB) also produce certain antioxidants that remove stress induced oxidants and prevent cell damage, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase that minimizes stress-induced ethylene level, thereby, improves root and shoot elongation during stress (Jha, Gupta, Jha, & Mehrotra, 2013).

Furthermore, these bacteria may possess carbonic anhydrase (CA) that have ability to capture the atmospheric carbon dioxide. CA is the primary enzyme for carbon dioxide concentrating mechanism, involved in photosynthesis where it facilitates the reversible conversion of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) at carboxylation site in chloroplast (Lazova, Naidenova, & Velinova, 2004). It regulates the diffusion of CO_2 for phosphoenol pyruvate carboxylase (PEPC) in C4 plants and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in C3 plants (Tiwari, Kumar, Singh, & Ansari, 2005). An uncatalyzed interconversion between CO_2 and HCO_3 is 10^4 times slower in comparison to CO_2 flux in photosynthesis (Badger & Price, 1994). Moreover, CA activity is required for CO_2-mediated regulation of stomata in guard cells and may provide protection to plant against stress conditions. Any change in plant CA activity directly influences CO_2 assimilation under CO_2-limiting environment. So, it would be interesting to enhance the photosynthetic CO_2 assimilation in C3 plants by artificial regulation of CA expression (Sun, Wu, Sun, Wu, & Wen, 2014).

Wheat is classified as C3 plant and its growth is badly affected due to drought stress. Moreover, competition of CO_2 and O_2 at the site of Rubisco causes loss of fixed carbon. Drought-tolerant endophytic bacteria containing CA may provide promising approach for plant protection against drought stress by enhancing photosynthetic rate. Therefore, present study has been conducted to investigate the impact of endophytic bacteria containing CA on growth and physiological attributes of wheat under water-deficit stress.

Materials and methods

Plant material and isolation of culturable endophytic bacteria

Healthy and disease-free wheat plants were randomly collected from five different locations at University of Agriculture, Faisalabad. For isolation of endophytic bacteria, each plant was separated into different parts i.e. root, shoot and leaves and washed thoroughly with tap water to remove adhering dust particles. Plant samples were surface sterilized by dipping in 70% ethanol for 30 s, 3% sodium hypochlorite (NaClO) for 3 min and washing with sterilized distilled water. After sterilization, samples were crushed and serially diluted in 0.85% sodium chloride (NaCl) solution. One hundred microliters from appropriate dilutions were plated in triplicate on two different media, viz Tryptic Soy Agar (TSA) and Luria Bertani (LB). Plates were placed in an incubator at 28°C for 3–7 days to recover bacterial endophytes. Colonies that appeared morphological different were selected, purified and preserved in glycerol stock at −80°C for long-term use. Sterility check was also performed to confirm the absence of any bacteria after sterilization by plating the last washing of plant samples on both media.

Drought tolerance ability

Total 150 isolates were tested for drought tolerance by using different levels of PEG-6000 in LB media. Isolates were grown in 100 mL conical flasks containing 50 mL LB media and left for 3 days in shaking incubator at 28°C and 100 rpm. Bacterial cells were harvested by centrifugating the culture at 4000 × *g* for 15 min and uniform cell density (10^7–10^8 CFU mL^{-1}) was maintained in LB media. Freshly prepared bacterial culture (0.5 mL) was inoculated into test tubes containing 7 mL

LB media with different osmotic potentials and left for 3 days at shaking incubator. Osmotic potential of −0.31, −0.61, −1.09, −1.91 and −3.20 MPa were developed by adding different level of polyethylene glycol (0, 10, 20, 30 and 40%) in LB broth media measured by Cryoscopic Osmometer (OSMOMAT-030-D, Gonotec, Germany). Osmotic potential was measured before and after autoclaving the LB media. Uninoculated control was also maintained at same osmotic potentials with three repeats. Drought tolerance ability was measured by spectrophotometer at 600 nm after 3 days.

CA activity

Fifty drought-tolerant isolates were further tested for CA activity following the method described by Achal and Pan (2011) and Zhang et al. (2011) with some modifications. Bacterial isolates were grown in LB broth and kept at 28°C for 3 days. Then, these isolates were inoculated in flask containing 100 mL CA-producing medium [yeast extract, 0.7 g; KNO_3, 0.3g; $(NH_4)_2SO_4$, 0.3 g; $MgSO_4$, 0.05 g; Na_2MoO_4, 0.05 g; $Na_2B_4O_7.10H_2O$, 0.1 g; Glycerol, 40 mL; 3.5-dintrosalicyclic acid, 0.02 g dissolved in 1 liter] and incubated at 32°C in mechanical shaker at 150 rpm for 4 days. Cell cultures of bacterial isolates were centrifuged at 8000 × *g* for 10 min and cell pallets were suspended in Tris-EDTA (pH 8.0) buffer containing 0.01 mg of RNaseI/mL, and kept at 37°C for 1 h. These bacterial lysates were centrifuged and supernatant was used as CA enzyme solution. The assay mixture consisted of 0.8 mL tris buffer (pH 7.5), 0.1 mL enzyme and 1 mL 4-Nitrophenyl acetate (p-NPA) dissolved in acetonitrile. The released p-nitrophenol was determined at 400 nm by spectrophotometer using *p*-nitrophenol as standard. Distilled water was used as a blank. One unit enzyme activity represented the amount of enzyme to produce 1 µmol p-nitrophenol per mL.

Plant growth promotion assay

Ten drought-tolerant isolates with higher CA activity were used for plant growth promotion assay. Seeds of wheat variety (Uqab-2000) were surface sterilized with 70% ethanol for 30 s and 3.5% NaClO for 5 min followed by four washings with sterile distilled water. Sterilized seeds were imbibed for overnight in sterilized distilled water to germinate. Four pre-germinated surface-sterilized seeds were dipped in inoculum for 10 min and placed in sterile growth pouch containing half-strength Hoagland solution. Inoculum was prepared in LB media and sterilized broth was used for control. The experiment was conducted with three replicates. Water-deficit stress was induced by dissolving various amounts of PEG-6000 into half strength Hoagland solution to develop −0.04, −1.09 and −1.23 MPa. After 5 days of germination, plants were irrigated with PEG-containing Hoagland solutions for inducing water-deficit stress. The growth chamber was maintained at 25 ± 1 °C and light and dark period was adjusted at 10 and 14 h, respectively. Light intensity of 275 µmol m^{-2} s^{-1} was maintained. Plants were harvested after 21 days and data regarding maximum root length, shoot length, root dry biomass, and shoot dry biomass were collected. Data regarding shoot and maximum root length were measured with the help of meter rod (Khan, Ahmad, Singh, & Nazar, 2006). Dry biomass was determined by drying the sample at 72°C for 72h.

Physiological parameters

Physiological parameters such as CO_2 assimilation rate (*A*), transpiration rate (*E*), stomatal conductance (g_s) and substomatal CO_2 concentration (C_i) were measured 20 days after sowing on fully expanded leaves of wheat seedling using CIRAS 3(PP system, Amesbury, MA, USA) between 10:00 a.m and 2:00 p.m. Intact leaves from each treatment were selected for photosynthetic measurements. The gas-exchange analysis was performed at 25 ± 1°C temperature and 50 ± 3% relative humidity. The reference level of CO_2 was 380 µmol mol^{-1} and light intensity (photosynthetic active radiation) was 250 µmol m^{-2} s^{-1}. Chlorophyll content was measured using chlorophyll meter (SPAD-502, Minolta, Japan).

Relative water content (RWC) and electrolyte leakage (EL)

For relative water content, fresh leaf samples were weighed and placed in distilled water for 24 h in refrigerator at 4°C. Then, turgid weight was measured. Dry weight of leaf samples was also noted after placing them in an oven for 24 h. The leaf relative water content was determined following equation as described by Teulat, Zoumarou-Wallis, Rotter, Bahri, and This (2003).

$$\%RWC = \frac{(\text{Fresh weight} - \text{Dry weight})}{(\text{Fully turgid weight} - \text{Dry weight})} \times 100$$

Leaf disc of plants from each treatment were transferred into test tubes containing 5 mL deionized water to determine electrolyte leakage. Test tubes were placed in shaking incubator (25 °C) for 4 h at 150 rpm

and electrical conductivity (EC) was recorded with EC meter (Jenway Conductivity Meter Model 4070). Then, same tubes were autoclaved at 121°C for 20 min and EC was determined. Electrolyte leakage was determined with following equation.

$$\%EL = \frac{EC\ before\ autoclaving}{EC\ after\ autoclaving} \times 100$$

CA activity in plant

The CA activity was determined following the method described by Dwivedi and Randhawa (1974). Leaf samples were cut, dipped in cystein hydrochloride solution and incubated for 20 min at 4°C. The leaf samples were blotted and transferred to test tube containing 0.2 M phosphate buffer (pH = 6.8) followed by 0.2 M alkaline bicarbonate ($NaHCO_3$) and 0.002% bromothymol indicator. These test tubes were incubated at 4°C for 20 min and titrated against HCl using methyl red as indicator. Results are expressed as mol (CO_2) kg^{-1} Fresh Mass (F.M.) s^{-1}.

Phenotypic and biochemical characterization of endophytic bacteria

Selected bacterial isolates were streaked on LB agar plates and placed overnight at 28°C. After 24 h, color and shape of colony was observed. Catalase activity was determined by spreading a loopful culture on glass slide. After spreading, a drop of 35% H_2O_2 was placed by following the Macfaddin (1980) method. Production of bubbles was considered as positive for catalase. Oxidase activity was performed by rubbing a bacterial culture on filter paper containing Kovacs reagent. Change in color from blue to purple was considered positive for oxidase (Steel, 1961).

Growth promoting traits of selected endophytic bacteria

Indole-3-acetic acid production was observed with and without L-tryptophan using Salkowski reagent and measured at 535 nm by spectrophotometer and indole acetic acid production was calculated by standard curve (Sarwar, Arshad, Martens, & Frankenberger, 1992). Ability of bacterial strains to solubilize phosphate was determined by spotting 10 µL of overnight culture of each strain on NBRI-PBP media (Mehta & Nautiyal, 2001).

For hydrocyanide production (HCN), isolates were grown in King B medium supplemented with 4.4 gL^{-1} glycine (Lorck, 1948). Hydrocyanic acid was observed with change in color of filter paper previously dipped in sodium carbonate solution prepared in picric acid from yellow to brown. Ability of bacterial isolates to produce chitinase was observed in LB media supplemented with chitin (Chernin et al., 1998). Loopful amount of inocula was placed on four different places in Petri plates and put in incubator at 28 ± 1°C. After 3 days of incubation, halo zone was observed around the colonies, where presence of halos was indicator for chitinase activity. Similarly, loopful of bacterial culture was placed at four places on Petri plates containing RCV-glucose media (Ashraf, Berge, & Mahmood, 2004). Plates were examined for mucoid growth after 96 h of incubation and colonies showing mucoid growth were considered positive for exopolysaccharides (EPS) production.

Identification of efficient isolates

Efficient endophytic bacterial isolates (WR2, WS11 and WL19) on the basis plant growth stimulation were selected for identification. These isolates were identified by 16S rRNA gene sequencing. Sequencing process of bacterial isolates was performed by Macrogen Inc. (Korea). The partial sequences of nucleotide were analyzed using Basic Local Alignment Search Tool (BLAST) program on National Center for Biotechnology Information site and identified on the basis of closest homology.

Statistical analysis

Data were analyzed according to completely randomized design (Steel, Torrie, & Dicky, 1997) whereas difference among the means was analyzed by Tukey test using Statistic 8.1 software.

Results

Endophytic bacterial isolates and their drought tolerance ability

A total of 150 endophytic bacterial isolates from different tissues of wheat plants were isolated using LB and TSA media. After isolation, these isolates were cultured in LB medium at different osmotic potentials to assess their survival ability. Out of 150 isolates, only 50 were able to grow at osmotic potentials ranging from −0.31 to −3.20 MPa. Among the 50 isolates, 10 isolates WR2, WS7, WS11, WS22, WS23, WL9, WL13, WL16, WL19 and WL20 which possessed higher CA activity are shown in Table 1 for their growth and survival ability. Out of 10 isolates, isolate WR2, WS11, WL13 and WL19 showed higher drought tolerance ability at any PEG

Table 1. Endophytic bacterial isolates and their ability to tolerate drought.

	Optical density at 600 nm				
Isolates	−0.31MPa	−0.61MPa	−1.09MPa	−1.91MPa	−3.20MPa
WR2	1.134 a	0.618 g	0.401 k-m	0.201 st	0.099 vw
WS7	0.920 e	0.462 j	0.293 op	0.186 s-u	0.062 w
WS11	1.046 b	0.628 g	0.387 l-m	0.231 q-s	0.100 vw
WS22	0.814 f	0.537 h	0.254 p-r	0.140 uv	0.071 w
WS23	0.943 de	0.436 j-l	0.323 no	0.201 st	0.087 w
WL9	0.845 f	0.439 jk	0.217 rs	0.161 tu	0.079 w
WL13	1.118 a	0.641 g	0.321 no	0.209 r-t	0.102 vw
WL16	1.036 bc	0.483 ij	0.276 o-q	0.197 st	0.089 w
WL19	0.989 cd	0.662 g	0.368 mn	0.230 q-s	0.100 vw
WL20	0.828 f	0.522 hi	0.253 p-r	0.140 uv	0.067 w

Means sharing similar letters do not differ significantly at $p \leq 0.05$ (Tukey's test). The data are average of three replicates.

concentrations compared to other isolates. However, their optical density decreased with increasing the PEG-induced osmotic stress.

CA activity

Fifty bacterial isolates showing tolerance to drought were further screened for CA activity (Supplemental data). Among the 50 bacterial isolates, 10 isolates which showed higher CA activity of are given in Figure 1. Maximum CA activity was observed by bacterial endophytes WL19, WS11 and WS23 and followed by WR2 and WL9, respectively. Results also revealed that isolates (WR2, WS11 and WL19) having drought tolerance ability showed better CA activity.

Effect of endophytic bacteria on plant growth

Ten drought-tolerant CA-containing endophytic bacterial isolates were tested for improving growth and physiology of wheat under water-deficit stress.

Table 2. Effect of drought-tolerant CA-containing endophytic bacterial isolates on maximum root length and shoot length of wheat under non-stressed and PEG-induced water-deficit conditions.

	Maximum root length (cm)			Shoot length (cm)		
Isolates	−0.04 MPa	−1.09 MPa	−1.23 MPa	−0.04 MPa	−1.09 MPa	−1.23 MPa
Control	17.7 no	14.2 r	12.3 s	24.8 g-j	20.2 l-m	15.5 n
WR2	26.2 a	21.9 d-f	19.5 h-m	32.7 *ab*	26.6 e-h	22.6 i-k
WS7	22.4 de	18.1 m-o	15.2 qr	31.9 a-c	24.9 g-j	20.7 k-m
WS11	26.4 a	22.5 d	18.6 k-n	32.9 a	27.5 e-g	21.8 j-m
WS22	20.8 e-h	18.4 k-o	15.6 p-r	26.1 e-h	23.3 h-k	19.3 lm
WS23	24.6 bc	20.4 f-i	18.7 j-n	28.5 d-f	26.8 e-g	20.1 k-m
WL9	24.3 c	19.8 h-l	16.9 op	32.3 a-c	26.5 e-h	19.8 lm
WL13	22.3 de	18.1 m-o	15.4 p-r	29.4 b-e	25.7 f-i	18.5 mn
WL16	21.8 d-g	19.9 h-k	18.3 l-o	29.1 c-f	24.5 g-j	20.2 k-m
WL19	26.1 *ab*	22.5 d	20.3 g-j	31.8 a-d	26.8 e-g	22.5 i-l
WL20	22.4 de	18.9 i-n	15.8 pq	27.7 e-g	24.9 g-j	19.4 lm

Means sharing similar letters do not differ significantly at $p \leq 0.05$ (Tukey's test). The data are average of three replicates.

Results showed that water deficit caused significant reduction in maximum root length but inoculation with drought-tolerant CA-containing endophytic bacteria enhanced maximum root length compared to uninoculated control. All the isolates showed significant increase in maximum root length under non-stressed as well as stressed conditions (Table 2). Isolates WR2, WS11 and WL19 performed significantly better compared to others and uninoculated control under non-stressed (−0.04 MPa) as well as stressed conditions (−1.09MPa). However, isolate WL19 showed significant increase in maximum root length compared to other isolates under severe water-deficit conditions (−1.23 MPa). Highest increase up to 65.0% in maximum root length was recorded by the inoculation of isolate WL19 under PEG-induced water-deficit stress (−1.23 MPa) compared to respective control. Inoculation with endophytic bacterial isolates significantly improved the

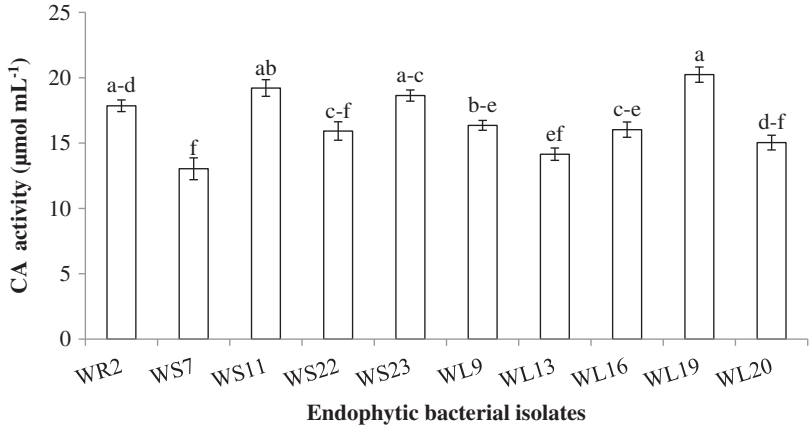

Figure 1. Drought-tolerant endophytic bacterial isolates from wheat with high carbonic anhydrase activity. Means sharing similar letters do not differ significantly at $p \leq 0.05$ (Tukey's test). The data are average of three replicates.

shoot length under non-stressed as well as stressed conditions (Table 2). Isolate WR2, WS7, WS11, WL9 and WL19 showed significant increase in shoot length compared to uninoculated control under non-stressed conditions (−0.04 MPa). Maximum increase up to 31.8% in shoot length was observed by the inoculation of isolate WR2 under non-stressed conditions (−0.04 MPa). Under PEG-mediated water-deficit stress (−1.09MPa), highest shoot length was observed with isolate WR2, WS11, WS23, WL9 and WL19 compared to other isolates. Isolate WR2 and WL19 significantly enhanced the shoot length compared to others at −1.23 MPa of PEG-induced water-deficit stress. Isolate WR2 showed 48.5% increase in shoot length at PEG-mediated water-deficit stress (−1.23 MPa). Water deficit also caused significant reduction in root and shoot dry weight but inoculation with endophytic bacteria improved their weight (Table 3). Under non-stressed conditions (−0.04 MPa), isolate WR2, WS23 and WL19 showed significant increase in root dry weight compared to other isolates and uninoculated control plants. Root dry weight was increased up to 36.0 and 45.5% by the isolate WR2 and WL19 under non-stressed conditions (−0.04 MPa). Maximum increase of 48.7% was recorded by the inoculation of isolate WL19 compared to uninoculated control under PEG-mediated water-deficit conditions (−1.09 MPa). Isolate WL19 caused 53.6% increase in root dry weight followed by WR2 and WS23 under severe stress (−1.23MPa) compared to uninoculated control. Shoot dry weight also decreased under PEG-induced water-deficit conditions but bacterial inoculation also improved the shoot dry weight (Table 3). Significant enhancement of 38.1, 47.0 and 48.8% in shoot dry weight was observed by isolate WL19 followed by WR2 and WS11 under non-stressed as well as water stressed conditions (−0.04, −1.09 and −1.23 MPa).

Effect of bacterial endophytes on physiological attributes

Chlorophyll content, CA activity, net photosynthetic assimilation, stomatal conductance and transpiration rate was also determined in wheat plant. Water-deficit stress significantly decreased the leaf chlorophyll content but inoculation with bacterial isolate WL19 showed 39.1 and 48.5% increase at −0.04 and −1.09 MPa, respectively (Table 3). However, under severe water-deficit conditions (−1.23 MPa), bacterial isolate WL9 showed 57.2% increase in chlorophyll compared to control plants followed by WL19 and WS11, respectively. CA activity was also improved in bacterial inoculated plants under non-stressed as well as stressed conditions.

Isolates WR2, WS11 and WL19 significantly enhanced CA activity compared to uninoculated control under non-stressed (−0.04 MPa) as well as stressed conditions (−1.09, −1.23 MPa) (Table 3). Inoculation with isolate

Table 3. Effect of drought-tolerant CA-containing endophytic bacterial isolates on root dry weight, shoot dry weight, chlorophyll content and carbonic anhydrase activity of wheat under non-stressed and PEG-induced water-deficit conditions.

	Root dry weight (mg plant^{-1})			Shoot dry weight (mg plant^{-1})		
Isolates	−0.04 MPa	−1.09 MPa	−1.23 MPa	−0.04 MPa	−1.09 MPa	−1.23 MPa
Control	17.7 f-i	13.4 l-o	9.50 q	25.7 f	16.8 n-q	12.9 s
WR2	24.1 *ab*	19.0 e-h	13.8 l-o	34.1 *ab*	24.0 f-h	18.5 l-o
WS7	20.9 c-e	16.7 h-k	13.3 l-o	30.0 de	22.0 h-j	15.0 q-s
WS11	23.1 bc	18.2 f-i	11.7 n-q	32.9 bc	23.6 f-h	18.2 m-p
WS22	19.5 d-g	19.6 d-g	10.6 pq	28.4 e	21.0 i-k	16.7 o-q
WS23	23.4 *ab*	17.0 h-j	13.9 l-n	30.8 cd	20.8 i-l	15.8 p-r
WL9	22.5 bc	16.5 i-k	12.7 m-p	31.5 cd	22.0 h-j	18.0 m-p
WL13	19.9 d-f	15.3 k-l	11.5 o-q	30.8 cd	19.4 k-m	13.7 q-s
WL16	21.8 b-d	17.3 g-j	12.6 m-p	32.4 bc	23.2 g-i	18.4 m-o
WL19	25.8 a	19.8 d-f	14.6 k-m	35.5 a	24.7 fg	19.2 k-n
WL20	23.1 bc	18.4 f-i	13.1 l-o	29.3 de	20.3 j-m	16.1o-r
	Chlorophyll Content (SPAD Value)			CA activity (mol CO_2 Kg^{-1} leaf F.M s^{-1})		
Control	31.9 ij	24.9 mn	18.5 o	1.027 d-g	0.760 j-m	0.501o
WR2	43.2 a-c	36.4 f-h	28.3 kl	1.496 a	1.127 b-e	0.799 i-l
WS7	39.3 d-f	33.7 hi	26.8 k-n	1.149 b-e	1.012 e-h	0.608 m-o
WS11	42.7 a-c	34.5 g-i	28.5 k	1.461 a	1.148 b-e	0.825 i-k
WS22	38.7 d-f	32.3 i	23.9 n	1.139 b-e	0.859 g-j	0.566 no
WS23	43.6 *ab*	33.2 i	27.4 k-m	1.251 b	1.171 b-e	0.761 j-m
WL9	37.7 ef	32.1 i	29.1 jk	1.273 b	0.956 f-i	0.637 l-o
WL13	38.8 d-f	32.9 i	25.5 l-n	1.183 b-d	0.903 f-j	0.680 k-n
WL16	40.3 c-e	34.0 hi	27.7 k-m	1.247 b	1.048 c-f	0.581 no
WL19	44.4 a	37.0 fg	28.8 k	1.549 a	1.204 bc	0.843 h-k
WL20	40.8 b-d	34.2 g-i	27.4 k-m	1.152 b-e	0.872 g-j	0.754 j-m

Means sharing similar letters do not differ significantly at $p \leq 0.05$ (Tukey's test). The data are average of three replicates.

WR2, WS11 and WL19 showed significant increase in CA activity up to 59.5, 64.6 and 68.2%, respectively, compared to uninoculated control under water-deficit conditions (−1.23 MPa). Water-deficit stress also decreased the net photosynthetic assimilation, stomatal conductance and transpiration efficiency but inoculation with bacterial endophytes improved these physiological parameters under mild as well as severe water-deficit conditions (−1.09, −1.23 MPa). Isolate WR2, WL19, WS11 and WS23 showed significant increase in photosynthetic rate compared to other isolates and uninoculated control (Table 4) under non-stressed conditions (−0.04MPa). At PEG-mediated water-deficit stress (−1.09, −1.23MPa), maximum increase in photosynthetic rate was observed by the inoculation of isolates WR2 and WL19. Isolate WL19 significantly improved the net photosynthetic assimilation by 39.4, 44.2 and 47.2% at −0.04, −1.09 and −1.23 MPa, respectively, compared to uninoculated control plants. Transpiration rate was markedly decreased under drought stress (Table 4). Inoculation with bacterial endophytes WR2, WS11 and WL19 significantly improved the transpiration rate compared to control under stressed conditions (−1.09 and −1.23 MPa). Isolate WR2, WS11, WS23 and WL19 improved transpiration rate by 40.5, 37.1, 41.9 and 44.5% compared to uninoculated control at −1.23 MPa, respectively. Inoculation with endophytic bacterial isolate WL19 increased the stomatal conductance up to 40.4% under non-stressed conditions (Table 4). However, at PEG-induced water-deficit stress (−1.09MPa) isolate WR2 showed significant increase up to 44.4% compared to others. Isolate WL19 improved stomatal conductance by 60.3% under PEG-induced water-deficit conditions of −1.23 MPa (Table 4). Substomatal CO_2 concentration was significantly decreased by the inoculation of bacterial isolate WR2, WS11 and WL19 under non-stressed and stressed conditions. Isolate WL19 showed 47.2% decrease in substomatal CO_2 concentration under water-deficit conditions (−1.23MPa) compared to respective control (Table 4). Results also showed the significant positive correlation between bacterial CA and photosynthesis under non-stressed as well as stressed conditions (Figure 2).

Relative water content, electrolyte leakage

Water-deficit conditions significantly decreased the RWC and increased the electrolyte leakage in non-inoculated plants (Table 5). Inoculation with drought-tolerant CA-containing bacterial endophytes considerably improved the RWC compared to respective control under non-stressed as well as stressed conditions. Isolate WL19 enhanced the RWC by 34.6, 37.0 and 43.0% at −0.04, −1.09 and −1.23 MPa of PEG-induced water-deficit conditions, respectively, when compared with non-inoculated plants. Limited water conditions also caused noticeable increase in electrolyte leakage

Table 4. Effect of drought-tolerant CA-containing endophytic bacterial isolates on photosynthetic rate, transpiration rate, stomatal conductance and substomatal CO_2 concentration of wheat under non-stressed and PEG-induced water-deficit conditions.

	Photosynthetic rate (μmol CO_2 m^{-2} s^{-1})			Transpiration rate (mmol H_2O m^{-2} s^{-1})		
Isolates	−0.04 MPa	−1.09 MPa	−1.23 MPa	−0.04 MPa	−1.09 MPa	−1.23 MPa
Control	7.6 e	5.2 i	3.6 l	2.13 hi	1.83 m	1.48 n
WR2	10.3 a	7.1 e-g	5.0 ij	2.72 a	2.52 bc	2.08 i-k
WS7	9.0 cd	6.1 h	3.7 kl	2.49 cd	2.45 c-e	1.95 k-m
WS11	10.0 *ab*	6.9 fg	4.8 ij	2.70 a	2.51 c	2.03 i-l
WS22	8.8 d	6.2 h	3.6 l	2.26 gh	1.96 k-m	1.57 n
WS23	10.0 *ab*	7.1 e-g	4.9 ij	2.66 *ab*	2.44 c-f	2.10 ij
WL9	9.5 bc	6.9 e-g	4.3 jk	2.45 c-e	2.36 d-g	2.04 i-k
WL13	9.2 cd	6.4 gh	4.0 kl	2.67 a	2.33 e-g	1.90 lm
WL16	9.5 bc	6.5 gh	4.1 kl	2.72 a	2.42 c-f	1.96 j-m
WL19	10.6 a	7.5 ef	5.3 i	2.73 a	2.52 bc	2.14 hi
WL20	8.8 d	6.0 h	4.0 kl	2.52 bc	2.30 fg	1.95 k-m
	Stomatal Conductance (mol H_2O m^{-2} s^{-1})			Substomatal CO_2 Concentration (μmol mol^{-1})		
Control	0.09 ef	0.05 j-n	0.03 o	235 cd	276 b	328 a
WR2	0.12 a	0.07 fg	0.05 j-n	174 k-m	183 h-m	189 g-l
WS7	0.09 de	0.06 g-k	0.03 o	180 j-m	204 e-i	214 d-f
WS11	0.11 a-c	0.07 gh	0.05 k-o	175 k-m	179 j-m	186 g-m
WS22	0.10 c-e	0.06 g-j	0.04 no	192 f-k	205 e-h	214 d-f
WS23	0.11 *ab*	0.07 gh	0.05 j-m	179 j-m	184 h-m	198 e-j
WL9	0.10 b-d	0.07 gh	0.04 m-o	199 e-j	220 de	242 cd
WL13	0.09 de	0.06 h-l	0.04 no	190 g-l	191 g-l	236 cd
WL16	0.10 b-d	0.07 g-i	0.05 l-o	181 i-m	188 g-l	207 eg
WL19	0.12 a	0.07 gh	0.05 i-m	165 m	169 lm	172 k-m
WL20	0.09 de	0.06 g-k	0.04 m-o	182 h-m	190 g-l	200 e-j

Means sharing similar letters do not differ significantly at $p \leq 0.05$ (Tukey's test). The data are average of three replicates.

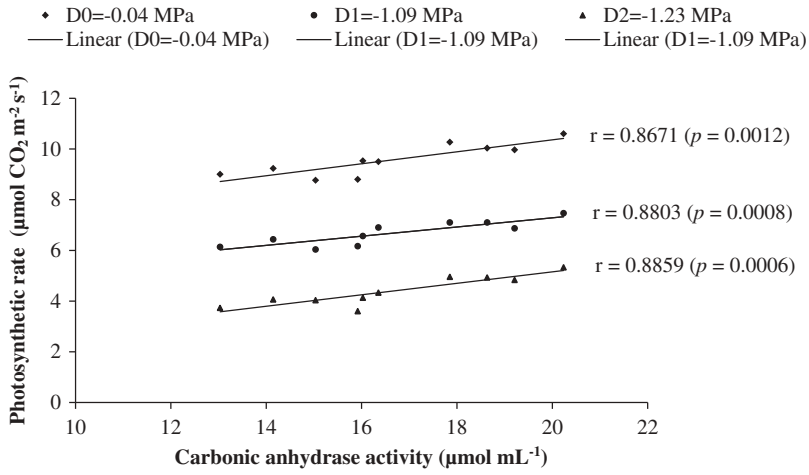

Figure 2. Relationship between photosynthetic rate and carbonic anhydrase activity exhibited by drought-tolerant endophytic bacterial isolates.

Table 5. Effect of drought-tolerant CA-containing endophytic bacterial isolates on relative water content and electrolyte leakage of wheat under non-stressed and PEG-induced water-deficit conditions.

	Relative water content (%)			Electrolyte leakage (%)		
Isolates	−0.04 MPa	−1.09 MPa	−1.23 MPa	−0.04 MPa	−1.09 MPa	−1.23 MPa
Control	65.2 i-l	54.2 o	44.8 p	9.79 f	26.13 cd	41.01 a
WR2	84.5 *ab*	72.1 f-h	62.0 k-n	8.43 f	19.75 e	26.90 cd
WS7	75.0 d-f	68.4 g-j	59.7 l-o	9.17 f	20.32 e	27.28 cd
WS11	85.2 *ab*	70.3 f-i	61.9 k-n	8.41 f	19.49 e	25.93 d
WS22	78.3 c-e	69.8 f-i	58.5 m-o	9.37 f	21.30 e	31.71 b
WS23	81.7 bc	70.7 f-i	61.9 k-n	8.78 f	19.70 e	26.94 cd
WL9	72.3 f-h	66.8 h-k	59.5 m-o	9.14 f	21.07 e	27.87 c
WL13	78.5 c-e	71.4 f-h	54.9 o	8.57 f	20.30 e	26.72 cd
WL16	79.9 b-d	67.5 h-k	59.4 m-o	8.62 f	19.97 e	27.33 cd
WL19	87.8 a	74.3 d-f	64.1 j-m	8.33 f	19.41 e	25.70 d
WL20	73.8 e-g	66.9 h-k	58.2 no	9.20 f	20.87 e	32.30 b

Means sharing similar letters do not differ significantly at $p \leq 0.05$ (Tukey's test). The data are average of three replicates.

in wheat plant (Table 5). However, inoculation with endophytic bacterial isolates caused significant reduction in electrolyte leakage under PEG-mediated water-deficit conditions (−1.09, −1.23MPa). Maximum electrolyte leakage was observed in control while minimum was observed in inoculated plants in severe water-deficit stress (−1.23 MPa). Isolate WL19 showed 37.3% inhibition in electrolyte leakage followed by WS11, WL13 and WR2 under severe water-deficit stress (−1.23MPa) compared to respective control plants.

Characterization and identification of efficient isolates

Plant growth-promoting characteristics of efficient endophytic bacterial isolates (WR2, WS11, WL19) are shown in Table 6. These isolates were positive for catalase activity. Isolate WL19 was positive while isolates

Table 6. Characterization and identification of efficient endophytic bacterial isolates.

Isolates	WR2	WS11	WL19
Colony color	Yellow	White	White
Colony shape	Round	Round	Round
Catalase	+	+	+
Oxidase	-	-	+
Phosphate solubilization	+	+	+
Auxin production µg mL^{-1}			
Without L-TRP	10.94 ± 0.58	13.62 ± 0.19	14.65 ± 1.78
With L-TRP	54.8 ± 2.17	35.0 ± 0.59	73.7 ± 1.68
HCN production	+	+	+
EPS production	+	+	+
Chitinase activity	+	+	+
16S rRNA Closest match in Genbank	*Bacillus* sp. (*Bacillus marisflavi* strain TF-11)	*Bacillus* sp. (*Bacillus thuringiensis* strain IAM 12,077)	*Bacillus* sp. (*Bacillus subtilis* strain JCM 1465)
Similarity index	96%	96%	97%

Means are given with standard error of three replicates (Tukey's test)

WR2 and WS11 were negative for oxidase test. Isolates WR2, WS11 and WL19 were able to solubilize the tricalcium phosphate. These isolates produced IAA from 10.94 to 73.7 µg mL^{-1} in the presence as well as absence of L-tryptophan and maximum IAA production was observed by bacterial isolate WL19. These isolates were also found positive for HCN production and chitinase activity. Moreover, EPS production was observed in all isolates. These isolates were identified by 16S rRNA as *Bacillus marisflavi* (WR2) *Bacillus thuringiensis* (WS11) and *Bacillus subtilis* (WL19).

Discussion

Plants under natural conditions are exposed to several biotic and abiotic stresses where water deficit is a major

constraint to plant growth and crop productivity affecting agricultural production. Endophytic bacteria have recently been reported for their potent role in improving the plant growth under stressed environment. However, potential of drought-tolerant endophytic bacteria to alleviate drought stress in plant is least explored. In the present study, potential of bacterial endophytes to adapt to drought stress were assessed by growing them at −0.31, −0.61, −1.09, −1.91 and −3.20 MPa osmotic potential with PEG-6000 induced drought stress. Among the 150 isolates, 50 isolates showed highest growth at different PEG concentrations. However, their growth decreased with increasing PEG concentration. These drought-tolerant endophytic bacteria also contained high CA activity. Out of 50, ten isolates which showed highest CA activities were selected (Figure 1). These isolates also had ability to tolerate highest level of drought stress (Table 1). These finding are in accordance to the observation of previous reports where PGPB showed growth at −0.73 MPa (Vardharajula et al., 2011) and *Shinorhizobium* sp. SK27 and SK36 at −3.5 MPa (induced with PEG) (Abolhasani, Lakzian, Tajabadipour, & Haghnia, 2010). Survival ability of bacteria under stress condition might be due to exopolysaccharides production that protects the microbes from water scarcity by improving water retention and modulating the diffusion of organic carbon (Chenu & Roberson, 1996). Results of present study showed that efficient isolates were found positive for EPS production (Table 6). Moreover, ability to produce oxidase, catalase and exopolysaccharide by bacteria has also been attributed to their survival under severe water-deficit condition (Goyal, Chetal, & Nainawatee, 1986). The results of current study also showed that isolates WR2, WS11 and WL19 were positive for catalase production while isolate WL19 was positive for oxidase test (Table 6).

Plant growth and development is reduced under drought condition due to disruption in biochemical and physiological processes. Microbial inoculants can help plants to sustain their growth under stress environment. In the present study, inoculation with endophytic bacteria improved plant growth by increasing maximum root and shoot length (Table 2), root and shoot dry weight (Table 3) under non-stressed as well as stressed conditions. Increase in root growth has been reported by inoculation of PGPB in many plants (Dawwam, Elbeltagy, Emara, Abbas, & Hassan, 2013; Delshadi, Ebrahimi, & Shirmohammadi, 2017). Synthesis of auxins by bacteria affects the root system through increasing the size and number of the adventitious roots (Gutierrez et al., 2012) resulting in improved water and nutrient uptake that may have positive influences on plant growth. In this study, endophytic bacterial isolate WL19 produced IAA of 73.7 µg mL^{-1} (Table 6) and showed maximum root growth compared to other isolates. Ability of bacterial isolates to produce IAA reveals their potential for use as growth promoter (Majeed, Abbasi, Hameed, Imran, & Rahim, 2015). Improved phosphorus nutrition has been proposed to influence root development and plant growth (Jones & Darrah, 1994). Our investigation showed isolate WR2, WS11 and WL19 have ability to solubilize tricalcium phosphate $Ca_3(PO_4)_2$ (Table 6). Of the 10 isolates evaluated in wheat, isolates WR2, WS11 and WL19 significantly enhanced shoot biomass compared to control plants. Increase in shoot weight might be due to CA activity of endophytic bacteria that supplied higher photoassimilate to plant by increasing the photosynthetic rate. There could be several possible reasons of increasing growth as has been found in other bacterial endophytes such as hormone production, enhanced phosphorus utilization, increased nitrogen fixation and iron acquisition (Hardoim, Overbeek, & Elsas, 2008). Researchers have also reported that plant biomass increased up to 78% in bacterially primed plant under drought stress (Timmusk et al., 2014).

In the present study, inoculation with endophytic bacterial isolates improved chlorophyll content, CA activity (Table 3), photosynthesis, stomatal conductance, substomatal CO_2 concentration and transpiration rate (Table 4) compared to un-inoculated control plants. These results are in line with Shi, Lou, and Li (2010) where endophyte-infected sugar beet showed significant increase in carbon assimilation, chlorophyll content, stomatal conductance and transpiration rate. Increase in photosynthetic activity was due to increase in cholorophyll content that consequently enhanced the carbohydrate synthesis, might be due to enhanced metabolism of chloroplast by endophytic infection. Another possible explanation can be bacterially produced CA. However, role of bacterial produced CA in plant photosynthesis is not understood. This enzyme is involved in several physiological processes such as photosynthesis and CO_2 transport (Lazova et al., 2004) and regulates the stomatal conductance by maintaining equilibrium between CO_2 and HCO_3 (Tiwari et al., 2005). Results also showed that photosynthetic rate was significantly correlated with CA activity in bacteria under non-stressed as well as stressed conditions. However, correlation was more positive under stressed conditions (Figure 2). It has been reported that CA activity was inhibited in ethoxyzolamide-treated C3 plant and caused 80–90% reduction in photosynthesis at low CO_2 concentration showing the key role of CA (Badger & Pfanz, 1995). In addition, Perez-Martin et al. (2014)

studied the role of CA enzyme in the stomatal and mesophyll conductance and found that CA enzyme had small but important role in stomatal conductance in 5-year-old olive plant under water stress. Furthermore, CA activity is required for CO_2 regulated stomatal opening and closing and can be alternative approach to provide protection against unfavorable conditions (Sun et al., 2014).

Relative water content decreased under stressed and non-stressed conditions but inoculation with bacterial endophytes improved the relative water content (Table 5). The relative water content is considered an indicator of plant water-deficit stress tolerance. Decrease in RWC has been observed in many plants under drought stress. Inoculated plants minimize the inhibitory effect of water stress on plant root and develop more effective root system (Dodd et al., 2010). In contrast to RWC, water-deficit stress also stimulated the electrolyte leakage in inoculated and uninoculated plants but inoculation with endophytic bacterial isolates helped to reduce the damage under water-deficit stress compared to uninoculated seedlings. Vardharajula et al. (2011) found that a direct correlation exists between membrane damage and drought sensitivity; bacterial inoculation decreased the membrane damage caused by water-deficit stress.

Moreover, increase in growth and physiological parameters by the application of drought-tolerant CA-containing endophytic bacteria was more with bacterial inoculation compared to uninoculated control under non-stressed and stressed conditions (Table 7).

Table 7. ANOVA shows the drought tolerance ability, maximum root length, shoot length, root and shoot dry weight, chlorophyll content, CA activity in plant, photosynthetic rate, transpiration rate, stomatal conductance, substomatal CO_2 concentration, relative water content and electrolyte leakage.

	Isolates	Levels	Isolates*Levels
Drought tolerance ability	212.56*	16955.1*	38.19*
Maximum root length	216*	1314.25*	5.33*
Shoot length	37.38*	702.70*	3.04*
Root dry weight	49.07*	1359.90*	6.62*
Shoot dry weight	83.20*	3197.87*	4.30*
Chlorophyll Content	101.37*	1774.41*	4.90*
CA activity in plant	59.66*	1019.63*	4.88*
Photosynthetic rate	93.90*	4718.02*	3.27*
Transpiration rate	215.07*	1781*	6.10*
Stomatal conductance	35.28*	1658*	2.09*
Substomatal CO_2 concentration	165*	159.56*	10.32*
Relative water content	86.63*	1060.85*	4.98*
Electrolyte leakage	128.26*	9537*	37.81*

Asterisks show significant difference at * 5% according to Tukey HSD comparison test.

Conclusion

This study provides an evidence that drought-tolerant endophytic bacteria having higher CA activity enhanced plant growth under water-deficit stress. Based on our results, we conclude that endophytic bacterial isolates WR2, WS11 and WL19 improved seedling growth, physiology and relative water content more efficiently compared to uninoculated control under non-stressed as well as stressed conditions. We have also demonstrated that these bacteria possess characteristics i.e. IAA production and phosphate solubilization which are considered important plant growth-promoting traits. Multi-site experiments are needed to further evaluate potential of CA-containing endophytic bacteria under natural/field conditions.

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