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ORIGINAL ARTICLE

Evaluation of plant-growth-promoting activities of rhizobacterium *Pseudomonas putida* under herbicide stress

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Abstract The aim of this study was to evaluate the effect of selected herbicides (quizalafop-p-ethyl, clodinafop, metribuzin and glyphosate) at one, two and three times the recommended field rates on the plant-growth-promoting (PGP) traits of *Pseudomonas putida* strain PS9 isolated from the mustard rhizosphere. *P. putida* strain PS9 was selected for its high herbicide-tolerance and its production of substantial amounts of indole acetic acid, siderophores (salicylic acid and 2,3 dihydroxy benzoic acid) and exo-polysaccharides. It also exhibits HCN and ammonia releasing ability. A progressive, herbicide-concentration-dependent, decline in PGP properties (except exo-polysaccharide production) of *P. putida* strain PS9 was observed when the strain was grown in the presence of increasing concentrations of each herbicide. Generally, among the selected herbicides, the maximum toxicity to PGP traits of *P. putida* strain PS9 was shown by quizalafop-p-ethyl at three times the recommended dose. This study concludes that herbicides should be screened before field application for their degree of toxicity to PGP traits of beneficial microorganisms.

Keywords *Pseudomonas putida* · Herbicide · Plant growth promoting rhizobacteria · Toxicity · Pest management

Introduction

Soil ecosystems are governed by the numerous and diverse interactions involving their physical, chemical, and biological components (Buscot 2005). The various genetic and functional activities of the heterogeneous microbial populations have an especially crucial impact on soil functions; as such, microbes are considered a powerful force in specific fundamental enzyme-mediated metabolic processes (Ahemad and Khan 2011). Moreover, these microorganisms affect the biogeochemical cycling of nutrients and consequently help plants to grow better both under normal and stressed soil conditions (Barea et al. 2004; Khan et al. 2009). The physiological activities of the soil bacteria that inhabit the space in or around the root surface are of enormous importance with regard to plant growth and development, and are often referred to as plant growth-promoting rhizobacteria (PGPR) (Chen et al. 2008; Kloepper et al. 1991). In general, PGPR facilitate plant growth through N_2 fixation (Masson-Boivin et al. 2009), solubilization of insoluble phosphorus (Zaidi et al. 2009), production of siderophores (Tian et al. 2009; Wani et al. 2008), production of phytohormones (Tank and Saraf 2010), lowering of ethylene concentration (Tank and Saraf 2010; Rodrigues et al. 2008), production of antibiotics and antifungal metabolites, and induced systemic resistance (Saravanakumar et al. 2007; Cazorla et al. 2007).

Among various pesticides, herbicides are used in agricultural fields to overcome noxious weeds that are responsible to a great extent for decreased crop productivity (Ahemad et al. 2009; Vasileva and Ilieva 2007). However, injudicious application of herbicides in different production systems beyond certain threshold levels poses a serious threat to both rhizospheric organisms and associated biotic processes (Das et al. 2003; Sándor et al. 2007) and plants (Ahemad and Khan 2010). The effect on soil microflora and plants of herbicide application is influenced by the dose rates, activities and persistence of these chemicals in soils (Ahemad et al. 2009).

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Although *Pseudomonas putida* has been used as a representative organism to study a range of plant growth promoting (PGP) traits, information regarding the impact of herbicides on PGP traits of *P. putida* in the rhizospere-niche have not been explored. Further, the most direct approach to analyzing the specific agrochemical (including herbicides)-induced changes in microbial communities is the use of microorganisms that are tolerant to that agrochemical (Oves et al. 2009). In addition, studies on the effect of various herbicides have been focused largely on changes in populations of soil microflora including PGPR. However, reports on in vitro PGP activities of phosphate (P) solubilizing *P. putida* in the presence of herbicides are rare. Considering these scientific gaps, the present study was designed to evaluate the effects of four herbicides, namely, quizalafop-p-ethyl, clodinafop, metribuzin, and glyphosate (Table 1), at the recommended (1X), double (2X) and three times (3X) the recommended rates on in vitro PGP activities of the herbicide-tolerant rhizobacterium *P. putida*. In pest management practices, an in vitro assessment of the eco-toxicological effects of herbicides towards the functional activities of rhizobacteria prior to their field application is almost always lacking. From this perspective, the results of this study would further contribute towards the more efficient implementation of effective pest management strategies.

Materials and methods

Isolation and screening of phosphate-solubilizing bacteria

Three rhizosphere soil samples (10 g each) of mustard (*Brassica compestris*) cultivated in an experimental field (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, water holding capacity 0.44 ml g^{-1}, cation exchange capacity 11.7 cmol kg^{-1} and anion exchange capacity 5.1 cmol kg^{-1}) at the 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 mixed thoroughly. In order to identify phosphate-solubilizing bacteria, a serial dilution assay was carried out in 0.9% NaCl solution; 10 μl diluted suspension was spread plated on Pikovskaya agar medium (Pikovskaya 1948) [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]. Plates were incubated at 28±2°C for 7 days. Isolates showing a clear halo within 7 days around bacterial colonies were considered as P-solubilizers. A total of 50 P-solubilizing isolates with maximum halo sizes and different pigmentations and morphological parameters were selected.

Assessment of bacterial strains for herbicide-tolerance

The bacterial strains were tested further for sensitivity/resistance against four herbicides (quizalafop-p-ethyl, clodinafop, metribuzin, and glyphosate) (Table 1), by the agar plate dilution method using minimal salt agar medium (g/l: KH_2PO_4 1; K_2HPO_4 1; NH_4NO_3 1; $MgSO_4 \cdot 7H_2O$ 0.2; $CaCl_2 \cdot 2H_2O$ 0.02; $FeSO_4 \cdot 7H_2O$ 0.01; agar 15; pH 6.5). Freshly prepared agar plates amended separately with increasing concentrations (0–3,200 μg ml^{-1}; at a two-fold dilution interval) of herbicides were spot inoculated with 10 μl of 10^8 cells ml^{-1} bacterial strains. Plates were incubated at 28±2°C for 3 days and the highest concentration of herbicides 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) against the herbicides 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 selected for further study. Morphological, physiological and biochemical properties of strain PS9, including Gram reaction, citrate

Table 1 Herbicides used in the present study

Common name	Grade and purity	Chemical name	Chemical family	Recommended dose (1X)
Quizalafop-p-ethyl	Technical (98% w/w)	(*RS*)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionic acid	Aryloxyphenoxy	40 μg kg^{-1}
Clodinafop	Technical (98%w/w)	(*R*)-2-[4-(5-chloro-3-fluoro-2-pyridyloxy)phenoxy]propionic acid	Aryloxyphenoxy	400 μg kg^{-1}
Metribuzin	Commercial (70%w/w)	4-amino-6-*tert*-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one	Triazinone	850 μg kg^{-1}
Glyphosate	Commercial (71% w/w)	*N*-(phosphonomethyl)glycine	Organophosphorus	1,444 μg kg^{-1}

utilization test, indole production test, methyl red test, nitrate reduction, Voges Proskaur, catalase test, carbohydrate (dextrose, mannitol and sucrose) utilization test, starch hydrolysis, and gelatin liquefaction test, were determined according to standard methods (*Bergey's Manual of Determinative Bacteriology*; Holt et al. 1994).

16S rDNA based identification

Sequencing of the 16S rDNA of strain PS9 was carried commercially by a DNA sequencing service (Macrogen, Seoul, South Korea) using universal primers, 518 F (5′ CCAGCAGCCGCGGTAATACG3′) and 800R (5′ TACCAGGGTATCTAATCC3′). Nucleotide sequence data was deposited with the GenBank sequence database.

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

Time course of growth of the bacterial strain

The recommended field doses of quizalafop-p-ethyl (40 μg kg^{-1}), clodinafop (400 μg kg^{-1}), metribuzin (850 μg kg^{-1}), and glyphosate (1,444 μg kg^{-1}) were used. Since all the in vitro experiments in this study were performed in liquid media, herbicide doses were simply converted micrograms per kilogram to micrograms per liter. Stock solutions of herbicides were prepared in HPLC grade DMSO (dimethyl sulfoxide). Exponentially grown cultures of the test organisms were inoculated into minimal salt medium treated with 0 (control), the recommended dose (1X), two times (2X) and three times (3X) the recommended dose of herbicides and incubated at 30°C in rotary shaker (150 rpm) for different time intervals. A solvent control was also run simultaneously. Growth was determined turbidometrically at different time intervals by measuring optical density (OD) at 540 nm.

Bioassay of PGP activities under herbicide-stress

Various PGP activities (P-solubilization, indole acetic acid, siderophore, exo-polysaccharides, hydrogen cyanide and ammonia production) of the P-solubilizing bacterial strain PS9 were assayed both in the presence and absence of the selected herbicides under in vitro conditions.

Phosphate solubilization

The bacterial strain PS9 showing P-solubilizing activity was inoculated into Pikovskaya agar medium supplemented with 0, 1X, 2X and 3X of the recommended rate of each herbicide and incubated at 28 ± 2°C for 7 days and observed for halo formation. The halo formed around the 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). Briefly, 100 ml Pikovskaya broth treated with 0, 1X, 2X and 3X of each herbicide, was inoculated with 1 ml of 10^8 cells/ml of culture. The flasks were incubated for 7 days with shaking (120 rpm) at 28 ± 2°C. A 20 ml culture broth from each flask was removed and centrifuged (9,000 *g*) for 30 min and the amount of soluble P released into the supernatant. To 10 ml supernatant, 10 ml chloromolybdic acid and 5 drops of chlorostannous acid was added and the volume adjusted to 50 ml with distilled water. The absorbance of developing blue color was read at 600 nm. The amount of P solubilized was calculated using the calibration curve of KH_2PO_4. The change in pH following tri-calcium phosphate (TCP) solubilization was also recorded.

Bioassay for indole-3-acetic acid production

Indole-3-acetic acid (IAA) was analyzed quantitatively by the method of Gordon and Weber (1951), as later modified by Brick et al. (1991). Briefly, the phosphate solubilizing bacterial strain PS9 was grown in Luria Bertani (LB) broth (g l^{-1}: tryptone 10; yeast extract 5; NaCl 10 and pH 7.5). A 100 ml aliquot of LB broth with 100 μg tryptophan/ml supplemented with 0, 1X, 2X and 3X of each herbicide was inoculated with 1 ml culture containing 10^8 cells/ml bacterial isolate and incubated for 24 h at 28±2°C with shaking at 125 rpm. After 24 h, 5 ml culture was centrifuged at 9,000 *g* for 15 min and 2 ml Salkowsky reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) was added to 2 ml supernatant and incubated at 28°C in darkness for 1 h. The IAA concentration in the supernatant was determined against a standard curve using a spectrophotometer (λ 540 nm).

Bioassay for siderophore production

Chrome azurol S (CAS) agar plates supplemented with 0, 1X, 2X and 3X of each herbicide were prepared separately and spot inoculated with 10 μl of 10^8 cells ml^{-1}. Plates were incubated at 28±2°C for 4 days. Development of a yellow-to-orange halo around the growth indicated siderophore production (Alexander and Zuberer 1991).

The siderophore produced by the test strain was also assayed quantitatively using Modi medium (g l^{-1}: K_2HPO_4 0.5; $MgSO_4$ 0.4; NaCl 0.1; mannitol 10; glutamine 1; NH_4NO_3 1). (Standardization). Modi medium amended with 0, 1X, 2X and 3X of each herbicide was inoculated with 100 μl of 10^8 cells ml^{-1} bacterial strain PS9 and

incubated at 28±2°C for 5 days. The culture was centrifuged (4,528*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 supernatant twice with an equal volume of solvent (ethyl acetate) at pH 2. Hathway's reagent was prepared by adding 1 ml 0.1 M ferric chloride in 0.1 *N* HCl to 100 ml distilled water and to this was added 1 ml 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 strain PS9 was grown in 100 ml basal medium with 5% sucrose supplemented with 0, 1X, 2X and 3X of each herbicide and incubated for 5 days at 28±2°C on shaker (100 rpm). Culture broth was spun (5,433*g*) for 30 min and EPS was extracted by adding three volumes of chilled acetone to one volume of supernatant. The precipitated EPS was washed repeatedly three times alternately with distilled water and acetone, transferred to a filter paper and weighed after drying overnight (Mody et al. 1989).

Bioassays for hydrogen cyanide and ammonia production

The bacterial strain PS9 was also tested for the synthesis of hydrogen cyanide (HCN) by adopting the method of Bakker and Schipper (1987). Briefly, each bacterial strain was grown in HCN induction medium (g l^{-1}: tryptic soy broth 30; glycine 4.4; agar 15) supplemented with 0, 1X, 2X and 3X of each herbicide and was incubated at 28±2°C for 4 days. Bacterial strain PS9 was then streaked on HCN induction plates. A Whatman filter paper No.1 soaked in 2% sodium carbonate prepared in 0.5% picric acid solution was placed on the top of the plate. Plates were sealed with Parafilm and incubated at 28±2°C for 4 days. Development of an orange-to-red color indicated HCN production.

Synthesis of ammonia by the bacterial strain PS9 was detected using peptone water supplemented separately with 0, 1X, 2X and 3X of each herbicide. Freshly grown bacterial strains (200 µl of 10^8 cells ml^{-1}) was inoculated in 20 ml peptone water in tubes and incubated at 28±2°C for 4 days. Nessler reagent (1 ml) was added to each tube. Development of yellow color indicated a positive test for ammonia production (Dye 1962).

Each individual experiment was replicated three times.

Statistical analysis

All 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 recovered from mustard rhizosphere was characterized and identified by standard morphological, physiological and biochemical tests. The characteristics of the strain of PS9 are described in Table 2. On the basis of these features, PS9 was 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 the 16S

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

Characteristics	Strain PS9
Morphological	
Gram reaction	-[a]
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)	
Quizalafop-p-ethyl	1,600 µg ml^{-1}
Clodinafop	1,600 µg ml^{-1}
Metribuzin	3,000 µg ml^{-1}
Glyphosate	2,800 µg ml^{-1}

[a] + positive reaction, – negative reaction

rDNA of this strain was submitted to GenBank (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 the NCBI database. Such high similarity values confirmed strain PS9 rhizobacterium as *Pseudomonas putida*.

Herbicide tolerance

In this study, the rhizobacterium *Pseudomonas putida* PS9 tolerated a considerable amount of herbicide (quizalafop-p-ethyl, clodinafop, metribuzin, glyphosate) when grown on C- and N-source-deficient minimal salt agar plates amended with the graded concentrations (0–3,200 μg ml^{-1}) of each herbicide. The tolerance levels of *P. putida* strain PS9 against herbicides ranged between 1,600 μg ml^{-1} and 3,000 μg ml^{-1}. Among the selected herbicides, *P. putida* PS9 displayed the maximum tolerance against metribuzin (3,000 μg ml^{-1}). However, the MTL values of the *P. putida* PS9 against each herbicide were remarkably high.

Growth pattern of *P. putida* strain PS9

Growth of *P. putida* strain PS9 in minimal salt medium amended with three doses of each herbicide was monitored at different incubation intervals (Fig. 1). In general, the highest tested doses of herbicides showed the maximum toxicity to bacterial growth.

PGP activities of *P. putida* strain PS9

In our study, the rhizobacterium *P. putida* strain PS9 displayed PGP activities in both conventional and herbicide-stressed media. In the control, *P. putida* strain PS9

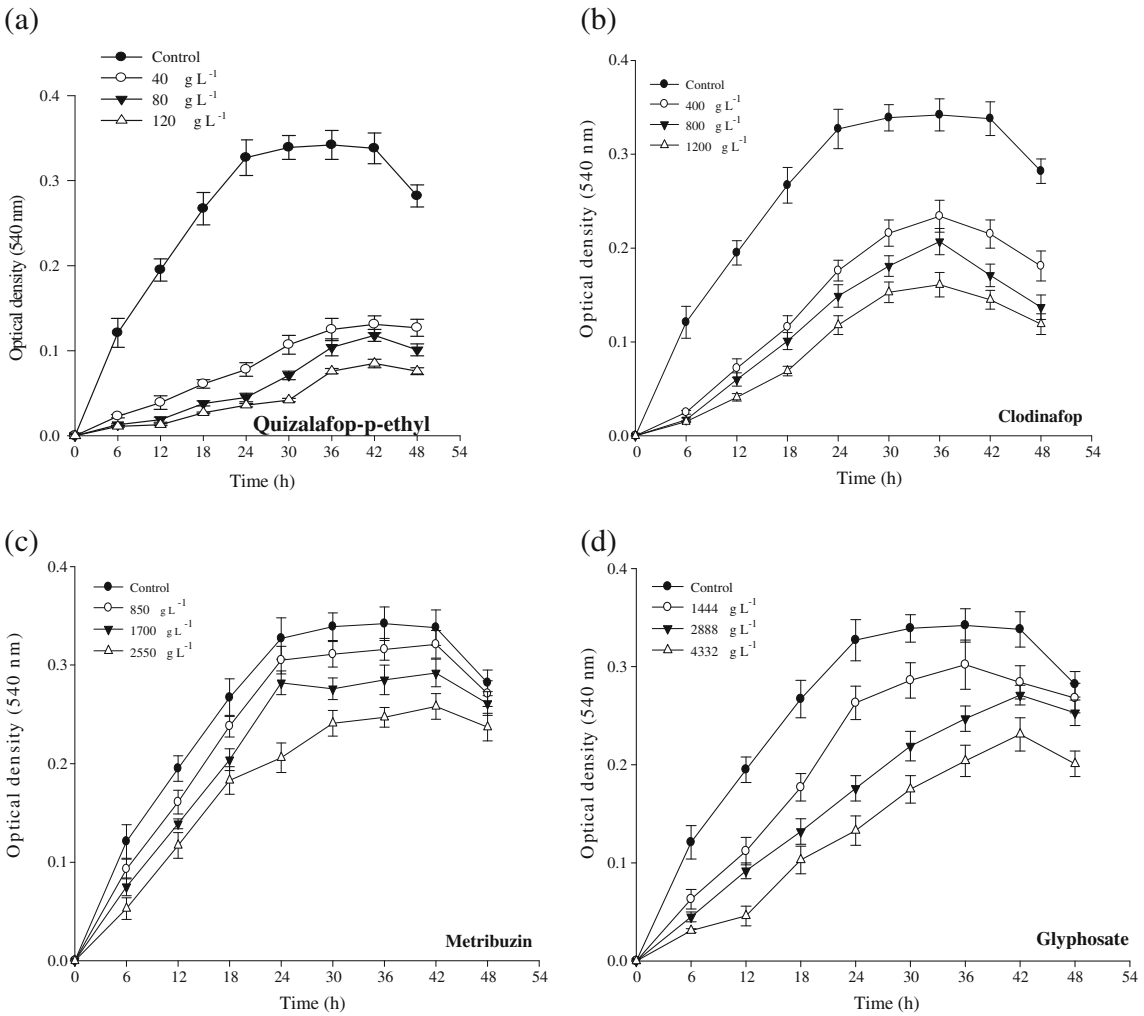

Fig. 1 Effect of the recommended (*open circles*), double (*inverted filled triangles*) and three times (*upright open triangles*) the recommended dose of quizalafop-p-ethyl (**a**), clodinafop (**b**), metribuzin (**c**) and glyphosate (**d**) on the *Pseudomonas putida* strain PS9 grown in minimal salt medium

solubilized inorganic P to a great extent and produced IAA, siderophores and EPS in substantial amounts. When subjected to herbicide-stress, a significant decline in PGP activities was observed in rhizobacterial strain PS9.

Phosphate solubilization under herbicide-stress

The P-solubilizing efficiency of *P. putida* PS9 in the presence of three concentrations (the recommended dose, two times and three times the recommended dose) of each herbicide, was evaluated both qualitatively and quantitatively using Pikovskaya medium (Table 3). The P-halo on Pikovskaya agar medium reflecting P-solubilizing ability of *P. putida* PS9 decreased in size significantly but in a non-linear manner. When the P-solubilizing ability of two organisms is compared, the P-solubilizing zone does not give an accurate estimation of which organism produces the greater sized P-zone because the size of the P-zone also depends on growth of bacterial colony, and may be smaller or greater depending upon spot-inoculation and the bacterial growth rate. A more accurate assessment of comparative P-solubilizing ability is revealed by the solubilization index (SI). The effect of 1X and 2X of all herbicides on SI of the *P. putida* PS9 was less deleterious, while the highest concentration (3X) had the maximum adverse impact on P-halo formation. The order of toxicity of herbicides at 3X on SI was: quizalafop-p-ethyl>clodinafop> metribuzin=glyphosate (Table 3).

In the control, *P. putida* PS9 solubilized a considerable amount of TCP in Pikovskaya broth after 7 days of incubation, with a decline in medium pH from 7.0 to 4.4. A progressive herbicide-concentration-dependent inhibition in the P-solubilizing trait of the *P. putida* PS9 was observed. The amount of P-solubilized in Pikovskaya broth decreased with increasing concentration of each herbicide from the recommended dose to three times the recommended rate. Generally, the pH of the herbicide-amended Pikovskaya broth increased with the graded increment of each herbicide. The correlation (R^2=0.82) between the pH of the herbicide-amended medium and extent of P-solubilization was not strong. The maximum inhibition of the P-solubilizing activity of the *P. putida* PS9 was seen with quizalafop-p-ethyl, which decreased the solubilized P compared to untreated control by 89, 92 and 95% at 1X, 2X and 3X, respectively. At 3X of each herbicide, the order of herbicidhe-toxicity (percent decline relative to control) was: quizalafop-p-ethyl (95)>clodinafop (92)>glyphosate (86)>metribuzin (75). No correlation (R^2=0.48) was observed between SI and P solubilized in broth (Table 3).

Table 3 Plant growth promoting (PGP) activities of phosphate solubilizing bacterium *P. putida* in the presence of varying concentrations of herbicides. Values indicate the mean of three replicates. Mean values (± SD) followed by different letters within a row or column are significantly different at $P \leq 0.05$ according to Tukey test. *CAS* Chrome azurol S agar; *SA* salicylic acid; *DHBA* 2,3-dihydroxy benzoic acid; *IAA* indole acetic acid; *T* Tryptophan concentration (μg/ml); *EPS* exopolysaccharides; *HCN* hydrogen cyanide

Herbicide	Dose rate (μg l^{-1})	Phosphate solubilized			PGP activities					HCN	Ammonia
		Liquid medium (μg ml^{-1})	pH	SI[a]	Siderophores			IAA (μg ml^{-1}) 100T	EPS (μg ml^{-1})		
					Zone on CAS agar (mm)	SA (μg ml^{-1})	DHBA (μg ml^{-1})				
Quizalafop-p-ethyl	40	33±3 i	6.5	1.3	10±1.2 b	38±2.1 b	11±1.2 b	13±1.1 fg	19±1.5 fg	+	+
	80	25±2 j	6.8	1.0	9±1.4 c	34±1.2 d	8±0.5 e	9±0.5 i	21±1.3 de	+	+
	120	17±2 k	6.8	0.8	8±1.0 d	22±1.3 i	2±0.6 i	4±0.6 j	24±2.7 ab	+	+
Clodinafop	400	135±6 c	5.7	1.5	11±1.0 a	35±1.6 cd	11±0.5 b	19±1.2 de	19±1.6 fg	+	+
	800	75±5 e	6.2	1.5	10±1.0 b	31±2.7 e	3±0.5 h	12±0.8 gh	22±1.2 cd	+	+
	1,200	53±3 g	6.5	1.3	10±1.0 b	22±1.5 i	2±0.4 i	10±0.6 hi	25±2.6 a	+	+
Metribuzin	850	201±8 b	5.7	1.8	11±1.5 a	27±1.4 fg	9±0.2 d	25±1.4 b	18±1.2 gh	+	+
	1,700	116±6 d	6.3	1.8	11±1.1 a	24±1.5 h	8±0.5 e	17±1.2 e	20±1.1 ef	+	+
	2,550	74±4 e	6.5	1.5	10±1.2 b	19±1.6 j	6±0.2 f	12±1.1 gh	22±1.2 cd	+	+
Glyphosate	1,444	86±5 e	6.0	1.8	11±1.0 a	26±1.2 g	10±0.3 c	22±1.2 c	18±1.4 gh	+	+
	2,888	69±3 f	6.4	1.5	11±1.5 a	18±1.2 k	6±0.2 f	13±1.3 fg	21±1.5 de	+	+
	4,332	41±2 h	6.7	1.5	10±1.0 b	15±1.3 l	4±0.2 g	9±0.5 i	25±1.5 a	+	+
Control (without herbicide)		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 h	+	+
F value (treatment)		632.3	-	-	47.4	274.6	171.5	128.2	108	-	-

[a] SI=[(zone size including colony diameter − colony diameter)/ colony diameter]

Siderophore production under herbicide stress

Pseudomonas putida PS9 was also evaluated qualitatively for the production of iron-binding molecules—siderophores—on CAS agar plates in both the presence and absence of herbicides. The siderophore-producing ability of strain PS9 was manifested through the formation of an orange-colored zone around the rhizobacterial growth on CAS agar plates. Without herbicide-stress, strain PS9 produced a siderophore-zone of 11-mm diameter in CAS agar medium. However, when the organism was grown in the same medium amended with 1X, 2X and 3X doses of each herbicide, the siderophore-zone remained generally unaffected relative to control at 1X and 2X herbicide doses. In contrast, the 3X dose rate of each herbicide decreased the siderophore-zone but only to a small extent. Among herbicides, the highest reduction in the siderophore-zone was observed in the presence of quizalafop-p-ethyl at all rates. The order of percent decline in zone diameter relative to untreated control for the tested herbicides at 3X was: quizalafop-p-ethyl (27)>clodinafop (10)=metribuzin (10)=glyphosate (10).

Furthermore, *P. putida* PS9 was grown in Modi medium to quantify production of siderophore molecules (SA and DHBA) in both the presence and absence of the tested herbicides. In the absence of herbicide stress, the strain PS9 released 41 μg ml^{-1} SA and 17 μg ml^{-1} DHBA into the supernatant. In contrast, a herbicide-concentration-dependent progressive decline in production of both siderophore molecules SA and DHBA was observed when strain PS9 was grown in Modi medium amended with the graded concentrations of each herbicide from 1X to 3X. Of the selected herbicides, glyphosate displayed the greatest toxicity to SA biosynthesis at all concentrations. Glyphosate at 1X, 2X and 3X decreased SA by 37, 56 and 63%, respectively, compared to control. At 3X, the order of percent decline in SA synthesis by each herbicide was: glyphosate (63)>metribuzin (54)>quizalafop-p-ethyl (46)=clodinafop (10). The trend of herbicide-toxicity to DHBA biosynthesis was found to be very similar (Table 3).

Production of IAA, EPS, HCN and ammonia under herbicide-stress

Pseudomonas putida PS9 produced a substantial amount of IAA in both the absence and presence of herbicides. In LB medium untreated with herbicides, *P. putida* PS9 produced IAA (42 μg ml^{-1}), which, however, decreased progressively with increasing concentrations of each herbicide from 1X to 3X. Comparing the effects of the tested herbicides at 3X, quizalafop-p-ethyl reduced the IAA production maximally by 88% while metribuzin showed least toxicity, decreasing IAA by 65% over the untreated control (Table 3). Unlike other PGP substances, EPS synthesis by strain PS9 increased gradually with regular enhancement of herbicide concentrations. At 3X, EPS secretion (percent increase over control) was found, in order, as follows: clodinafop (47)= glyphosate (47)>quizalafop-p-ethyl (41)>metribuzin (29). Finally the three concentrations of each herbicide did not affect production of HCN or ammonia by *P. putida* strain PS9 (Table 3).

Discussion

Herbicide-tolerance

In our study, *P. putida* strain PS9 displayed the highest tolerance against the selected herbicides among the isolated strains. The MTL values of herbicides ranged between 1,600 g ml^{-1} to 3,000 μg ml^{-1}. Tolerance or resistance in microorganisms against pesticides including herbicides is a complex process that is regulated both physiologically and genetically by the microorganism. Hence, microorganisms that have developed resistance to pesticides are frequently capable of biodegrading them (Kumar et al. 1996; Ortiz-Hernández and Sánchez-Salinas 2010). Temporary resistance (tolerance) against pesticides in general is attributed to physiological changes that induce microbial metabolism to form a new metabolic pathway to bypass a biochemical reaction inhibited by a specific pesticide (Bellinaso et al. 2003). Permanent resistance, on the other hand, depends upon genetic modifications, inherited by subsequent generations of microbes (Johnsen et al. 2001; Herman et al. 2005). It is general practice to isolate pesticide-degrading bacteria on minimal/ mineral salt agar medium (Bano and Musarrat 2003). Since the medium used in our study to assess the MTL values and growth curves of *P. putida* strain PS9 did not contain any carbon and nitrogen sources except the tested herbicides, it is inferred that *P. putida* strain PS9 might have utilized these herbicides amended in minimal salt agar medium as a sole energy source through a biodegradation process and hence, showed high tolerance levels against herbicides.

In vitro production of PGP substances

In this study, *P. putida* strain PS9 exhibited PGP traits like P-solubilization, production of siderophores, phytohormone and EPS in considerable amounts in both the absence and presence of herbicide stress.

Under normal conditions, *P. putida* strain PS9 solubilized inorganic phosphate considerably, with a concomitant decline in medium pH. In contrast, strain PS9 solubilized inorganic phosphate even in the presence of the recommended and higher doses of herbicides but with an increase in medium pH. It is reported that the phosphate solubilizing property in rhizobacteria 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). The increase in pH of the medium under herbicide stress is consistent with the decline in P-solubilizing efficiency of strain PS9. The increment in pH of the medium may possibly be due to a reduction in bacterial organic acid secretion, or the degraded by-products of herbicides; indeed, both factors might be involved in elevating the pH of herbicide-amended medium.

In aerobic environments, 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 (Miethke and Marahiel 2007). The production by *P. putida* strain PS9 of a substantial amount of siderophores under herbicide stress in our study revealed that this strain could be used to suppress plant-pathogen-mediated diseases by conferring a competitive advantage on bio-control agents for the limited supply of essential trace minerals in natural habitats.

The phytohormone IAA, synthesized from transamination and decarboxylation of tryptophan, controls cell division, root initiation, phototropism, geotropism and apical dominance in plants (Khan et al. 2010). In general, the IAA produced by rhizobacteria promotes root growth by directly stimulating plant cell elongation or cell division. A low level of IAA produced by rhizosphere bacteria promotes primary root elongation whereas a high level of IAA stimulates lateral and adventitious root formation but inhibits primary root growth (Ma et al. 2009). In our study, *P. putida* strain PS9 produced a high amount of IAA (37 μg ml^{-1}) in LB medium amended with 100 μg ml^{-1} tryptophan and without herbicides, while, in the presence of three concentrations of each herbicide, IAA released by *P. putida* strain PS9 decreased significantly.

The EPS helps to shield bacteria against environmental stresses like desiccation, phagocytosis and phage attack besides supporting N_2 fixation by preventing high oxygen tension (Tank and Saraf 2003). The EPS so excessively synthesized by *P. putida* strain PS9 is likely to provide protection against stress factors like herbicides in soils.

Rhizobacteria protect growing plants from phytopathogen attack by killing parasites directly with the production of HCN (Kang et al. 2010). In agreement with our report, Devi et al. (2007) also reported excretion of HCN into the rhizosphere by rhizobacterial strains. In contrast, the ammonia released by rhizobacterial strains plays a signaling role in the interaction between rhizobacteria and plants, and also increases glutamine synthetase activity (Chitra et al. 2002). In our study, HCN and ammonia production by *P. putida* strain PS9 remained unaffected despite elevated concentrations of herbicides up to three times the recommended dose. Hence, strain PS9 may be exploited as a bio-control agent.

Each PGP trait of bacteria is the result of sequential metabolic reactions mediated by various specific functional proteins (enzymes) along a defined metabolic pathway. Depending on the type of PGP substance and the bacterial genus/species, there may be more than one metabolic pathway for a specific PGP trait. Pesticides and herbicides adversely affect protein synthesis and metabolic enzymes (Kapoor and Arora 1996; Boldt and Jacobsen 1998). Therefore, it seems probable that the herbicides employed in this study might have inhibited the functioning of enzymes participating in different metabolic pathways of PGP traits in *P. putida* strain PS9. Additionally, pesticides not only damage structural proteins essential for growth of the organism but are also responsible for geno-toxicity (Pham et al. 2004), and eventually lead to the decreased functioning and survival of organisms exposed to high concentration of pesticides (Kumar et al. 2010). Interestingly, the amount of EPS secreted by *P. putida* strain PS9, unlike other PGP traits in this study, increased with the gradual increase in herbicide concentrations. The reason for this abnormal trend is unknown. Nevertheless, the increase in EPS following increased concentration of each herbicide suggested that the herbicides might have acted as inducers of EPS biosynthesis. EPS provides protection to soil bacteria against environmental stresses; hence it is possible that strain PS9 secreted higher EPS under herbicide stress to safeguard itself against these chemicals in a manner proportional to the herbicide concentration.

In conclusion, graded concentrations of each herbicide, including the recommended field rates, added to culture media was found to have suppressing effect on PGP activities [IAA and siderophores (SA and DHBA) except EPS] of *P. putida* strain PS9. Our study suggests that, prior to their application on agriculture fields, appropriate attention should be paid to screen herbicides on the basis of their degree of toxicity to PGP traits of soil micro-flora, specifically beneficial microorganisms. The implementation of this approach (along with present measures and strategies of pest management employed in agricultural practices) could possibly lead to more efficacious pest management.

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