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Pesticide tolerant and phosphorus solubilizing *Pseudomonas* sp. strain SGRAJ09 isolated from pesticides treated *Achillea clavennae* rhizosphere soil

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Abstract In this study, an attempt was made to identify an effective phosphate solubilizing bacteria from pesticide polluted field soil. Based on the formation of solubilization halo on Pikovskaya's agar, six isolates were selected and screened for pesticide tolerance and phosphate (P) solubilization ability through liquid assay. The results showed that only one strain (SGRAJ09) obtained from *Achillea clavennae* was found to tolerate maximum level of the pesticides tested and it was phylogenetically identified as *Pseudomonas* sp. It possessed a wide range of pesticide tolerance, ranging from 117 μg mL^{-1} for alphamethrin to 2,600 μg mL^{-1} for endosulfan. The available P concentrations increased with the maximum and double the maximum dose of monocrotophos and imidacloprid, respectively. On subjected to FT-IR and HPLC analysis, the presence of organic acids functional group in the culture broth and the production of gluconic acid as dominant acid aiding the P solubilization were identified. On comparison with control broth, monocrotophos and imidacloprid added culture broth showed quantitatively high organic acids production. In addition to gluconic acid production, citric and acetic acids were also observed in the pesticide amended broth. Furthermore, the *Pseudomonas* sp. strain SGRAJ09 possessed all the plant growth promoting traits tested. In presence of monocrotophos and imidacloprid, its plant growth promoting activities were lower than that of the pesticides unamended treatment.

Keywords HPLC · *Pseudomonas* sp. · Organic acid · Pesticide · P solubilization · Gluconic acid

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Introduction

Phosphorus (P) is an essential major nutrient required for growth and development of plants. P nutrition is important for the root growth and to obtain a maximum productivity, plants require available P concentration of 30 μMol L^{-1} in the soil solution. Owing to the low solubility of P minerals and constant fixation of available P as inorganic phosphate (calcium, aluminium and iron phosphates), the P availability in soil is lesser than 1 μMol L^{-1}. The role of microorganisms is inevitable in enhancing the P availability of the soil environment as they solubilizes insoluble P, mineralizes the organic P and plays a major role in P nutrition. Solubilization of insoluble P had been previously documented in rhizosphere soil bacteria like *Enterobacter*, *Azospirillum*, *Pseudomonas*, *Bacillus*, *Serratia*, *Pantoea* and *Rhizobium*; those bacteria were also studied for the plant growth promotion (PGP) traits (Hwangbo et al. 2003; Rodríguez et al. 2004; Son et al. 2006; Hameeda et al. 2008; Park et al. 2009; Vyas and Gulati 2009; Ahemad and Khan 2012).

Pesticides are xenobiotic compounds that are deliberately spread into the environment to control the pest that affects crop production. On application into the soil, it may harm the native microbial population, affects bacterial diversity and influences the soil biochemical processes including degradation of organic matter, nitrogen fixation, nitrification, denitrification, ammonification and P solubilization (Niewiadomska 2004). Recently, few studies had

elucidated the effect of pesticides on the bacterial growth and their role in phosphate solubilization (Ramani 2011; Ahemad and Khan 2011; Ahemad and Khan 2012). Furthermore, reduction in the P solubilizing activity of *Klebsiella* sp. was noticed in single or double or triple the recommended dose of pesticides like pyriproxyfen, fipronil, imidacloprid and thiomethoxam. In particular, in the triple recommended dose of pyriproxyfen treatment, more than 90 % reduction in P solubilizing activity was noted (Ahemad and Khan 2011). Similar results were observed in P solubilizing *Pseudomonas putida*, when the medium was added with triple the recommended dosage of fungicides like tebuconazole, hexaconazole, metalaxyl and ketazin (Ahemad and Khan 2012). Conversely, in the presence of chlorpyrifos, P solubilizing activity of *Bacillus sphaericus* and *Burkholderia cepacia* were noticed higher than that of the control treatment (Ramani 2011). In another study, the population of phosphate solubilizing bacteria (PSB) was significantly increased when insecticides like phorate and BHC were added to the soil, the authors speculated that the PSB may utilized those pesticides as their energy and nutrients source (Das and Mukherjee 1998).

Phosphorus solubilization is mediated through the decrease in the pH of the medium and by the complexing ability of the cation which is bound to the PO_4^{2-} ions. The decrease in pH is associated with organic acid excretion and proton extrusion accompanying ammonium ion assimilation (Vassilev et al. 2006; Park et al. 2009). Organic acids mediated P solubilization and chelation was proposed as a prevalent mechanism aiding P availability (Park et al. 2009). During metabolic processes, various types of organic acids were excreted by the soil dwelling microbes as byproduct of metabolism, these includes gluconic, 2-keto gluconic, 5-ketogluconic, tartaric, acetic, formic, oxalic, malic, alpha ketoglutaric, succinic, citric, propionic and lactic acid (Saravanan et al. 2007a; Park et al. 2009; Vyas and Gulati 2009). In general, mineral P solubilization property is common among gram negative bacteria than gram positive. This is due to the fact that it expresses extracellular aldose oxidation pathway in the periplasmic space that helps in catabolism of hexose sugar like glucose, hence driving large amount of organic acids (gluconates and their derivatives) into the surrounding medium, which might play a vital role in mineral phosphate solubilization (Goldstein et al. 1993; Rodríguez and Fraga 1999). These acids possess certain unusual chelating properties like both their COOH and OH group can form complexes with metals and ions. It was previously reported that sugar acid like 2-ketogluconic acid have multiple conformations and therefore may chelate ions by unusual molecular mechanism (Goldstein 1995).

The *Pseudomonas* spp. has a long standing history of plant associated occurrence, especially in rhizosphere and thus they impart plant growth promoting activities (Lugtenberg and Dekkers 1999; Ahmad et al. 2008). Particularly, their P nutrition potential for the crop plants had been studied by various research groups (Hameeda et al. 2008; Park et al. 2009; Vyas and Gulati 2009). In the present study, pesticides tolerating and P solubilizing *Pseudomonas* sp. strain SGRAJ09 was recovered from the rhizosphere soil of *Achillea clavennae* and its PGP abilities were studied in the presence of monocrotophos and imidacloprid. Furthermore, this is a significant study that depicts the effect of pesticides on the qualitative and quantitative production of organic acids.

Materials and methods

Isolation and screening of PSB

Rhizosphere soil samples of *Solanum lycopersicum*, *Acalypha coryloides* and *A. clavennae* grown in pesticides treated fields around Vellore Institute of Technology, Vellore, Tamil Nadu, India were collected using a sterile container. The collected samples were transported to the laboratory immediately for isolating the PSB. The isolation of PSB from the soil samples were performed using spread plate assay. For this, 100 µL of appropriate soil dilutions were spread on freshly prepared Pikovskaya agar medium (g L^{-1}: glucose 10; $Ca_3(PO_4)_2$ 5; $(NH_4)_2SO_4$ 0.5; NaCl 0.2; $MgSO_4 \cdot 7H_2O$ 0.1; KCl 0.1; yeast extract 0.5; $MnSO_4$ and $FeSO_4$ trace; agar 15; pH 7.0). All the plates were incubated at 28 ± 2 °C for 7 days and colonies showing clear halo against an opaque background were considered as P solubilizers. Further the isolates were purified in Pikovskaya agar and maintained in glycerol stock at −80 °C.

Assessment of phosphate solubilization by the bacterial strains in presence and absence of pesticides

The P solubilization potential of the strains were assessed in the National Botanical Research Institute's phosphate (NBRIP) broth (g L^{-1}: glucose 10; $Ca_3(PO_4)_2$ 5; $MgSO_4 \cdot 7H_2O$ 0.25; $MgCl_2 \cdot 6H_2O$ 0.5; KCl 0.2; $(NH_4)_2SO_4$ 0.1; pH 7.0) both in presence and absence of the pesticides. For pesticide amendment in the broth, both commercial and technical grade pesticides were used in this study; they were received as gift from the Pest Control India, Mumbai, India (Table 2). The P solubilization under pesticides presence were tested using, pesticide unsupplemented broth as control, broth containing highest recommended dosage of pesticide (1×) and double the maximum dosage (2×) as treatments. Further, PSB strains were inoculated in pesticide amended broth and incubated at 28 ± 2 °C at 120 rpm for 6 days. The available P level in various

treatments were measured in the culture supernatants on the 2nd, 4th and 6th day of experiment using vanado-molybdate method as described by Gulati et al. (2008). The amount of available P was calculated using the standard curve prepared with KH_2PO_4. The concomitant change in pH following tri-calcium phosphate (TCP) solubilization was also recorded.

Screening of bacterial supernatants for organic acids by FT-IR and HPLC analysis

To test the type of organic acids produced during P solubilization, the culture supernatants of NBRIP broth in the presence and absence of monocrotophos and imidacloprid were analyzed in FT-IR. Six-days-old NBRIP culture broth was centrifuged (10,000 rpm, 20 min) and the clear supernatant was lyophilized at −50 °C for further use. Using a pestle and mortar, appropriate micrograms of lyophilized supernatant were finely ground with 300 mg of KBr. The homogenized mixture was placed into a stainless steel holder and was made into pellets by applying pressure ranging from 7,500 to 1,500 cm^{-2} for 3 min. The infrared spectrum of each sample was recorded using IR Affinity-1 (Shimadzu) equipped with DLATGS detector and temperature control mechanism. High energy ceramic light source was employed and the acquisition parameters were in 4 cm^{-1} resolution within the range of 4,000–400 cm^{-1}.

The qualitative and quantitative production of organic acids in the presence and absence of the pesticides were confirmed using HPLC. Thus for organic acid analysis, the broth was harvested after 6 days of incubation and centrifuged at 10,000 rpm for 10 min. The supernatants were filtered through a 0.22 µm Millipore filter and the filtrate was injected to Waters 1525 binary HPLC pump equipped with C18 column (150 mm × 4.5 µm) with waters 2487 dual λ absorbance detector. The chromatograms were developed using a mobile phase consisting of 50 mM KH_2PO_4 moving at a constant flow rate of 0.7 mL min^{-1} in isocratic mode. Retention time of each signal was recorded at a wavelength of 210 nm. The production of organic acids was quantitatively determined by comparing the sample peak area with that of standard organic acids.

Evaluation of *Pseudomonas* sp. strain SGRAJ09 for highest pesticide tolerance and phylogenetic identification

The pesticides (alphamethrin, cypermethrin, endosulfan, imidacloprid, carbendazim, mancozeb, triazophos, chlorpyrifos and monocrotophos) tolerance potential of the strain SGRAJ09 was tested using the minimal salt medium (MSM) (g L^{-1}: glucose 3; $(NH_4)_2SO_4$ 2.0; $Fe_2SO_4 \cdot 7H_2O$ 0.001; $MgSO_4 \cdot 7H_2O$ 0.2; $CaCl_2 \cdot 2H_2O$ 0.01; $Na_2HPO_4 \cdot 12H_2O$ 1.5; KH_2PO_4 1.5; pH 7.0). The strain SGRAJ09 was initially grown in 100 mL MSM supplemented with 0.3 % glucose and in each treatment a single pesticide was added with highest concentration used under field conditions (Table 2). The flasks were further incubated at 28 ± 2 °C in 120 rpm for 4 days. For assessing the viability of the cells, 100 µL bacterial suspensions from each pesticide added flask was spread on MSM media. Based on the bacterial growth at highest pesticide concentration tested, the maximum tolerance limit of the strain SGRAJ09 was determined.

Using the bacterial universal primers 27F (AGAGTT TGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTA CGACTT), the 16S rRNA gene of the strain SGRAJ09 was sequenced at Chromous Biotech, DNA Sequencing Service, Tamil Nadu, India. The Ribosomal Database Project (RDP) II SEQMATCH (Cole et al. 2009) program was used to find the phylogenetically related sequences from available taxonomic information at RDP website (http://rdp.cme.msu.edu/). Later, the nucleotide sequence data was deposited in GenBank under the accession number JN257136.

Assessment of PGP activities

The strain SGRAJ09 showing highest pesticide tolerance and P solubilization potential was further tested for other PGP activities including indole-3-acetic acid, siderophore, NH_3, HCN production and ACC deaminase activity in the presence and absence of single and double dose of monocrotophos and imidacloprid.

Production of indole-3-acetic acid (IAA)

The isolated strain was screened for the conversion ability of tryptophan into IAA compounds by the method of Brick et al. (1991). Briefly, for testing IAA synthesis, the Luria–Bertani broth was supplemented with 100 µg tryptophan mL^{-1}, the flask was inoculated with *Pseudomonas* sp. strain SGRAJ09 and incubated at 28 ± 2 °C for 24 h in an incubator shaker at 125 rpm. After incubation, 5 mL of culture filtrate was centrifuged at 10,000 rpm for 15 min and to their supernatant, 2 mL of Salkowski's reagent (2 mL of 0.5 M $FeCl_3$ mixed with 98 mL of 35 % $HClO_4$) was added and incubated at 28 °C in darkness for 1 h. The intensity of color was measured at 530 nm in UV-Spectrophotometer. For IAA quantification, standard curve was constructed using pure IAA.

Siderophore production

Siderophore secretion by the strain was detected by employing the universal method of Schwyn and Neilands

(1987) using the Chrome azurol S dye containing agar plates. Briefly, 10 µL culture of *Pseudomonas* sp. strain SGRAJ09 was spot inoculated on to the centre of the plate and colony with orange halo against blue agar background indicates the siderophore production.

NH_3 production

The NH_3 production was tested for *Pseudomonas* sp. strain SGRAJ09 using the peptone water medium. Freshly grown culture was inoculated in 10 mL peptone water and incubated for 48–72 h at 28 ± 2 °C. On addition of 0.5 mL of Nessler's reagent, a change from brown to yellow color was noted therefore confirming the NH_3 production (Cappuccino and Sherman 1992).

HCN production

Briefly the strain SGRAJ09 was streaked on the nutrient agar amended with glycine (4.4 g L^{-1}). A Whatman filter paper No. 1 soaked in 2 % sodium carbonate prepared in 0.5 % picric acid solution was placed to the top of the plate. The plates were sealed with parafilm and incubated at 28 ± 2 °C for 4 days. Change of filter paper colour from orange to red indicates HCN production (Lorck 1948).

Aminocyclopropane-1-carboxylate (ACC) deaminase activity

Assessment of ACC deaminase activity was carried out as per the method of Saleh and Glick (2001). In brief, bacterial suspension of 200 µL was washed and added to 25 µL of toluene and vortexed vigorously for 30 s. Further, 20 µL of 0.5 M ACC was added to the mixture and incubated for 15 min; later to this, 1 mL of 0.56 N HCl was added. After centrifugation (10,000 rpm, 10 min), bacterial lysate of 1 mL was added to 800 µL of 0.56 N HCl and 300 µL of 2,4- dinitrophenylhydrazine (0.2 g in 100 mL of 2 N HCl). The mixture was incubated at 30 °C for 30 min to which 2 mL of 2 N NaOH was added and the absorbance was recorded at 540 nm. The ACC deaminase activity of *Pseudomonas* sp. strain SGRAJ09 was quantified by measuring the amount of α-ketobutyrate produced by the deamination of ACC and hence expressed in µMol of α-ketobutyrate mg $protein^{-1}$ h^{-1}.

Statistical analysis

All the experiments were conducted in multiples ($n = 4$) using same treatment. The data were subjected to statistical analysis and significant differences were calculated at $P \leq 0.05$ by two-way ANOVA using Graphpad Prism, v5.03.

Results

Isolation of PSB from pesticides treated rhizosphere soil

The PSB were recovered from the pesticides treated rhizosphere soil of *A. clavennae*, while they were absent in the rhizosphere of *S. lycopersicum* and *A. coryloides*. The PSB were identified by observing colonies with clearing zones against an opaque background. On total, 138 colonies noticed in the plate, but only six colonies were positive for the phosphorus solubilization activity. Based on the morphological

Table 1 Screening and isolation details of bacterial cultures recovered from pesticide treated *A. clavennae* rhizosphere soil

Sample source	Types of pesticide treated	Dilution factor	Total colonies detected	Strain number and diameter of the solubilization zone (mm)[a]	P-liberated in broth assay (µg mL^{-1})[b]	pH	Gram reaction	Colony morphology
A. clavennae	Monocrotophos, Endosulfan, Cypermethrin, Chlorpyrifos and Imidacloprid	10^{-5}	347	–				
		10^{-6}	138		11 ± 2[c]	7.0		
				SGRAJ01 (11 ± 1.0)	121 ± 7	4.0	Positive	Pale white, serrated margins
				SGRAJ02 (15 ± 1.1)	192 ± 6	4.2	Negative	White circular
				SGRAJ05 (11 ± 0.5)	221 ± 8	4.0	Negative	Smooth mucoid
				SGRAJ06 (13 ± 1.1)	168 ± 5	4.4	Negative	Pinhead colony
				SGRAJ09 (17 ± 0.5)	338 ± 8	3.8	Negative	Pale yellowish round
				SGRAJ12 (8 ± 0.5)	98 ± 6	4.1	Negative	White circular transparent colony

Values represent mean ± SD ($n = 4$). "–" indicates the absence of P solubilizing bacterial strains

[a] Zone size of TCP solubilization in Pikovaskaya agar after subtracting the colony diameter

[b] Amount of P liberated in NBRIP broth after 4 days of incubation without pesticide amendment

[c] The value represents the uninoculated control and their respective pH was provided in next column

difference and P solubilizing potential, only one effective strain (SGRAJ09) was selected for further studies.

Phosphate solubilization by strain SGRAJ09 in presence and absence of pesticides

Based on the P solubilizing capability, the P availability in the pesticide unamended broth differed among the PSB strains (Table 1). In pesticide free broth, strain SGRAJ12 showed the least P availability (98 μg P mL^{-1}) whereas, the highest P availability (338 μg P mL^{-1}) was shown by the strain SGRAJ09. However, in pesticide amended broth, only the strain SGRAJ09 was capable of tolerating pesticides and solubilized P. All other isolates were unable to grow or solubilize phosphate even at 1× dosage of pesticides tested. In the 1× dosage of monocrotophos added broth, strain SGRAJ09 showed maximum P availability (348 μg P mL^{-1}), while the least (14 μg P mL^{-1}) was recorded in mancozeb added treatment. Additionally, in 2× dosage of imidacloprid treatment, a maximum of 406 μg P mL^{-1} was recorded, while the least (6 μg P mL^{-1}) was obtained in mancozeb added broth (Table 3).

Qualitative and quantitative screening for organic acids by FT-IR and HPLC analysis

The culture supernatant of pesticide free broth, monocrotophos and imidacloprid added NBRIP broth were screened for the presence of organic acids functional group using FT-IR. In the control treatment, the COOH group showed the IR spectrum range of 1,608 cm^{-1}. However, the shift in the spectrum was observed in the monocrotophos (1,627 cm^{-1}) and imidacloprid (1,610 cm^{-1}) added broth (Fig. 1).

Further, the production of organic acids in the NBRIP broth without pesticide and with monocrotophos and imidacloprid addition was confirmed by HPLC. On qualitative analysis of organic acids in the control broth and pesticide added broth, gluconic acid was found to be the dominant acid (Fig. 2). In the imidacloprid added broth, in addition to gluconic acid, citric acid and formic acid peak were also noticed; similarly in monocrotophos added broth in addition to gluconic acid peak, a peak pertaining to acetic acid was observed. On quantitative basis, in the control and pesticide added broth, gluconic acid was found to be the dominant organic acid. In terms of total organic acids production, compared to control (15,176 μg mL^{-1}), slightly increased production of organic acids was observed both in the monocrotophos (15,813 μg mL^{-1}) and imidacloprid (16,962 μg mL^{-1}) added flasks. Compared to control treatment (14,846 μg mL^{-1}), a slight increase in the production of gluconic acid (14,853 μg mL^{-1}) was noticed in the monocrotophos added broth. Likewise, maximum amount of organic acids production was observed in the imidacloprid added broth. Formic acid

Table 2 Chemical details of pesticides used in this study and their maximum tolerance level of *Pseudomonas* sp. strain SGRAJ09

Pesticide grade and their active ingredient	Chemical name	Molecular formula	Chemical group	MTL (μg mL^{-1})
Chlorpyrifos (98 % w/w)	*O*,*O*-diethyl O-3,5,6- trichloropyridin-2-yl phosphorothioate	$C_9H_{11}C_{13}NO_3PS$	Organophosphate	2,000
Imidacloprid (97 % w/w)	*N*-[1-[(6-Chloro-3-pyridyl)methyl]-4, 5-dihydroimidazol-2-yl]nitramide	$C_9H_{10}ClN_5O_2$	Neonicotinoid	606
Triazophos (97 % w/w)	*O*,*O*-diethyl *O*-1-phenyl-1*H*-1,2, 4-triazol-3-yl phosphorothioate	$C_{12}H_{16}N_3O_3PS$	Organophosphate	1,600
Endosulfan (96 % w/w)	α-β-1,2,3,4,7-Hexachloro bicycle 2,2,1)-hepten-2bisoxymethylene-5,6-sulphite	$C_9H_6C_{l6}O_3S$	Organochlorine	2,600
Alphamethrin (97 % w/w)	α-cyano-3 = phenoxybenzyl (1S, 3S)-3-(2, 2-dichlorovinyl-2, 2-dimethylpropanecarboxylate	$C_{22}H_{19}C_{l2}NO_3$	Pyrethroid	117
Monocrotophos (78 % w/w)	Dimethyl (*E*)-1-methyl-2-(methylcarbamoyl) vinyl phosphate	$C_7H_{14}NO_5P$	Organophosphate	1,488
Cypermethrin (94 % w/w)	RS9-alpha-cyano-3-phenoxybenzyl (1RS) *cis*–trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane carboxylate	$C_{22}H_{19}Cl_2NO_3$	Pyrethroid	359
Carbendazim[a] (50 % WP)	Methyl *1H*-benzimidazol-2-ylcarbamate	$C_9H_9N_3O_2$	Benzimidazole carbamate	1,375
Mancozeb[a] (75 % WP)	Manganese ethylene bis(dithiocarbamate) (polymeric)	$C_8H_{12}MnN_4S_8Zn$	Ethylene (bis) dithiocarbamate	1,125

MTL maximum tolerance level, *WP* wettable powder

[a] Indicates the usage of commercial grade fungicides and all other pesticides used were of technical grade

Table 3 Effect of maximum recommended and double the maximum recommended dosage of pesticides on TCP solubilization by *Pseudomonas* sp. strain SGRAJ09 in NBRIP liquid broth

Pesticides	P availability with 1× concentration of pesticides amendment						P availability with 2× concentration of pesticides amendment					
	Day 2		Day 4		Day 6		Day 2		Day 4		Day 6	
	P-liberated (μg mL^{-1})	pH	P-liberated (μg mL^{-1})	pH	P-liberated (μg mL^{-1})	pH	P-liberated (μg mL^{-1})	pH	P-liberated (μg mL^{-1})	pH	P-liberated (μg mL^{-1})	pH
Control	314 ± 2	3.9	345 ± 3	3.7	336 ± 2	3.9	314 ± 2	3.9	345 ± 3	3.7	336 ± 2	3.9
Alphamethrin	308 ± 2*	3.9	339 ± 6*	3.9	290 ± 6*	4.1	174 ± 3*	3.9	266 ± 4*	3.6	352 ± 4*	3.8
Endosulfan	314 ± 2*	4.1	262 ± 3*	4.1	193 ± 4*	4.4	317 ± 9*	4.1	311 ± 3*	4.1	168 ± 2*	4.4
Chlorphyrifos	287 ± 4*	4.5	160 ± 13c	4.0	226 ± 4*	4.3	122 ± 3c	4.5	158 ± 3c	4.4	154 ± 3c	4.5
Cypermethrin	290 ± 4*	4.0	344 ± 3*	4.0	337 ± 4*	3.8	290 ± 4*	3.6	337 ± 4*	3.8	298 ± 1*	4.4
Monocrotophos	343 ± 4*	3.8	289 ± 6*	3.7	348 ± 6*	3.6	88 ± 2c	3.6	102 ± 2b	3.8	115 ± 2c	3.6
Imidacloprid	318 ± 8*	3.9	335 ± 3*	3.8	310 ± 2*	4.0	264 ± 2*	3.7	406 ± 3*	3.7	374 ± 3*	3.7
Triazophos	121 ± 3c	4.6	128 ± 3b	4.6	145 ± 4c	4.5	148 ± 2*	4.5	117 ± 3b	5.5	154 ± 3c	4.7
Carbendazim	18 ± 1a	6.5	142 ± 3b	4.5	142 ± 4c	6.3	15 ± 1a	6.4	8 ± 2a	6.5	14 ± 1a	6.5
Mancozeb	14 ± 2a	6.5	22 ± 2a	6.4	24 ± 2a	6.3	11 ± 1a	6.5	6 ± 1a	6.4	61 ± 3b	6.3

Each value represents the mean ± SD of four replicates per treatment. In the same column, significant differences at $P \leq 0.05$ levels over control are indicated by different letters according to two-way ANOVA. Values followed by the asterisk (*) denote non-significant values over control

production was noticed to be common in control and imidacloprid added broth at 330 μg mL^{-1} and 200 μg mL^{-1}, respectively. Compared to control, in the imidacloprid and monocrotophos added broth, significant quantity of citric acid (5,167 μg mL^{-1}) and acetic acid (960 μg mL^{-1}) production was observed. Certain unknown peaks in the control and also in imidacloprid added broth were observed that do not pertain to any of the organic acids standard tested in the present study.

Assessment of highest pesticide tolerance level by the strain SGRAJ09 and its phylogenetic position

The strain SGRAJ09 was further subjected to concentration dependent tolerance of the different pesticides that were tested for P solubilization. The strain SGRAJ09 was found to tolerate a wide range of pesticide level ranging from 117 (alphamethrin) to 2,600 μg mL^{-1} (endosulfan) (Table 2). Using the RDP II SEQMATCH, the strain SGRAJ09 was phylogenetically identified as nearest to the type strain *P. fulva* (AB06996) with 94.4 % similarity (Fig. 3). Since the similarity is <98.7 %, the strain (SGRAJ09) could be further processed to elevate it to a new species (Stackebrandt and Ebers 2006).

Assessment of other PGP activities

The *Pseudomonas* sp. strain SGRAJ09 that showed promising result in the P solubilization assay was tested for various other PGP traits including IAA synthesis, Siderophore secretion, NH_3, HCN production and ACC deaminase activity (Table 4). The synthesis of IAA was comparatively higher in monocrotophos added broth than in imidacloprid broth at 1× and 2× dose, but the pesticide unamended broth recorded the highest value of 38 μg mL^{-1}. The ACC deaminase activity was observed more only in 1× dose of imidacloprid than 2× imidacloprid or 1× and 2× dose of monocrotophos. On testing siderophore production, a maximum zone size was noticed in 1× dose of imidacloprid compared to monocrotophos treatment. The HCN and NH_3 production was unaffected irrespective of the concentrations of the pesticides tested. However, in all PGP traits checked in the presence of pesticides, compared to control, significant reduction in their activities were observed.

Discussion

PSB influences the available phosphorus concentration in the soil system. They are numerically dominant in rhizosphere soil; however, when exposed to pesticides, their population is drastically reduced. Compared with the total heterotrophic bacterial count of the soil system, the PSB population in a pesticide stressed environment may be less. Among six bacterial isolates recovered in the present study, only one strain was capable of tolerating pesticides and solubilized P. Similarly, a recent study reports that, out of 50 PSB isolates obtained from rhizosphere of *Brassica campestris*, only 18 strains were highly tolerant towards most of the pesticides tested. Among them, only one strain (*Klebsiella* sp.) tolerated high concentration of pesticides (fipronil, pyriproxyfen, imidacloprid and thiamethoxam) and produced prominent halo in the insoluble PO_4^{2-} added

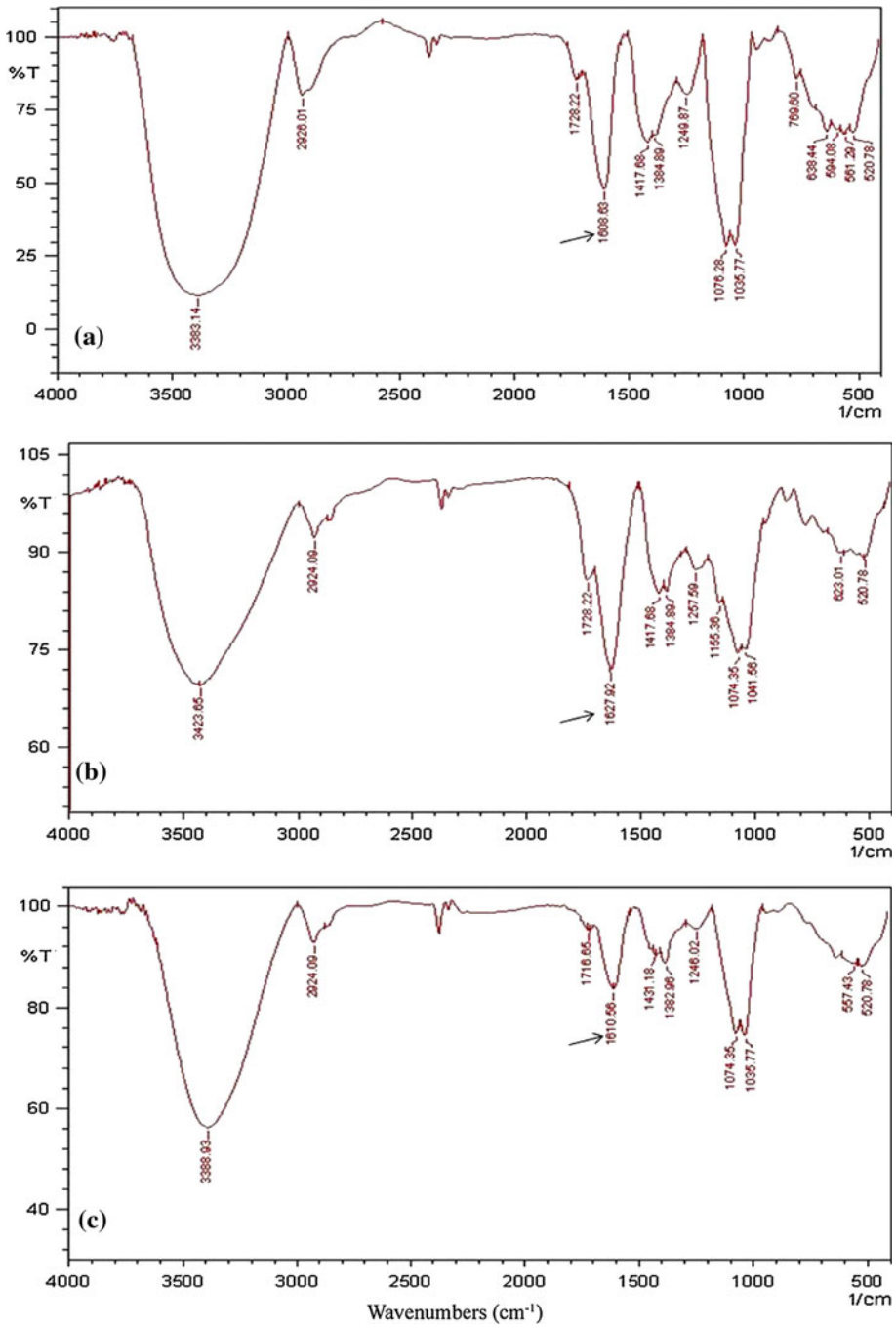

Fig. 1 FT-IR analysis of organic acids functional group in culture supernatants. The presence of organic acids functional group in the NBRIP broth inoculated with *Pseudomonas* sp. strain SGRAJ09 was identified using FT-IR analysis. (**a**) Lyophilized supernatant of pesticide free NBRIP broth (**b**) monocrotophos amended NBRIP culture supernatant (**c**) imidacloprid amended NBRIP culture supernatant. Note the IR spectrum showing the possible presence of COOH group in the unamended broth, indicated by a *peak* at 1,608.63 cm^{-1} (**a**) however, a shift in the IR spectrum was noticed for both the pesticides tested that were indicated by *arrows* (**b**, **c**)

media. The soil inherently possesses PSB population that also has potential pesticides tolerance and PGP traits that might play a significant role in the PGP activity (Ahemad and Khan 2011).

In the present study, only a *Pseudomonas* sp. strain SGRAJ09 tolerated high concentration of pesticides, solubilized P and also possessed the PGP traits tested. Even a previous study showed that the PSB tolerating high concentration of pesticides were capable of possessing various PGP traits. A *P. putida*, capable of solubilizing P and tolerating high concentration of fungicides were found to possess other PGP traits like siderophore production, IAA, exopolysaccharide, HCN and NH_3 production (Ahemad and Khan 2012).

Among different strains tested for P solubilization under single and double the dose of pesticides, only the *Pseudomonas* sp. strain SGRAJ09 was capable of solubilizing insoluble P in the liquid medium, while the other strains were unable to grow or solubilize P. This effect may be due to the toxicity of pesticides on the other PSB strains.

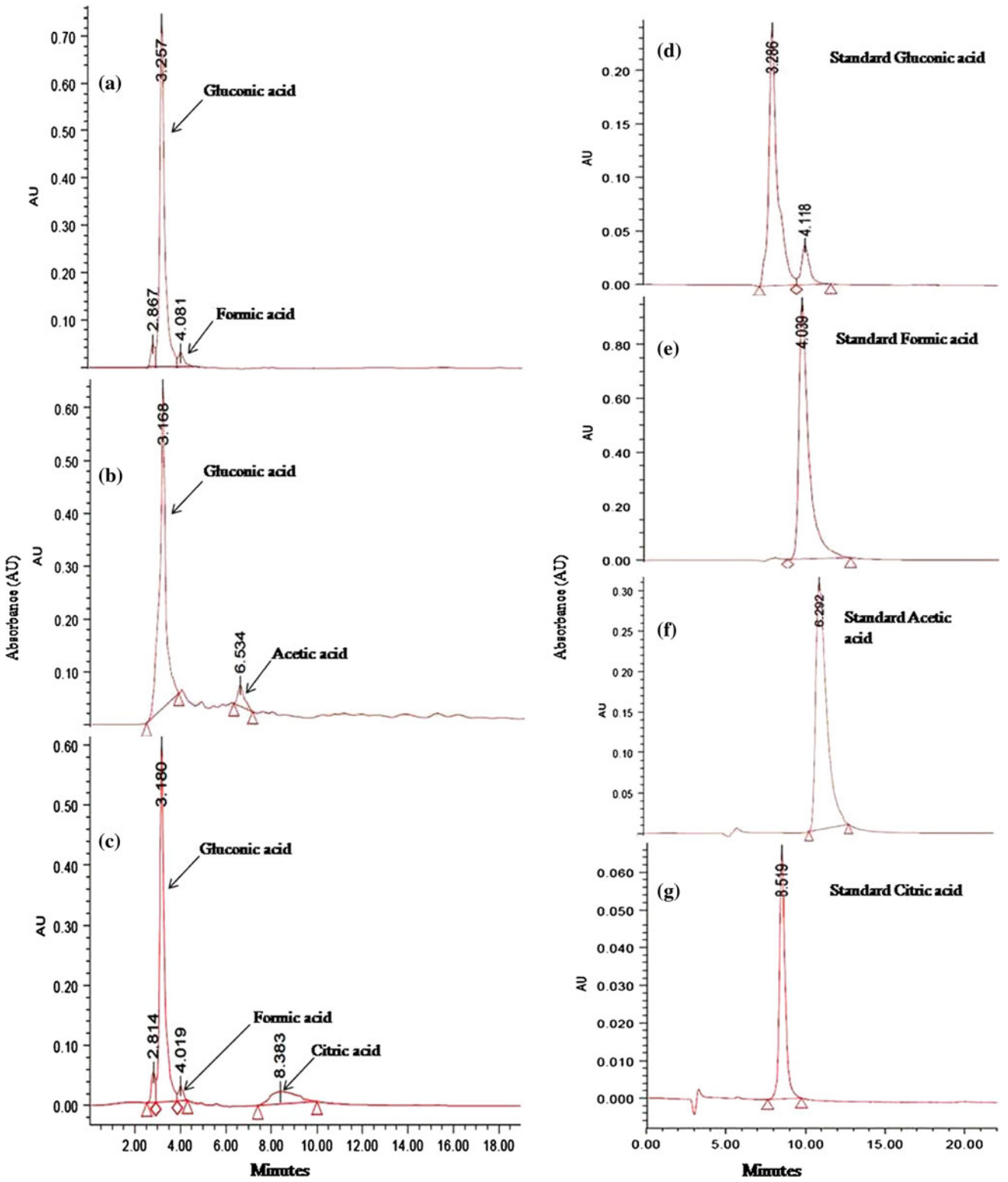

Fig. 2 HPLC chromatogram of culture supernatants in the absence and presence of the pesticides. HPLC chromatogram peaks of organic acids obtained (on 6th day) from the NBRIP broth inoculated with *Pseudomonas* sp. strain SGRAJ09. (**a**) Pesticide free culture broth showing the presence of gluconic and formic acids (**b**) culture broth amended with monocrotophos showing gluconic and acetic acid production (**c**) culture broth amended with imidacloprid showing gluconic, formic and citric acids production. (**d**–**g**) Standard chromatogram peak of gluconic, formic, acetic and citric acids

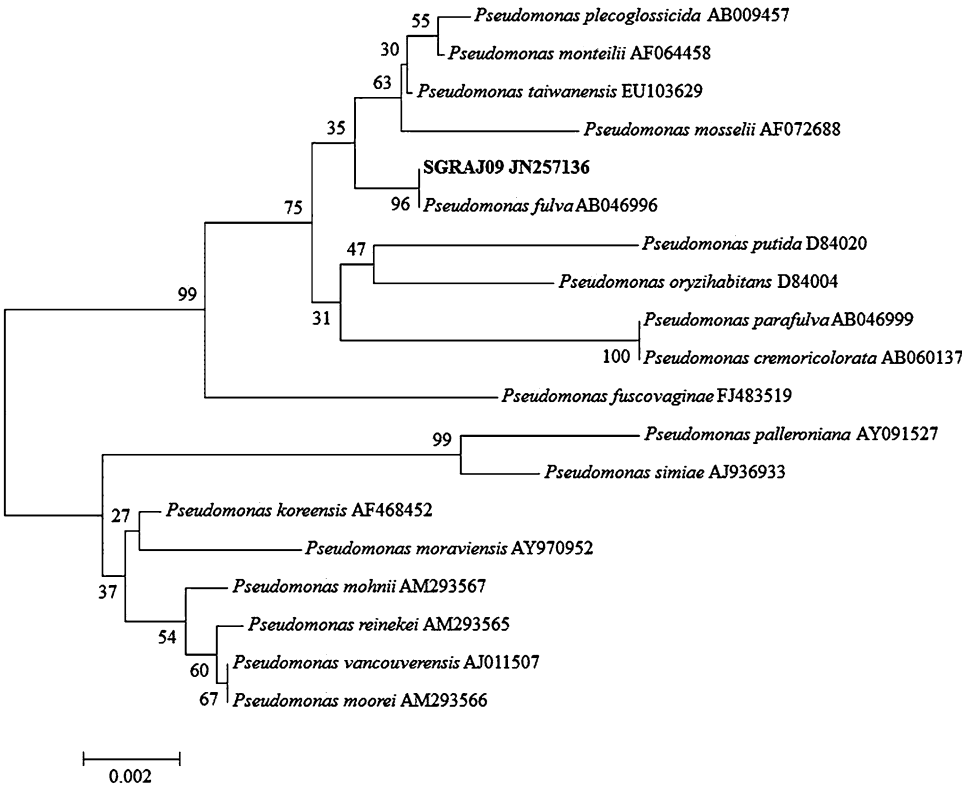

Fig. 3 Phylogenetic analysis of 16S rRNA gene of *Pseudomonas* sp. strain SGRAJ09. The phylogeny was reconstructed for the *Pseudomonas* sp. strain SGRAJ09 obtained from the *Achillea clavennae* using the 16S rRNA gene sequence employing the Neighbor joining method. The *numbers* at the nodes are percentage indicating the levels of bootstrap support, based on analysis of 1,000 resampled datasets

However, when the P solubilization by different strains was tested in the absence of pesticides, all the PSB strains solubilized P, whereas the available P was higher in the *Pseudomonas* sp. strain SGRAJ09 inoculated flask (Table 1). Noticeably, the pH reduction was significant in this particular strain inoculated flask which may be a reason behind the increased P availability compared to other PSBs. Besides possessing several PGP traits and tolerance to pesticide, the strain SGRAJ09 was a fast grower on Pikovaskaya agar showing clear halo within 24 h of inoculation. In addition, the organic acids secretion potential of this strain was not affected in presence of monocrotophos or imidacloprid. The availability of P due to *Pseudomonas* sp. strain SGRAJ09 inoculation was slightly higher in single and double the dose of monocrotophos and imidacloprid amended broth respectively, compared to control. In contrast, culture growth and P solubilization were reduced in single and double the dose of fungicides (carbendazim and mancozeb) added broth. This may be due to the toxic effect of fungicides on the *Pseudomonas* sp. strain SGRAJ09. Earlier studies showed that fungicides carbendazim and mancozeb were exerting an adverse effect on various soil bacteria including *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* (Castro et al. 1997; Niewiadomska 2004; Niewiadomska and Klama 2005). Additionally, a long term inhibitory effect of mancozeb on the diazotrophic population of the soil was also documented (Doneche et al. 1983). Published evidence suggests that, degraded end products of carbendazim were microbicidal and were moderately toxic to *P. fluorescens* (Virág et al. 2007). Furthermore, fungicides including ridomil and hinosan were identified as inhibitors of P and Zn solubilizing bacteria (Madhaiyan et al. 2006).

The P solubilization by *Pseudomonas* sp. strain SGRAJ09 was effected by organic acids production, mainly through gluconic acid excretion. Previous reports had clearly depicted the presence of extracellular aldose oxidation pathway, a non phosphorylating but an important pathway operating in *Pseudomonas* spp. While operating this pathway, gluconic acid is produced as a major metabolic product (Kim and Gadd 2008) and similarly in the present study too, gluconic acid was identified as one of the dominant acid produced during P solubilization.

During P solubilization, the overall decrease in the pH of pesticide free and added (monocrotophos and imidacloprid) broth may be attributed due to organic acids production. The FT-IR analysis showed the presence of COOH group in the culture supernatant, in addition, shift in the absorption spectrum was observed in the culture supernatants of monocrotophos and imidacloprid added broth. The change in the IR spectrum may be due to the additional binding of pesticide residues to the free carboxyl terminal of the organic acids. A similar shift in IR spectra denoting the chelation of Zn^{2+} ions to COOH group was already reported when Zn metal was solubilized by *Gluconacetobacter diazotrophicus* (Saravanan et al. 2007b).

Table 4 PGP activity of phosphate solubilizing *Pseudomonas* sp. strain SGRAJ09 under the presence of imidacloprid and monocrotophos

Treatments	IAA (μg mL^{-1})	Siderophore zone size (mm)	ACC (μMol α-KB mg^{-1} of protein h^{-1})	HCN	NH_3
Control	38 ± 0.0	13 ± 0.5	7.4 ± 0.1	+	+
Monocrotophos 1×	31 ± 0.5[a]	9 ± 0.5[a]	5.7 ± 0.1[a]	+	+
Monocrotophos 2×	27 ± 0.5[a]	8 ± 1.1[a]	5.1 ± 0.1[a]	+	+
Imidacloprid 1×	29 ± 0.5[a]	10 ± 0.5[b]	6.0 ± 0.1[a]	+	+
Imidacloprid 2×	21 ± 0.5[a]	7 ± 0.5[a]	5.2 ± 0.1[a]	+	+

Each value represents the mean ± SD of four replicates per treatment. In the same column according to Dunnett test significant differences at $P \leq 0.05$ levels over control are indicated by different letters. "+" indicates the presence of activity

Using HPLC, the organic acids profile was analyzed qualitatively and quantitatively in pesticide free and monocrotophos and imidacloprid added culture broth. Gluconic acid was identified as major as well as common organic acid in all the treatments, in addition, the presence of acetic and citric acid was also observed in the monocrotophos and imidacloprid added broth. Interestingly, a study stated that during the degradation of endosulfan by *P. spinosa* and *P. aeruginosa*, reduction in pH and possible organic acids excretion was observed (Hussain et al. 2007). In the present study, the pesticide degradation process or their end products were not quantified, however in presence of pesticides, significant amount of organic acids were detected that may in turn aided the solubilization of inorganic phosphorus. The free protons released from gluconic acid, may diffuse out from periplasm, aids the solubilization by replacing the Ca^{2+} in the insoluble calcium phosphates [e.g. $Ca_3(HPO_4)_2$, $Ca_5(PO_4)_3F$] separating Ca^{2+} and HPO_4^{2-}ions. Further, the gluconate, which in polyhydroxy carboxylate form, can successfully chelate Ca^{2+} ions under low pH (Goldstein 1995). A previous study had shown that the hydroxyl and carboxylate moieties of gluconate form a bidentate coordination (chelation) of the cation (Ca^{2+}) (Goldstein 2000).

Rhizosphere dwelling or free living soil bacteria are noteworthy for their PGP activities that directly or indirectly enhance the plant growth (Dobbelaere et al. 2003; Ahmad et al. 2008). However, in soil bacteria including *Rhizobium* sp., *G. diazotrophicus*, *Klebsiella* sp. and *Pseudomonas* sp. reduced PGP activities were recorded in the presence of pesticides (Castro et al. 1997; Madhaiyan et al. 2006; Ahemad and Khan 2011; Ahemad and Khan 2012). Similarly, in the present study also both single and double the dose of monocrotophos and imidacloprid had reduced the PGP activities. This reduction may be attributed to the adverse effect of pesticides on the metabolic enzymes and protein synthesis as reported by Kapoor and Arora (1996) and Boldt and Jacobsen (1998).

In presence of pesticides, the *Pseudomonas* sp. strain SGRAJ09 solubilized P by synthesizing significant levels of organic acids and also possessed several PGP traits, in presence of pesticides, changes in the qualitative or quantitative profile of organic acid production was observed. These properties make *Pseudomonas* sp. strain SGRAJ09 as a unique candidate for P nutrition even in monocrotophos and imidacloprid treated crop fields.

Conclusions

Difference in the P solubilization and P availability was noticed with different pesticides tested. A *Pseudomonas* sp. strain SGRAJ09 was able to tolerate the pesticides tested, solubilized P and possessed different PGP properties. When this strain was inoculated in the NBRIP broth, added either with single dose of monocrotophos or double dose of imidacloprid, the P solubilization was not affected. With those pesticides added, qualitative and quantitative changes in organic acids production was observed during P solubilization. Compared to pesticides, fungicides were found to have adverse effect on P solubilization of *Pseudomonas* sp. strain SGRAJ09.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Ahemad M, Khan MS (2011) Effects of insecticides on plant-growth-promoting activities of phosphate solubilizing rhizobacterium *Klebsiella* sp. strain PS19. Pestic Biochem Phys 100:51–56

Ahemad M, Khan MS (2012) Effect of fungicides on plant growth promoting activities of phosphate solubilizing *Pseudomonas putida* isolated from mustard (*Brassica compestris*) rhizosphere. Chemosphere 86:945–950

Ahmad F, Ahmad I, Khan MS (2008) Screening of free living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res 163:173–181

Boldt TS, Jacobsen CS (1998) Different toxic effects of the sulphonylurea herbicides metsulfuron methyl chlorsulfuron and thifensulfuron methyl on fluorescent pseudomonads isolated from an agricultural soil. FEMS Microbiol Lett 161:29–35

Brick JM, Bostock RM, Silverstone SE (1991) Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. Appl Environ Microbiol 57:535–538

Cappuccino JC, Sherman N (1992) Microbiology: a laboratory manual, 3rd edn. Benjamin/cummings Pub, New York, pp 125–179

Castro S, Vinocur M, Permigiani M, Halle C, Taurian T, Fabra A (1997) Interaction of the fungicide Mancozeb and *Rhizobium* sp. in pure culture and under field conditions. Biol Fertil Soils 25:147–151

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Mohideen ASKS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37(Database issue): D141–D145

Das AC, Mukherjee D (1998) Insecticidal effects on soil microorganisms and their biochemical processes related to soil fertility. World J Microbiol Biotechnol 14:903–909

Dobbelaere S, Vanderleyden J, Okon Y (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Crit Rev Plant Sci 22:107–149

Doneche B, Seguin G, Ribereau-Gayon P (1983) Mancozeb effect on soil microorganisms and its degradation in soils. Soil Sci 135: 361–366

Goldstein AH (1995) Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by Gram negative bacteria. Biol Agric Hortic 12:185–193

Goldstein AH (2000) Bioprocessing of rock phosphate ore: essential technical considerations for the development of a successful commercial technology. Proceedings of the 4th International Fertilizer Association Technical Conference, IFA, Paris

Goldstein AH, Rogers RD, Mead G (1993) Mining by microbes. Biotechnology 11:1250–1254

Gulati A, Rahi P, Vyas P (2008) Characterization of phosphate-solubilizing fluorescent pseudomonads from the rhizosphere of sea buckthorn growing in the cold deserts of Himalayas. Curr Microbiol 56:73–79

Hameeda B, Harini G, Rupel OP, Wani SP, Reddy G (2008) Growth promotion of maize by phosphate solubilizing bacteria isolated from composts and macrofauna. Microbiol Res 163:234–242

Hussain S, Arshad M, Saleem M, Khalid A (2007) Biodegradation of α- and β-endosulfan by soil bacteria. Biodegradation 18:731–740

Hwangbo H, Park RD, Kim YW, Rim YS, Park KH (2003) 2-Ketogluconic acid production and phosphate solubilization by *Enterobacter intermedium*. Curr Microbiol 47:87–92

Kapoor K, Arora L (1996) Observations on growth responses of cyanobacteria under the influence of herbicides. Pollut Res 15:343–351

Kim BH, Gadd GM (2008) Bacterial physiology and metabolism. Cambridge University Press, Cambridge, p 553

Lorck H (1948) Production of hydrocyanic acid by bacteria. Physiol Plant 1:142–146

Lugtenberg BJJ, Dekkers LC (1999) What makes *Pseudomonas* bacteria rhizosphere competent? Environ Microbiol 1:9–13

Madhaiyan M, Poonguzhali S, Hari K, Saravanan VS, Sa TM (2006) Influence of pesticides on the growth rate and plant growth promoting traits of *Gluconacetobacter diazotrophicus*. Pestic Biochem Phys 84:143–154

Niewiadomska A (2004) Effect of carbendazim, Imazetapir and thiram on nitrogenase activity, the number of microorganisms in soil and yield of red clover (*Trifolium pretense* L). Pol J Environ Stud 13:403–410

Niewiadomska A, Klama J (2005) Pesticide side effect on the symbiotic efficiency and nitrogenase activity of *Rhizobiaceae* bacteria family. Pol J Microbiol 54:43–48

Park KH, Lee CY, Son HJ (2009) Mechanism of insoluble phosphate solubilization by *Pseudomonas fluorescens* RAF15 isolated from ginseng rhizosphere and its plant growth promoting activities. Lett Appl Microbiol 49:222–228

Ramani V (2011) Effect of pesticides on phosphate solubilization by *Bacillus sphaericus* and *Pseudomonas cepacia*. Pestic Biochem Phys 99:232–236

Rodríguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17: 319–339

Rodríguez H, Gonzalez T, Goire I, Bashan Y (2004) Gluconic acid production and phosphate solubilization by the plant growth promoting bacterium *Azospirillum* spp. Naturwissenschaften 91:552–555

Saleh SS, Glick BR (2001) Involvement of gacS and rpoS in enhancement of the plant growth promoting capabilities of *Enterobacter cloacae* CAL2 and UW4. Can J Microbiol 47: 698–705

Saravanan VS, Madhaiyan M, Thangaraju M (2007a) Solubilization of zinc compounds by the diazotrophic, plant growth promoting bacterium *Gluconacetobacter diazotrophicus*. Chemosphere 66:1794–1798

Saravanan VS, Osborne J, Madhiyan M, Mathew L, Chung J, Kisup AHN, Sa TM (2007b) Zinc metal solubilization by *Gluconacetobacter diazotrophicus* and induction of pleomorphic cells. J Microbiol Biotechnol 17:1477–1482

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Son HJ, Park GT, Cha MS, Heo MS (2006) Solubilization of insoluble inorganic phosphates by a novel salt and pH tolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere. Bioresour Technol 97:204–210

Stackebrandt E, Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 33:152–155

Vassilev N, Vassileva M, Nikolaeva I (2006) Simultaneous P-solubilizing and biocontrol activity of microorganisms: potentials and future trends. Appl Microbiol Biotechnol 71:137–144

Virág D, Naár Z, Kiss A (2007) Microbial toxicity of pesticide derivatives produced with UV-photodegradation. Bull Environ Contam Toxicol 79:356–359

Vyas P, Gulati A (2009) Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. BMC Microbiol 9:174–188