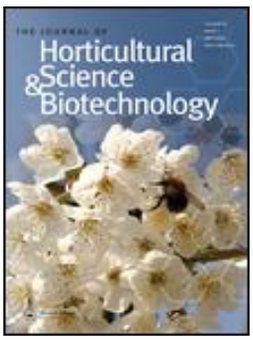

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Enzyme activities and growth promotion of spinach by indole-3-acetic acid-producing rhizobacteria

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SUMMARY
The objective of this study was to evaluate the effects of twelve plant growth-promoting rhizobacteria (PGPR; *Bacillus mycoides* FD07, *B. sphaericus* RC12, *B. pumilus* RC19, *B. cereus* RC18, *Variovorax paradoxus* RC21, *Paenibacillus polymyxa* RC35, *Pseudomonas putida* RC06, *B. megaterium* RC07, *B. megaterium* M-3, *B. licheniformis* RC08, *B. subtilis* RC11, and *B. subtilis* OSU-142) used as biofertilisers, on various enzyme activities [glucose-6-phosphate dehydrogenase (G6PD); 6-phosphogluconate dehydrogenase (6PGD); glutathione reductase (GR); and glutathione S-transferase (GST)] and on seedling growth in spinach (*Spinacia oleracea* L.). Enhanced plant growth could result from rhizobacterial production of indole-3-acetic acid (IAA). The highest IAA-producing rhizobacteria (RC35 and RC06) produced the highest root and shoot weights. PGPR improved N and P nutrition in spinach, and therefore stimulated plant growth and key enzyme activities. The responses to inoculation, compared to uninoculated control plants, were: –1.9% to +36.4% for shoot fresh weights (FWs), –5.5% to +30.1% for root FWs, –3.5% to +29.8% for shoot dry weights (DWs), –3.8% to +38.5% for root DWs, and –5.9% to +30.1% for leaf areas. Plant growth responses were variable and dependent on the inoculant strain used, as well as on the enzyme activity and growth parameter being evaluated. Close correlations between plant shoot growth, PGPR inoculation, and G6PD (r = 0.28*), 6PGD (r = 0.55**), GR (r = 0.73**), and GST (r = 0. 64**) enzyme activities in spinach have been demonstrated.

Nitrogen (N) and phosphorus (P) are essential for plant growth and development, and often limit productivity. Increments in mineral N rates caused an increase in the NO_3^- content of spinach (Gülser, 2005), and NO_3^- accumulation warrants serious attention because of its hazardous effects on human health. Plant growth-promoting rhizobacteria (PGPR) have been considered as a possible alternative to inorganic fertiliser for promoting plant growth. PGPR may affect plant growth directly by synthesising phytohormones and vitamins, through N fixation for plant use, by improvements in nutrient uptake, by enhanced stress resistance, and/or by the solubilisation of inorganic phosphate (Dobbelaere *et al*., 2003).

These physiological changes are linked to increases in various enzyme activities, and the mRNAs and protein levels required for NO_3^- assimilation into amino acids. Nitrogen assimilation in plants consists of three processes. First, NO_3^- is reduced to NO_2^- by nitrate reductase. Second is the reduction of NO_2^- to NH_4^+ by nitrite reductase. Third, NH_4^+ is assimilated into amino acids. An improved understanding of nitrogen assimilation is vital if improvements in crop N-use efficiency (NUE) are to be made in order to reduce the need for fertiliser inputs.

The capacity of the oxidative pentose phosphate pathway (OPPP) in plant tissues is increased during nitrate assimilation, which requires reducing power for nitrate and nitrite reduction (Bowsher *et al*., 1989). The primary regulated enzyme of the OPPP is glucose-6-phosphate dehydrogenase (G6PD), which catalyses the first reaction in the cycle (Esposito *et al*., 2001) and provides NADPH for nitrate reduction (Savidov *et al*., 1998). These reactions have been implicated in the provision of reducing agents for a wide range of processes including inorganic N assimilation (Neuhaus and Emes, 2000), responses to oxidative and field drought stress, and pathogen infection (Chen *et al*., 2004). G6PD and 6-phosphogluconate dehydrogenase (6PGD) may play important roles in cell division and salt responses in plants (Huang *et al*., 2003). Glutathione reductase (GR) catalyses the NADPH-dependent reduction of the oxidised form of glutathione to reduced glutathione (GSH; Medici *et al*., 2004), therefore GR appears to be important during the regeneration of ascorbate and GSH (Tanaka *et al*., 1994). GR plays a key role in anti-oxidant defense processes and also appears to be related to tolerance to environmental stresses (Tanaka *et al*., 1994; Chen *et al*., 2004). Glutathione S-transferase (GST) can protect cells from a wide variety of biotic and abiotic stresses (Zeng *et al*., 2005; Gong *et al*., 2005). There have been several studies on the different isoforms of G6PD and GR in leaf tissues, and their relationship with N metabolism (Wright *et al*., 1997;

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Savidov *et al*., 1998; Rios-Gonzales *et al*., 2002; Medici *et al*., 2004).

To date, many studies on the introduction of PGPR have focussed on economically important agricultural and wild plants (de Freitas *et al*., 1997; Şahin *et al*., 2004; Orhan *et al*., 2007). However, the effects of PGPR on the activities of anti-oxidant and OPPP cycle enzymes in plants have not been considered. Recently, our studies demonstrated, for the first time, that PGPR could enhance GR, GST, 6PGD and G6PD activities, together with the growth of plants (Çakmakçı *et al*., 2007a). The beneficial effect of PGPR on plant growth varied, depending on the species, and are strain specific (Şahin *et al*., 2004).

In this paper, we examine the effects of PGPR on the activities of anti-oxidant (GR and GST) and OPPP (G6PD and 6PGD) enzymes in the leaves of spinach.

MATERIALS AND METHODS

Bacterial species and strains, isolation, and identification of bacteria

Pseudomonas putida RC06, *Bacillus megaterium* RC07, and *B. licheniformis* RC08 were isolated from the rhizosphere of wheat and barley (Çakmakçı *et al*., 2006). *Bacillus subtilis* RC11 and *B. cereus* RC18 were initially isolated from the rhizosphere of wild red raspberries (Çakmakçı *et al*., 2007a) and characterised as PGPR that could promote plant growth, solubilise P, and fix N_2. *Bacillus subtilis* OSU-142 was originally isolated from tomato plants, and *B. megaterium* M-3 was isolated from pepper (Şahin *et al*., 2004). Five bacterial strains were first isolated from the rhizosphere of wild red raspberries and identified as *Paenibacillus polymyxa* RC35, *B. mycoides* FD07, *B. sphaericus* RC12, *B. pumilus* RC19, and *Variovorax paradoxus* RC21 with similarity indices of 0.729, 0.621, 0.769, 0.488, and 0.661, respectively. These indices were based on whole-cell fatty acid methyl ester (FAMEs) analysis (de Freitas *et al*., 1997) using the Sherlock Microbial Identification System (Version 4.5) and Biolog microplate assays (Biolog Inc., Hayward, CA, USA). All bacterial strains were maintained in nutrient broth (NB) with 15% (v/v) glycerol at –86°C for further tests.

Quantification of IAA production

The PGPR were tested for indole-3-acetic acid (IAA)-like auxin production using the method of Bent *et al*. (2001). Flasks (125 ml) containing 40 ml half-strength TSB were incubated for 18 h at 27°C on a 100 rpm rotary shaker supplemented with 0, 0.1, or 25 µg tryptophan ml^{-1}, then each was inoculated with 1 ml of each PGPR. After incubation for 48, 72, or 168 h, the density of each culture was measured spectrophotometrically at 600 nm. The bacterial cells were removed from the culture medium by centrifugation (5.500 × *g* for 10 min). The level of indoles present in the culture fluid was estimated colorimetrically. The concentration of IAA in the culture medium was measured using Salkowski's reagent [50 ml 35% (v/v) $HClO_4^+$ containing 1 ml 0.5 M $FeCl_3$; Gordon and Weber, 1951]. The absorbance was measured at 530 nm in a Shimadzu UV-1208 spectrophotometer (Tokyo, Japan). Bacterial cells were separated from the supernatant by centrifugation at 10.000 × *g* for 30 min at 4°C. The concentration of IAA in each culture medium was determined by comparison with a standard curve.

Greenhouse experiment and growth conditions

Spinach (*Spinacia oleracea* L.) seeds were surface-sterilised in 70% (v/v) ethanol for 2 min, and rinsed ten-times in sterile tap water. For this application, pure cultures were grown in NB at 28°C and diluted to a final concentration of 10^9 colony-forming units (cfu) ml^{-1} in sterile distilled water containing 0.025% (v/v) Tween-20. Surface-sterile seeds were inoculated by immersion in the appropriate PGPR suspension (at 10^9 cfu ml^{-1}) for 2 h on a rotary shaker at 81 rpm, air-dried, and sown immediately. The cell densities in the PGPR suspensions were adjusted to a final density of approx. 10^8 cfu $seed^{-1}$.

The experimental design consisted of three completely randomised blocks in a factorial arrangement with 13 treatments: twelve PGPR applications, and an uninoculated control. Pots were sterilised with 20% (v/v) sodium hypochlorite solution and filled with a loamy soil with an organic matter content of 2.1% (w/w), a pH of 6.9, an available Olsen-P content of 13.4 mg kg^{-1}, and NH_4^+-N and NO_3^--N contents of 10.1 and 8.9 mg kg^{-1}, respectively. Spinach seeds were sown in plastic pots filled with 5 kg of field soil. Twelve seeds were sown per pot, at six points (two seeds at each point), then thinned to six uniform plants per pot 10 d after sowing.

The spinach seedlings were grown in a greenhouse under a 13 h natural light photoperiod at 18° – 14°C, and 65% relative humidity. Pots were watered to 60% of their maximum water-holding capacity and were maintained at this moisture content by watering to weight every 2 – 3 d. Plants were harvested on day-28 and day-50, after emergence of the seedlings, and separated into shoots and roots. Each root system was washed in deionised water, and its fresh weight (FW) was determined immediately after each harvest. The leaf area of each plant was recorded using an electronic planimeter (Licor-3000; LICOR, Lincoln, NE, USA). Plant materials were then oven-dried at 70°C for 72 h, and dry weights (DWs) were determined for all shoots and roots. The macronutrient (N, P, K, Ca and Mg) and micronutrient (Fe, Mn, Zn and Cu) contents of spinach seedlings were determined according to the Association of Official Analytical Chemists (Helrich, 1990).

Extraction of enzymes

Spinach leaves were harvested, wrapped separately in aluminium foil, then frozen in liquid nitrogen and kept at –80°C prior to use. Two g of each sample, frozen in liquid nitrogen, was powdered, then added to 10 ml of extraction buffer (50 mM Tris-HCl, containing 1 mM EDTA and 1 mM DTT, pH 7.5) and mixed. Each mixture was centrifuged at 20,000 × *g* at 4°C for 20 min, and the precipitate was removed. The supernatants were used as crude extracts for the determination of enzyme activities.

Measurements of enzyme activities and protein concentrations

Glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44) activities were determined according to the method of Beutler (1984). The assay

TABLE I
Production of IAA (μg ml^{-1} OD$_{600}$ unit^{-1}) by PGPR in the presence of various concentrations of tryptophan

PGPR strain*	Tryptophan (μg ml^{-1}) 0	0.1	25	Reference
FD07	1.6 ± 0.4 g†	4.3 ± 0.5 e	13.4 ± 1.4 f	This study
RC12	5.1 ± 0.6 cd	6.2 ± 0.7 d	21.4 ± 1.6 de	This study
RC19	4.9 ± 0.3 cd	6.8 ± 0.6 cd	24.7 ± 1.3 bc	This study
RC21	ND	ND	ND	This study
M-3	3.7 ± 0.1 e	4.9 ± 0.3 e	18.6 ± 1.1 e	This study
RC35	6.6 ± 0.9 a	10.9 ± 1.1 a	32.8 ± 2.7 a	This study
RC06	5.7 ± 0.4 bc	9.7 ± 0.8 b	26.8 ± 2.7 b	Çakmakçı *et al*. (2007a)
RC07	5.6 ± 0.5 bc	6.4 ± 0.4 d	25.3 ± 1.7 bc	Çakmakçı *et al*. (2007a)
RC08	2.6 ± 0.3 f	4.9 ± 0.5 e	13.6 ± 1.3 f	Çakmakçı *et al*. (2007a)
RC11	4.3 ± 0.7 de	7.9 ± 0.9 c	20.4 ± 1.6 de	Çakmakçı *et al*. (2007a)
RC18	1.9 ± 0.3 fg	3.7 ± 0.5 e	12.4 ± 1.5 f	Çakmakçı *et al*. (2007a)
OSU-142	6.3 ± 0.8 ab	9.6 ± 0.9 b	22.4 ± 2.1 cd	Çakmakçı *et al*. (2007a)

**Bacillus mycoides* FD07; *B. sphaericus* RC12; *B. pumilus* RC19; *Variovorax paradoxus* RC21; *Paenibacillus polymyxa* RC35; *Pseudomonas putida* RC06; *B. megaterium* RC07; *B. licheniformis* RC08; *B. subtilis* RC11; *B. cereus* RC18; *B. megaterium* M-3; and *B. subtilis* OSU-142.
†Values are means ± SE from three separate experiments. Numbers followed by the same lower-case letters in a column are not statistically different according to Duncan's multiple range test ($P \leq 0.05$). Data are the means of three replicates for IAA production in 48, 72, and 168 h cultures.
ND; not determined.

system contained 0.1 mM Tris-HCl buffer, pH 7.5, 0.5 mM EDTA, 0.2 mM NADP$^+$, and 0.6 mM G6P for G6PD, or 0.6 mM 6PGA for 6PGD, in a total volume of 1 ml. The increase in A_{340} was monitored over 3 min. One unit of enzyme activity was defined as the reduction of 1 μmol NADP$^+$ min^{-1} under the assay conditions.

Glutathione reductase (GR; EC 1.8.1.7) activity was assayed according to the method of Carlberg and Mannervik (1985). The assay system contained 0.75 mM Tris-HCl buffer, pH 7.0, 1 mM EDTA, 1 mM GSSG, and 0.1 mM NADPH, in a total volume of 1 ml. The decrease in A_{340} was monitored for 3 min. One unit of enzyme activity was defined as the oxidation of 1 μmol NADPH min^{-1} under the assay conditions.

Glutathione S-transferase (GST; EC 2.5.1.18) activity was determined as described by Habig and Jacoby (1981). The reaction medium contained 0.1 M potassium phosphate buffer, pH 6.5, 1.0 mM GSH, 1.0 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 1% (v/v) absolute ethanol, in a total volume of 1.0 ml. The reaction was monitored by the increase in A_{340} for 3 min.

All reactions were initiated by addition of the enzyme solution. All enzymatic activities were determined spectrophotometrically at 25°C using a Shimadzu 1208 UV spectrophotometer (Kyoto, Japan). One unit of enzyme activity was defined as the formation of 1.0 μmol product min^{-1} (extinction coefficients at 340 nm = 6.2 mM^{-1} cm^{-1} for NADPH, and 9.6 mM^{-1} cm^{-1} for the glutathione-2,4-dinitrobenzene conjugate). Protein concentrations were calculated from measurements of the absorbance at 595 nm according to the method of Bradford (1976) with bovine serum albumin as a standard.

TABLE II
Effect of PGPR on the activities of the anti-oxidant enzymes (GR and GST) and the pentose phosphate oxidative cycle enzymes (G6PD and 6PGD) in spinach leaves

Treatment	6PGD* (Units mg^{-1} protein)	G6PD (Units mg^{-1} protein)	GST (Units mg^{-1} protein)	GR (Units mg^{-1} protein)
Control	1.70 ef†	0.83 de	2.62 c	4.26 g
FD07#	1.52 f	0.99 b	2.14 e	4.17 gh
RC12	2.40 c	0.70 f	2.95 ab	5.03 cd
RC19	1.62 f	0.58 g	1.93 f	4.56 f
RC21	2.39 c	0.84 de	2.06 ef	5.20 c
RC35	2.26 c	0.59 g	1.71 g	4.29 g
RC06	3.01 a	1.21 a	3.08 a	4.74 ef
RC07	1.84 de	0.80 ef	2.17 e	4.99 cd
RC08	1.95 d	0.89 cd	2.59 c	4.15 gh
RC11	2.46 c	0.99 b	3.10 a	6.00 a
RC18	2.30 c	0.88 d	2.89 b	5.54 b
OSU-142	2.74 b	0.72 f	2.63 c	4.92 de
M-3	1.85 de	0.96 bc	2.42 d	3.97 h

*G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; GR, glutathione reductase; GST, glutathione S-transferase.
†Values followed by different lower-case letters in a column were significantly different ($P \leq 0.01$) using Duncan's multiple range test. Values are the average from two separate experiments and two harvests (on day-28 and day-50) with three replications (3 samples per replicate). (n = 36).
#Bacterial strains are explained in Table I.

Statistical analysis

The experiments were performed in a completely randomised design with three replicates. Each replicate consisted of six spinach seedlings at each harvest. The experiment was repeated twice. Enzyme activities were determined on three samples from each replicate. The data were subjected to analysis of variance using STATISTICA 5.1 (Stat Soft Inc., Tulsa, OK, USA) and the means were separated according to Duncan's Multiple Range Test.

RESULTS

Except for *V. paradoxus* RC21, all species and strains of PGPR used were able to produce plant growth-promoting phytohormones (Table I). The levels of IAA produced by the various PGPR tested, in the presence of 25 μg ml^{-1} tryptophan, ranged from 12.4 μg ml^{-1} for *B. cereus* RC18, to 33.2 μg ml^{-1} for *P. polymyxa* RC35. In the absence of tryptophan, all PGPR produced low levels of IAA (ranging from 1.6 to 6.6 μg IAA ml^{-1} culture).

Different rhizobacteria had variable effects (both negative and positive) on the measured enzyme activities and on the FWs and DWs of shoots and roots in spinach (Table II; Table III). Except for PGPR strains FD07, RC07, RC19, and M-3, bacterial inoculation significantly increased 6PGD activities in spinach. Strains FD07, RC06, RC11, and M-3 increased G6PD activity levels. GR and GST activities were greatest with the application of RC11, whereas the highest levels of 6PGD and G6PD activities were determined in treatments with RC06 (Table II). Four and eight of the 12 PGPR strains test selectively increased GST and GR activities in the leaves of spinach plants.

TABLE III
Effects of inoculation of spinach with plant growth-promoting rhizobacteria (PGPR) on leaf area, whole plant weight, and shoot and root fresh and dry weights

Harvest date	Treatment	Whole plant weight (g plant^{-1})	Shoot fresh weight (g plant^{-1})	Root fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Leaf area (cm^2 plant^{-1})
Day-28[†]	Control	3.43 h[*]	3.06 g	0.37 fgh	0.40 h	0.08	66 g
	FD07	3.29 h	2.96 g	0.33 h	0.38 h	0.07	63 g
	RC12	3.83 gh	3.47 fg	0.36 fgh	0.44 gh	0.09	72 fg
	RC19	3.31 h	2.97 g	0.34 gh	0.40 h	0.08	64 g
	RC21	3.98 gh	3.67 fg	0.32 h	0.47 gh	0.06	76 fg
	RC35	5.04 g	4.56 f	0.47 f	0.55 g	0.11	95 ef
	RC06	4.80 g	4.35 f	0.45 fg	0.53 gh	0.10	87 f
	RC07	4.57 g	4.19 f	0.38 fgh	0.56 g	0.08	85 fg
	RC08	4.35 gh	3.89 fg	0.46 fg	0.51 gh	0.11	81 fg
	RC11	3.98 gh	3.63 fg	0.35 gh	0.46 gh	0.09	75 fg
	RC18	4.07 gh	3.60 fg	0.46 fg	0.43 gh	0.11	75 fg
	OSU-142	4.60 g	4.19 f	0.42 fgh	0.50 gh	0.10	91 f
	M-3	4.00 gh	3.54 fg	0.46 fg	0.50 gh	0.11	76 fg
Day-50	Control	15.21 f	14.12 e	1.09 de	1.89 f	0.44	305 e
	FD07	14.94 f	13.89 e	1.05 e	1.82 f	0.45	288 e
	RC12	17.18 e	16.01 d	1.17 d	2.05 e	0.47	333 d
	RC19	15.17 f	14.07 e	1.11 de	1.85 f	0.43	297 e
	RC21	18.86 bcd	17.69 bc	1.17 d	2.26 bcd	0.47	368 bc
	RC35	20.47 a	19.07 a	1.40 ab	2.40 ab	0.59	390 ab
	RC06	20.41 a	19.09 a	1.33 abc	2.42 a	0.62	385 ab
	RC07	18.98 bcd	17.62 bc	1.36 abc	2.35 ab	0.52	352 cd
	RC08	18.13 de	16.97 cd	1.16 de	2.18 d	0.49	353 cd
	RC11	19.44 abc	18.15 ab	1.29 c	2.39 ab	0.49	377 ab
	RC18	19.96 a	18.52 ab	1.44 a	2.36 ab	0.60	380 ab
	OSU-142	19.36 abc	18.04 abc	1.32 bc	2.42 a	0.46	390 ab
	M-3	18.77 cd	17.41 bc	1.36 abc	2.22 cd	0.57	351 cd
Average[#]	Control	9.32 d	8.59 e	0.73 ef	1.14 e	0.26 c	186 f
	FD07	9.12 d	8.43 e	0.69 f	1.10 e	0.25 c	175 f
	RC12	10.51 c	9.74 d	0.77 def	1.25 d	0.28 b	203 e
	RC19	9.24 d	8.52 e	0.72 f	1.12 e	0.26 c	180 f
	RC21	11.42 b	10.68 c	0.74 def	1.36 bc	0.26 c	222 cd
	RC35	12.75 a	11.82 a	0.93 ab	1.48 a	0.35 a	242 a
	RC06	12.60 a	11.72 ab	0.89 abc	1.47 a	0.36 a	236 abc
	RC07	11.78 b	10.90 c	0.87 abc	1.45 ab	0.30 b	218 d
	RC08	11.24 b	10.43 cd	0.81 cde	1.35 c	0.30 b	217 de
	RC11	11.71 b	10.89 c	0.82 cd	1.43 abc	0.29 b	226 bcd
	RC18	12.01 ab	11.06 bc	0.95 a	1.40 abc	0.36 a	228 abcd
	OSU-142	11.98 ab	11.11 abc	0.87 bc	1.46 a	0.28 b	240 ab
	M-3	11.38 b	10.48 cd	0.91 ab	1.36 bc	0.31 b	213 de

[*]Values followed by different lower-case letters in a column were significantly different ($P \leq 0.05$) using Duncan's multiple range test.
[†]Values are the averages from the two experiments with three replications.
[#]Average of 28- and 50-day harvests.

Apart from FD07 and RC19, the ten remaining PGPR strains tested significantly increased whole plant weight (WPW), shoot fresh weight (SFW) and leaf area (LA) in spinach plants compared to the controls (Table III). The maximum SFW and shoot DWs in spinach were found after RC35 inoculation, followed by RC06 and OSU-142. The highest root FWs and DWs were observed after RC18 inoculation, followed by RC35 and RC06 treatments. In general, inoculation with RC35, RC06, RC18, or OSU-142 resulted in higher yields in terms of WPW and LA (Table III).

Four of the PGPR strains (FD07, RC12, RC19, and RC07) did not change the N content of spinach plants. On the other hand, the other eight strains significantly increased N concentrations in spinach. In addition, six of the strains (RC35, RC06, RC07, RC08, RC11, and RC21) significantly increased the P content of spinach plants, but not K, Ca, Mn, Zn and Fe concentrations (Table IV). The maximum N concentration in spinach leaves was found after RC11 treatment, followed by RC35, RC06, and OSU-142 treatments. In the case of increasing P concentrations, RC07 and RC35 were the

TABLE IV
Effect of plant growth-promoting rhizobacteria (PGPR) on macro- and micro-nutrient concentrations in spinach leaves

	Macro-nutrient (g kg^{-1} DW)					Micro-nutrient (mg kg^{-1} DW)			
Treatment	N	P	K	Ca	Mg	Fe	Mn	Zn	Cu
Control	18.9 c[*]	5.5 c	6.9 a–c	10.4 a	7.6 bc	101 a	112 a	77 a	10.1 c
FD07	19.0 c	5.2 c	6.7 bc	10.6 a	7.5 c	104 a	113 a	74 a	10.1 c
RC12	18.5 c	5.1 c	6.9 a–c	10.4 a	7.8 bc	102 a	110 a	73 a	10.3 c
RC19	18.1 c	5.1 c	7.0 a–c	9.9 a	8.0 ac	101 a	113 a	78 a	10.6 bc
RC21	20.9 b	6.0 b	7.2 a	10.2 a	8.6 a	99 a	105 a	77a	10.5 bc
RC35	24.0 a	6.9 a	7.3 a	10.0 a	8.3 ab	108 a	104 a	72 a	11.0 ab
RC06	24.0 a	6.0 b	7.0 a–c	10.1 a	8.5 a	103 a	107 a	72 a	10.4 c
RC07	18.6 c	6.7 a	7.0 a–c	10.3 a	8.5 a	104 a	113 a	76 a	11.2 a
RC08	21.1 b	6.2 b	6.7 bc	9.9 a	7.9 ac	102 a	105 a	76 a	11.6 a
RC11	24.1 a	6.1 b	6.9 a–c	10.5 a	7.5 c	107 a	111 a	73 a	11.5 a
RC18	20.6 b	5.4 c	6.8 bc	9.8 a	8.6 a	102 a	105 a	77 a	11.2 a
OSU-142	23.9 a	5.4 c	7.2 ab	10.5 a	8.2 a–c	107 a	106 a	74 a	10.6 bc
M-3	20.2 b	5.5 c	7.0 a–c	10.1 a	7.7 bc	104 a	105 a	74 a	10.5 c

[*]Mean values in the same column followed by the same lower-case letters did not differ significantly by Duncan's multiple range test at $P \leq 0.05$.

most effective, followed by RC08, RC11, RC06 and RC21.

DISCUSSION

The present study showed that 11 of the PGPR strains tested were able to produce and secrete IAA under *in vitro* conditions. *Paenibacillus polymyxa* RC35 was determined to be the best in terms of IAA production, and all plant yield parameters, compared to the other treatments. A positive relationship between the amount of IAA secreted and plant yield values was also observed for some of the other PGPR treatments such as RC06, RC07, and OSU-142 (Table I; Table III). This result confirmed the evidence in previous studies suggesting that the production of hormones is one of the mechanisms by which PGPR stimulate plant growth (Steenhoudt and Vanderleyden, 2000; Çakmakçı *et al*., 2007b). In contrast, RC18 was one of the lowest IAA producing PGPR strains, but good for yield parameters (Table I; Table III). This observation suggests that production of IAA is not the only PGPR trait responsible for the enhancement of spinach growth. As reported previously, the effect of PGPR is a complex process, and depends on the bacterial strain and population, on the plant-bacterial strain combination, the plant genotype, the growth parameters evaluated, and environmental conditions (Şahin *et al*., 2004; Çakmakçı *et al*., 2006). In addition, our results showed that some PGPR (e.g., RC35, RC06, RC11, OSU-142, RC21, RC07, RC18, and M3) treatments significantly increased N and/or P uptake in a similar way to their effects on increasing the growth and yield parameters of spinach plants.

Under greenhouse conditions, our previously isolated IAA-producing and P-solubilising PGPR strain, RC11, caused the maximum enhancement in GR and GST activities in spinach, while the N_2-fixing and IAA-producing strain RC06 was the most effective promoter of G6PD and 6PGD activities. This provides clear evidence for increased GR activity induced by PGPR inoculation in spinach leaves. Medici *et al*. (2004) showed, in three plant species, that GR activity increased at high levels of N supply compared with low N. Ammonium-fed plants showed higher GR activities in maize and sunflower leaves, with the highest GST activity in maize (Rios-Gonzalez *et al*., 2002). Other studies have indicated that G6PD and 6PGD activities are higher in roots supplied with NO_3^- (Bowsher *et al*., 1989; Wright *et al*., 1997) and these activities depend on changes in plant growth and development (Esposito *et al*., 2001).

The observed changes in enzyme activities appeared to be triggered by the PGPR strain selected. Since PGPR inoculation caused a differential increase in leaf 6PGD activity, as well as activation of other plant enzymes, this may indicate that activation of these enzymes in spinach leaves would be differentially affected by different PGPR strain. Thus, the growth and yield parameters of spinach seedlings could be enhanced by PGPR treatment due to increases in the activities of enzymes which have an important role in nitrate assimilation as well as in water and nutrient use efficiency. Additional studies are required to confirm the effects of PGPR strains on the different enzyme activities responsible for plant nutrient uptake in other plant species under different conditions.

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