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Influence of Selective Herbicides on Plant Growth Promoting Traits of Phosphate Solubilizing *Enterobacter asburiae* Strain PS2

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Abstract: This study examines the effect of four herbicides, quizalafop-p-ethyl, clodinafop, metribuzin and glyphosate, on plant growth promoting activities, like, phosphate solubilization, siderophores, indole acetic acid, exo-polysaccharides, hydrogen cyanide and ammonia production by herbicide tolerant *Enterobacter asburiae* strain PS2 isolated from mustard rhizosphere. The selected herbicides were applied at recommended, two and three times the recommended rates. The activities of *E. asburiae* strain PS2 observed under *in vitro* environment were persistent for all herbicides at lower rates which however, decreased regularly, but not lost completely, as the concentration of each herbicide was increased from lower to higher one. Herbicides at recommended dose had less inhibitory effect while the dose higher than the recommended one adversely affected the plant growth promoting traits of *E. asburiae* strain PS2. Among all herbicides, quizalafop-p-ethyl generally, showed maximum toxicity to plant growth promoting activities of this bacterium. The order of herbicide toxicity at highest dose rate for each herbicide was observed as quizalafop-p-ethyl>clodinafop>glyphosate>metribuzin for phosphate solubilizing potential; quizalafop-p-ethyl>glyphosate>clodinafop> metribuzin for salicylic acid synthesis; quizalafop-p-ethyl>clodinafop = glyphosate >metribuzin for 2, 3-dihydroxybenzoic acid and quizalafop-p-ethyl>clodinafop> glyphosate>metribuzin for indole acetic acid production. In contrast *E. asburiae* strain PS2 produced higher exo-polysaccharides on increasing concentration of each herbicide. At three times the recommended rate of each herbicide, the order of induction in exo-polysaccharides secretion by *E. asburiae* strain PS2 was found as clodinafop>quizalafop-p-ethyl>metribuzin>glyphosate. The herbicide tolerance together with growth promoting activities shown under herbicide stress suggests that *E. asburiae* strain PS2 could be used as inoculant for raising the productivity of crops even in soils poisoned with herbicides.

Key words: *Enterobacter*, herbicide, phosphate solubilization, plant growth promoting rhizobacteria, toxicity

INTRODUCTION

Microbial communities inhabiting soils catalyzes many processes important for soil fertility and plant growth (Zaidi *et al*., 2009). Such processes include cycling of nutrients and transfer of nutrients, like, nitrogen, phosphorus and iron etc. directly to crops and production

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of specific chemical compounds such as, organic acids, siderophores and phytohormones (Khan *et al*., 2010). However, the beneficial microbial communities of soils largely involving Plant Growth Promoting Rhizobacteria (PGPR) are greatly influenced by various factors including the agrochemicals (e.g., herbicides), which are applied in modern agricultural practices by agronomists to offset the noxious weeds and consequently to augment the productivity of crops (Ahemad *et al*., 2009).

However, the intensive, expensive and erratic application of herbicides leads to their accumulation in soils to a dangerous level that adversely affects both the quality and biological composition of soils (Srinivas *et al*., 2008; Zahran, 1999). The naturally abundant PGPR are metabolically inactivated by uptaking herbicides, applied excessively to soils (Singh and Wright, 2002; Bellinaso *et al*., 2003; Ahemad and Khan, 2009). In contrast, a few microorganism can be tolerant or resistant (slightly or not affected) towards a specific herbicide. Moreover, herbicides not only adversely affect the important soil microbial communities including rhizobacteria and their functional activities but also the growing plants (Barriuso *et al*., 2010; Niemi *et al*., 2009; Ratcliff *et al*., 2006; Hess, 2000). Globally, the greater concern is therefore, as to how to minimize or reduce the effects of herbicides so that the consequential impact of these chemicals on the microorganisms involved in nutrient cycling, vis-a-vis the productivity of crops could be saved. Furthermore, among soil microbes, rhizobacteria residing in the vicinity of plant roots play a very important role in growth promotion of plants. Rhizobacteria belonging to genera *Enterobacter* have been reported as phosphate solubilizing PGPR by many authors (Chung *et al*., 2005; Hwangbo *et al*., 2003). Through this perspective, this study was designed to test the hypothesis that herbicides, quizalafop-p-ethyl, clodinafop, metribuzin and glyphosate, when applied at recommended, double and three times the recommended rate, affect the survival and *in vitro* Plant Growth Promoting (PGP) activities of rhizobacteria.

MATERIALS AND METHODS

Three soil samples of 10 g each, in September, 2006, collected from rhizosphere of mustard (*Brassica compestris*), cultivated in the Experimental Fields of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, Uttar Pradesh, India were thoroughly mixed and serially diluted. Phosphate solubilizing bacteria were isolated using Pikovskaya agar medium and the isolates demonstrating clear halo around bacterial growth were considered as phosphate (P) solubilizers. A total of 50 P-solubilizing isolates with larger halo size were selected. The bacterial strains were tested further for their sensitivity/resistance to various concentrations of four herbicides (Table 1) by agar plate dilution method using minimal salt agar medium amended separately with increasing concentration of quizalafop-p-ethyl, clodinafop, metribuzin and glyphosate (Fig. 1a-d). The

Table 1: Herbicides used in the present study

Common name	Grade and purity	Chemical name	Chemical family	Recommended dose (1X) ($\mu g\ kg^{-1}$)
Quizalafop-p-ethyl	Technical (98% w/w)	(RS)-2-[4-(6-chloroquinoxalin-2-yloxy) phenoxy]propionic acid	Aryloxyphenoxy	40
Clodinafop	Technical (98% w/w)	(R)-2-[4-(5-chloro-3-fluoro-2-pyridyloxy)phenoxy]propionic acid	Aryloxyphenoxy	400
Metribuzin	Commercial (70% w/w)	4-amino-6-*tert*-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one	Triazinone	850
Glyphosate	Commercial (71% w/w)	N-(phosphonomethyl)glycine	Organophosphorus	1444

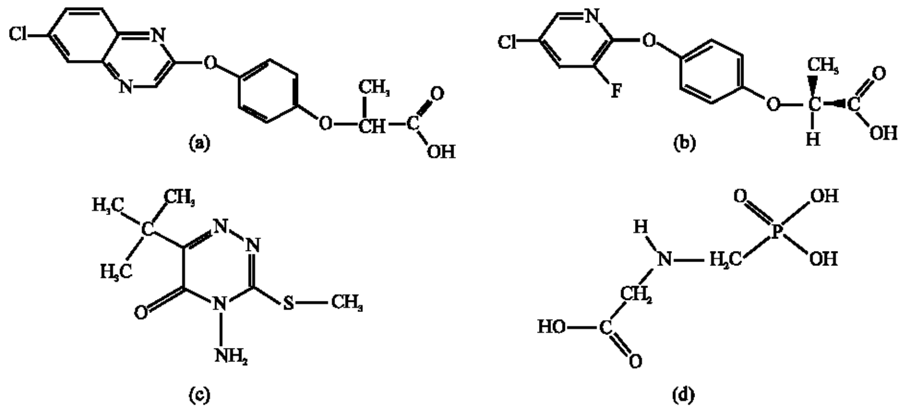

Fig. 1: Chemical structure of herbicides used in the present study, (a) Quizalafop-p-ethyl, (b) Clodinafop, (c) Metribuzin and (d) Glyphosate

highest concentration of herbicides supporting bacterial growth was defined as the Maximum Resistance Level (MRL). Out of 50, a total of 18 bacterial isolates showing higher MRL values were selected and identified using morphological and biochemical tests (Holt *et al*., 1994). Of the 18 P-solubilizing isolates, isolate PS2 showing higher MRL and P-solubilization was further, identified commercially at molecular level by Macrogen Inc., Seoul, South Korea, through 16S-rDNA sequencing using universal primers, 518F (5'CCAGCAGCCGCGGTAATACG3') and 800R (5'TACCAGGGTATCTAATCC3').

The bacterial strains showing P-solubilizing activity were inoculated into Pikovskaya medium supplemented with 0, 1X (recommended dose), 2X (two times the recommended dose) and 3X (three times the recommended dose) of each herbicide and P-solubilized and change in pH of the medium was assessed (King, 1932; Jackson, 1967). For quantitative assay of Indole Acetic Acid (IAA), the bacterial strains were grown in Luria Bertani (LB) broth. Luria Bertani broth (100 mL) having fixed concentration of tryptophan (100 μg mL^{-1}) supplemented with 1X, 2X and 3X of recommended rate of each herbicide and without herbicide (control) was inoculated with one ml culture (10^8 cells mL^{-1}) of bacterial isolates and was incubated for seven days at 28±2°C with shaking at 125 rpm. The IAA concentration in the supernatant was determined by the method of Gordon and Weber (1951), later modified by Bric *et al*. (1991). The bacterial strains were further tested for siderophore [salicylic acid (SA) and 2,3-dihydroxybenzoic acid (DHBA)] production using Chrome Azurol S (CAS) agar medium and Modi medium supplemented with 0, 1X, 2X and 3X of herbicides following the method of Alexander and Zuberer (1991) and Reeves *et al*. (1983), respectively. Hydrogen cyanide (HCN) and ammonia production by bacterial strains was detected by the method of Bakker and Schipper (1987) and Dye (1962), respectively. The exo-polysaccharide (EPS) produced by the bacterial strains was determined under *in vitro* conditions as suggested by Mody *et al*. (1989). Each experiment was replicated three times.

RESULTS

Molecular Identification and Tolerance of Bacteria to Herbicides

In the present study, strain PS2 exhibiting higher MRL to four herbicides, quizalafop-p-ethyl, clodinafop, metribuzin and glyphosate, was characterized morphologically and biochemically (Table 2) and later subjected to 16S rDNA sequencing. This strain was

Table 2: Morphological and biochemical characteristics of *Enterobacter asburiae* strain PS2

Characteristics	Strain PS2
Morphological	
Gram reaction	G-ve
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 resistance levels (μg mL^{-1})	
Quizalafop-p-ethyl	1200
Clodinafop	1600
Metribuzin	3000
Glyphosate	2800

+: Positive reactions, -: Negative reactions

identified as *Enterobacter asburiae* (Gene Bank accession number FJ705887) whose rDNA sequence was found 99% similar to that of *Enterobacter asburiae* strain J2S4 (accession number EU221358) stored in NCBI database. In our study, *E. asburiae* strain PS2 displayed higher tolerance to herbicides and hence, exhibited an exceptionally higher MRL (e.g., 1200, 1600, 3000 and 2800 μg mL^{-1}, respectively, for quizalafop-p-ethyl, clodinafop, metribuzin and glyphosate) to varying concentrations of each herbicide.

Phosphate Solubilization

The P-solubilizing capacity of *E. asburiae* strain PS2 in the presence of varying concentrations of herbicides was assayed both qualitatively and quantitatively using solid and liquid Pikovskaya medium. Generally, when the concentration of each herbicide was increased from 1X to 3X, size of halo decreased considerably (Table 3). The effect of 1X and 2X of all herbicides on zone diameter was less offensive but the highest concentration (3X) had the most hazardous effect on halo formation. The order of toxicity of herbicides at 3X on halo size (solubilization index) was: quizalafop-p-ethyl>clodinafop>glyphosate>metribuzin (Table 3). In addition, the amount of P-solubilized in liquid medium also decreased with increasing concentration of each herbicide from recommended to three times the recommended rate. Among all herbicides, the highest toxic effect was shown by quizalafop-p-ethyl which decreased P-solubilizing activity of *E. asburiae* strain PS2 in broth by 72, 91 and 94% at 40, 80 and 120 μg L^{-1}, respectively, over control. The order of herbicide toxicity (percent decrease over control) at highest dose rate for each herbicide was observed as: quizalafop-p-ethyl (94)>clodinafop (79)>glyphosate (74)>metribuzin (50). No correlation was distinguished between the halo size and P-solubilized in broth (Table 3).

Siderophore Production

Similar to the herbicide-concentration dependent reduction of P-solubilization, the size of siderophore zone also decreased with increasing concentrations of each herbicide. The

Table 3: Plant growth promoting activities of phosphate solubilizing bacterium *Enterobacter asburiae* strain PS2 in the presence of varying concentrations of herbicides

					Plant growth promoting activities						
		Phosphate solubilized			Siderophores						
Herbicides	Dose rate (µg L^{-1})	Liquid medium (µg mL^{-1})	pH	SI*	Zone on CAS^{A} agar (mm)	SA^{B} (µg mL^{-1})	DHBA^{C} (µg mL^{-1})	IAA^{D} (µg mL^{-1}) 100T^{E}	EPS^{F} (µg mL^{-1})	Ammonia	HCN^{G}
Quizalafop-p-ethyl	40	71 ± 3^{e}	6.5	1.5	11 ± 1.0^{ab}	21 ± 1.3^{c}	5 ± 0.5^{c}	11 ± 1.2^{f}	20 ± 1.0^{e}	+	+
	80	23 ± 2^{g}	6.8	1.3	10 ± 1.0^{b}	14 ± 1.6^{g}	4 ± 0.7^{d}	4 ± 0.5^{i}	23 ± 1.0^{c}	+	+
	120	15 ± 2^{h}	6.8	0.8	9 ± 1.0^{c}	9 ± 1.5^{h}	2 ± 0.4^{f}	3 ± 0.6^{j}	25 ± 1.0^{b}	+	+
Clodinafop	400	198 ± 7^{bc}	5.1	2.0	12 ± 1.0^{a}	23 ± 2.5^{b}	5 ± 0.4^{c}	15 ± 1.5^{d}	22 ± 1.0^{d}	+	+
	800	97 ± 5^{d}	6.3	1.8	11 ± 1.0^{ab}	21 ± 1.3^{c}	4 ± 0.6^{d}	9 ± 0.4^{gh}	25 ± 1.0^{b}	+	+
	1200	55 ± 4^{f}	6.5	1.3	10 ± 1.5^{b}	15 ± 1.2^{f}	3 ± 0.7^{e}	7 ± 0.3^{h}	28 ± 1.5^{a}	+	+
Metribuzin	850	212 ± 7^{b}	5.6	2.0	12 ± 1.0^{a}	20 ± 1.1^{cd}	7 ± 0.5^{b}	21 ± 1.3^{b}	18 ± 1.0^{fg}	+	+
	1700	195 ± 8^{bc}	6.1	2.0	12 ± 1.3^{a}	19 ± 2.3^{d}	5 ± 0.8^{c}	15 ± 1.4^{d}	21 ± 1.3^{de}	+	+
	2550	128 ± 5^{d}	6.5	1.8	11 ± 1.0^{ab}	16 ± 2.2^{ef}	4 ± 0.9^{d}	13 ± 1.5^{e}	24 ± 1.0^{bc}	+	+
Glyphosate	1444	184 ± 4^{c}	5.4	2.0	12 ± 1.0^{a}	18 ± 1.4^{e}	6 ± 0.6^{bc}	19 ± 1.6^{c}	19 ± 1.0^{f}	+	+
	2888	112 ± 3^{cd}	5.7	1.8	11 ± 1.0^{ab}	15 ± 1.5^{f}	5 ± 0.7^{c}	13 ± 1.5^{e}	21 ± 1.0^{de}	+	+
	4332	67 ± 6^{ef}	6.2	1.5	11 ± 1.0^{ab}	13 ± 1.2^{gh}	3 ± 0.4^{e}	10 ± 1.4^{g}	23 ± 1.0^{c}	+	+
Control (without herbicide)		258 ± 3^{a}	4.7	2.0	12 ± 1.5^{a}	28 ± 2.2^{a}	9 ± 0.4^{a}	32 ± 1.5^{a}	16 ± 1.4^{g}	+	+
F-value (treatment)		244.8	-	-	12.7	37.6	17.9	83.5	139.4	-	-

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

highest drop in siderophore synthesis by *E. asburiae* expressed as zone on CAS agar plates supplemented with three doses of each herbicide was displayed in the presence of 3X of quizalafop-p-ethyl which decreased the siderophore zone by 25% compared to untreated bacterial sample. The order of percent decline in zone diameter relative to untreated control for all herbicides at 3X was: quizalafop-p-ethyl (25)>clodinafop (17)>metribuzin (8) = glyphosate (8). The siderophores (both SA and DHBA) produced by *E. asburiae* strain PS2 in the supernatant also decreased consistently with increasing dose of each herbicide (Table 3). Quizalafop-p-ethyl at 3X showed highest toxic effect on the synthesis of both SA and DHBA and decreased it maximally by 68 and 78%, respectively compared to untreated control. At three times the recommended rate for each herbicide, the sequence of toxicity on SA synthesis (percent decline over control) was: quizalafop-p-ethyl (68) >glyphosate (54) > clodinafop (46)>metribuzin (43) (Table 3). Moreover, trend of toxicity of herbicides on bacterial DHBA biosynthesis (percent decline over control) was observed as: quizalafop-p-ethyl (78)>clodinafop (67) = glyphosate (67)>metribuzin (56) (Table 3).

Indole Acetic Acid, Exo-Polysaccharides, HCN and Ammonia Production

E. asburiae strain PS2 produced a substantial amount of IAA in LB broth supplemented with 100 µg mL^{-1} tryptophan both in the absence and presence of herbicides. In the medium untreated with herbicides, *E. asburiae* strain PS2 produced a maximum (32 µg mL^{-1}) amount of IAA. However, IAA released by the *E. asburiae* strain PS2 decreased progressively with increase in concentration of each herbicide. While comparing the effects of all herbicides at 3X, quizalafop-p-ethyl reduced the IAA production maximally by 91% while metribuzin exhibiting least toxicity decreased IAA by 59% above the untreated control. Trend of toxicity of herbicides on IAA biosynthesis (percent decline over control) was observed in an order as: quizalafop-p-ethyl (91)>clodinafop (78)>glyphosate (69)>metribuzin (59) (Table 3). Unlike other PGP substances, EPS synthesized by strain PS2 increased progressively with gradual enhancement of each herbicide concentration. At 3X, the maximum induction in EPS secretion (percent increase over control) was found as clodinafop (75)>quizalafop-p-ethyl (56)>metribuzin (50)>glyphosate (44) (Table 3).

DISCUSSION

Tolerance to Herbicides

In present study, phosphate solubilizing *E. asburiae* strain PS2 displayed considerably higher MRL value for the selected herbicides of various chemical groups. In a similar study, Ahemad and Khan (2009) also reported that phosphate-solubilizing *Pseudomonas aeruginosa* strain PS1 tolerated quizalafop-p-ethyl and clodinafop to a level of 1600 μg mL^{-1} when grown in a minimal salts medium supplemented with increasing concentrations of quizalafop-p-ethyl and clodinafop. The ability of microorganisms to grow at higher concentrations of herbicides belonging to any specific chemical group may be temporary or permanent. The development of pesticide tolerance is however, a complex process occurring both at physiological or genetic level of microorganism or its inhabiting niche. And hence, the microorganisms that developed resistance to pesticides are frequently capable of biodegrading them (Kumar *et al*., 1996). The temporary resistance towards herbicides shown by microbial communities in general is largely due to physiological changes that induce the microbial metabolism for the formation of a new metabolic pathway to bypass a biochemical reaction inhibited by a specific toxic substance (Herman *et al*., 2005). Permanent resistance, on the other hand, occurs due to genetic modifications, inherited by the subsequent generation of microbes (Bellinaso *et al*., 2003; Johnsen *et al*., 2001).

In vitro Production of Plant Growth Promoting Substances

In the present study, *E. asburiae* strain PS2 exhibited plant growth promoting traits like inorganic phosphate solubilization, production of siderophores, phytohormone and exo-polysaccharides in substantial amount in both the absence and presence of herbicide-stress. Rhizobacteria solubilize mineral P in the rhizosphere and hence, provide soluble P to plants. Cause of mineral P solubilization could be the secretion of organic acids, such as, gluconic, 2-ketogluconic, oxalic, citric, acetic, malic and succinic, etc. (Zaidi *et al*., 2009). In another study, progressive decline in phosphate solubilizing potential of *Pseudomonas aeruginosa* strain PS1 was also observed by Ahemad and Khan (2009) when quizalafop-p-ethyl and clodinafop at recommended and higher dose were supplemented into Pikovskaya medium.

Similarly, Ahemad and Khan (2009) in a study reported that quizalafop-p-ethyl mediated percent decrease in zone size of siderophores on CAS agar plates, SA and DHBA secreted by *P. aeruginosa* strain PS1 was 27, 35 and 48, respectively, whereas clodinafop decreased the same traits by 14, 30 and 72%, respectively, at three times of recommended rate, relative to the herbicide free control. Siderophores synthesized by microbial communities of soil supply iron to plants that possess the mechanisms for its uptake under iron-deficient conditions (Indiragandhi *et al*., 2008).

The phytohormone, IAA synthesized from transamination and decarboxylation of tryptophan, primarily in young leaves and seeds, controls cell division, root initiation, phototropism, geotropism and apical dominance in plants (Khan *et al*., 2010). Bacterial IAA has the potential to interfere with any of these processes by input of IAA into the plant's auxin pool. The EPS production is an important trait of bacteria as it helps bacteria to protect itself against desiccation, phagocytosis and phage attack besides supporting N_2 fixation by preventing high oxygen tension (Tank and Saraf, 2003). Interestingly, the three concentrations of each herbicide did not affect negatively HCN and ammonia synthesized by *E. asburiae* strain PS2 (Table 3). The ammonia released by the rhizobacterial strain plays a signaling role in the interaction between PGPR and plants and also increase the glutamine synthetase activity (Chitra *et al*., 2002). In agreement to our report, Devi *et al*. (2007) also reported the excretion of HCN by the rhizobacterial strains into the rhizosphere. Study on the effect of herbicides on PGP activities of rhizobacteria is scarce. However, Madhaiyan *et al*. (2006) and Wani *et al*. (2005) reported that phytohormones production, nitrogenase activity, zinc and P-solubilization of Gram-negative bacteria decreased considerably in the presence of different groups of pesticides including herbicides.

CONCLUSIONS

Selected herbicides at all tested rates displayed varying degree of toxicity to PGP traits (except EPS) of *E. asburiae* strain PS2. However, toxicity to these traits was less prominent at recommended rate than that of higher dose rate of each herbicide. The present study revealed the toxicological the effects of indiscriminate and injudicious application of herbicides on functions and activities of PGPR. The strain PS2 with inherent ability to produce growth regulators even in the presence of herbicides can be exploited as bio-inoculant to increase the productivity of crops grown in herbicide contaminated soils.

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