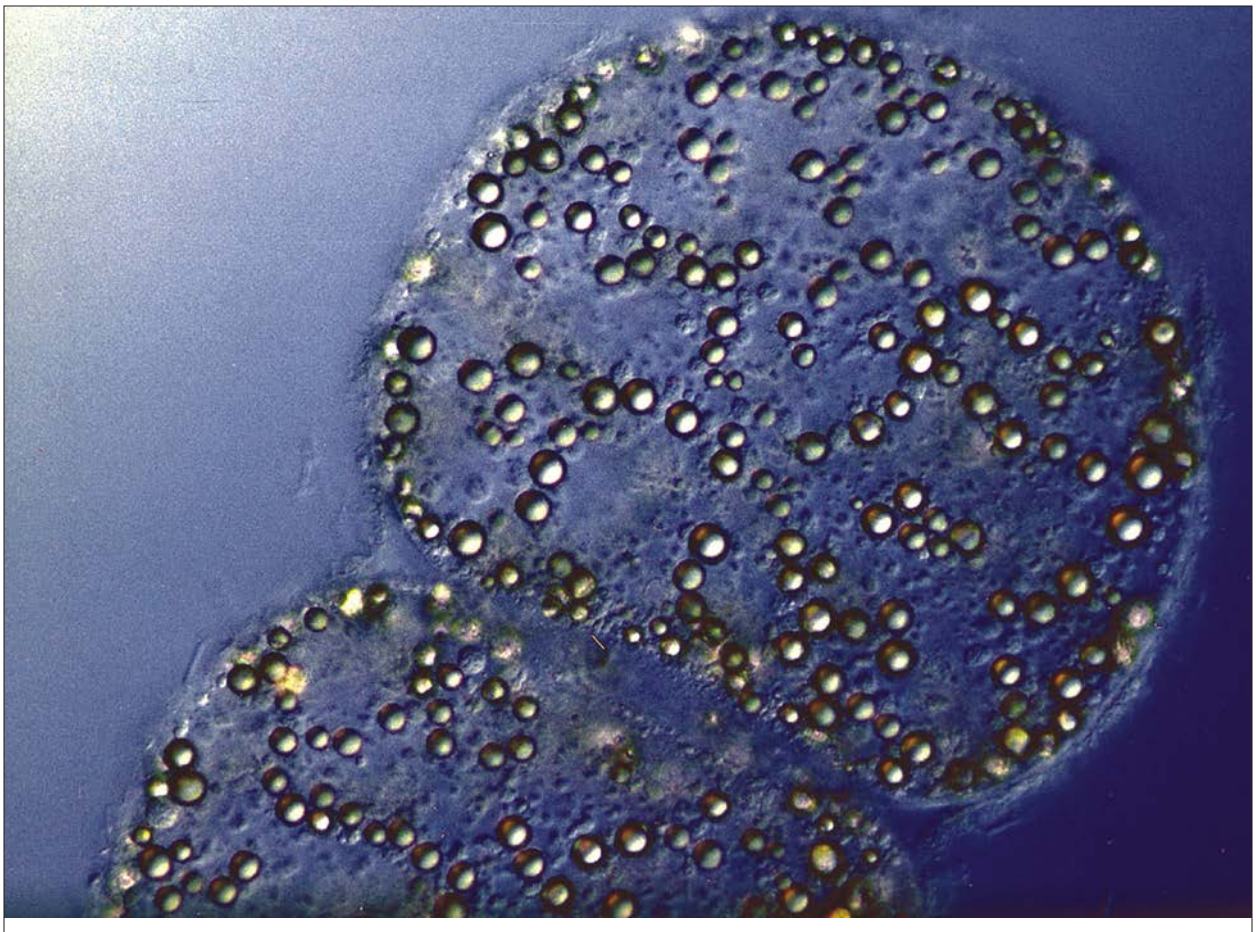

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Assessment of Plant Growth Promoting Activities of Rhizobacterium *Pseudomonas putida* under Insecticide-Stress

Munees Ahemad and Mohammad Saghir Khan
Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh-202002, U.P., India

Corresponding Author: Mohammad Saghir Khan, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, A.M.U, Aligarh-202002, U.P., India Tel: +91-571-2702945

ABSTRACT

The aim of this study was to evaluate the effect of the recommended field rates as well as two and three times the recommended field rates of insecticides (fipronil, pyriproxyfen, imidacloprid, thiamethoxam) on plant growth promoting traits of the rhizobacterium *Pseudomonas putida* strain PS9. The insecticide-tolerant and phosphate solubilizing *Pseudomonas putida* strain PS9 was isolated from the mustard (*Brassica compestris*) rhizosphere and identified following 16S rDNA sequencing. The *Pseudomonas putida* strain PS9 possessed considerable insecticide-tolerance and indole acetic acid, siderophores (salicylic acid and 2, 3 dihydroxy benzoic acid), exo-polysaccharides, HCN and ammonia producing traits. As the concentration of each insecticide was increased from the recommended dose rate to the higher one, a progressive decline in all plant growth promoting properties (excluding exo-polysaccharides) of the *Pseudomonas putida* strain PS9 was observed. Insecticides at the recommended dose had less inhibitory impact while the doses of insecticides higher than the recommended dose reduced the plant growth promoting traits significantly. The study concluded that insecticides should be screened based on their degree of toxicity to plant growth promoting properties of rhizobacteria before their field application.

Key words: *Pseudomonas putida*, insecticide, plant growth promoting rhizobacteria, toxicity, pest management

INTRODUCTION

In soil ecosystem, microorganisms including Plant Growth-Promoting Rhizobacteria (PGPR) participate in a myriad of recycling processes of nutrients to sustain the soil fertility (Barea *et al*., 2004; Vikram and Hamzehzarghani, 2008; Oyeyiola, 2010; Woyessa and Assefa, 2011). Soil microorganisms, specifically PGPR facilitate plant growth by: (1) solubilizing insoluble phosphates; (2) fixing atmospheric N and transporting it to plants; (3) facilitating uptake of other plant nutrients and (4) synthesizing siderophores and phytohormones (Yadav *et al*., 2011; Zaidi *et al*., 2009; Saber *et al*., 2009). Since soil microorganisms are very sensitive to environmental change and a significant degradation of the microbial community can occur following drastic disturbance, both in terms of total biomass and species composition (Lin *et al*., 2007). For instance, due to extensive use of agricultural chemicals including various insecticides of different chemical families in crop production, these beneficial microorganisms and their physiological activities important to soil fertility are adversely affected (Wani *et al*., 2005; Srinivas *et al*., 2008; Ahemad and Khan, 2010a) and ultimately is influenced the soil fertility and plant growth (Ahemad and Khan, 2010c).

Although *Pseudomonas putida* has been used as a representative to study a range of plant growth promoting traits, the information regarding the impact of insecticides on the plant growth promoting traits of *Pseudomonas putida* of rhizospere-niche have not comprehensively been explored. Further, the most direct approach to analyze the specific agrochemical (including insecticides) induced changes in microbial community is the use of tolerant microorganisms against the same agrochemical (Oves *et al.*, 2009). In addition, studies on the effect of various insecticides have largely been focused on the changes in populations of soil microflora including PGPR. However, the reports on *in vitro* Plant Growth Promoting (PGP) activities of Phosphate (P) solubilizing *Pseudomonas putida* in the presence of insecticides are rare. Considering these scientific gaps, the present study was, therefore, designed to evaluate the effects of four insecticides, namely, fipronil, pyriproxyfen, imidacloprid, thiamethoxam (Table 1), at the recommended (1X), double (2X) and three times the recommended rates (3X) on *in vitro* PGP activities of insecticide-tolerant rhizobacterium *Pseudomonas putida*.

MATERIALS AND METHODS

Isolation and screening of phosphate solubilizing bacteria: Three rhizosphere soil samples (10 g each) of mustard (*Brassica compestris*) cultivated in Experimental Fields (alluvial sandy clay loam, sand 667 g kg^{-1}, silt 190 g kg^{-1}, clay143 g kg^{-1}, organic matter 6.2 g kg^{-1}, Kjeldahl N 0.75 g kg^{-1}, Olsen P 16 mg kg^{-1}, pH 7.2 and water holding capacity 0.44 mL g^{-1}, cation exchange capacity 11.7 and 5.1 cmol kg^{-1} anion exchange capacity) of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh (27° 29' latitude and 72° 29' longitude), Uttar Pradesh, India were collected in sterile polythene bags (15×12 cm^2) and thoroughly mixed. In order to identify the phosphate solubilizing bacteria, a serial dilution assay was carried out in 0.9% NaCl solution and 10 µL of diluted suspension was spread plated on Pikovskaya agar medium (Pikovskaya, 1948) (gL^{-1}: glucose 10; $Ca_3(PO_4)_2$ 5; $(NH_4)_2SO_4$ 0.5; NaCl 0.2; $MgSO_4.7H_2O$ 0.1; KCl 0.1; yeast extract 0.5; $MnSO_4$ and $FeSO_4$ trace; agar 15; pH 7.0). Plates were incubated at 28±2°C for seven days. The isolates showing clear halo within seven days around bacterial colonies were considered as P-solubilizers. A total of 50 P-solubilizing isolates with maximum halo sizes, different pigmentations and morphological parameters were selected.

Assessment of bacterial strains for insecticide-tolerance: The bacterial strains were tested further for their sensitivity/resistance to four insecticides (fipronil, pyriproxyfen, imidacloprid, thiamethoxam) (Table 1), by agar plate dilution method (Gupta *et al.*, 1994) using minimal salt agar medium (g L^{-1}: KH_2PO_4 1; K_2HPO_4 1; NH_4NO_3 1; $MgSO_4.7H_2O$ 0.2; $CaCl_2.2H_2O$ 0.02; $FeSO_4.7H2O$ 0.01; agar 15; pH 6.5). The freshly prepared agar plates amended separately with increasing concentration (0 to 3200 µg mL^{-1}; at a two fold dilution interval) of insecticides were spot inoculated with 10 µL of 10^8 cells mL^{-1} of bacterial strains. Plates were incubated at 28±2°C for three days and the highest concentration of insecticides supporting bacterial growth was defined as the Maximum Tolerance Level (MTL). Out of 50, a total of 18 bacterial isolates showing higher MTL values (>600 µg mL^{-1}) against the insecticides as well as greater halo size (>4 mm) were selected and maintained on solid Pikovskaya agar medium until use.

Bacterial characterization: Among 18 bacterial strains, the strain PS9 showing higher MTL values and phosphate solubilization was further selected. Morphological, physiological and biochemical properties of the strain PS9, that included Gram reaction, citrate utilization test, indole production test, methyl red test, nitrate reduction, Voges Proskaur, catalase test, carbohydrates

Table 1: Insecticides used in the present study

Common name	Grade (purity)	Chemical name	Chemical family	Recommended dose (1X)
Fipronil	Technical (98%w/w)	5-amino-1-(2,6-dichloro-a,a,a-trifluoro-p-tolyl) -4-trifluoromethylsulfinylpyrazole-3-carbonitrile	Phenylpyrazole	200 μg L^{-1}
Pyriproxyfen	Technical (98%w/w)	4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether	Juvenile hormone mimics	1300 μg L^{-1}
Imidacloprid	Technical (100% EC)	(E)-1-(6-chloro-3-pyridylmethyl) -N-nitroimidazolidin-2-ylideneamine	Pyridylmethylamine	100 μg L^{-1}
Thiamethoxam	Technical (100%w/w)	(EZ)-3-(2-chloro-1,3-thiazol-5-ylmethyl)-5 -methyl-1,3,5-oxadiazinan-4-ylidene (nitro)amine	Thiazole	25 μg L^{-1}

(dextrose, mannitol and sucrose) utilization test, starch hydrolysis and gelatin liquefaction test was determined as per the standard methods according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

16S rDNA based identification: The sequencing of 16S rDNA of the strain PS9 was carried commercially by DNA Sequencing Service, Macrogen Inc., Seoul, South Korea using universal primers, 518F (5'CCAGCAGCCGCGGTAATACG3') and 800R (5'TACCAGGGTATCTAATCC3'). Later, nucleotide sequence data was deposited in the Gen-Bank sequence database.

The online program BLAST was used to find out the related sequences with known taxonomic information in the databank at NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) to accurately identify the strain PS9.

Bioassays of plant growth promoting activities under insecticide-stress: The PGP activities (P-solubilization, indole acetic acid, siderophore, exo-polysaccharides, hydrogen cyanide and ammonia production) of P-solubilizing bacteria were assayed both in the presence and absence of the selected insecticides under *in vitro* conditions.

Phosphate solubilization by bacterial strains: The bacterial strains showing P-solubilizing activity were inoculated into Pikovskaya agar medium supplemented with 0, 1, 2 and 3X of the recommended rate of each insecticide and incubated at 28±2°C for seven days and observed for halo formation. The halo formed around the each bacterial colony was measured and the bacterial strains were further used to determine the extent of P-solubilization in Pikovskaya broth by the chlorostannous-reduced molybdophosphoric acid blue method (King, 1932; Jackson, 1967).

Bioassay for indole-3-acetic acid production: The indole-3-acetic acid (IAA) was quantitatively analyzed by the method of Gordon and Weber (1951) later modified by Bric *et al.* (1991).

Bioassay for siderophore production: Chrome azurol S (CAS) agar plates supplemented with 0, 1, 2 and 3X of each insecticide were prepared separately and spot inoculated with 10 μL of 10^8 cells mL^{-1}. Plates were incubated at 28±2°C for four days. Development of yellow to orange halo around the growth indicated siderophore production (Alexander and Zuberer, 1991).

The siderophore produced by the test strains was also quantitatively assayed using Modi medium (K_2HPO_4 0.05%; MgSO4 0.04%; NaCl 0.01%; mannitol 1%; glutamine 0.1%; NH_4NO_3 0.1%). Modi medium amended with 0, 1, 2 and 3X of each insecticide was inoculated with 100 μL of 10^8 cells mL^{-1} of bacterial strains and incubated at 28±2°C for five days. Cultures were

centrifuged (4528 g) and the catechol type phenolates [salicylic acid (SA) and 2,3-dihydroxybenzoic acid (DHBA)] in the supernatant were measured using a modification of the ferric chloride-ferricyanide reagent of Hathway (Reeves *et al*., 1983). Briefly, ethyl acetate extracts was prepared by extracting 20 mL of supernatant twice with an equal volume of solvent (ethyl acetate) at pH 2. Hathway's reagent was prepared by adding 1 mL of 0.1 M ferric chloride in 0.1 N HCl to 100 mL of distilled water and to this, was added 1 mL of 0.1 M potassium ferricyanide. For the assay, one volume of the reagent was added to one volume of sample and absorbance was determined at 560 nm for salicylates with sodium salicylate as standard and at 700 nm for dihydroxy phenols with DHBA as standard.

Bioassay for exo-polysaccharides: For exo-polysaccharides (EPS), the bacterial strains were grown in 100 mL basal medium with 5% sucrose supplemented with 0, 1, 2 and 3X of each insecticide and incubated for five days at 28±2°C on shaker (100 rpm). Culture broth was spun (5433 g) for 30 min and EPS was extracted by adding three volumes of chilled acetone to one volume of supernatant. The precipitated EPS was repeatedly washed three times alternately with distilled water and acetone, t ransferred to a filter paper and weighed after overnight drying (Mody *et al*., 1989).

Bioassays for hydrogen cyanide and ammonia production: Bacterial strains were also tested for the synthesis of hydrogen cyanide (HCN) and ammonia by adopting the method of Bakker and Schippers (1987) and Dye (1962), respectively.

Each individual experiment was replicated three times.

Statistical analysis: The experiments were conducted in three replicates using the same treatments. The difference among the treatment means was compared by Honestly Significant Difference (HSD) using Tukey test at 5% probability level.

RESULTS

Characterization and molecular identification of the strain PS9: The strain PS9 was characterized and identified by using standard morphological, physiological and biochemical tests. The rhizobacterial strain PS9 was found to be positive for citrate utilization test, indole production test, nitrate reduction, catalase test, dextrose utilization test and gelatin liquefaction test while the strain PS9 showed negative response to Gram reaction, methyl red test, Voges Proskaur test, mannitol and sucrose utilization test and starch hydrolysis (Table 2). On the basis of these features, PS9 was tentatively identified as *Pseudomonas*.

To further consolidate the identity of the strain PS9, 16S rDNA sequence analysis this strain was performed. The nucleotide sequence of 16S rDNA of PS9 was found to be approximately 845 bp in size. The sequence of 16S rDNA of this strain was submitted to Gen-Bank (Gen-Bank accession number FJ705888). A similar search was performed by using the BLAST program that indicated the strain PS9 shared a close relationship with the rDNA sequence of *Pseudomonas putida* strain ATCC 17514 (16S: 99% similarity with the reference strain AF094741) in NCBI database. Such high similar values confirmed the strain PS9 to be *Pseudomonas putida*.

Tolerance against insecticides: In this study, the growth of rhizobacterium the *Pseudomonas putida* strain PS9 recovered from the mustard rhizosphere was monitored under insecticide-stress

Table 2: Morphological and biochemical characteristics of *Pseudomonas putida* strain PS9

Characteristics	Strain PS9
Morphological	
Gram reaction	-
Cell shape	Rods
Colony morphology	Mucoid, smooth margin
Biochemical	
Citrate utilization	+
Indole	+
Methyl red	-
Nitrate reduction	+
Voges Proskaur	-
Catalase	+
Carbohydrate utilization	
Dextrose	+
Mannitol	-
Sucrose	-
Hydrolysis	
Starch	-
Gelatin	+
Maximum Tolerance Level (MTL)	
Fipronil	1600 μg mL^{-1}
Pyriproxyfen	1400 μg mL^{-1}
Imidacloprid	2200 μg mL^{-1}
Thiamethoxam	2400 μg mL^{-1}

+: Positive reaction, –: Negative reaction

by exposing the culture to the graded concentrations (0 to 3200 μg mL^{-1}) of each selected insecticide on C and N sources-deficient minimal salt agar medium. The *Pseudomonas putida* strain PS9 grew well with the varying concentrations of insecticides and showed a variable tolerance to the tested chemicals. The tolerance levels of *Pseudomonas putida* strain PS9 against the insecticides ranged between 1400 μg mL^{-1} (pyriproxyfen) to 2400 μg mL^{-1} (thiamethoxam). However, the MTL values of the *Pseudomonas putida* strain PS9 against each insecticide were evidently high.

Plant growth promoting activities of the Pseudomonas putida strain PS9: In present study, rhizobacterium *Pseudomonas putida* strain PS9 demonstrated the PGP activities under both normal and insecticide-stressed conditions. The test strain under normal condition (in the absence of insecticides) however, produced the PGP substances (solubilized P, IAA, siderophores and EPS) extensively.

Phosphate solubilization under insecticide-stress: The TCP-solubilizing activity of the *Pseudomonas putida* PS9 in the presence of three concentrations of each insecticide was evaluated both qualitatively and quantitatively in Pikovskaya medium (Table 3). Generally, with graded increment of each insecticide from normal to three times the recommended rate, the halo-size in term of Solubilization Index (SI) decreased with minor fraction while the highest concentration (3X) had the most adverse effect on halo formation. Thiamethoxam did not affect the P-halo at all. The order of toxicity of other three insecticides at 3X to halo formation was: pyriproxyfen>fipronil>imidacloprid (Table 3).

Table 3: Plant growth promoting activities of phosphate solubilizing bacterium Pseudomonas putida in the presence of varying concentrations of insecticides

		Plant growth promoting activities									
		Phosphate solubilized			Siderophores				IAA[D]		
Insecticides	Dose rate ($\mu g\ L^{-1}$)	Liquid medium ($\mu g\ mL^{-1}$)	pH	SI*	Zone on CAS[A] agar (mm)	SA[B] ($\mu g\ mL^{-1}$)	DHBA[C] ($\mu g\ mL^{-1}$)	($\mu g\ mL^{-1}$) 100T[E]	EPS[F] ($\mu g\ mL^{-1}$)	HCN[G]	Ammonia
Fipronil	200	205±9[b]	5.6	1.5	11±1.0[a]	36±1.4[b]	15±1.2[b]	26±1.2[b]	21±1.7[de]	+	+
	400	125±7[d]	5.8	1.3	10±1.0[b]	33±2.1[cd]	7±0.2[e]	15±1.4[ef]	23±1.6[bc]	+	+
	600	75±3[f]	6.1	1.3	9±1.0[c]	28±1.3[e]	4±0.3[gh]	9±1.2[h]	25±1.3[a]	+	+
Pyriproxyfen	1300	192±8[c]	5.8	1.5	10±1.5[b]	34±1.2[bc]	11±0.7[c]	24±2.0[c]	20±1.2[e]	+	+
	2600	37±4[i]	6.8	1.3	9±1.0[c]	31±1.4[d]	4±0.8[gh]	14±1.4[fg]	21±1.2[de]	+	+
	3900	22±3[k]	6.8	1.0	9±1.0[c]	25±1.3[f]	3±0.6[h]	7±0.6[i]	23±1.2[bc]	+	+
Imidacloprid	100	75±2[f]	6.2	1.8	11±1.5[a]	22±1.2[gh]	7±0.5[e]	16±2.1[de]	20±1.6[e]	+	+
	200	38±4[h]	6.6	1.5	11±1.8[a]	17±1.2[ij]	4±0.4[gh]	7±0.8[i]	20±1.5[e]	+	+
	300	25±6[j]	6.8	1.5	10±1.5[b]	15±1.1[k]	3±0.6[h]	4±0.6[k]	22±1.7[cd]	+	+
Thiamethoxam	25	83±7[e]	6.7	1.8	11±1.0[a]	21±1.1[h]	8±0.8[de]	13±1.5[g]	20±1.4[e]	+	+
	50	56±3[g]	6.7	1.8	10±1.0[b]	16±1.2[ik]	5±0.6[fg]	9±0.6[h]	22±1.3[cd]	+	+
	75	37±2[i]	6.8	1.8	9±1.0[c]	13±1.5[l]	3±0.5[h]	5±0.8[ik]	23±2.2[bc]	+	+
Control (without insecticide)		298±7[a]	4.4	1.8	11±1.0[a]	41±1.5[a]	17±1.4[a]	34±1.2[a]	17±1.1[f]	+	+
LSD ($p \leq 0.05$)		3.62	-	-	0.65	3.51	2.37	2.11	1.02	-	-
F value (treatment)		972.6	-	-	27.3	124.4	211.5	74.5	34.8	-	-

Values indicate mean of three replicates. Mean values (Mean±SD) followed by different letters are significantly different within a row or column at $p \leq 0.05$ according to Tukey test. *SI = [(Zone size including colony Diameter-Colony diameter)/Colony diameter], [A]Chrome azurol S agar, [B]Salicylic acid, [C]2,3 Dihydroxy benzoic acid, [D]Indole acetic acid, [E]Tryptophan concentration ($\mu g\ mL^{-1}$), Exopolysaccharides; [G]Hydrogen cyanide, +: Positive reaction, –: Negative reaction

Furthermore, insecticide-concentration dependent progressive decline in the *Pseudomonas putida* PS9-mediated solubilization of TCP amended in Pikovskaya broth was observed. Among tested insecticides, the maximum toxicity to P-solubilization at the highest tested dose rate was observed in the presence of pyriproxyfen. The P-solubilizing activity of the *Pseudomonas putida* strain PS9 in the presence of pyriproxyfen at 1, 2 and 3X was inhibited by 36, 88 and 93%, respectively compared to control. At 3X of each insecticide, the order of toxicity (percent inhibition over control) was observed as: pyriproxyfen (93)>imidacloprid (92)>thiamethoxam (88)>fipronil (75). No correlation ($R^2 = 0.06$) was observed between SI and P solubilized in broth (Table 3).

Siderophore production under insecticide-stress: Additionally, the size of siderophores-zone also declined with increasing concentrations of each insecticide in CAS agar medium. A reduction in siderophore-zone generally, was not observed in the presence of the recommended doses of each insecticide. In contrast, 3X of each insecticide suppressed greater the siderophore-zone over untreated control. The order of percent decline in zone diameter relative to control for the tested insecticides at 3X was- pyriproxyfen (19) = fipronil (19) = thiamethoxam (19)>imidacloprid (10) (Table 3). In case of quantitative estimation of siderophores, the amount of both SA and DHBA in the supernatant of *Pseudomonas putida* PS9 also declined progressively with increasing dose rate of each insecticide (Table 3). At three times the recommended rates, the order of percent decline in SA synthesis by each insecticide was: thiamethoxam (69)>Imidacloprid (64)>pyriproxyfen (39)>fipronil (32). In contrast, the trend of toxicity to DHBA secretion at the highest dose rate of each insecticide was found approximately similar (Table 3).

Indole-3-acetic acid, exo-polysaccharides, hydrogen cyanide and ammonia production under insecticide-stress: Furthermore, the *Pseudomonas putida* strain PS9 released considerable IAA in LB medium both in the absence and the presence of insecticides. In the medium devoid of insecticides, the *Pseudomonas putida* strain PS9 produced IAA 34 μg mL^{-1} which decreased progressively with increasing concentrations of each insecticide. Among the tested insecticides, the effect of thiamethoxam on IAA biosynthesis was most inhibitory. Thiamethoxam at 1, 2 and 3X decreased IAA by 62, 74 and 84%, respectively above control (Table 3). Unlike other PGP activities, EPS secreted by the strain PS9 with gradual addition of insecticides in medium increased progressively. At 3X, the trend of EPS secretion (percent increase over control) was: fipronil (47)>pyriproxyfen (35) = thiamethoxam (35)>imidacloprid (29). Moreover, the three concentrations of each insecticide did not affect HCN and ammonia synthesis by the *Pseudomonas putida* PS9 (Table 3).

DISCUSSION

Insecticide-tolerance: In this study, rhizobacterium *Pseudomonas putida* PS9 grew well with the varying concentrations of insecticides and showed a varying degree of tolerance levels against the tested insecticides. In agreement to this finding, other Gram negative bacteria, *Pseudomonas* and *Flavobacterium* have also shown resistance to organophosphorus insecticides, guthion, methyl parathion and dimethoate, as reported by Nazarian and Mousawi (2005). To overcome the toxic effects of pesticides including insecticides, rhizobacteria may either biodegrade or hydrolyze pesticide enzymatically (Ahemad and Khan, 2010c). The variation in sensitivity/tolerance of a wide array of rhizobacteria to a specific pesticide may be attributed to their metabolic pathways which detoxify these xenobiotic compounds or break up/modify them into other forms that may be further more or less deleterious (Johnsen *et al.*, 2001). In this study, *Pseudomonas putida* strain PS9 however, demonstrated abnormally higher tolerance levels against the selected insecticides amended in minimal salt agar medium. Since the medium used to assess the MTL values of the *Pseudomonas putida* strain PS9 did not contain any C and N sources except the tested insecticides, the *Pseudomonas putida* strain PS9 might have utilized these insecticides as the only energy source.

***In vitro* production of plant growth promoting substances:** In the present study, the *Pseudomonas putida* strain PS9 exhibited the PGP traits like phosphate solubilization, production of siderophores, phytohormone and exo-polysaccharides in substantial amount.

Phosphate solubilization: Rhizospheric bacteria promote the plant growth by different mechanisms. One of the important mechanisms is the solubilization of mineral phosphate in the rhizosphere and provides soluble P to plants (Zaidi *et al.*, 2009). The amount of soluble P in soil is generally very low, normally at levels of 1 mg kg^{-1} or less (Goldstein, 1994). Phosphate solubilizing bacteria play a crucial role in making available solublized fraction of various phosphate minerals in soils to growing plants. The phosphate solubilizing property is due to a drop in pH which has been associated with their ability to secrete low molecular weight organic acids such as gluconic, 2-ketogluconic, oxalic, citric, acetic, malic and succinic, etc. (Zaidi *et al.*, 2009). In present study, the *Pseudomonas putida* strain PS9 solubilized the inorganic phosphate considerably even in the presence of the recommended and the higher doses of insecticides.

Siderophore production: In the aerobic environment, iron occurs principally as Fe^{3+} and is likely to form insoluble hydroxides and oxyhydroxides, thus making it generally inaccessible to microorganisms. To acquire sufficient iron, the most commonly found strategy in bacteria is the secretion of siderophores, low-molecular mass iron chelators with high association constants for complexing iron. Thus, siderophores act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation (Indiragandhi *et al.*, 2008). The production of siderophores like SA and DHBA by the *Pseudomonas putida* strain PS9 indicated that this bacterial strain may be used as a bio-control against soil borne phytopathogens.

Production of indole-3-acetic acid: Plant growth enhancing hormone, IAA synthesized by PGPR affect many physiological activities of plants, such as, cell enlargement, cell division, root initiation, growth rate, phototropism, geotropisms and apical dominance etc. (Ahemad and Khan, 2010b). Moreover, this phytohormone also acts as a signaling molecule during the onset of symbiosis in legumes (Barker and Tagu, 2000). In our study, IAA was also produced by the *Pseudomonas putida* strain PS9 exposed even to three times the recommended dose of each insecticide.

Production of exo-polysaccarides, HCN and ammonia: The EPS production is an important trait of rhizobacteria because it protects the bacterial cell against desiccation, phagocytosis and phage attack and also helps in nitrogen fixation by preventing high oxygen tension (Tank and Saraf, 2003). The EPS synthesized excessively by the *Pseudomonas putida* strain PS9 is likely to provide it protection against the stressed environments.

In present study, HCN and ammonia production by the *Pseudomonas putida* strain PS9 remained unaffected whether insecticides were added into media or not. The release of HCN by rhizospheric bacteria into the soil can be toxic to subterranean animals and phytopathogenic organisms (Guo *et al.*, 2007). Similarly, ammonia production by rhizobacterial strains is reported elsewhere (Wani *et al.*, 2008). However, there is no such report wherein the effect of these insecticides was assessed on these PGP activities of rhizobacterium *Pseudomonas putida*.

Each PGP trait of bacteria is the result of sequential metabolic reactions mediated by various specific functional proteins (enzymes) along the defined metabolic pathway. The metabolic pathways for any specific PGP trait may be more than one depending upon the type of the PGP substances and bacterial genera/species. Pesticides including insecticides adversely affect protein synthesis and the metabolic enzymes (Ahemad and Khan, 2010c). Additionally, these chemical agents not only damage structural proteins essential for growth of the organism but also responsible for geno-toxicity (Pham *et al.*, 2004) and eventually leads to the decreased functioning and survival of organisms (Kumar *et al.*, 2010). Therefore, it seems probable that insecticides employed in this study might have inhibited the functioning of the enzymes involved in metabolic pathways of PGP traits in the *Pseudomonas putida* strain PS9. Interestingly, the amount of EPS secreted by the *Pseudomonas putida* strain PS9 in this study increased progressively with gradual amplification in insecticide-concentrations. The reason for this abnormal trend is unknown. Nevertheless, the increase in EPS following increased concentration of each insecticide suggested that the insecticides might have acted as inducers of EPS biosynthesis. EPS provides protection to soil bacteria against environmental stresses; hence it is possible that the *Pseudomonas putida* strain PS9 secreted more EPS under insecticide-stress to shield themselves against these chemicals in a proportion to the pesticide-concentrations.

In conclusion, the selected insecticides at all tested rates showed varying degree of toxicity towards PGP traits (except EPS) of the *Pseudomonas putida* strain PS9. Toxicity to these traits was less pronounced at the recommended rate and increased as the dose rate of insecticides was enhanced further. This study revealed that prior to the application of insecticides in agricultural fields, an appropriate attention should be paid to screen them on the basis of their degree of toxicity to PGP traits of soil micro-flora specifically the beneficial microorganisms. The study also provides an explanation to the possible cause of the reduced crop-productivity with regard to indiscriminately applied insecticides in agricultural practices.

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