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Short communication

Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions

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Abstract

This study was conducted with sugar beet in greenhouse and field at two soil type with different organic matter (containing 2.4 and 15.9% OM, referred as the low- and high-OM soil) conditions in order to investigate seed inoculation of sugar beet, with five N_2-fixing and two phosphate solubilizing bacteria in comparison to control and mineral fertilizers (N and P) application. Three bacterial strains dissolved P; all bacterial strains fixed N_2 and significantly increased growth of sugar beet. In the greenhouse, inoculations with PGPR increased sugar beet root weight by 2.8–46.7% depending on the species. Leaf, root and sugar yield were increased by the bacterial inoculation by 15.5–20.8, 12.3–16.1, and 9.8–14.7%, respectively, in the experiment of low- and high-OM soil. Plant growth responses were variable and dependent on the inoculants strain, soil organic matter content, growing stage, harvest date and growth parameter evaluated. The effect of PGPR was greater at early growth stages than at the later. Effective *Bacillus* species, such as OSU-142, RC07 and M-13, *Paenibacillus polymyxa* RC05, *Pseudomonas putida* RC06 and *Rhodobacter capsulatus* RC04 may be used in organic and sustainable agriculture.

Keywords: Plant growth-promoting bacteria; Nitrogen fixation; Phosphate solubilization; Organic matter; Sugar beet; *Bacillus*; *Paenibacillus*; *Rhodobacter*; *Pseudomonas*

Nitrogen and phosphorus are essential nutrients for plant growth and development. Intensive agriculture entails the risk of excessive fertilization. Microorganisms are important in agriculture in order to promote the circulation of plant nutrients and reduce the need for chemical fertilizers as much as possible. Plant growth-promoting rhizobacteria (PGPR) are able to exert a beneficial effect upon plant growth. N_2-fixing and P-solubilizing bacteria may be important for plant nutrition by increasing N and P uptake by the plants, and playing a significant role as PGPR in the biofertilization of crops. Biological N fixation (BNF) provides a major source of nitrogen for plants as part of environmental friendly agricultural practices.

Trials with rhizosphere-associated plant growth-promoting N_2-fixing and P-solubilizing *Bacillus* species indicated yield increases in rice (Sudha et al., 1999), sugar beet (Çakmakçi et al., 1999), wheat (de Freitas, 2000), canola (de Freitas et al., 1997), maize (Pal, 1998), and conifer species (Bent et al., 2002). One of the most often reported plant growth-promoting rhizobacteria (PGPR) is *Bacillus polymyxa*, now named *Paenibacillus polymyxa* (Timmusk et al., 1999). It has a range of reported properties, including nitrogen fixation (Coelho et al., 2003); P-solubilization (de Freitas et al., 1997), production of antibiotic (Rosado and Seldin, 1993), cytokinin (Timmusk et al., 1999), hydrolytic enzymes (Nielsen and Sørensen, 1997), colonization hair and cortical cells (Shishido et al., 1999), and increased root and shoot growth of crops (Sudha et al., 1999). Some strains of *Rhodobacter* are known to fix N_2 (Drepper et al., 2002), but they have not been extensively studied (Gallon, 2001). *Pseudomonas* inoculants significantly increased root dry weight in spring wheat (Walley and Germida, 1997), yield in sugar beet (Çakmakçi et al., 2001), colonized winter wheat roots (de Freitas and Germida, 1992), could effectively adapt to new environments (Misko

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and Germida, 2002), and promoted the growth of the spinach (Urashima and Hori, 2003).

The aim of this study was to evaluate the efficiency of novel N_2-fixing and P-solubilizing bacterial strains isolated from barley and wheat rhizosphere soils, and previously identified strains. We, therefore, investigated the effectiveness of PGPR in sugar beet in the greenhouse; and evaluate the effect of inoculation on the yield and quality of sugar beet at two soil organic matter content in field.

Five bacterial strains were isolated from the rhizosphere field-grown crops and identified as *Bacillus megaterium* RC07, *Bacillus licheniformis* RC08, *Paenibacillus polymyxa* RC05, *Rhodobacter capsulatus* RC04 and *Pseudomonas putida* RC06 (with MIS similarity index (SIM) of 0.786, 0.643, 0.809, 0.841 and 0.776, respectively) based on fatty acid methyl ester analysis using MIDI system. The bacterial strains, *Bacillus* OSU-142, were originally isolated from tomato plants at the Ohio State University (USA), and *Bacillus* M-13 was isolated from pepper plants at Atatürk University, Turkey (Şahin et al., 2004). *Bacillus* OSU-142 was the most effective N_2-fixing bacteria in the previous field experiments with sugar beet and barley (Çakmakçi et al., 2001). In the present study, P-solubilizing activities of five new and two previously isolated strains (positive control) were measured according to the qualitative and quantitative methods (Pal, 1998; Mehta and Nautiyal, 2001). All the isolates were tested for their phosphate solubilizing capacities in sucrose–tricalcium phosphate agar media (Pikovskaya, 1948) by inoculating 1 ml of 6 days old culture (density 4×10^9) in 250 ml of Erlenmear flasks in triplicate containing 500 μg/ml of P as Rock Phosphate (RP) at 30 ± 1 °C. It was also observed that three strains had highest capacity for RP in media. *Bacillus* M-13, RC07 and RC08 isolates were capable of dissolving insoluble P, respectively, by 38.3, 35.7 and 26.2 μg P solubilized per ml culture per day. The bacterial strains were characterized by morphological, biochemical and physiological tests including pigment production on nutrient agar medium and the Gram reaction (Forbes et al., 1998). Nitrogen fixation of the isolates was determined in nitrogen free medium by the acetylene reduction assay (Hardy et al., 1968). Cultures for the acetylene reduction assay were prepared as Holguin and Bashan (1996) and incubated at 30 °C for 24 and 48 h without agitation. Ethylene production was measured using a Hewlett Packard gas chromatograph (Model 6890, USA). Among the seven strains, the strains RC05, isolated from wheat soil, exhibited highest nitrogenase activity (0.68 ± 0.15 nmol C_2H_4, 10^7 cfu/h) and for other strains, the activity ranged from 0.17 to 0.54 nmol C_2H_4/h. All isolates were oxidase, nitrate reduction, acetylene reduction assay (ARA) and catalase positive and were also able to grow in N-free basal medium.

Bacterial strains were initially isolated from the rhizosphere of wheat and barley. Plants were uprooted along with good amount of non-rhizosphere soil, brought immediately to the laboratory in polythene bags and air-dried. The non-rhizosphere sandy-loam soil was removed by gentle shaking, whereas the soil adhering strongly to the root was referred to as rhizosphere soil. Ten grams soil from each sample was aseptically weighed and transferred to an Erlenmeyer flask with 100 ml sterile water, and was shaken for 30 min at 150 rpm. Immediately after shaking, a series of 10-fold dilutions of the suspension was made for each sample by pipetting 1-ml aliquots into 9 ml sterile water. The final dilution was 10^5-fold; 0.1 ml of each dilution of the series was placed onto a petri dish with NA. Three replicate dishes were made for each dilution. Dishes were placed in an incubator at 28 °C for 7 days. Rhizobacteria isolates were selected to represent distinct types based on differences in colony morphology including: colony form, elevation and pigment production. Isolates were restreaked on NA and checked for purity. Isolated bacterial strains were identified based on whole-cell fatty acids methyl esters (FAMEs) analysis (de Freitas et al., 1997) performed according to the method described by manufacturing manual MIDI system. The bacterial strains were maintained for long-term storage in nutrient broth with 15% glycerol at −80 °C for further tests. For this experiment, pure cultures were grown in nutrient broth (NB) at 28 °C and diluted to a final concentration of 10^9 cfu/ml in sterile distilled water containing 0.025% Tween 20. Sugar beet seeds were surface-sterilized in 70% ethanol for 2 min and 1.2% sodium hypochlorite for 10 min, and rinsed 10 times in sterile tap water. Cell densities in the suspension were adjusted to a final density of approximately 10^8 cfu/seed.

Pots were sterilized with 20% sodium hypochlorite solution, filled with soil and seeded. The soil was a loam with an organic matter content of 1.8 and 0.4% lime (pH 6.9). Available P and mineral NH_4^+–N and NO_3^-−N contents were 13.9, 10.9 and 8.5 mg/kg, respectively. Exchangeable K and Ca+Mg were 1.6 and 17.8 mequiv./100 g; available Fe, Mn, Zn and Cu contents were 4.7, 3.4, 2.6 and 1.8 ppm. The following treatments with three replicates were investigated: (1) control (without bacteria inoculation and mineral fertilizers), (2) N fertilizer (60 mg N/kg soil), (3) P fertilizer (25 mg P/kg soil), (4) *B. megaterium* RC07, (5) *Bacillus* M-13, (6) *B. licheniformis* RC08, (7) *R. capsulatus* RC04, (8) *P. polymyxa* RC05, (9) *P. putida* RC06, (10) *Bacillus* OSU-142. The pots were arranged in a completely randomized factorial design in the greenhouse. Seeds were placed at the same depth (approximately 2.5 cm below the soil surface) in all pots. The seedlings were grown in a greenhouse under natural light which provided a 15 h photoperiod, temperatures of 16–25 °C and relative humidity 55%. Sixteen pots were used for each treatment at 60, 75, 150 and 165 days after inoculation sugar beet seedling four pots all treatments at each sampling time were removed. The pots were watered to 60% water holding capacity and were maintained at this moisture content by watering to weight every 2–3 days.

Two years replicated field trials were conducted with the same treatments as used in pot experiment. In order to investigate the effects of seed inoculation with seven PGPR on the yield and yield components of sugar beet (*Beta vulguris* cv. *Loretta*) at two soil type with different organic matter (OM) content, 400 m apart from each other, were done in 2003 and 2004. The experimental soil was a sandy loam with organic matter content of 15.9 and 1.1% lime content (pH 6.8). At site

I, available P_2O_5 and K_2O contents were 216 and 1268 kg/ha. At site II, soil was a sandy loam with 2.4% organic matter and 2.5 lime content (pH 7.5). Available P_2O_5 and K_2O contents were 62 and 1165 kg/ha, respectively. The soil with 2.4% OM was referred as the low-OM soil and that with 15.9% as the high-OM soil.

The experimental design consisted of four completely randomized blocks in a factorial arrangement having 11 treatments as 7 PGPR, nitrogen (N) and phosphorus (P) fertilizer applications as well as a control treatment without inoculation and any fertilizer application. Sugar beet received 110 kg N/ha in urea form (N) plots and 90 kg P_2O_5/ha in the form of triple super phosphate (P) plots. N fertilizer was applied during disk-harrowing in spring and before the first hoeing in equal amounts. Triple super phosphate was applied during deep ploughing in autumn. Sugar beet seeds were hand sown (with a plot drill) in 6 m$\times$2.25 m plots so as to give 45 inter- and 5 cm intra-row spacing in five rows on 1 May in 2003 and 2004 following the bacterial inoculation of seeds depending on the treatment. When seedling reached 2–4 leaf-stage, hoeing was done by hand and repeated as required. At 4–6 leaf-stage, thinning to 20–25 cm intra-row plant spaces was performed. Weeds were removed by hand. Plots were irrigated five times depending on the visual inspection of plants starting in the first half of July until 2–3 weeks prior to harvest. Harvesting was done on the 15th of October in both years excluding one side row and 1 m from each end of plots. Total yield, sugar content and the concentration of impurities, α-amino N, Na and K contents in beet were determined by standard methods (Last et al., 1976). Data were also made at harvest on leaf yield, root yield (RY), sugar content (SC), α-amino N, sodium and potassium contents. White sugar content (WSC) and white sugar yield (WSY) were calculated as $\mathrm{WSC} = \mathrm{SC} - [0.343(\mathrm{Na} + \mathrm{K}) + 0.094\alpha - \text{amino N} + 0.29]$ and $\mathrm{WSY} = (\mathrm{WSC}/100)\text{root yield (t/ha)}$ (Reinefeld et al., 1974). The data were subjected to analysis of variance using Statistica 5.1 and means were separated according to Duncan Multiple Range Test (at $P < 0.05$).

In the pot, trial PGPR inoculation increased the weight of both tops and roots throughout the season, although the difference in weight of tops and root diminished during the end of the growing period (Table 1). The first sampling data weight of per plant were greater or equally between N fertilizer and six PGPR strains but especially after day 150, growth appeared to have showed down PGPR responses as the differences were compared to N fertilizer. At the final harvest in pots, leaf, root and sugar weight of sugar beet were significantly greater than the control in all treatments except the *B. licheniformis* RC08 inoculation. The root sugar weight in the inoculated with OSU-142, RC05, RC07 and M-13 were significantly greater than other treatments in the first sampling date. Sugar beet plants inoculated with four PGPR strains (RC07, M-13, RC05 and OSU-142) generally grew better and had a higher total sugar content 60 days after sowing than did plants fertilized with N or P. This work indicated that growth promotion effects were seen early in plant development, and these subsequently translated into higher yields. Similar results were reported in some of

Table 1
The effect of PGPR and fertilizer application on the leaf, root and sugar weight in the individual plant of sugar beet in the greenhouse with time (average of four replications)

Treatment	Days after inoculation											
	60 days			75 days			150 days			165 days		
	LW	RW	SW	LW	RW	SW	LW	RW	SW	LW	RW	SW
Control	70 c	30 f	3.0 e	168 f	78 f	9.2 e	315 g	497 e	89 f	282 e	607 f	115 e
N	90 a	38 cde	3.7 cd	223 a	106 ab	11.7 bc	449 a	689 a	116 a	427 a	835 a	148 a
P	72 bc	35 de	3.4 de	180 e	85 e	9.6 e	362 e	560 d	96 e	318 d	677 d	124 d
RC07	88 a	42 abc	4.3 ab	213 bc	98 cd	11.5 cd	394 d	617 c	110 c	354 c	752 c	141 b
M-13	84 ab	40 bc	4.2 ab	189 d	93 d	10.9 d	342 f	550 d	97 e	308 d	670 d	125 d
RC08	74 bc	34 e	3.4 de	169 f	84 e	9.6 e	319 g	507 e	89 f	280 e	624 e	113 f
RC04	89 a	36 de	3.6 cd	209 c	104 bc	12.0 bc	395 d	613 c	107 d	355 c	750 c	138 c
RC05	90 a	43 ab	4.4 a	219 ab	106 ab	12.3 ab	427 b	661 b	115 a	382 b	808 b	149 a
RC06	85 ab	39 cd	3.9 bc	208 c	99 c	11.4 cd	403 c	619 c	108 c	385 b	751 c	139 c
OSU-142	91 a	45 a	4.5 a	220 ab	111 a	12.7 a	428 b	657 b	114 b	356 c	802 b	148 a

LW, leaf weight (g/plant); RW, root weight (g/plant); SW, sugar weight (g/plant); RC07, *Bacillus megaterium* RC07; M-13, *Bacillus* M-13; RC08, *Bacillus licheniformis* RC08; RC04, *Rhodobacter capsulatus* RC04; RC05, *Paenibacillus polymyxa* RC05; RC06, *Pseudomonas putida* RC06; OSU-142, *Bacillus* OSU-142.

the previous studies showing that inoculation was found to affect early plant and root development, plant and root dry weight, grain yield and the N-uptake efficiency of plants (Dobbelaere et al., 2002). PGPR inoculation strongly influenced the weight of root, leaf and sugar during the early stages of growth. The early leaves in the PGPR grew to larger than fertilizer. Faster early response in PGPR caused leaves to expand faster, but in addition to root on the plants also reached faster 'growth point date'. Start of full ground coverage is approximated by the 'growth point date', which is defined as the date on which a beet contains 4 g of sugar (Spitters et al., 1990). With PGPR growth point date was reached 60 days after sowing. This point also marks the start of secondary thickening of the tap root and is important for later growth. Inoculated plants reached 'growth point date' faster than fertilizers and control plants. These results indicated that the PGPR promote plant growth and protection will remain high during the early stages of growth after sowing. This is the period when young seedlings and plants are so vulnerable to environmental stresses. Also, it is when the greatest loss in potential crop yield and quality can occur.

As all selected PGPR had promising positive effects on growth and yield parameters of sugar beet under greenhouse conditions, all the PGPR except RC08 increased leaf, root and sugar yield of sugar beet under both field conditions (Table 2). In the field, PGPR isolate *P. polymyxa* RC05 caused maximum enhancement in the leaf yield of sugar beet, while *Bacillus* OSU-142 was the most effective promoter of root and sugar yield. Inoculations with *P. polymyxa* enhanced grain yield of wheat (de Freitas, 2000), and stimulated growth, the growth of rice (Sudha et al., 1999) and conifer species (Bent et al., 2002). Recently, Xavier and Germida (2003) reported that *Bacillus pabuli* could have enhanced the shoot growth of pea.

Our data showed that two of the bacterial strains tested, RC07 and M-13, gave similar increases in root and sugar yield consistently to sole P application. This result in the present study may be explained with P-dissolving as well as N_2-fixation capabilities of PGPR strains reported in our previous studies (Çakmakçi et al., 2001; Şahin et al., 2004). Some of the bacteria may solubilize inorganic P due to excretion of organic acids (Hoberg et al., 2005). Plant response to these bacteria could be associated with other mechanisms, rather than by direct N_2-fixation and P solubilization. Plant growth parameters were generally enhanced by PGPR inoculation, whereas in pot and two soils experiments responses varied, it is suggested that bacterial interactions within the rhizosphere may have played an important role in restricting expression of growth promotion. It is particularly interesting that sugar and white sugar content of sugar beet grown in two soils was significantly enhanced by PGPR than

Table 2
Yield and yield components of sugar beet in response to inoculation with PGPR and mineral fertilizers in high-OM and low-OM soil in the field as an average of 2003 and 2004

Sites	Treatments	Leaf yield (t/ha)	Root yield (t/ha)	Sugar content (%)	White sugar content (%)	White sugar yield (t/ha)
I High-OM soil	Control	25.0 gh	49.8 f	18.74 bc	16.16 d	8.03 hi
	N fertilizer	33.0 a	62.1 a	17.97 g	15.36 g	9.54 a
	P fertilizer	27.9 de	55.5 d	18.41 ef	16.01 e	8.88 e
	RC07	27.8 de	56.9 c	18.55 de	16.19 cd	9.21 b
	M-13	27.4 e	55.6 d	18.58 cd	16.17 d	8.99 de
	RC08	26.0 f	50.3 ef	18.56 de	16.16 d	8.17 g
	RC04	28.4 d	56.0 d	18.36 f	15.96 e	8.93 de
	RC05	30.2 b	57.7 b	18.39 ef	15.86 f	9.15 bc
	RC06	29.4 c	56.8 c	18.41 ef	15.90 e	9.03 cd
	OSU-142	28.5 d	57.8 b	18.40 ef	15.89 e	9.19 b
II Low-OM soil	Control	22.0 j	44.8 h	18.98 a	16.62 a	7.44 j
	N fertilizer	29.8 bc	55.6 d	18.07 g	15.55 f	8.64 f
	P fertilizer	25.2 gh	49.7 f	18.76 b	16.39 b	8.13 gh
	RC07	24.1 i	48.6 g	18.76 b	16.39 b	7.97 i
	M-13	24.1 i	48.3 g	18.68 bcd	16.34 bc	7.90 i
	RC08	21.7 j	44.9 h	18.73 bc	16.28 bcd	7.31 j
	RC04	24.5 hi	48.7 g	18.61 bcd	16.29 bcd	7.93 i
	RC05	25.4 fg	50.0 ef	18.58 cd	16.22 cd	8.11 gh
	RC06	25.3 fg	48.9 g	18.64 bcd	16.18 cd	7.91 i
	OSU-142	25.3 fg	50.3 ef	18.71 bcd	16.26 bcd	8.17 g
Average	Control	23.5 g	47.3 e	18.86 a	16.39 a	7.73 e
	N fertilizer	31.4 a	58.9 a	18.02 e	15.45 e	9.09 a
	P fertilizer	26.5 d	52.6 c	18.59 bcd	16.20 bc	8.51 cd
	RC07	25.9 ef	52.8 c	18.66 b	16.29 ab	8.59 bc
	M-13	25.8 f	52.0 d	18.63 bc	16.26 b	8.44 d
	RC08	23.9 g	47.7 e	18.65 b	16.23 b	7.74 e
	RC04	26.4 de	52.3 cd	18.49 d	16.12 cd	8.43 d
	RC05	27.8 b	53.9 b	18.49 d	16.06 d	8.63 b
	RC06	27.3 bc	52.8 c	18.52 cd	16.04 d	8.47 d
	OSU-142	26.9 cd	54.0 b	18.56 bcd	16.07 d	8.68 b

Values followed by different letters in a column were significantly different ($P<0.05$), using Duncan's multiple range test.

N fertilizers. These observations indicate that mechanisms of growth promotion other than N_2 fixation, such as phytohormone production, improved nutrient uptake balance, may be attributable to these PGPR. Some other studies have reported that plant growth may be affected by the synthesis of phytohormones and vitamins, inhibition of plant ethylene synthesis, improved nutrient uptake and solubilization of inorganic phosphate (Dobbelaere et al., 2003; Lucy et al., 2004).

Bacterial inoculations and fertilizer application also affected quality parameters investigated. N fertilizer significantly reduced sugar content compared with the control and the PGPR. Late season uptake of N increases soluble N in harvested beet by increasing the amount of N in the crop at a time when it cannot be fully utilized for growth, resulting in the decrease of the efficiency of sucrose extraction within the factory. In addition, Na and K are also impurities, which may be increased by the application of N fertilizer at high rates (Allison et al., 1996). Moreover, lower leaf/root ratio in bacterial inoculation in contrast with N application could be of importance in indicating earliest harvest maturity dates in the areas with relatively shorter vegetation period. More balanced uptake of minerals in the presence of N_2 fixation rectified the quality as compared with reduced quality in the plots receiving N fertilizers. N application enhances leaf growth but reduced sugar and white sugar content compared with PGPR. In particularly, beet given too much fertilizer N contains smaller content of sugar and higher impurity content, both of which decrease the efficiency of sugar extraction.

Two years of trials under different field conditions showed that treatments including bacterial seed inoculations and fertilizer applications significantly affected the parameters investigated compared with control in sugar beet depending on the years and soil types. As an average of the years PGPR inoculation increased leaf yield by 4.0–20.8% and 1.4–15.5% at the high- (15.9% OM) and low-OM (2.4% OM) soil, whereas, N and P fertilizer increased leaf yield by 32.0 and 11.6% and 31.4 and 14.5%, respectively, compared with control (Table 2). The beet from the low-OM soil had significantly lower yields and higher sugar content in their root portions than beet from the high-OM soil. Sugar content of beet grown in the high-OM soil was generally lower than in the low-OM soil. The plant growth-promoting ability of PGPR inoculation varied with soil organic matter content. On the other hand, growth promotion of sugar beet with tested PGPR strains strongly depend on soil organic matter content. Organic compound can be used as carbon and energy sources by microorganisms, microbial growth and activity is particularly intense in rhizosphere. In general, the number, diversity, and activity of soil organisms are influenced by soil organic matter properties (Kobabe et al., 2004). Organic matter content may have contributed to the development of different microbial community structures in the soils (Clegg et al., 2003; Marschner et al., 2003). Also, the growth and metabolic activity of soil microorganisms are limited by the availability of nutrients (Welbaum et al., 2004). Mineral nutritional factors can affect the number of bacteria in the rhizosphere (Marschner et al., 1999).

Plant growth responses were variable and dependent on bacterial strains, harvest dates and growth parameters evaluated. It was found that out of seven PGPR strains that promoted the growth, only one was ineffective in promoting the growth of sugar beet. We have observed different responses of plants to inoculation with PGPR in regard to growth conditions and different soils. Our study demonstrates the importance of evaluating potential growth-promoting bacteria under a variety of experimental conditions and plant stages and/or soil OM content.

The results of the trials reported in this study indicate that from soils with PGPR inoculation a higher yield potential can be expected. In particular, newly N_2-fixing bacterial strain *P. polymyxa* RC05, *P. putida* RC06 and *R. capsulatus* RC04 and P-solubilizing *B. megaterium* RC07 have great potential being formulated and used as biofertilizer. In view of environmental pollution due to excessive use of fertilizers and high costs of the production of N fertilizers, bacteria tested during our study may well be used to achieve more sustainable and environmental friendly agricultural production. The experiment revealed that the PGPR inoculation was an effective treatment to improve the parameters measured of sugar beet, especially with reference to the increase growth responses early in the season. As free living bacteria depend on soil organic matter as a food source, addition of organic matter to the soil may be increased nitrogen fixing and plant growth-promoting activity of PGPR. The favourable effect of the inoculation on plant growth and improved N and P nutrition may be due to growth-promoting substances by plant growth rhizobacteria.

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