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ORIGINAL PAPER

Drought stress amelioration in wheat through inoculation with *Burkholderia phytofirmans* strain PsJN

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Abstract Plant growth promoting endophytic bacteria *Burkholderia phytofirmans* PsJN was used to investigate the potential to ameliorate the effects of drought stress on growth, physiology and yield of wheat (*Triticum aestivum* L.) under natural field conditions. Inoculated and uninoculated (control) seeds of wheat cultivar Sahar 2006 was sown in the field. The plants were exposed to drought stress at different stages of growth (tillering stage and flowering stage) by skipping the respective irrigation. The results showed that drought stress adversely affected the physiological, biochemical and growth parameters of wheat seedlings. It decreased the CO_2 assimilation, stomatal conductance, relative water content, transpiration rate and chlorophyll contents in wheat. Inoculation of wheat with PsJN significantly diluted the adverse effects of drought on relative water contents and CO_2 assimilation rate thus improving the photosynthetic rate, water use efficiency and chlorophyll content over the uninoculated control. Grain yield was also decreased when plants were exposed to drought stress at the tillering and flowering stage, but inoculation resulted in better grain yield (up to 21 and 18 % higher, respectively) than the respective uninoculated control. Similarly, inoculation improved the ionic balance, antioxidant levels, and also increased the nitrogen, phosphorus, potassium and protein concentration in the grains of wheat. The results suggested that *B. phytofirmans* strain PsJN could be effectively used to improve the growth, physiology and quality of wheat under drought conditions.

Keywords Physiology · Growth stages · *Burkholderia phytofirmans* PsJN · Drought stress · Wheat

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Introduction

Plants are constantly exposed to a wide range of environmental stresses which limit plant productivity. Sustaining agricultural production under adverse environmental conditions, such as drought and high salinity, represents a major challenge. Drought is expected to cause serious plant growth problems for more than 50 % of the arable lands by 2050 (Vinocur and Altman 2005). Moreover, with global climate change, i.e. rising temperature and altered soil moisture, there is potential for long-lasting droughts across the globe in the near future (Overpeck and Cole 2006). Beneficial soil microorganisms such as bacteria and/or arbuscular mycorrhizal fungi can adapt to specific environmental conditions and develop tolerance to stressful conditions. The role of these microorganisms in plant abiotic stress tolerance (such as drought stress) is known and has been studied in the context of providing a biological understanding of the adaptation of living organisms to extreme environments (Marulanda et al. 2009).

In order to maintain or increase crop productivity, it has become necessary to evolve efficient low-cost technologies for abiotic stress management. Several strategies have been suggested for controlling the negative effects of drought stress in plants where breeding for tolerant varieties and genetic engineering are the most explored approaches (Warren 1998) along with resource management practices

(Venkateswarulu and Shanker 2009). However, most of these techniques are time consuming and cost-intensive besides being not accepted well in some regions (Wahid et al. 2007). An alternative strategy is to induce stress tolerance by using beneficial microorganisms. Soil microorganisms with a potential for alleviation of abiotic stresses in combination with plant growth promotion would be extremely useful tools in sustainable agriculture.

Plant growth promoting rhizobacteria (PGPR) and/or endophytes that colonize the rhizosphere or plant interior enhance the plant growth by a variety of mechanisms. They can exert a beneficial effect on plant growth and nutrition probably due to fixation of atmospheric nitrogen (Compant et al. 2005; Watanabe et al. 1979), synthesis of phytohormones (Patten and Glick 2002), synergism with other bacteria-plant interactions (Zahir et al. 2011), inhibition of plant ethylene synthesis (Glick et al. 1998), as well as increasing availability of nutrients like phosphorus, iron and other micro-elements (Costa and Loper 1994; Rodriguez and Fraga 1999), and growth enhancement by volatile compounds (Kai et al. 2009). It is accepted that the role of PGPR in contributing to plant establishment, growth, and drought tolerance when growing under water-stress conditions is the result of the sum of nutritional, physiological, and cellular effects (Saravanakumar et al. 2011; Vardharajula et al. 2011; Kasim et al. 2013).

Burkholderia phytofirmans PsJN is one of the most studied bacterial endophyte so far and is able to establish rhizospheric and endophytic populations associated with a variety of genetically unrelated plants. Originally isolated from surface-sterilized *Glomus vesiculiferum*-infected onion roots (Frommel et al. 1991), strain PsJN has been shown to colonize a wide variety of plants [e.g. potato, tomato, peat moss and grapevines (Compant et al. 2008)] and it stimulates plant growth and vitality in many of its host plants under lab and greenhouse conditions. However, little is known about the inoculation response of PsJN to host plant under field conditions. Present study was conducted to evaluate the potential of *B. phytofirmans* strain PsJN for improving physiology, growth and yield of wheat under drought stress applied at different growth stages in the field conditions.

Materials and methods

Bacterial inoculum and plant bacterization

The bacterial inoculum was produced by transferring a loop of *B. phytofirmans* strain PsJN to 200 mL of LB liquid medium in a 500-mL Erlenmeyer flask incubated for 48 h at 28 °C and 150 rpm. The optical density of the broth was adjusted to 0.5 measured at λ 535 nm using spectrophotometer (Gene Quant Pro, Gemini BV, The Netherlands) to obtain a uniform population of bacteria (10^8–10^9 colony-forming units (CFU) mL^{-1}) in the broth at the time of inoculation. For inoculation, the obtained suspension of inoculum was mixed with sterilized peat (200 mL kg^{-1} peat) and incubated for 24 h at 28 ± 1 °C before being used for seed coating (seed to peat ratio 1.25:1 w/w). Wheat seed dressing was done with the inoculated peat mixed with 10 % sterilized sugar (sucrose) solution in 10:1 ratio. In the case of the noninoculated control, the seeds were coated with the sterilized peat treated with sterilized broth and 10 % sterilized sugar solution.

Wheat (*Triticum aestivum* L. cv. Saher) seeds were provided by the Wheat Research Institute, Ayub Agricultural Research Institute, Faisalabad, Pakistan. The *B. phytofirmans* strain PsJN was obtained from the Culture Collection Section/Bioresource Unit, AIT—Austrian Institute of Technology GmbH, 3430-Tulln, Austria.

Field experiment under drought stress

A field trial was conducted at the Experimental Farm, Institute of Soil and Environmental Sciences, University of Agriculture (UAF), Faisalabad to assess the efficacy of *B. phytofirmans* strain PsJN for improving growth and yield of wheat under drought stress during October to April 2011–2012. The soil samples from the field were collected for analysis of various physicochemical characteristics. The soil was sandy clay loam (Typic Haplocambid) having pH 7.8; electrical conductivity of a saturated soil extract (ECe), 2.11 dS m^{-1}; organic matter, 0.84 %; total nitrogen, 0.06 %; available phosphorus, 6.9 mg kg^{-1}; extractable potassium, 102 mg kg^{-1} and 36 % saturation percentage.

Seeds of wheat were inoculated (coated) with bacterial culture-injected peat based-slurry. Inoculated and non-inoculated seeds were sown in the well-prepared field at 120 kg ha^{-1} with a plot size of 10 m^2. The seed was sown with Rabi drill and treatments were arranged in randomized complete block design with two factor factorial settings and four repeats. Recommended doses of NPK fertilizers at 120–90–60 kg ha^{-1} were applied as urea, diammonium phosphate, and muriate of potash, respectively. Phosphorus and potassium were applied as a basal dose, while nitrogen was applied in splits (at tillering and booting stage). Weather conditions i.e. precipitation and temperature were recorded by "Meteorological department, UAF" during the crop growth period and described in Fig. 1. Field was irrigated with canal water and rainfall contributed only 24.2 mm from October 2011 to April 2012. There were five irrigations applied (normal irrigation; IN) during the crop growth period. The drought stress was applied by skipping irrigation at tillering (IST) and flowering (ISF) growth stages of the crop.

Data of plant physiology, water relations and antioxidant contents of flag leaves were recorded from normal

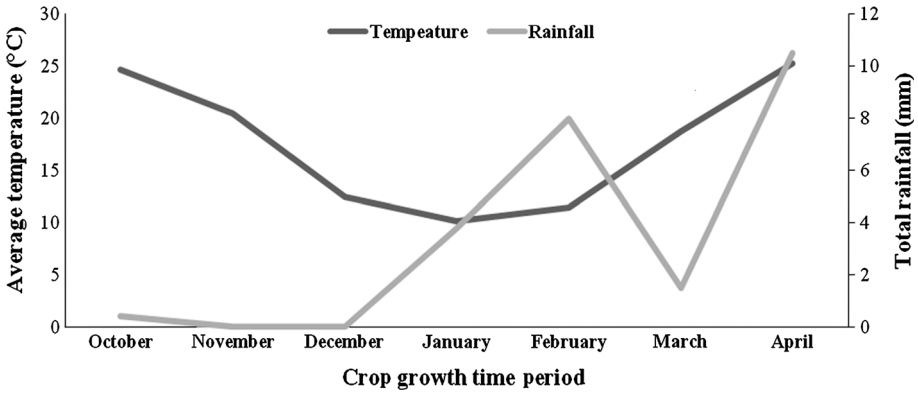

Fig. 1 Weather conditions data during the crop growth period obtained from "Meteorological department" UAF, Pakistan

irrigation (IN) and reduced irrigation (IST and ISF) plots. Growth and yield contributing parameters were recorded after maturity. Grain and straw samples were analyzed for nitrogen, phosphorus, and potassium content (Ryan et al. 2001).

Physiological and biochemical traits of plant

Physiological measurements

The plant physiological parameters were recorded at midday (between 10:00 and 14:00) of both irrigated and drought-stressed plots. Portable infra-red gas analyzer [IRGA (LCA-4) Germany] was used (at 1,200–1,400 μmol m^{-2} s^{-1} photosynthetic photon flux density) to measure transpiration rate (E), stomatal conductance (g_s), CO_2 assimilation rate (A) and sub-stomatal CO_2 concentration (C_i). Fully expanded flag leaves were selected for gaseous exchange measurements. Chlorophyll content was measured using SPAD-502 meter (Konica-Minolta, Japan). Readings were recorded with four repeats from each treatment. Water use efficiency (WUE) was derived by dividing photosynthetic rate (A) with transpiration rate (E).

Relative water contents and electrolyte leakage

Flag leaves were used for measuring the relative water content (RWC) and percentage of electrolyte leakage. After measuring the fresh weights, leaves were placed in distilled water for 24 h at 4 °C in darkness and the turgid weight was recorded. Dry weights were obtained after oven drying the leave samples at 72 °C for 24 h. Relative water contents were determined following the Eq. 1 described by Teulat et al. (2003). For electrolyte leakage, leaf discs were transferred in 5 mL de-ionized water and electrical conductivity (EC) (R1) was recorded with EC meter (Jenway Conductivity Meter Model 4070) after 4 h incubation at 28 ± 1 °C and 100 rpm in orbital shaking incubator (Firstek Scientific, Tokyo, Japan). The same samples were autoclaved at 121 °C for 20 min to determine EC (R2). Electrolyte leakage (EL) was measured following the protocol described by Jambunathan (2010) using Eq. 2.

$$\text{RWC} = \frac{(\text{Fresh weight} - \text{Dry weight})}{(\text{Fully turgid weight} - \text{Dry weight})} \quad (1)$$

$$\%\text{EL} = \frac{\text{EC before autoclaving (R1)}}{\text{EC after autoclaving (R2)}} \times 100 \quad (2)$$

Analysis of stress-related metabolites

Total soluble sugars were measured by anthrone reagent (0.2 %) following Sadasivam and Manickam (1992). A reaction mixture (10 mL) consisting of 200 μL leaf extract (1 g leaf homogenized in de-ionized water), 1,800 μL DI water and 8 mL anthrone reagent was heated for 10 min in boiling water and cooled in ice bath to stop the reaction. Absorbance was measured at 630 nm and total soluble sugar concentration (μg mL^{-1}) was calculated using glucose standard curve.

Proline content was measured following Bates et al. (1973). A reaction mixture consisting of leaf extract (1 g leaf homogenized with 3 % sulphosalicylic acid), ninhydrin acid and glacial acetic acid was heated at 100 °C for 1 h in water bath. The reaction was stopped by cooling in ice bath and absorbance was recorded at 520 nm after mixture extraction with toluene. Proline content (μg mL^{-1}) was calculated following standard curve of L-proline.

Bradford (1976) method was followed to measure protein contents in green leaves. A reaction mixture consisting of 200 μL leaf extract (1 g leaf homogenized in de-ionized water), 1,800 μL DI water and 2 mL of Bradford reagent was incubated at room temperature for 10–20 min and spectrophotometric absorbance was measured at 595 nm afterwards. Bovine serum albumin standard curve was used to calculate the protein concentration (μg mL^{-1}).

Enzymatic/non enzymatic antioxidant activity assays

The enzymes were extracted by homogenizing frozen fresh leaf material in ice-cold solution containing potassium phosphate buffer (0.2 M, pH 7) having 0.1 mM EDTA.

For glutathione reductase (GR) activity, increase in spectrophotometer absorbance at 412 nm was observed due to reduction of DTNB (5,5′-dithiobis (2-nitrobenzoic acid)) into TNB (2-nitro-5-thiobenzoic acid). Thirty microliters of enzyme extract [extracted in potassium phosphate buffer (50 mM, pH 7.8) with 2 mM EDTA] was resuspended in three milliliters reaction mixture containing 0.75 mM DTNB, 0.1 mM NADPH and 1 mM GSSG (oxidized glutathione). GR activity was calculated in µmol TNB min^{-1} g^{-1} leaf fresh weight at 25 ± 2 °C following Smith et al. (1988). Ascorbate peroxidase (APX) activity was determined by tracking the ascorbate reduction through H_2O_2 with decrease in spectrophotometer absorbance at 290 nm (Nakano and Asada 1981). Two milliliters reaction mixture consisting of 20 µL crude leaf extract, 660 µL ascorbic acid solution, 660 µL potassium phosphate buffer (pH 7.0, 50 mM) and 660 µL H_2O_2 was used to measure APX activity. Decrease in absorbance was monitored for 3 min just after the addition of H_2O_2. Enzyme activity was calculated in the form of µmol ascorbate min^{-1} g^{-1} leaf fresh weight. For catalase (CAT), 2 mL of 200 times diluted enzyme extract in potassium phosphate buffer (50 mM, pH 7.0) and 1 mL of 10 mM H_2O_2 was used following the method Cakmak and Marschner (1992). Decrease in absorbance at 240 nm due to H_2O_2 loss was observed for 3 min. CAT activity was calculated in µmol H_2O_2 min^{-1} g^{-1} fresh weight at 25 ± 2 °C.

Lipid peroxidation or malondialdehyde (MDA) concentration was determined following the method described by Jambunathan (2010). A reaction mixture (2.5 mL) consisting of leaf extract (0.5 mL, extracted in 0.1 % TCA), trichloroacetic acid (20 %) and thiobarbituric acid (0.5 %) was heated at 95 °C for 30 min in a fume hood and cooled in ice bath. Then absorbance was measured by spectrophotometer at 600 and 532 nm. The concentration of MDA was calculated using Beer and Lambert's law following the difference in absorbance (Abs at 532–Abs 600). The concentration was expressed as µmol g^{-1} fresh weight of leaf. For total phenolics, 2 mL reaction mixture consisting of 20 µL leaf extract, 300 µL Na_2CO_3 (1 N), 1,580 µL DI water and 100 µL Folin Ciocalteu's reagent (0.25 N) was incubated in the dark at room temperature for 2 h. Then absorbance was recorded at 760 nm and concentration in µg mL^{-1} was calculated following gallic acid standard curve (Singleton et al. 1999).

Mineral nutrients measurement

Grain and straw (dry) samples (0.1 g) were ground and digested with sulphuric acid and hydrogen peroxide (2:1 ratio) following Wolf (1982), and final volume made up to 50 mL with de-ionized water. Nitrogen content was determined with Kjeldhal method (Jackson 1962). For phosphorus, the extracted material (5 mL) was mixed in 10 ml of Barton reagent and total volume was made 50 mL. The samples were kept for half an hour and phosphorus contents were measured by spectrophotometer (Shimadzu, Japan) at λ 420 nm using standard curve. The Barton reagent was prepared as described by Ashraf et al. (1992). Potassium content was recorded by flame photometer using a standard curve (Ryan et al. 2001).

Enumeration of *B. phytofirmans* PsJN in the rhizosphere and root

The persistence of PsJN in the rhizosphere and root of wheat seedlings was monitored in a pot experiment. We used a genetic variant of the bacterial strain *B. phytofirmans* PsJN::*gusA* that carries a beta-glucuronidase reporter gene (*gusA*) that allows specific visualization of bacterial cells upon color formation (Compant et al. 2005). Wheat seeds were surface-sterilized with 70 % ethanol (3 min), treated with 2 % sodium hypochlorite (NaClO) (5 min), followed by repeated washing with sterile distilled water (3 times for 1 min). The PsJN inoculated seeds were sown in the pot having 1 kg soil (collected from the natural field). Four moisture levels were used i.e. normal water, 75 % FC, 50 % FC and 25 % of field capacity in the pots. The plants were harvested 25 days after sowing and rhizosphere/root colonization was recorded.

For the isolation of bacteria, 3 g rhizosphere soil and 1 g of surface-sterilized root material was homogenized in 5 mL of 0.85 % (w/v) NaCl solution. The material was shaken for 30 min at room temperature. After settlement of the material, serial dilutions up to 10^{-4} were plated onto selective LB medium containing spectinomycin (100 µg mL^{-1}), 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (XGlcA) (100 µg mL^{-1}), and isopropyl-β-D-galactopyranoside (100 µg mL^{-1}). The plates were incubated at 28 ± 1 °C for 48 h and then transferred to 4 °C for 3 days. Blue colonies were counted on each plate and survival efficiency was calculated.

Statistical analysis

Two way analysis of variance was used to analyze the data and Tukey's test was used to compare treatment means using Statistix 8.1 software (Copy right 2005, Analytical Software, USA). The means and standard errors were calculated using Microsoft Excel 2010.

Results

Growth physiology and agronomic yield

Skipping IST or ISF stage of wheat disturbed the growth and yield of the crop either inoculated or uninoculated

Table 1 Growth physiology and yield of wheat with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition

Drought stress	Uninoculated	PsJN inoculated	Uninoculated	PsJN inoculated
	Photosynthetic rate (A) (μmol CO_2 m^{-2} s^{-1})		Transpiration rate (E) (mmol H_2O m^{-2} s^{-1})	
IN	14.95 ± 0.41 a	15.62 ± 0.96 a	6.05 ± 0.89 bcd	7.29 ± 0.36 a
IST	9.48 ± 0.24 c	12.94 ± 0.46 b	5.40 ± 0.11 d	6.83 ± 0.12 bc
ISF	12.19 ± 1.29 b	13.16 ± 1.19 b	5.78 ± 0.09 cd	7.00 ± 0.25 ab
HSD	1.31		1.16	
	Water use efficiency (WUE = A/E)		Substomatal CO_2 content (Ci)(vpm)	
IN	2.47 ± 0.14 a	2.15 ± 0.13 ab	247 ± 4.38 bc	304 ± 5.29 a
IST	1.76 ± 0.05 b	1.90 ± 0.08 b	230 ± 5.33 c	267 ± 2.94 b
ISF	2.14 ± 0.04 ab	1.89 ± 0.10 b	227 ± 3.35 c	265 ± 4.91 b
HSD	0.46		24.91	
	Stomatal conductance (g_s) (mol H_2O m^{-2} s^{-1})		1,000 grain weight (g)	
IN	0.32 ± 0.06 ab	0.38 ± 0.02 a	39.22 ± 1.13 b	42.05 ± 1.02 a
IST	0.25 ± 0.01 c	0.38 ± 0.02 a	34.56 ± 0.46 c	39.68 ± 0.80 b
ISF	0.29 ± 0.02 bc	0.36 ± 0.01 a	35.37 ± 0.82 c	41.08 ± 0.59 ab
HSD	0.07		1.90	
	Straw yield (Mg ha^{-1})		Grain yield (Mg ha^{-1})	
IN	5.16 ± 1.05 b	6.16 ± 1.06 a	3.31 ± 1.02 b	3.91 ± 0.95 a
IST	4.20 ± 1.13 c	5.60 ± 1.09 ab	2.38 ± 0.82 c	2.87 ± 0.87 bc
ISF	4.98 ± 1.14 b	6.12 ± 1.02 a	2.43 ± 0.92 c	2.87 ± 0.65 bc
HSD	0.71		0.60	

Quantities sharing similar letters are statistically similar to each other at $p \leq 0.01$

Data are average of four replicates ± Standard error (SE)

IN irrigation normal, *IST* irrigation skipped at tillering, *ISF* irrigation skipped at flowering, *HSD* Tukey's honestly significant difference

(Table 1). However, improvement in the growth has been observed with dilution of stress impact on the crop due to *B. phytofirmans* PsJN inoculation. A significant improvement in CO_2 assimilation rate (A) was recorded with PsJN inoculation compared to respective control at IST growth stage whereas "A" was similar between respective inoculated and uninoculated plants under normal irrigations (IN) or ISF. Inoculation showed increment in the transpiration rate (E) of the crop during IN and skipped irrigation (IST or ISF) situations up to 21, 27 and 21 %, respectively, over their respective controls. When WUE was calculated from A and E of the plants, there was no significant difference between inoculation and control. Sub-stomatal CO_2 concentration (C_i) changed due to water deficit stress and PsJN inoculation, where 23, 16 and 18 % increase was recorded due to inoculation at IN, IST and ISF stages, respectively, in contrast to the uninoculated control. Inoculation of PsJN improved stomatal conductance (g_s) significantly about 52 and 24 %, respectively, during water deficit at IST and ISF stages with respect to the corresponding non-inoculated controls. Although, "g_s" was similar at unstressed and water deficit stressed treatments with the inoculation of *B. phytofirmans* PsJN. More chlorophyll content in wheat plants was observed due to PsJN inoculation but water deficit reduced it in both inoculated and uninoculated plants (Fig. 2). About 11, 3 and 14 %, increase in chlorophyll content was recorded with PsJN inoculation at IN, IST and ISF growth stages, respectively, compared to the respective uninoculated controls.

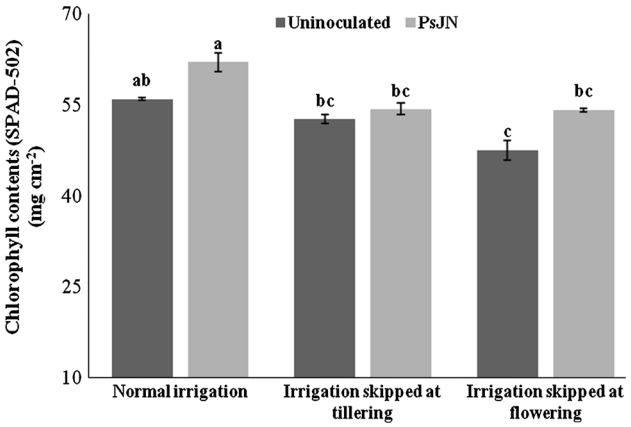

Fig. 2 Chlorophyll contents of wheat flag leaf with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition. Note: *Bars* sharing similar letters are statistically similar at $p \leq 0.01$

As for as agronomic yield of the wheat crop under reduced water system, inoculation of the plant growth promoting endophyte *B. phytofirmans* PsJN increased 1,000 grain weight, straw and grain yields of the crop (Table 1). Under normal irrigation, inoculation enhanced up to 7, 19 and 18 %, the 1,000 grain weight, straw and grain yields respectively, over control. Water deficit at IST reduced the grain yield approximately with 28 and 26 % in uninoculated and PsJN inoculated crops, respectively. On the other hand, with water limitation through ISF, PsJN inoculation caused 18, 16 and 23 % increase in grain yield, 1,000 grain weight and straw yield respectively, over respective control treatment.

Enzymatic and non-enzymatic antioxidant activity

Increase in activity of glutathione reductase (GR) was observed due to inoculation of endophyte PsJN (Table 2). Maximum increase (115 %) in GR was recorded with PsJN inoculation under no water deficit conditions compared to respective control whereas in conditions of drought at IST stage, inocculation resulted in 77 % more GR in the plants in contrast to the respective uninoculated control. Stress at the ISF stage was relieved due to inoculation of PsJN with a 58 % increase in GR activity with respect to the corresponding uninoculated control. Similarly, catalase activity was improved due to PsJN inoculation at normal as well as skipped irrigation systems compared to respective control treatments. However, maximum increase (about 78 %) in catalase activity due to inoculation compared to control was measured at ISF stage. Ascorbate peroxidase activity was 3.2-fold higher in PsJN inoculated plants compared to control when drought was applied at the tillering growth stage of the crop (IST). Lipid peroxidation increased under stress and inoculation further improved malodialdehyde contents up to 81 and 40 % at IST and ISF with respect to corresponding non-inoculated controls.

Accumulation of osmolytes (non-enzymatic anti-oxidants) in flag leaf changed due to the inoculation of *B. phytofirmans* PsJN and application of water deficit stress during tillering or flowering growth stage of wheat crop (Table 2). Although changes in total soluble sugars (TSS) remained statistically non-significant for drought or inoculation treatments, even so, drought increased TSS and inoculation decreased TSS compared to the respective non stressed or uninoculated controls. Protein concentration in

Table 2 Antioxidant activity (enzymatic and non-enzymatic) of wheat with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition

Drought stress	Uninoculated	PsJN inoculated	Uninoculated	PsJN inoculated
	Glutathione reductase (GR) (μmol TNB min^{-1} g^{-1} fw)		Catalase (CAT) (μmol H_2O_2 min^{-1} g^{-1} fw)	
IN	4.00 ± 0.23 e	8.61 ± 0.29 cd	190 ± 6.12 d	232 ± 8.42 d
IST	7.36 ± 0.52 d	13.07 ± 0.32 b	317 ± 7.43 c	482 ± 6.54 b
ISF	9.79 ± 0.85 c	15.48 ± 0.98 a	332 ± 5.98 c	590 ± 6.72 a
HSD	2.21		49	
	Ascorbate peroxidase (APX) (μmol ascorbate min^{-1} g^{-1} fw)		Malondialdehyde (MDA) (μmol MDA g^{-1} fw)	
IN	46 ± 0.36 e	63 ± 0.63 de	1.44 ± 0.05 d	3.03 ± 0.03 c
IST	95 ± 1.08 d	405 ± 1.22 a	3.66 ± 0.13 c	6.63 ± 0.22 a
ISF	154 ± 1.17 c	241 ± 0.98 b	4.55 ± 0.29 b	6.35 ± 0.32 a
HSD	42		0.71	
	Protein contents (μg g^{-1})		Proline contents (μg g^{-1})	
IN	1.40 ± 0.04 c	1.68 ± 0.06 a	0.50 ± 0.05 c	0.39 ± 0.03 d
IST	1.39 ± 0.05 c	1.61 ± 0.06 a	0.71 ± 0.08 a	0.42 ± 0.04 cd
ISF	1.48 ± 0.03 b	1.64 ± 0.05 a	0.61 ± 0.02 b	0.40 ± 0.04 d
HSD	0.08		0.09	
	Total phenolics (μg g^{-1})		Total soluble sugars (μg g^{-1})	
IN	90.77 ± 5.12 e	62.83 ± 5.62 f	3.41 ± 0.15 a	3.31 ± 0.13 a
IST	177.53 ± 7.61 a	151.75 ± 6.52 c	3.99 ± 0.24 a	3.58 ± 0.31 a
ISF	162.50 ± 5.28 b	109.57 ± 4.98 d	4.03 ± 0.54 a	3.45 ± 0.43 a
HSD	7.44		1.54	

Quantities sharing similar letters are statistically similar to each other at $p \leq 0.01$

Data are average of four replicates ± Standard error (SE)

IN irrigation normal, *IST* irrigation skipped at tillering, *ISF* irrigation skipped at flowering, *HSD* Tukey's honestly significant difference

leaves was enhanced due to endophyte inoculation and PsJN also eliminated the impact of drought on the protein contents. Almost 11 % more proteins were recorded in leaves of inoculated plants compared to respective control at ISF whereas 16 % increased protein content with inoculation was noted in IST treatment compared to respective control. Proline and total phenolic contents were highest in both uninoculated and inoculated plant leaves at IST in contrast to respective non-stressed plants. However, inoculation reduced the total phenolic and proline contents up to 15 and 41 %, respectively during this stage and about 33 and 34 %, respectively at ISF over respective uninoculated controls.

Mineral nutrition

Improvement in the mineral nutrition of wheat was recorded due to *B. phytofirmans* PsJN inoculation in normal as well as water limited conditions (Table 3). Both wheat straw and grain acquired high amounts of nitrogen, phosphorus and potassium due to water deficit and endophyte inoculation but the drought treatment results remained almost similar for inoculated and uninoculated plants with respect to their corresponding controls. However, maximum grain N, K and P contents of 1.70, 0.19 and 0.21 % respectively, were found in plants inoculated with PsJN and minimum values of 0.99, 0.14 and 0.14 %, respectively, in the grains of non-inoculated crop plants. Straw nitrogen content was high (0.93 %) in the inoculated plant subjected to ISF but maximum straw potassium (0.44 %) was observed in PsJN treated plant subjected to IST. Maximum phosphorus in wheat straw was collected from the crop inoculated with PsJN and treated with IST.

Water relations

Relative water contents RWC demonstrate the genetic capability of the crop plants to combat or abide under water limited conditions where inoculation can be helpful (Fig. 3). Under normal irrigated conditions inoculated *B. phytofirmans* PsJN improved the RWC about 9 % compared to un-stressed control. Water deficit treatments, IST and ISF, reduced the RWC of uninoculated control from 0.87 to 0.73 and 0.78 respectively, but inoculated plant leaves demonstrated no change with respect to inoculated and unstressed plants. An increase in electrolyte leakage (Fig. 4) was observed due to IST and ISF treatment of wheat but inoculation of *B. phytofirmans* PsJN rescued plant growth under stress and reduced the electrolyte leakage up to 5, 7 and 8 % after IN, IST and ISF treatment respectively, compared to the respective uninoculated controls.

Detection and enumeration of PsJN in the rhizosphere and root

The inoculant strain (PsJN) efficiently survived and colonized the rhizosphere and root interior of wheat seedlings

Table 3 Mineral nutrition of wheat straw and grain with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition

Drought stress	Uninoculated Grain nitrogen (%)	PsJN inoculated	Uninoculated Straw nitrogen (%)	PsJN inoculated
IN	1.29 ± 0.09 b	1.64 ± 0.08 a	0.26 ± 0.05 c	0.79 ± 0.06 b
IST	0.99 ± 0.14 c	1.56 ± 0.12 a	0.72 ± 0.02 b	0.80 ± 0.07 b
ISF	1.19 ± 0.08 b	1.70 ± 0.10 a	0.74 ± 0.02 b	0.93 ± 0.05 a
HSD	0.15		0.09	
	Grain potassium (%)		Straw potassium (%)	
IN	0.14 ± 0.01 c	0.20 ± 0.02 a	0.27 ± 0.01 d	0.37 ± 0.02 ab
IST	0.17 ± 0.01 bc	0.19 ± 0.01 ab	0.34 ± 0.01 bc	0.44 ± 0.03 a
ISF	0.15 ± 0.02 bc	0.18 ± 0.01 ab	0.29 ± 0.02 cd	0.41 ± 0.01 a
HSD	0.04		0.07	
	Grain phosphorus (%)		Straw phosphorus (%)	
IN	0.14 ± 0.01 b	0.21 ± 0.02 a	0.15 ± 0.01 c	0.20 ± 0.02 bc
IST	0.15 ± 0.01 ab	0.19 ± 0.01 ab	0.17 ± 0.03 c	0.26 ± 0.01 a
ISF	0.16 ± 0.02 ab	0.21 ± 0.02 a	0.17 ± 0.01 bc	0.21 ± 0.02 ab
HSD	0.07		0.05	

Quantities sharing similar letters are statistically similar to each other at $p \leq 0.01$

Data are average of four replicates ± Standard error (SE)

IN irrigation normal, *IST* irrigation skipped at tillering, *ISF* irrigation skipped at flowering, *HSD* Tukey's honestly significant difference

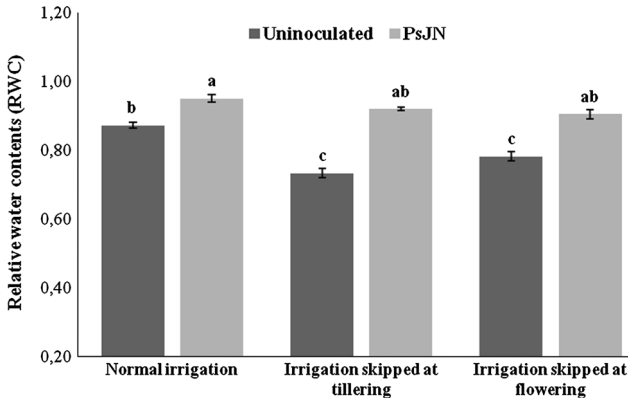

Fig. 3 Relative water contents of wheat flag leaf with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition. Note: *Bars* sharing similar letters are statistically similar at $p \leq 0.01$

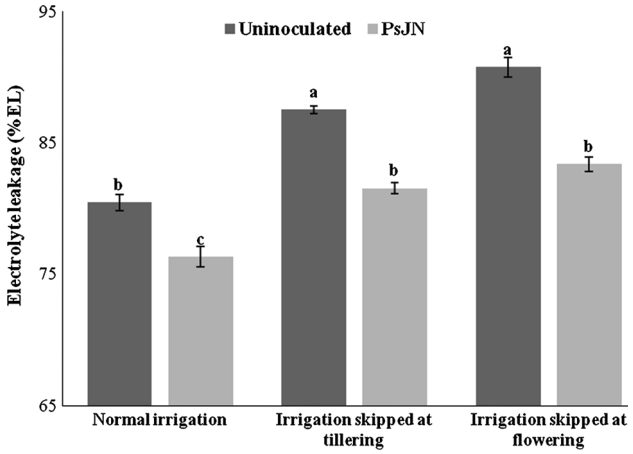

Fig. 4 Electrolyte leakages of wheat flag leaf with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition. Note: *Bars* sharing similar letters are statistically similar at $p \leq 0.01$

(Fig. 5). The persistence of PsJN in the rhizosphere and root differed non significantly at normal water and at 75 % of the field capacity. However, relatively less CFU (colony forming unit) of PsJN was recorded at 50 % of field capacity conditions compared to normal watering. The lowest CFU was recovered at 25 % of field capacity from the rhizosphere of wheat seedlings. Overall, as the moisture stress increased the inoculant strains preferred to colonize in the roots compared to soil.

Discussion

In the changing climate, plants are constantly exposed to abiotic stress, such as drought, which is one of the most serious problems associated with plant growth and development affecting agricultural demands. Inoculation with

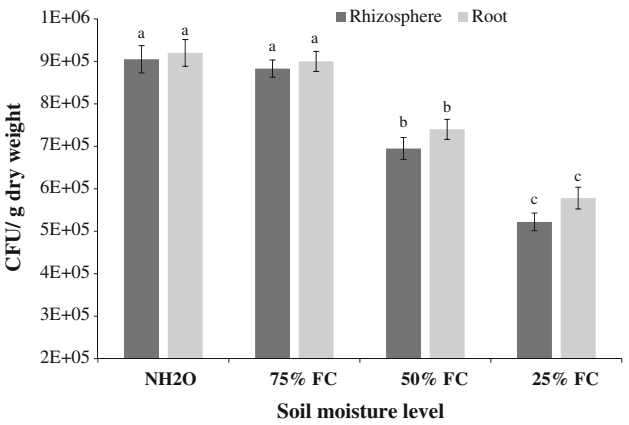

Fig. 5 Survival efficiency of PsJN in the rhizosphere and root interior of wheat seedlings (NH_2O, normal watering; FC, field capacity). Note: *Bars* sharing similar letters are statistically similar at $p \leq 0.01$

PGPR has been found effective under drought stress environment (Chanway and Holl 1994) to increase productivity. Beneficial plant–microbe (PGPR) interactions, impact of microbial inoculation on plant growth and differential mechanisms underlying growth promotion under stress conditions have been documented by various researchers (Saravanakumar et al. 2011; Kasim et al. 2013).

In the present investigation, the potential of the endophytic bacteria *B. phytofirmans* strain PsJN for improving physiology, antioxidant activity, growth and yield of wheat was evaluated under drought stress applied at different growth stages in field conditions. PsJN was originally isolated as a contaminant from *G. vesiculiferum*-infected onion roots (Frommel et al. 1991). It stimulates plant growth in many of its host plants. Metabolic activities suggested to be involved in these functions include phytohormone production, ACC deaminase activity and siderophore production (Sessitsch et al. 2005). Impact assessment of endophytic bacteria on plant growth promotion and underlying physiological and biochemical mechanisms is scarcely documented. Therefore, an understanding of the interactions between host plant and endophytic bacteria having influence on plant growth, physicochemical changes, yield and drought stress tolerance is required.

The results of present study revealed that in general, drought stress applied at any stage of growth (tillering and flowering stage) had strong negative effects on the growth of uninoculated wheat plants, but the magnitude of severity varied with the growth stage. Drought stress applied at the tillering stage had relatively stronger negative effects on shoot biomass and grain yield whereas drought stress applied at the flowering stages had a more negative effect on chlorophyll content. Similarly, changes in different plant physiological and biochemical processes were

observed due to drought stress that might have contributed to the growth and development processes of the wheat plants. This premise was supported by the fact that plants showed variable responses to water deficit faced in their various development periods (Mogensen et al. 1985; Gupta et al. 2001). Wheat, one of the most important crop species, is known to be susceptible to even mild or moderate drought particularly at the booting stage; however, unfavorable soil water conditions at the beginning of the plant growth may also dramatically limit the biomass production and the photosynthetic ability of leaves and thus indirectly negatively affect the formation of reproductive organs and yield parameters (Mogensen et al. 1985; Gupta et al. 2001; Kettlewell et al. 2010). In the present study, PsJN inoculations gave better response to wheat at tillering stage and resulted in significant increase in plant biomass, photosynthesis and grain yield compared to control as was evident from the data documented in Tables 1 and 2. This might be because of the suppression of stress-induced accelerated synthesis of ethylene by the ACC-deaminase activity of PsJN in the inoculated plants. Sharp increases in ACC levels and consequently, ethylene synthesis in plants under drought stress conditions have been frequently reported (Mayak et al. 2004; Zahir et al. 2008). Also, PsJN was previously reported as a potent root colonizers (also observed in our lab study, Fig. 5); it is highly probable that PsJN while living inside plant tissues evoked various physiological and metabolic processes to help the plants to sustain their growth under stress conditions (Vardharajula et al. 2011; Theocharis et al. 2012; Fernandez et al. 2012).

The beneficial influence of bacterial inoculations was also apparent in terms of improved physiological and biochemical changes. Under drought stress, the photosynthetic activity in term of CO_2 assimilation rate (A) was markedly reduced at IST stage in the uninoculated and PsJN treated plants without disruption of stomatal conductance (g_s) while in the latter case, the photosynthetic rate was effected only to a limited extent. The cessation of growth resulting from drought stress reduces the capacity for energy utilization which, in turn, probably results in feedback inhibition of photosynthetic rate (Wang et al. 2003). The PsJN inoculation increased photosynthetic parameters e.g. CO_2 assimilation, transpiration rate, and stomatal conductance under drought stress compared to control. Furthermore, bacterization affected chlorophyll content but not the water use efficiency (Fig. 2; Table 1). Effects on photosynthesis parameters have been described in the literature for other beneficial plant–microbe interactions (Sandhya et al. 2010; Vardharajula et al. 2011; Yandigeri et al. 2012).

Drought stress is accompanied by the formation of reactive oxygen species (ROS) such as O_2, H_2O_2, and OH (Mittler 2002), which damage membranes and macromolecules. Plants have developed several antioxidation strategies to scavenge these toxic compounds. Enhancement of antioxidant defense in plants can thus increase tolerance to different stress factors. Antioxidants (ROS scavengers) include enzymes such as catalase, superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR), as well as non-enzyme molecules such as ascorbate, glutathione, carotenoids, and anthocyanins. Additional compounds, such as carbohydrates, sugars and phenolics, can also function as ROS scavengers (Wang et al. 2003; Theocharis et al. 2012; Fernandez et al. 2012). In our study, PsJN inoculation showed higher antioxidant activity of plants compared to control under drought stress. However, phenolics contents decreased in the bacterized plants compared to control while sugar contents remained non-significant at both growth stages under normal and reduced irrigation. Very recently, Chakraborty et al. (2013) reported increased antioxidant levels in drought-stressed wheat plants inoculated with beneficial bacteria. Similarly, Fernandez et al. (2012) reported higher sugars content in PsJN treated grapevine plantlets under stress conditions.

Drought-stressed plants accumulate various molecules such as proline, glycine, betaine etc., thereby protecting enzyme activity (Saravanakumar et al. 2011). Proline, the best-characterized stress-responsive molecule, is often synthesized by plants in response to diverse abiotic or biotic stresses. Moreover, the accumulation of a compatible solute (e.g. proline) is an energy-consuming process in addition to the already existing metabolic costs. We found that the proline concentration in plant leaves increased with drought but that inoculation with PsJN under drought stress decreased the proline content. In the present study, the proline content was higher in the non-bacterized plantlets than in the PsJN-inoculated plantlets under stress exposure; in conflict with the results of Theocharis et al. (2012) who reported increased proline content in the inoculated grapevine plantlets under stress environment.

The relative water content (RWC) and lower electrolyte ion leakage (EL) in plants exposed to drought, has been considered indicative of a relative tolerance to water stress (Fisher 2000; Pereyra et al. 2006). In our study, RWC declined while %EL increased in both inoculated and uninoculated seedlings under drought stress compared to normal irrigation. However, bacterial inoculation did help plants to increase their RWC and to decrease their %EL as compared with uninoculated plants in drought stress. The relatively higher water content and low EL as evident from Figs. 3 and 4 in the inoculated seedlings compared to control under drought stress, indicate that endophyte inoculation gives tolerance to plants under reduced irrigation. This may be due to a reduction in the inhibitory effect of drought on roots and the development of a more

effective root system for water uptake in the inoculated plants (Dodd et al. 2004; Zahir et al. 2008). Similarly, a positive correlation between drought stress sensitivity and membrane damage (EL) were observed by Vardharajula et al. (2011) and Sandhya et al. (2010), and the bacterial inoculation reduced the membrane damage in drought stressed plants compared to control.

The bacterial inoculations were also effective in improving the nitrogen, phosphorus, potassium, and protein content of various plant components. Under stress conditions, nutrient (NPK) contents of plant tissues were increased in response to inoculation, most likely due to increased root growth that exploited more soil volume for efficient uptake of nutrients by the plants, resulting in more biomass production. Enhanced nutrient concentrations in plant tissues were reported by bacterial inoculation under stress conditions (Vivas et al. 2003; Nadeem et al. 2006).

Soil is a complex system and various biotic and abiotic factors may influence the behavior of particular strains in this environment. In our pot study, we used non-sterilized soil and observed that endophytic population was more suppressed and the viable cell number dropped more drastically in soil than in root at lower moisture levels while at higher moisture levels, viability of endophytic bacteria seemed hardly affected (Fig. 5). It is well known that various stress factors frequently impact the plant and thus alter the allocation of photosynthates in the rhizosphere that may lead to changes in below-ground microbial communities and their interaction with the plant (Compant et al. 2008). Strain PsJN was able to successfully compete with the natural microflora and successfully colonized the plant environment (Fig. 5) in addition to promoting plant growth. In our field investigation, the PsJN inoculation improved physiology and growth parameters of wheat under natural field conditions. It is likely that bacteria colonization (inside plant tissues) evoked various physiological processes to help the plants to sustain photosynthesis and plant growth under natural soil condition.

In conclusion, *B. phytofirmans* inoculation modulates biochemical and physiological parameters of wheat seedlings under drought stress conditions. Based on our results we conclude that application of PsJN is effective to improve physiology, relative water content and biomass of wheat under reduced irrigation. The improved plant physiological and antioxidant activity ultimately leads to enhanced crop yield and quality. Thus, inoculation with *B. phytofirmans* strain PsJN could be efficiently used to partially or completely eliminate the effects of drought stress on growth and yield of wheat.

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