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Plant Growth-Promoting Rhizobacteria Enhance Abiotic Stress Tolerance in *Solanum tuberosum* Through Inducing Changes in the Expression of ROS-Scavenging Enzymes and Improved Photosynthetic Performance

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Abstract In this report we address the changes in the expression of the genes involved in ROS scavenging and ethylene biosynthesis induced by the inoculation of plant growth-promoting rhizobacteria (PGPR) isolated from potato rhizosphere. The two *Bacillus* isolates used in this investigation had earlier demonstrated a striking influence on potato tuberization. These isolates showed enhanced 1-aminocyclopropane-1-carboxylic acid deaminase activity, phosphate solubilization, and siderophore production. Potato plants inoculated with these PGPR isolates were subjected to salt, drought, and heavy-metal stresses. The enhanced mRNA expression levels of the various ROS-scavenging enzymes and higher proline content in tubers induced by PGPR-treated plants contributed to increased plant tolerance to these abiotic stresses. Furthermore, the photosynthetic performance indices of PGPR-inoculated plants clearly exhibited a positive influence of these bacterial strains on the PSII photochemistry of the plants. Overall, these results suggest that the PGPR isolates used in this study are able to confer abiotic stress tolerance in potato plants.

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Introduction

Plant growth-promoting rhizobacteria (PGPR) are plant-associated microorganisms that are known to induce plant defenses and confer beneficial effects such as increased plant growth and low susceptibility to diseases caused by pathogens (Kloepper and others 2004a; van Loon and Glick 2004; Dimkpa and others 2009b). PGPR have been proven to counteract the activities of other harmful soilborne microorganisms, thus promoting plant growth (Glick 1995). Some PGPR also elicit physical or chemical changes related to plant defense, a process called "induced systemic resistance" (ISR) (van Loon and Glick 2004). Although it is well known that ISR triggered by PGPR confers resistance against pathogen-induced plant diseases, a few published reports suggest the role of PGPR as elicitors of abiotic stresses in plants (Yang and others 2009). Strains with plant growth-promoting activity have been identified from various genera of which *Pseudomonas* and *Bacillus* are the most extensively studied (Kumar and others 2011). It has been proposed that these bacteria produce phytohormones, antibiotics, and siderophores in the rhizosphere, thus inducing systemic resistance to plant pathogens (Gutierrez-Manero and others 2001; Whipps 2001; Idris and others 2007; Richardson and others 2009). The underlying mechanisms of plant growth promotion by PGPR have been comprehensively described in several articles (van Loon 2007; Kloepper and others 2004b; Yang and others 2009). PGPR belonging to *Bacillus* spp. are frequently isolated from the rhizosphere, and most have shown favorable effects on plant growth, higher yield, and

disease tolerance (Vessey 2003; Compant and others 2005; Wahyudi and others 2011). *Bacillus mucilaginosus* has been observed particularly for potassium- (Wu and others 2005) and phosphate-solubilizing abilities (Idriss and others 2002). Inoculation of *Azotobacter* and *Bacillus* increased the yield of wheat by 30 and 43 %, respectively (Kloepper and others 1991). Similarly, the inoculation of *Bacillus* with *Bradyrhizobium japonicum* was found to enhance the growth of soybean plants to increasing nodulation levels (Bai and others 2003). In another report, Woitke and others (2004) demonstrated the ability of *B. subtilis* to induce stress tolerance to salinity in hydroponically grown tomato plants.

Potato is the third most common food crop in the world, with a high yield per hectare; however, the potato has been relatively sensitive to yield losses resulting from soil salinity, drought, and low nutrient availability (van der Linden and others 2011). Although *Bacillus* strains have been previously isolated from the potato rhizosphere (Sessitsch and others 2003; Berg and others 2005; Calvo and others 2010), there have been no reports suggesting their role in abiotic stress tolerance in potato plants. Earlier, we had identified 13 PGPR isolates from the potato rhizosphere and demonstrated their influence on in vitro potato tuberization (Nookaraju and others 2011). Next, we selected two *Bacillus* isolates from among the 13 exerting a significant positive influence on in vitro potato tuberization to investigate their influence on abiotic stress tolerance in potato plants. The putative changes in the antioxidant pathway gene expression and photosynthetic efficiency, conferred by inoculation with PGPR, were also studied.

Materials and Methods

Plant Material and Growth Conditions

Potato cultivar 'Taedong Valley' was propagated in culture tubes (25 × 150 mm) containing semisolid basal MS medium (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose. The pH of the medium was set at 5.7 and gelled with 8 g l^{-1} agar (Duchefa, Germany). The cultures were maintained in a growth chamber at 22 ± 2 °C and a 16 h light period with a light intensity of 100 µmol m^{-2} s^{-1} using white fluorescent lamps. Four-week-old in vitro rooted shoots were planted in pots (10 cm wide and 10 cm deep) containing sterilized biopeat (Seminis Asia Ltd., Korea) and transferred to the greenhouse for hardening.

Screening of PGPR Isolates for Stress Tolerance

All the PGPR isolates (described by Nookaraju and others 2011) were screened for abiotic stress tolerance as described by Upadhyay and others (2011). The intrinsic resistance of these PGPR isolates was determined by monitoring their growth on nutrient agar medium supplemented with various concentrations of NaCl (50–400 mM). A control plate containing 0.05 % NaCl (w/v) was also maintained and incubated for 48 h at 28 °C. A sensitivity test against heavy-metal toxicity was performed using different concentrations of $ZnCl_2$ (10–50 mM). The effect of $ZnCl_2$ on bacterial growth was determined by spectrophotometry (560 nm) after 24 h of incubation at 28 °C in a nutrient broth. To determine the sensitivity of the PGPR isolates against polyethylene glycol (PEG6000), a tryptic soy broth (TSB) of varying PEG concentrations (0–50 %) was prepared and inoculated with 10 µl of overnight-grown bacterial cultures in TSB. The cultures were incubated at 28 °C for 24 h in a shaker at 180 rpm and growth was estimated by measuring the absorbance at 600 nm (Sandhya and others 2009).

Estimation of ACC Deaminase Activity and IAA Production by Bacterial Isolates

PGPR strains were assayed for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity by monitoring their growth on the DF medium (Dworkin and Foster 1958) supplemented with 3 mmol l^{-1} ACC as the only source of nitrogen (Penrose and Glick 2003). Plates containing DF medium without any nitrogen source were also kept, serving as the negative control to exclude the possibility of free nitrogen fixers. Plates were then inoculated with 10 µl of overnight-grown bacterial culture and incubated at 25 °C, and colonies were monitored daily for up to 3 days (Smyth and others 2011).

Bacterial auxin indole-3-acetic acid (IAA) production was tested using LB broth and the Salkowski colorimetric assay (Gutierrez and others 2009). PGPR isolates were grown in the LB broth supplemented with l-tryptophan (500 µg ml^{-1}) at 25 °C in darkness at 160 rpm for 5 days, and the supernatant was harvested by centrifugation on the sixth day. Then 1 ml supernatant was added to 1 ml Salkowski's reagent (12 g l^{-1} $FeCl_3$ in 429 ml l^{-1} H_2SO_4) (Glickman and Dessaux 1995). The mixture was kept for 30 min at room temperature and absorbance was measured at 535 nm. Auxin concentrations were estimated using the standard curve of IAA, prepared from serial dilutions of IAA (Sigma-Aldrich, St. Louis, MO, USA) stock solutions, with IAA concentration ranging from 0 to 2 mg l^{-1} and R^2 coefficient of 0.996.

Mineral Solubilization Activity of the Bacterial Strains

To determine the phosphate-solubilizing capabilities, the PGPR strains were analyzed, as described earlier (Miller

and others 2010), on Pikovskaya's agar (Sundara Rao and others 1963), whereas the phytate screening medium (PSM) agar (Kerovou and others 1998) was used to estimate the phytate-mineralizing capabilities. CAS (Chrome Azurol S) agar plates were prepared for the assessment of siderophore production capabilities, as described earlier (Schwyn and Neilands 1987).

Inoculation of Plants with PGPR Isolates and Stress Treatments

PGPR strains 4 (*Bacillus pumilus* str. DH-11) and 6 (*Bacillus firmus* str. 40) (Nookaraju and others 2011) were maintained at −80 °C in TSB amended with 20 % glycerol. The inocula were prepared by streaking the strains from −80 °C onto plate count agar (BD Biosciences, San Jose, CA, USA) plates, incubating the plates at 28 °C for 24 h, and scraping the bacterial cells off the plates in a sterile 10 mM $MgSO_4$ buffer. The sterile substrate was bacterized to achieve 10^8 CFU/g and PGPR were applied to the roots of potted plants growing under glasshouse conditions. A second inoculum dose was applied by a soil drench (10^8 CFU g^{-1} substrate) 1 week after the first inoculation. Control plants of all treatments were mock-inoculated with 10 mM $MgSO_4$ buffer and kept in parallel, as described by Barriuso and others (2008).

Two weeks after inoculation with PGPR strains, the plants were subjected to different abiotic stress treatments. Three plants were used for each category of stress treatment. For salt stress, the plants were watered with 200 mM NaCl solution; for drought stress, the plants were watered with 10 % PEG; and for heavy-metal challenge stress, the plants were watered with 20 mM $ZnCl_2$ solution for a week. Potato plants not inoculated with any of the PGPR isolates served as controls. Each treatment entailed a minimum of three replicates and the experiment was repeated three times. To examine the colonization of PGPR strains, plants were carefully uprooted and the soil adhering to the roots and tubers was collected for reisolation of PGPR strains (4 and 6) as described earlier (Nookaraju and others 2011). CFUs were determined on soil agar plates after an appropriate dilution in sterile distilled water and were counted after a 48 h incubation at 28 °C.

Determination of Hydrogen Peroxide Levels and Enzyme Activity

The ferrous ammonium sulfate/xylenol orange method, as described by Cheeseman (2006), was used to determine the H_2O_2 content of the leaf tissues from control and PGPR-inoculated potato plants. H_2O_2 concentrations were estimated using the standard curve prepared from serial dilutions of H_2O_2, with concentrations ranging from 0 to 6 µM and R^2 coefficient of 0.940. Data were normalized and expressed as micromolar H_2O_2/g fresh weight (FW) of leaves. For protein extraction, leaf samples were homogenized in the presence of liquid nitrogen. Protein was extracted with an extraction buffer containing 0.1 M potassium phosphate buffer (pH 7.5), 50 % (v/v) glycerol, 16 mM $MgSO_4$, 0.2 mM PMSF, and 0.2 % PVPP.

For the estimation of ascorbate peroxidase (APX) activity, the leaves were homogenized in 100 mM sodium phosphate buffer (pH 7.0) containing 5 mM ascorbate and 1 mM EDTA. The homogenate was filtered and centrifuged at 16,000×*g* for 20 min at 4 °C and the resulting supernatant was tested to determine the antioxidant enzyme activities. The protein content was determined based on the method of Lowry and others (1951) using bovine serum albumin (BSA) as the standard.

Catalase (CAT) activity was determined by monitoring the decomposition of H_2O_2 at 240 nm, as described by Aebi (1974). The reaction was initiated by adding 10 mM H_2O_2 to the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and plant extract, 3 ml in volume. One unit of CAT is defined as the amount of enzyme that liberates half the peroxide oxygen from 10 mM H_2O_2 solution in 100 s at 25 °C.

To determine the superoxide dismutase (SOD) activity, a method described by Beyer and Fridovich (1987) was followed. The reaction mixture (30.25 ml) contained 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM methionine, 57 mM nitroblue tetrazolium (NBT), and an appropriate volume of plant extract. The reaction was initiated by light illumination. One unit of SOD is defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical. APX activity was determined spectrophotometrically at 290 nm, as described by Chen and Asada (1989). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.2 mM H_2O_2, and a suitable volume of enzyme extract.

Glutathione reductase (GR) activity was determined by measuring the oxidation of NADPH at 340 nm (extinction coefficient of 6.2 mM cm^{-1}), as described by Rao and others (1989). The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM glutathione (oxidized form, GSSG) and the enzyme extract in 1 ml volume. The reaction was initiated by the addition of NADPH at 25 °C.

Dehydroascorbate reductase (DHAR) activity was determined as described by Nakano and Asada (1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 20 mM reduced glutathione, 2 mM dehydroascorbate, and 100 µl crude enzyme. DHAR activity was assayed at 25 °C by adhering to the absorbance of GSH-dependent production of AsA at 265 nm (extinction coefficient of 6.2 mM^{-1} cm^{-1}).

Expression Analysis of Stress-responsive Genes

Total RNA was isolated from the potato plant leaves using Tri reagent (Sigma) and treated with DNase I for reverse transcription reaction, and first-strand cDNA was synthesized using a random hexamer as the primer and SuperScript-II RT (Invitrogen, Carlsbad, CA, USA). The real-time PCR amplification of the ROS pathway genes (SOD, CAT, APX, DHAR, and GR) was carried out in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using gene-specific primers (Hemavathi and others 2011), as listed in Table 1. The PCR was performed using the SYBR® Green PCR kit (Bio-Rad) with actin as an internal control. The PCR conditions included 5-min initial denaturation at 95 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and an extension at 72 °C for 2 min. Comparative threshold (Ct) values were normalized to actin control and compared to obtain relative expression levels.

Estimation of Proline Content

Proline was estimated (Bates and others 1973) from the PGPR-inoculated and noninoculated control potato plants subjected to various abiotic stress treatments. Approximately 500 mg of tuber tissue was crushed to a fine powder in the presence of liquid nitrogen and homogenized in 10 ml of 3 % aqueous sulfosalicylic acid. The homogenate was then filtered and 2 ml of the filtrate was mixed with 2 ml acid-ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100 °C. The reaction was terminated by placing the test tubes on ice followed by proline extraction with 4 ml toluene. The chromophore-containing phase was collected and kept at room temperature for 15 min before measuring the absorbance at 520 nm. The proline concentration was determined from a standard curve and calculated on a fresh weight basis (μmol proline g^{-1} FW).

Estimation of Photosynthetic Performance

Chlorophyll fluorescence transients of dark-adapted intact leaves of the potato plants were measured using a Pocket PEA (Plant Efficiency Analyzer, Hansatech Ltd., Norfolk, UK). The 2 h dark adaptation was done with specially provided clips that fit onto the leaves. Data were recorded up to 1 s, at a rate of 10 μs for the first 2 ms and at the rate of 1 ms thereafter (Strasser and others 2000). The transients were induced by a red (650 nm) diode with 3,000 μE s^{-1} m^{-2}. The maximum fluorescence (F_m) and the minimum fluorescence (F_0) of dark-adapted tissues were used to calculate the F_v/F_m ratio. The parameter F_v/F_m $[(F_m-F_0)/F_m]$ reflects the maximum quantum yield of photosystem II (PSII) or the potential quantum efficiency if all the PSII centers were open (Maxwell and Johnson 2000). Thus, the measurement of F_v/F_m provides a measure of the intactness of the PSII/LHC (light harvesting complex) unit and indicates the probability of a trapped photon within the reaction center to cause a photochemical event. Any change in the state of PSII causes a decrease in the value of F_v/F_m (Maxwell and Johnson 2000). The optimal value of F_v/F_m varies between 0.79 and 0.83 for most plant species (Björkman and Demmig 1987; Johnson and others 1993), and lower values indicate that the plant is either under stress or lacking an optimal health state. The measured values of leaf samples were also used to calculate the performance index (PI_{total} or PI) through a series of JIP equations (Strasser and others 1999, 2000; Yusuf and others 2010; Gururani and others 2012) in the Biolyzer software program. The PI is the sum of overall expressions that represent a kind of internal force of the sample to resist

Table 1 Primer sets used for real-time PCR in the study

NCBI accession no.	Primer name	Sequence (5′–3′)
X55749	Actin	F: CTGGTGGTGCAACAACCTTA
		R: GAATGGAAGCAGCTGGAATC
AB041343	APX	F: ACCAATTGGCTGGTGTTGTT
		R: TCACAAACACGTCCCTCAAA
AY442179	Cat	F: TGCCCTTCTATTGTGGTTCC
		R: GATGAGCACACTTTGGAGGA
AF354748	SOD	F: GTTTGTGGCACCATCCTCTT
		R: GTGGTCCTGTTGACATGCAG
X76533	GR	F: GGATCCTCATACGGTGGATG
		R: TTAGGCTTCGTTGGCAAATC
DQ512964	DHAR	F: AGGTGAACCCAGAAGGGAAA
		R: TATTTTCGAGCCCACAGAGG

constraints from outside thereby indicating the vitality of the samples used.

Statistical Analysis

All treatments contained at least three replicates and the experiments were repeated three times. The means were analyzed using the Statistical Analysis Software package program 9.1 (SAS Institute, Cary, NC, USA). Statistical differences were determined using one-way analysis of variance, and standard error was calculated by using the *n* values of each experiment.

Results

Physiological Characterization of the Bacterial Strains

All the PGPR strains used in this study were isolated in our laboratory from the potato rhizosphere and were found to positively influence in vitro potato tuberization, suggesting the significant role of the bacteria inhabiting the potato rhizosphere in tuberization (Nookaraju and others 2011). Among the isolates, two *Bacillus* isolates (*Bacillus pumilus* str. DH-11 and *Bacillus firmus* str. 40) exerted a significant positive influence on potato tuberization under both in vitro and ex vitro conditions. All the isolated bacterial strains were tested in vitro for a range of activities that are attributed to the positive effects of plant growth-promoting bacteria. Five bacterial strains were able to mineralize phytates, whereas four isolates exhibited ACC deaminase activity (Table 2). *Bacillus* strains 4 and 6 produced the highest auxin level in vitro (Table 2). However, no correlation was found between auxin production and in vitro tuberization. All these bacterial strains were also screened for the abiotic stress tolerance as mentioned in the Materials and Methods section. Four isolated PGPR strains (isolates 4, 6, 7, and 12) showed stress tolerance in terms of better growth on the stress-inducing medium (data not shown). However, two *Bacillus* strains (4 and 6, showing maximum effect on tuberization) exhibited higher tolerance on different stress-inducing media. The growth of PGPR strain 4 was better on the medium with NaCl (400 mM), whereas strain 6 showed better growth on the medium containing $ZnCl_2$ (40 mM). Both these strains were able to grow on the medium supplemented with 20 % PEG. Higher concentrations of NaCl, $ZnCl_2$, and PEG were found to be lethal for all bacterial strains. It was also observed that the inoculation of these two strains of PGPR on potato facilitated better plant growth compared with the noninoculated plants. The inoculated plants showed better growth in terms of plant height, number of leaves, and number of tubers under stress conditions (Table 3).

Plants Inoculated with PGPR Showed Enhanced Tolerance to Different Abiotic Stresses

Two bacterial strains showing maximum tolerance to different abiotic stresses and exhibiting other superior characteristics, as indicated in Table 2, were used to inoculate the potato plants before applying the abiotic stress on the plants. The first inoculation was done at the time of potato hardening. After 1 week, the second inoculation of PGPR was done. After another 2 weeks, different abiotic stresses were applied (PEG 10 %, 200 mM NaCl, and 20 mM $ZnCl_2$). These stresses were observed to be toxic to the

Table 2 Plant growth promotion activities of rhizobacteria isolated from potato rhizosphere

Isolate no	Identified PGPR strains	ACC deaminase activity	IAA production ($\mu g\ ml^{-1}$)	Phosphate solubilization	Phytate mineralization	Siderophore production
1	*Chryseobacterium* sp. U3	−	2.23	−	−	+
2	*Agrobacterium tumefaciens* strain ISSDS-425	−	37.89	−	+	−
3	*Bacillus cereus* strain HZB	+	6.31	+	−	−
4	*Bacillus pumilus* strain DH-11	+	109.46	+	+	+
5	*Staphylococcus epidermidis* strain BQN1N-02d	−	3.03	−	−	−
6	*Bacillus firmus* strain 40	+	138.93	+	+	−
7	*Bacillus firmus* strain 17	−	59.78	−	−	+
8	Uncultured beta *proteobacterium* strain MS092A1 C05	−	12.22	−	+	+
9	*Variovorax* sp. T71	−	44.62	+	−	−
10	*Bacillus* sp. 77	+	30.31	−	−	+
11	*Bacillus thuringiensis* strain IS2	−	0.84	−	+	−
12	*Plantibacter* sp. WPCB192	−	46.31	+	−	−
13	*Variovorax paradoxus* strain SFWT	−	15.57	+	−	+

Table 3 Effect of PGPR inoculation on potato plant growth under different abiotic stress conditions in glasshouse conditions

Stress treatment											
PGPR treatment	PEG (10 %)				NaCl (200 mM)				$ZnCl_2$ (20 mM)			
	Plant height (cm)	No. leaves/ plant	No. tubers/ plant	Tuber yield/ plant (g)	Plant height (cm)	No. leaves/ plant	No. tubers/ plant	Tuber yield/ plant (g)	Plant height (cm)	No. leaves/ plant	No. tubers/ plant	Tuber yield/ plant (g)
Control	58.78 ± 3.17^a	19.83 ± 1.88^a	6.33 ± 1.17^a	59.3 ± 3.57^a	59.88 ± 3.2^a	19 ± 1.8^a	7.5 ± 1.23^a	59.9 ± 3.39^a	61.05 ± 3.27^a	19.83 ± 1.94^a	7.66 ± 1.38^a	59.16 ± 3.29^a
PGPR 4	64.0 ± 3.42^b	22.16 ± 1.97^b	9.66 ± 1.34^b	71.53 ± 3.61^b	63.65 ± 3.48^b	23.16 ± 2.15^b	10.5 ± 1.41^b	65.16 ± 3.38^b	65.4 ± 3.60^b	23.16 ± 2.13^b	10 ± 1.33^b	64.73 ± 3.42^b
PGPR 6	65.55 ± 3.67^b	22.33 ± 2.09^b	10.33 ± 1.38^b	70.35 ± 3.80^b	63.91 ± 3.47^b	22 ± 2.02^b	10 ± 1.37^b	66.26 ± 3.46^b	64.78 ± 3.77^b	22 ± 2.01^b	9.16 ± 1.37^b	65.66 ± 3.58^b

Means within a column followed by the same letter are not significantly different ($p \leq 0.05$) as calculated with the use of Tukey's test. The data shown are the means of six replicates ± SE

potato plant without PGPR inoculation. However, the PGPR-inoculated plants growing under stress conditions showed better growth and tolerated these stresses significantly better than the noninoculated plants. Interestingly, plant survival was better after inoculation with both strains (Fig. 1). However, overall plant growth, leaf number, tuber size, and tuber yield were better in potato plants inoculated with PGPR strain 4 (Table 3).

PGPR Inoculation Induces the Higher Activity of ROS-Scavenging Enzymes in the Plant

The analysis of the soil adhered to a plant's roots and tubers after harvesting indicated that PGPR (isolates 4 and 6) had successfully colonized the root system in all inoculated plants (10^4–10^7 CFU/g tissue on a fresh weight basis) growing under different stress conditions. To appreciate the effect of abiotic stress treatments (drought, salt, and heavy metal) on the antioxidant capacity of PGPR-inoculated plants (isolates 4 and 6), the hydrogen peroxide levels as well as the enzymatic activities of the major ROS scavengers were analyzed. Hydrogen peroxide levels were significantly higher in control plants under all the applied stress treatments than in the PGPR (isolates 4 and 6)-inoculated plants (Fig. 2). Plants inoculated with PGPR isolate 6 recorded the least amount of H_2O_2 accumulation under PEG (10 %) and NaCl (200 mM) stress conditions. The specific activity of APX increased 1.2 times in strain 4, including inoculated plants under drought stress conditions, 1.4 times under salt stress, and 1.3 times under heavy-metal stress (Fig. 3a). The specific activity of SOD significantly increased in the PGPR-inoculated plants growing under these stresses compared with that in non-inoculated plants under the same stress conditions. The SOD activity in the inoculated plants was approximately 1.7 times higher under PEG stress, 2.4 times higher under salt stress, and 1.7 times higher under heavy-metal stress (Fig. 3b). The specific activity of GR also showed a similar trend (1.6–2.1 times increase), with maximum expression in the inoculated plants under heavy-metal stress compared with that in noninoculated control plants (Fig. 3c). A significant increase in the specific activity of CAT (up to 1.8 times under drought stress, 1.8 times under salt stress, and up to 1.7 times higher under heavy-metal stress) was also observed in the PGPR-inoculated plants (both strains) growing under stress conditions compared with that in noninoculated control conditions (Fig. 3d). Similarly, the specific activity of DHAR also increased significantly (ca. 1.3–1.7 times) in the inoculated plants growing under stress conditions compared with that in the noninoculated plants (Fig. 3e). The specific activities of these antioxidant enzymes were positively correlated with their mRNA

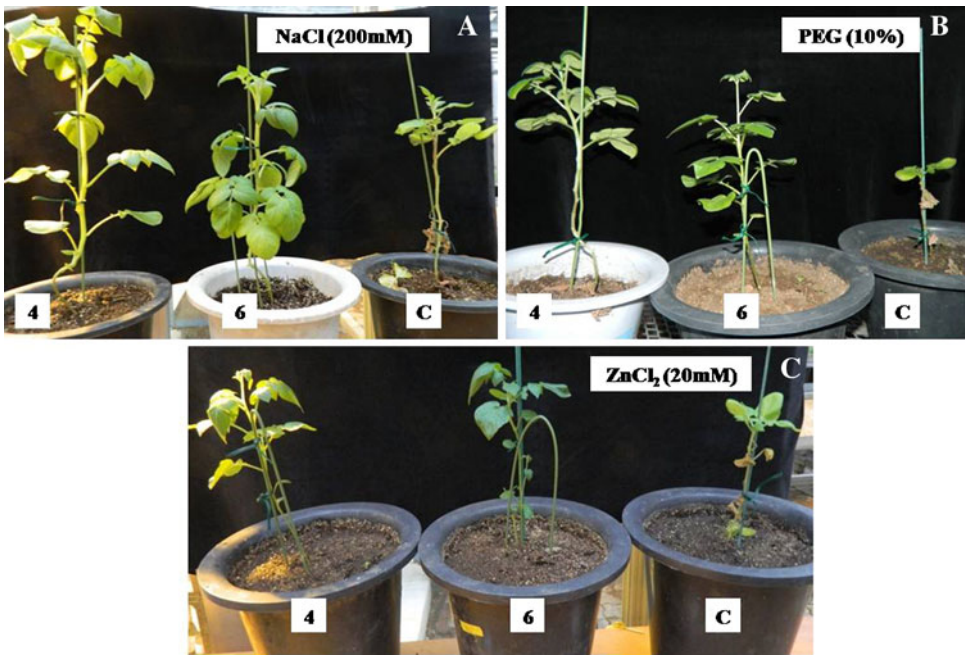

Fig. 1 Effect of PGPR (isolates *4* and *6*) inoculation on 4 week-old potato plants growing under different abiotic stress conditions induced by **a** 200 mM NaCl, **b** 10 % PEG, and **c** 20 mM $ZnCl_2$. *C* potato plants without any PGPR inoculation; *4* potato plants inoculated with PGPR isolate 4; *6* potato plants inoculated with PGPR isolate 6

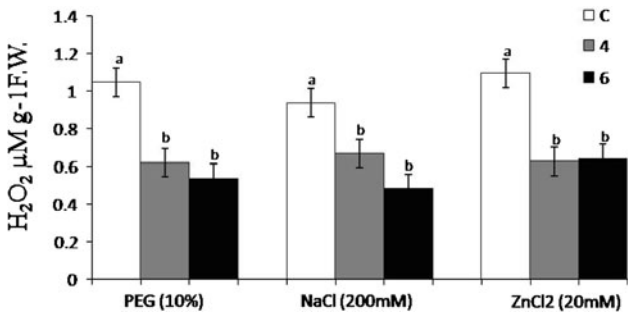

Fig. 2 Estimation of H_2O_2 in leaves of control potato plants (*C*), PGPR isolate 4-inoculated plants (*4*), and PGPR isolate 6-inoculated plants (*6*) sampled in the glasshouse. Values represent the mean ± SE from three independent assays with three replicates for each treatment. Different *letters* in each column indicate significant differences ($p \leq 0.05$) in H_2O_2 content between treatments after Tukey's test ($n = 5$)

expression levels as measured by quantitative real-time PCR analyses (Fig. 4a–e).

Accumulation of Higher Proline in PGPR-Inoculated Plant Tubers

Proline is a common osmolyte in higher plants and accumulates in response to stress. The change in the proline content was measured in the tubers from PGPR-inoculated and noninoculated plants growing under stress conditions. A significant increase in the proline content was recorded in PGPR-inoculated plant tubers (ca. 1.5–2.6 times) grown under stress conditions compared with that in noninoculated plant tubers (Table 4). The increase in proline content was greater in the inoculated plant tubers grown under salt stress than under PEG and heavy-metal stresses.

PGPR-Inoculated Potato Plants Exhibited an Increase in the Photosynthetic Efficiency

The two PGPR bacterial isolates were tested in *S. tuberosum* for their influence on the photosynthetic efficiency of plants under different abiotic stress conditions. The maximal photochemical efficiency (F_v/F_m) is the most common parameter used in fluorescence and is inversely proportional to damage in the PSII reaction centers (Farquhar and others 1989). The F_v/F_m ratio ranged between 0.4 and 0.8 and was observed to be most sensitive to drought treatment than to salt and heavy-metal treatments (Table 5). The F_v/F_m values of PGPR-inoculated plants were higher than those of the noninoculated control plants growing under the same stress conditions. This observation was further confirmed with the estimation of the performance index (PI), essentially an indicator of sample vitality. The PI value is considered an overall expression indicating a type of internal force of the sample to resist external constraints. The PI of PGPR-inoculated plants was significantly higher than that of the noninoculated control plants under abiotic stress conditions in all but one treatment (Fig. 5). The difference between PI values of plants inoculated with PGPR isolate 6 and control plants growing under salt stress was statistically nonsignificant. Interestingly, the PGPR isolate 4-inoculated potato plants had higher PI values under all the stress treatments than the PGPR isolate 6-inoculated and noninoculated control plants.

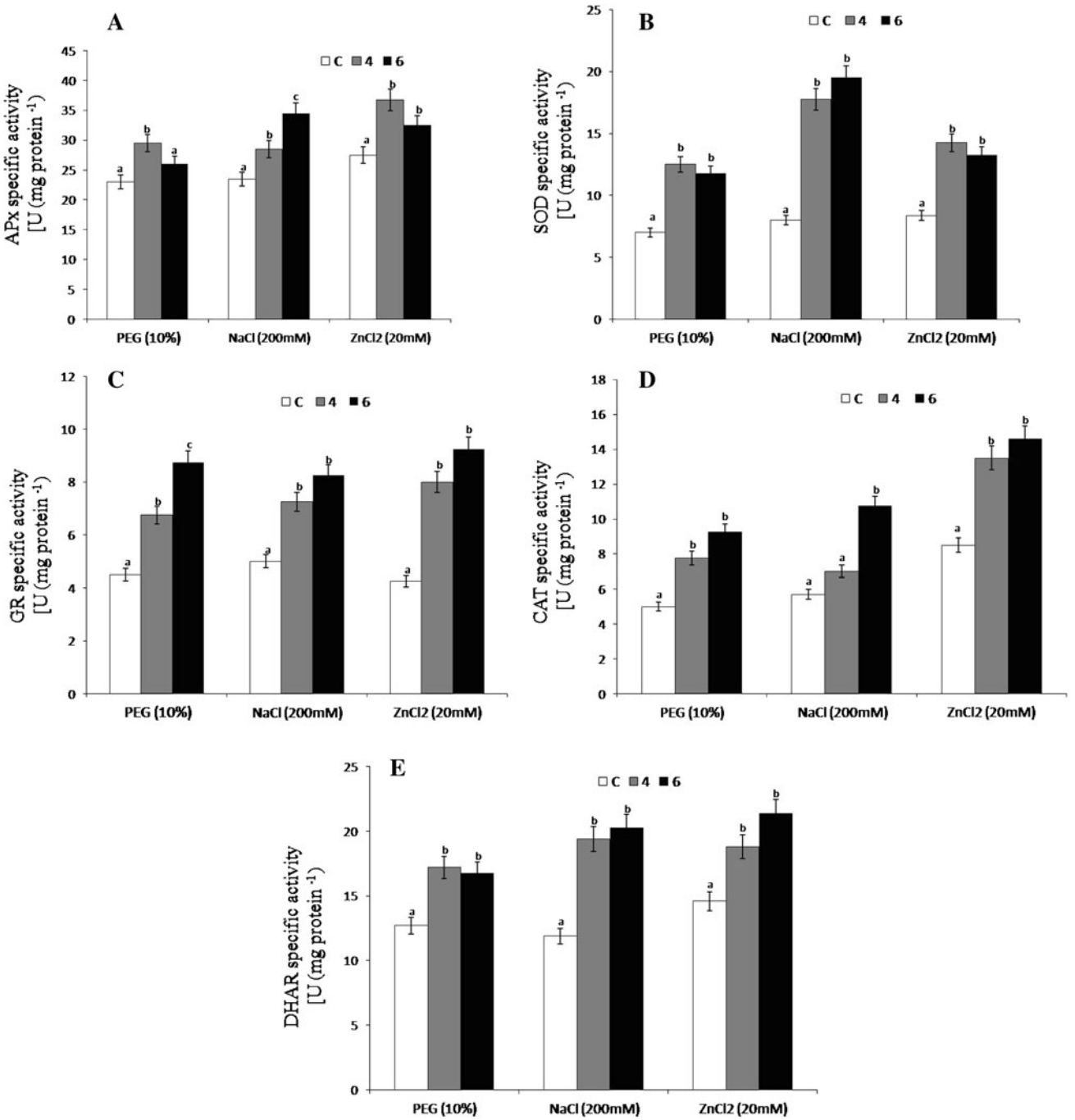

Fig. 3 Specific enzyme activities of APX (**a**), SOD (**b**), GR (**c**), CAT (**d**), and DHAR (**e**) in *S. tuberosum* stressed with PEG (10 %), NaCl (200 mM), and $ZnCl_2$ (20 mM). Values represent the mean ± SE from three independent assays with three replicates for each treatment. Different *letters* in each column indicate significant differences ($p \leq 0.05$) in enzyme activities between treatments after Tukey's test ($n = 5$). *C* potato plants without any PGPR inoculation; *4* potato plants inoculated with PGPR isolate 4; *6* potato plants inoculated with PGPR isolate 6

Discussion

Inoculation of potato plants with some of the rhizobacteria strains isolated and identified in our laboratory induced plant tolerance to different abiotic stresses. These bacteria revealed characteristics of plant growth-promoting bacteria such as ACC deaminase activity, IAA production, and mineral solubilization activity. Potato plants inoculated with two bacterial strains belonging to *Bacillus* showed significant tolerance to salinity, heavy-metal, and drought stresses induced by NaCl, $ZnCl_2$, and PEG, respectively. Inoculation with these bacteria not only induced significant

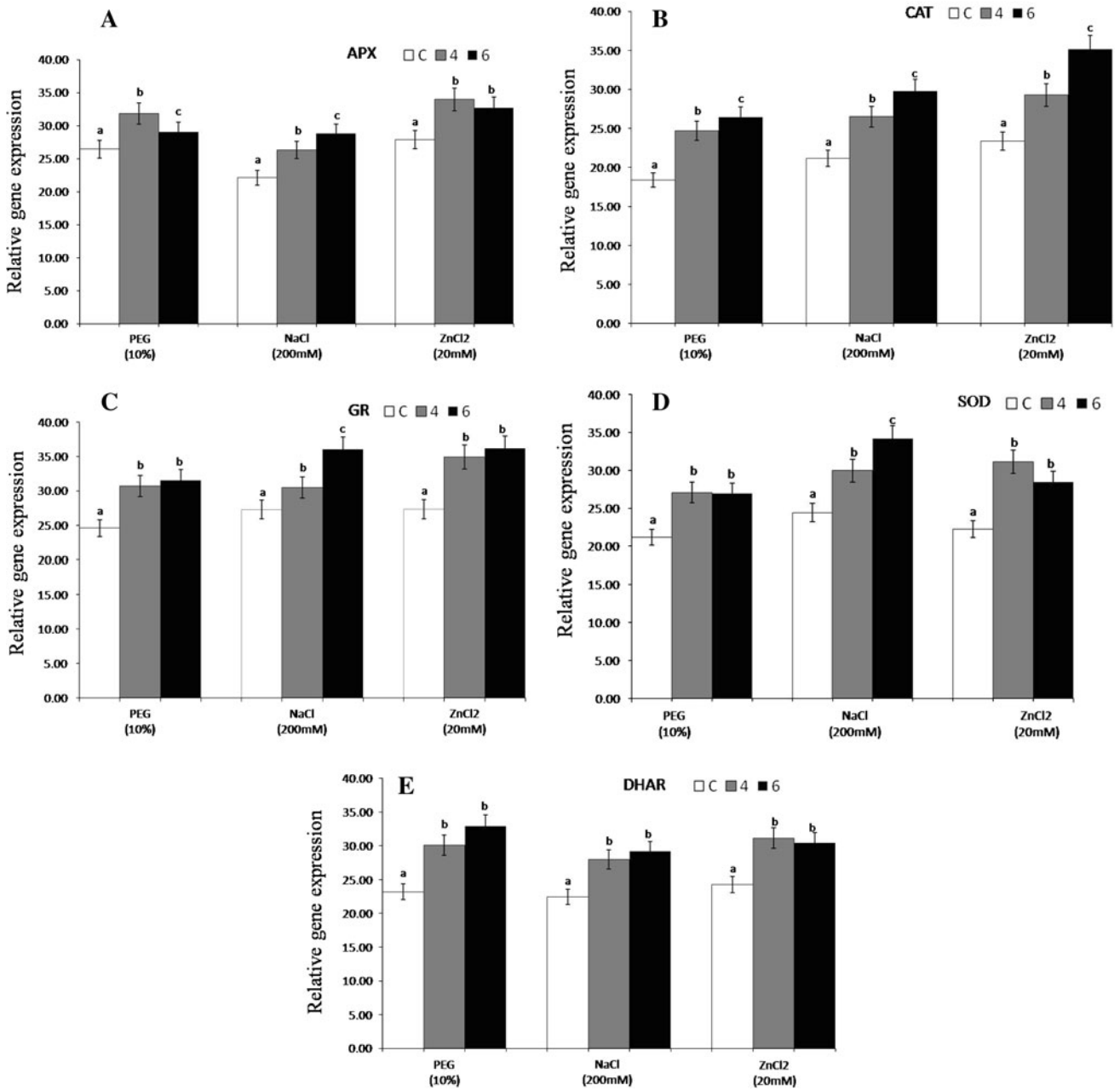

Fig. 4 Real-time mRNA expression of genes coding ROS-scavenging enzymes APX (**a**), CAT (**b**), GR (**c**), SOD (**d**), and DHAR (**e**) from PGPR-inoculated and noninoculated control (*C*) potato plants growing under various abiotic stress conditions. Fold expression values are normalized to those of actin control. Different *letters* in each column indicate significant differences ($p \leq 0.05$) in relative gene expression between treatments after Tukey's test ($n = 5$). *C* potato plants without any PGPR inoculation; *4* potato plants inoculated with PGPR isolate 4; *6* potato plants inoculated with PGPR isolate 6

changes in the gene expression of different ROS-scavenging enzymes but also enhanced the photosynthetic efficiency of inoculated plants, which proved to be a reason for the tolerance of abiotic stress. Earlier, these strains showed a significant influence on potato tuberization under in vitro and ex vitro conditions by inducing lipoxygenase (LOX) activity and other tuberization hormones (Nookaraju and others 2011; Upadhyaya and others 2011).

Abiotic stresses, including water deficiency, lead to ROS formation by misdirecting the electrons in the photosystems. Plants respond to water-deficit conditions by increasing their osmolyte production, which in turn increases the osmotic potential within the cell (Farooq and others 2009). Additionally, the compounds secreted by the rhizosphere bacteria belong to osmolytes. A positive correlation between glycine betaine production by PGPR and

Table 4 Proline content of potato plants inoculated with PGPR isolates 4 and 6 and untreated control after 1 month of growth

Stress treatment	Proline content (μmol g^{-1} FW)
PEG	
C	9.3 (±0.5)[a]
4	14.3 (±0.7)[b]
6	11.09 (±0.3)[a]
NaCl	
C	6.6 (±0.3)[a]
4	17.2 (±0.6)[b]
6	15.8 (±0.2)[b]
$ZnCl_2$	
C	11.3 (±0.6)[a]
4	21.8 (±0.4)[b]
6	18.6 (±0.6)[b]

Values represent the mean ± SE from three independent assays with three replicates for each treatment

Different letters in each column indicate significant differences ($p \leq 0.05$) in proline content between treatments after Tukey's test

C = control potato plants (without PGPR inoculation); 4 = potato plants inoculated with PGPR isolate 4; 6 = potato plants inoculated with PGPR isolate 6

enhanced drought tolerance was reported in plants (Yuwono and others 2005). As mentioned earlier, these rhizobacteria revealed ACC deaminase activity, IAA production, and mineral solubilization activity. Earlier, it had been demonstrated that ACC deaminase activity of *Achromobacter piechaudii* confers tolerance to water deficit in tomato and pepper plants (Mayak and others 2004). Similarly, PGPR isolates belonging to *Pseudomonas* and *Bacillus* genera were reported to enhance resistance to water stress in green gram plants (Sarvanakumar and others 2011).

Table 5 Estimation of photochemical efficiency in terms of F_v/F_m for control potato plants and plants treated with PGPR isolates 4 and 6 under abiotic stress conditions induced by PEG (10 %), NaCl (100 mM), and $ZnCl_2$ (20 mM)

	F_v/F_m		
	PEG (10 %)	NaCl (200 mM)	$ZnCl_2$ (20 mM)
C	0.717 (±0.08)[a]	0.455 (±0.09)[a]	0.565 (±0.04)[a]
4	0.828 (±0.06)[b]	0.821 (±0.07)[b]	0.802 (±0.05)[b]
6	0.817 (±0.05)[b]	0.792 (±0.05)[b]	0.834 (±0.05)[b]

Data represent mean of F_v/F_m values of nine leaves from three individual plants per treatment ± SE

Different letters indicate significant differences ($p \leq 0.05$) in F_v/F_m values between treatments after Tukey's test ($n = 9$)

C = control potato plants (without PGPR inoculation); 4 = potato plants inoculated with PGPR isolate 4; 6 = potato plants inoculated with PGPR isolate 6

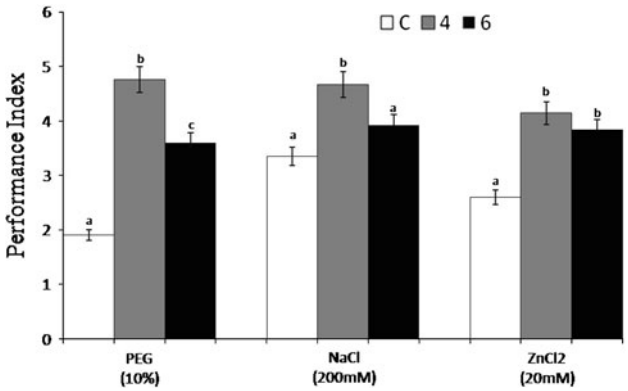

Fig. 5 Performance index (PI_{total}) of control (*C*, no PGPR inoculation) and PGPR (strains *4* and *6*)-inoculated intact leaves of potato plants growing under stress conditions induced by PEG (10 %), NaCl (200 mM), and $ZnCl_2$ (20 mM). Values represent the mean value from five leaves per plant and three replicates for each treatment. *C* potato plants without any PGPR inoculation; *4* potato plants inoculated with PGPR isolate 4; *6* potato plants inoculated with PGPR isolate 6

Ethylene is a plant hormone that is involved in the regulation of many physiological responses (Arshad and Frankenberger 2002; Owino and others 2006). Ethylene was originally regarded as a stress hormone because of its synthesis at accelerated rates in response to a variety of stress signals such as mechanical wounding, flooding, chemicals and metals, drought, extreme temperatures, and pathogen infection (Kende 1993; Johnson and Ecker 1998). ACC is the immediate precursor of ethylene in higher plants (Yang and Hoffman 1984) and its regulation has been described as the principal mechanism by which bacteria exert beneficial effects on plants under abiotic stress (Saleem and others 2007). Certain microorganisms have been reported to contain the enzyme ACC deaminase that hydrolyzes ACC into ammonia and α-ketobutyrate (Glick and others 1994, 1998; Mayak and others 1999; Shaharoona and others 2006) instead of converting it into ethylene. The cleavage of ACC by ACC deaminase-containing rhizobacteria reduces the ACC and ethylene levels in the rhizoplane, thereby providing a sink for the ACC. These reduced ACC levels in turn decrease the levels of endogenous ethylene, thus eliminating the potentially inhibitory effects of higher ethylene concentrations (Glick and others 1998). In our study, the two *Bacillus* strains used to inoculate the stressed potato plants showed significant ACC deaminase activity, which provides direct evidence of the beneficial effect of these bacteria on the plants.

The two PGPR isolates, strains 4 and 6, used in this study were identified as *Bacillus pumilus* strain DH-11 and *Bacillus firmus* strain 40, respectively (Nookaraju and others 2011). Besides other beneficial properties, *Bacillus* sp., as with most other bacteria, are known to reveal SOD

activities and mitigate the oxidative stress in living systems. The bacterial SODs play an important role in their survival in the rhizosphere by facilitating the removal of free radicals (Wang and others 2007). In general, the survival of the bacterial strains is attributed to their contribution to the alleviation of abiotic stress and plant growth promotion (Dimkpa and others 2009b). Abiotic stress conditions cause an increase in ROS formation at the cellular level. ROS such as superoxide radical (O_2^-), hydrogen peroxide, and hydroxyl radicals (OH) cause lipid peroxidation of membranes (Sgherri and others 2000; Hemavathi and others 2010). The antioxidant enzymes of the cell have the ability to remove the free radicals produced during abiotic stress conditions. These enzymes also protect the membranes and DNA from damage. To monitor the putative changes in the expression of genes encoding the ROS-scavenging enzymes, the PGPR-inoculated and noninoculated potato plants were subjected to different stresses induced by PEG, NaCl, and $ZnCl_2$. The mRNA expression of SOD and APX in bacteria-inoculated plants growing under stress conditions increased considerably when compared with that in the noninoculated stressed plants. Similarly, the mRNA expression levels of genes encoding for other antioxidative enzymes such as CAT, DHAR, and GR also increased in the bacteria-treated plants. These results were consistent with the semiquantitative RT-PCR results (data not shown) where the expression of the antioxidative pathway genes was more pronounced in the PGPR-inoculated plants than in the noninoculated plants.

Plant growth-promoting bacteria are known to release metal-chelating substances such as iron-chelating siderophores into the rhizosphere. It has been suggested that siderophore-producing bacteria influence the uptake by plants of various metals, including Fe, Zn, and Cu (Carrillo-Castaneda and others 2003, 2005; Egamberdiyeva 2007; Dimkpa and others 2008, 2009a). Similar to these findings, the bacterial isolates 4 and 6 showed enhanced siderophore production. These reports clearly indicate that the rhizobacteria can also influence the bioavailability of metal ions required by the plants. As soil conditions affect the metal valences, rhizobacteria and other microorganisms also impact the availability of the metals by acidifying the surrounding rhizosphere, as reported by Dimkpa and others (2009b).

Bacterial inoculation has also been reported to positively influence root growth, total aerial biomass, leaf area index, and proline accumulation in the leaves and roots. Higher proline accumulation in potato plants inoculated with bacteria indicated higher plant tolerance to water stress. Creus and others (2004) also reported reduced grain yield losses in the grains of *Azospirillum*-inoculated wheat exposed to drought conditions. Besides observing the increase in relative water content, water potential, and apoplastic water fraction, they also measured the volumetric cell wall moduli of elasticity and hypothesized the "elastic adjustment" as a crucial factor for increased drought tolerance (Creus and others 2004).

Photosynthesis is the most important process on earth, directly or indirectly influencing the growth and survival of every organism. Therefore, it cannot be examined simply as an isolated phenomenon but must be studied within the context of whole-plant regulation. To assess the changes in the photosynthetic machinery of potato plants subjected to various abiotic stresses, we measured the photosynthetic efficiency of these plants, represented by the expression F_v/F_m, which indicates the maximum quantum yield of PSII. It has been suggested that abiotic stresses such as drought and salinity decrease the quantum efficiency of PSII photochemistry (Briantais and others 1996). Earlier, several reports were published where these parameters were employed to examine the photosynthetic efficiency of different crop plants subjected to various stresses like drought, salinity, and heavy metal (de Ronde and others 2004; Yusuf and others 2010; Mathur and others 2011). The F_v/F_m values were in the range of 0.45–0.71 in all the stressed samples of untreated control plants, whereas it ranged from 0.79 to 0.83 in samples treated with either of the two PGPR strains (Table 5). Additionally, the performance index (PI) of the plants, which indicates the sample vitality, was also recorded. The overall PI represents a single multiparametric expression that combines all three independent functional steps of photosynthesis: the density of the reaction centers in the chlorophyll pool, trapped excitation energy, and its conversion to the electron transport (Strasser and others 1999; Tsimilli-Michael and others 2000). The PI values shown in Fig. 5 clearly indicate that the photosynthetic performance of PGPR-treated plants under all stress conditions significantly increased, indicating the positive influence of the PGPR isolates on the photosynthetic machinery of the plants. Potato plants treated with PGPR isolate 4 (*Bacillus pumilus* strain DH-11) exhibited a higher overall PI in all the stress treatment situations, whereas in all stress situations the overall PI of untreated control plants was significantly lower than that of the plants treated with either of the PGPR strains.

In conclusion, the results shown here demonstrate that the inoculations with rhizobacteria strains 4 and 6 protected *S. tuberosum* against abiotic stress factors like water deficit, salinity, and heavy-metal toxicity. Plant tolerance to these stresses correlated with the increased expression levels of ROS-scavenging enzymes APX, SOD, CAT, DHAR, and GR, suggesting that inoculation with these strains triggered abiotic stress-related defense pathways under stressed conditions. These observed effects were consistent with the relative mRNA expression levels as

determined by RT-PCR and real-time PCR. This study also proved that the use of photosynthetic parameters as a tool to investigate the changes in the photosynthetic machinery of the plants under stress conditions is a promising approach that can be applied to other crops as well. Furthermore, this study would facilitate follow-up studies on metabolites produced by these PGPR isolates that could be the potential candidate elicitors responsible for enhanced plant tolerance to various abiotic stresses.

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Conflict of interest The authors have no conflicts of interest to disclose.

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