Emir. J. Food Agric. 2012. 24 (4): 334-343
http://ejfa.info/

PLANT SCIENCE

Ecological assessment of biotoxicity of pesticides towards plant growth promoting activities of pea (*Pisum sativum*)-specific *Rhizobium* sp. strain MRP1

Munees Ahemad[1,2*] and Mohammad Saghir Khan[1]

[1]*Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh-202002, U.P., India*
[2]*Department of Biology, College of Science, Bahir Dar University, Bahir Dar, Ethiopia*

Abstract

This study was planned to assess the impact of pesticides [herbicides (metribuzin and glyphosate), insecticides (imidacloprid and thiamethoxam) and fungicides (hexaconazole, metalaxyl and kitazin)] at the recommended and the higher dose rates on plant growth promoting (PGP) traits of Rhizobium sp. strain MRP1 isolated from pea-nodules. Strain MRP1 was unambiguously selected due to high pesticide-tolerance and substantial production of indole acetic acid, siderophores, exo-polysaccharides, HCN and ammonia. Pesticide-concentration dependent progressive-decline for PGP properties of Rhizobium sp. strain MRP1 was observed except exo-polysaccharides which regularly increased on increasing concentration of each pesticide beyond the recommended dose. For example, hexaconazole at three times the recommended dose decreased salicylic acid and 2, 3-dihydroxy benzoic acid biosynthesis by 37% and 55%, respectively above control. Likewise, glyphosate, imidacloprid, and hexaconazole decreased indole acetic acid secretion by 28%, 19%, and 34%, respectively at three times the recommended dose. Among all tested pesticides, the greatest stimulatory effect on exo-polysaccharides secretion was shown by glyphosate which stimulated Rhizobium sp. strain MRP1 to secrete exo-polysaccharides by 40% higher with respect to untreated control. Generally, the maximum toxicity to PGP traits (excluding exo-polysaccharides) of Rhizobium was shown by glyphosate, imidacloprid and hexaconazole at three times the recommended rate among herbicides, insecticides and fungicides, respectively. The results of this study implied that prior to field-application pesticides must be tested in laboratory for the adverse impact on the physiological activities of plant-beneficial soil microorganisms. This study also revealed a circumlocutory mechanism of pesticide-mediated toxicity to plant growth.

Key words: Rhizobium, Pesticide, Plant Growth Promoting Rhizobacteria (PGPR), Toxicity, Tolerance, *Pisum sativum*

Introduction

In agriculture, the pesticides are recurrently applied for three major objectives- (i) to produce a larger yield (ii) to produce crops of high quality and (iii) to reduce the input of labor and energy into crop production (Ayansina, 2009). Millions of tons of pesticides are applied annually; however, less than 5% of these products are estimated to reach the target organism, with the remainder being deposited on the soil and non-target organisms, as well as moving into the atmosphere and water (de Oliveira et al., 2012; Ahemad and Khan, 2010a).

The surplus amount of pesticides, which does not reach the target organisms, is absorbed by the plants and hence, pesticide residues have been found in various fruits and vegetables; both raw and processed (González-Rodríguez et al., 2011). One of the most common routes of pesticide exposure in consumers is via food consumption (González-Rodríguez et al., 2008). They can have numerous negative health effects on human consumers owing to their continual exposure in the form of contaminated food products (López-Pérez et al., 2006).

The metabolic fate of pesticides is dependent on abiotic environmental conditions (temperature, moisture, soil pH, etc.), microbial community or plant species (or both), pesticide characteristics (hydrophilicity, p$K_{a/b}$, K_{ow}, etc.), and biological and chemical reactions. Abiotic degradation is due to chemical and physical transformations of the

Received 15 December 2011; Revised 09 February 2012; Accepted 21 February 2012

*Corresponding Author

Munees Ahemad
Department of Biology, College of Science, Bahir Dar University, Bahir Dar, Ethiopia

Email: muneesmicro@rediffmail.com

pesticide by processes such as photolysis, hydrolysis, oxidation, reduction, and rearrangements (Van Eerd et al., 2003). Further, pesticides may be biologically unavailable because of compartmentalization, which occurs as a result of pesticide adsorption to soil and soil colloids without altering the chemical structure of the original molecule (Van Eerd et al., 2003). In soils, these excess pesticides interact with rhizosphere microorganisms including nodule bacteria (Ahemad and Khan, 2010b) and restrict the root growth in that way lead to the reduction in the number of the root sites available for the rhizobial infection (Ahemad and Khan, 2010a). Although reports of pesticidal impact on microbes are conflicting, several studies have conclusively shown that these agrochemicals are incompatible with bacterial cultures (Singh and Wright, 2002; Aamil et al., 2005).

Pesticides like metribuzin and glyphosate (herbicides); imidacloprid and thiamethoxam (insecticides); and hexaconazole, metalaxyl and kitazin (fungicides) (Table 1) are used widely in legume and non-legume crop production. However, reports of their biotoxic impacts on plant growth promoting traits (siderophores, indole acetic acid, exo-polysaccharides, hydrogen cyanide and ammonia) of pea-specific rhizobia have been very limited and are almost completely lacking in the case of the above pesticides. An attempt has, therefore, been made in the present study to determine the effects of the these pesticides on viability as well as on plant growth promoting properties of *Rhizobium* sp. strain MRP1 isolated from pea (*Pisum sativum*) nodules. It is expected that the results of this investigation would further contribute towards the more efficacious implementation of an effective pest management.

Materials and Methods

Rhizobial strains and pesticide-tolerance

A total of 50 rhizobial strains were isolated from root nodules of pea plants grown in experimental fields of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh (27° 29' latitude and 72° 29' longitude), India using yeast extract mannitol (YEM) medium (g l^{-1}: mannitol 10; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.2; NaCl 0.1; yeast extract 1; $CaCO_3$ 1; pH 7) (Vincent, 1970). The experimental soil was an 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 cmol kg^{-1} and 5.1 cmol kg^{-1} anion exchange capacity). The rhizobial strains were characterized and identified following Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and host specificity test in sterile soils (Somasegaran and Hoben, 1994). The strains were tested for their sensitivity/tolerance to chemically and functionally diverse pesticides (metribuzin, glyphosate, imidacloprid, thiamethoxam, hexaconazole, metalaxyl and kitazin) by agar plate dilution method 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, FeSO4.7H2O 0.01, pH 6.5). The freshly prepared agar plates were amended separately with increasing concentrations of pesticides (0 to 3200 µg ml^{-1}; at a two-fold dilution interval). Later, plates were spot inoculated with 10 µl of 10^8 cells ml^{-1} rhizobial strains. Plates were incubated at 28 ±2 °C for 72 h and the highest concentration of each pesticide supporting rhizobial growth was defined as the maximum tolerance level (MTL). The experiment was replicated three times.

Quantitative assay of indole acetic acid

Indole-3-acetic acid (IAA) synthesized by rhizobial strains was quantitatively evaluated by the method of Gordon and Weber (1951), later modified by Brick et al. (1991). For this activity, the rhizobial strains were grown in Luria Bertani broth (g l^{-1}: tryptone 10; yeast extract 5; NaCl 10 and pH 7.5). Luria Bertani (LB) broth (100 ml) having fixed concentration of tryptophan (100 µg/ml) and supplemented with 0, the recommended dose (1X), two times the recommended dose (2X) and three times the recommended dose (3X) of each pesticide was inoculated with one ml culture (10^8 cells/ml) of rhizobial strains and incubated for seven days at 28±2 °C with shaking at 125 *g*. After seven days, a five milliliter culture from each treatment was centrifuged (9,000 *g*) for 15 min and an aliquot of two ml supernatant was mixed with 100 µl of orthophosphoric acid and four milliliter of Salkowsky reagent (2% 0.5M $FeCl_3$ in 35% per-chloric acid) and incubated at 28±2 ^oC in darkness for 1h. The absorbance of developed pink color was read at 530 nm. The IAA concentration in the supernatant was determined using a calibration curve of pure IAA as a standard.

Table 1. Pesticides used in the present study.

Category	Common name	Grade (purity)	Chemical name	Chemical family	Recommended dose	Source
Herbicides	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}	Singhal Pesticides, Mumbai, India
	Glyphosate	Commercial (71% w/w)	*N*-(phosphonomethyl)glycine	Organophosphate	1444 µg kg^{-1}	Excel Crop Core LTD., Mumbai, India
Insecticides	Imidacloprid	Technical (100% EC)	(*E*)-1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine	Pyridylmethylamine	100 µg L^{-1}	Parijat Agrochemicals, New Delhi, India
	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}	Parijat Agrochemicals, New Delhi, India
Fungicides	Hexaconazole	Technical (100%w/w)	(*RS*)-2-(2,4-dichlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol	Conazole	40 µg kg^{-1}	Parijat Agrochemicals, New Delhi, India
	Metalaxyl	Commercial (35%w/w)	methyl *N*-(methoxyacetyl)-*N*-(2,6-xylyl)-DL-alaninate	Anilide	1500 µg kg^{-1}	Tropical Agrosystem LTD., Chennai, India
	Kitazin	Commercial (48% EC)	O,O-Bis(1-methylethyl) S-phenylmethyl phosphorothioate	Organophosphate	96 µg kg^{-1}	P.I. Industries LTD., Rajasthan, India

Qualitative and quantitative estimation of siderophores

The rhizobial strains were further tested for siderophore production using Chrome Azurol S (CAS) agar medium following the method of Alexander and Zuberer (1991). Chrome Azurol S agar plates supplemented with 0, 1X, 2X and 3X of each pesticide were prepared separately and divided into equal sectors and spot inoculated with 10 µl of 10^8 cells/ml and incubated at 28±2 ^oC for five days. Development of yellow orange halo around the bacterial growth was considered as positive test for siderophores-biosynthesis. The production of siderophores by the test strains were further detected quantitatively using Modi medium (K_2HPO_4 0.05%; $MgSO_4$ 0.04%; NaCl 0.01%; mannitol 1%; glutamine 0.1%; NH_4NO_3 0.1%) (Reeves et al., 1983). Modi medium amended with 0, X, 2X and 3X of each pesticide, was inoculated with 10^8 cells ml^{-1} of bacterial cultures and incubated at 28±2 °C for five days. Catechol type phenolates were measured on ethyl acetate extracts of the culture supernatant using a modification of the ferric chloride-ferricyanide reagent of Hathway. Ethyl acetate extracts was prepared by extracting 20 ml of supernatant twice with an equal volume of solvent at pH 2. Hathway's reagent was prepared by adding one milliliter of 0.1 M ferric chloride in 0.1 N HCl to 100 ml of distilled water, and to this, was added one milliliter of 0.1 M potassium ferricyanide (Reeves et al., 1983). For the assay, one volume of the reagent was added to one volume of sample and absorbance was determined at 560 nm for salicylic acid (SA) with sodium salicylate as standard and at 700 nm for dihydroxy phenols with 2, 3- dihydroxy benzoic acid (DHBA) as standard.

Assay of hydrogen cyanide (HCN), ammonia and exo-polysaccharides

Hydrogen cyanide production by rhizobial strains was detected by the method of Bakker and Schipper (1987). For HCN production, all rhizobial strains were grown on an HCN induction medium (g l^{-1}: tryptic soy broth 30; glycine 4.4; agar 15) supplemented with 0, 1X, 2X and 3X of each pesticide at 28±2 °C for four days. Further, 100 µl of 10^8 cells/ml of each rhizobial strain was placed in the centre of the petri plates. A disk of Whatman filter paper No. 1 dipped in 0.5% picric acid and 2% Na_2CO_3 was placed at the lid of the petri plates. Plates were sealed with parafilm. After four days incubation at 28±2 °C, an orange brown color of the paper indicating HCN production was observed.

For ammonia assessment, the rhizobial strains were grown in peptone water with 0, 1X, 2X and 3X of each pesticide and incubated at 28±2 °C for four days. One milliliter of Nessler reagent was added to each tube and the development of yellow color indicating ammonia production was recorded following the method of Dye (1962).

The exo-polysaccharide (EPS) produced by the rhizobial strains was determined as suggested by Mody et al. (1989). For this, the bacterial strains were grown in 100 ml capacity flasks containing basal medium supplemented with 5% sucrose and treated with 0, 1X, 2X and 3X of each pesticide. Inoculated flasks were incubated for five days at 28±2°C on rotary shaker (100 *g*). Culture broth was spun (5433 *g*) for 30 min and EPS was extracted by adding three volumes of chilled acetone (CH_3COCH_3) to one volume of supernatant. The precipitated EPS was repeatedly washed three times alternately with distilled water and acetone, transferred to a filter paper and weighed after overnight drying at room temperature. Each individual experiment was repeated three times.

Statistical Analysis

The experiments were conducted in three replicates using the same treatments. The difference among treatment means was compared by high range statistical domain (HSD) using Tukey test at 5% probability level on SPSS 10 software.

Results

Characterization, identification and pesticide-tolerance

In the present study, a total of 50 rhizobial strains recovered from nodules of pea root systems were identified on the basis of morphological and biochemical tests and host-specificity test for nodulation in sterile soils and monitored further for pesticide-tolerate by exposing them to the graded concentrations of herbicides (metribuzin and glyphosate), insecticides (imidacloprid and thiamethoxam) and fungicides (hexaconazole, metalaxyl and kitazin) (Table 1) on minimal salt agar medium. Among these strains, *Rhizobium* sp. strain MRP1 was specifically selected due to the highest MTL for all selected herbicides, insecticides and fungicides (Figure 1, Table 2) and the maximum production of PGP substances (siderophores, IAA, EPS, HCN and ammonia) (Table 3).

Siderophore production

Production of siderophores by the pesticide-tolerant *Rhizobium* sp. strain MRP1 was determined on CAS agar plates supplemented with varying concentrations of pesticides. *Rhizobium* sp. strain MRP1 displayed siderophores-producing potential

by forming an orange zone of 11 mm size on pesticide free CAS agar medium.

Table 2. Morphological and biochemical characteristics of *Rhizobium* sp. strain MRP1.

Characteristics	Strain MRP1
Morphology	
Gram reaction	-
Shape	rods
Biochemical reactions	
Citrate utilization	-
Indole	+
Methyl red	+
Nitrate reduction	+
Oxidase	-
Voges Proskaur	+
Carbohydrate utilization	
Dextrose	-
Lactose	-
Mannitol	+
Sucrose	-
Hydrolysis	
Starch	+
Gelatin	-
Maximum tolerance level (MTL) to	
Metribuzin	3000 μg ml^{-1}
Glyphosate	2800 μg ml^{-1}
Imidacloprid	1600 μg ml^{-1}
Thiamethoxam	2200 μg ml^{-1}
Hexaconazole	2000 μg ml^{-1}
Metalaxyl	2600 μg ml^{-1}
Kitazin	3000 μg ml^{-1}

+ indicates positive and – indicates negative reactions

In general, addition of pesticides at the recommended field rates to the medium did not reduced the siderophore-zone formed by pure culture of *Rhizobium* sp. strain MRP1. At three times the recommended rates, the effect of imidacloprid, hexaconazole and metalaxyl was inhibitory to siderophore-zone. For example, fungicide hexaconazole reduced the siderophore-zone to the highest degree by 18% over untreated control. In addition, the degree of zone-inhibition was not co-related (R^2 = 0.009) with the concentration of each pesticide (Table 3).

Furthermore, the ethyl acetate extraction from culture supernatant of *Rhizobium* sp. strain MRP1 grown in the Modi medium devoid of pesticides yielded 32 and 22 μg/ml SA and DHBA type siderophores. Pesticide-concentration dependent progressive decline for both siderophore molecules was observed. Nevertheless, degree of pesticide-mediated decrease for SA and DHBA differed from the type and functional group of each pesticide. Within herbicide group, glyphosate showed the highest toxicity to the synthesis of SA and DHBA. For instance, glyphosate at 3X decreased SA and DHBA secretion by 19% and 32%, respectively compared to control. Among insecticides, thiamethoxam at 3X showed the most deleterious effect on the SA production while both imidacloprid and thiamethoxam at 3X most adversely affected the DHBA synthesis. For instance, three times the recommended dose of thiamethoxam decreased SA by 28% whereas three times the recommended dose of both imidacloprid and thiamethoxam decreased the DHBA secretion by 27% relative to control. Among fungicides, hexaconazole at three times the recommended dose triggered the maximum stress on siderophore-biosynthesis by *Rhizobium* sp. strain MRP1 and decreased SA and DHBA by 37% and 55%, respectively above control. Among all pesticides, hexaconazole at 3X in general, displayed the most toxic effect on SA and DHBA synthesis.

Indole acetic acid production

The effect of the three concentrations of each pesticide on IAA synthesized by *Rhizobium* sp. strain MRP1 varied considerably. In the absence of pesticides, *Rhizobium* sp. strain MRP1 produced a maximum (32 μg/ml) amount of IAA. In contrast, the amount of IAA released by *Rhizobium* sp. strain MRP1, however, decreased progressively with the graded addition of each pesticide in LB broth. Of herbicides, insecticides and fungicides, most severe effect on IAA synthesis was evident in the presence of glyphosate, imidacloprid and hexaconazole, respectively. For example, glyphosate decreased IAA by 12%, 22% and 28%, imidacloprid by 9%, 12% and 19% and hexaconazole by 12%, 22% and 34% at 1X, 2X and 3X, respectively. On comparing the toxicity of specific concentration of each pesticide, hexaconazole in general had the most toxic impact on IAA bio-synthesis by *Rhizobium* sp. strain MRP1 (Table 3).

Production of exo-polysaccharides, HCN and ammonia

Unlike other PGP substances produced by *Rhizobium* sp. strain MRP1 exposed to pesticide-stress, the amount of EPS synthesized increased progressively with gradual increment of each pesticide in culture medium. Among all tested pesticides, the greatest stimulatory effect on EPS secretion was shown by glyphosate which stimulated *Rhizobium* sp. strain MRP1 to secrete EPS by 40% higher with respect to untreated control (Table 3). Interestingly, the three concentrations of each herbicide, insecticide and fungicide did not affect negatively HCN and ammonia synthesis by *Rhizobium* sp. strain MRP1 (Table 3).

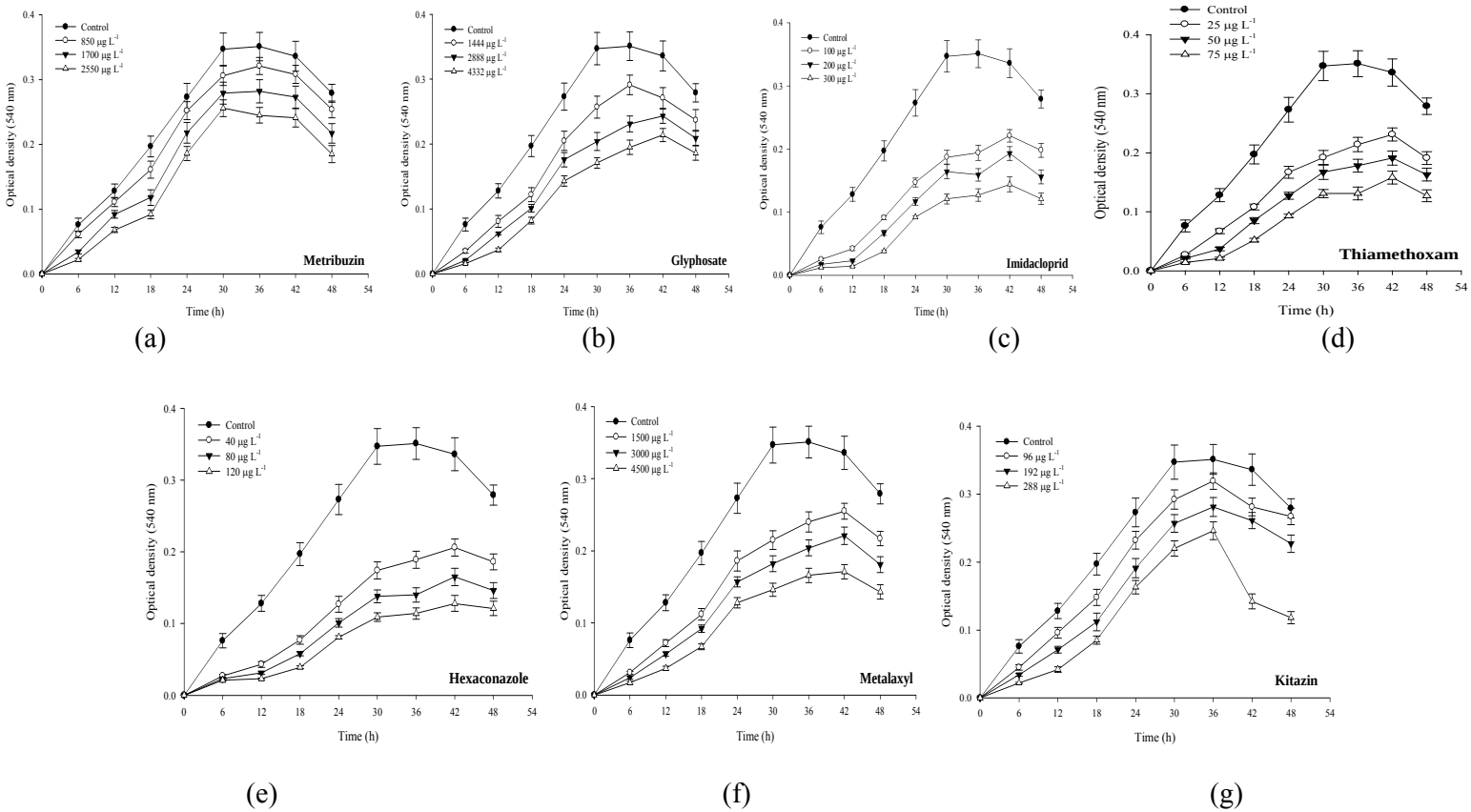

Figure 1. Impact of the recommended (*open circle*), double (*filled inverted triangle*) and three times (*open upright triangle*) the recommended rates of metribuzin (a), glyphosate (b), imidacloprid (c), thiamethoxam (d), hexaconazole (e), metalaxyl (f) and kitazin (g) on *Rhizobium* strain MRP1 (in terms of optical density) grown in minimal salt agar medium.

Table 3. Plant growth promoting activities of *Rhizobium* strain MRP1 in the presence of varying concentrations of pesticides.

Pesticides	Dose rate (µg l^{-1})	Plant growth promoting activities						
		Siderophores			IAA[d] (µg ml^{-1}) 100T[e]	EPS[f] (µg ml^{-1})	Ammonia	HCN[g]
		Zone on CAS[a] agar (mm)	SA[b] (µg ml^{-1})	DHBA[c] (µg ml^{-1})				
Metribuzin	850	11±2a	31±1.5ab	20±1.2b	30±1.8ab	22±2.1de	+	+
	1700	11±2a	29±1.6b	18±1.7d	27±1.4de	24±1.3bc	+	+
	2550	11±1a	27±1.3cd	16±1.6f	26±2.1e	27±2.5ab	+	+
Glyphosate	1444	11±2a	30±1.7ab	19±1.3cd	28±1.8cd	22±1.7de	+	+
	2888	11±1a	28±1.3b	17±1.3ef	25±1.9f	25±2.4bc	+	+
	4332	11±1a	26±1.2d	15±1.2gh	23±1.4g	28±1.2a	+	+
Imidacloprid	100	11±2a	28±1.4b	20±1.2b	29±1.3bc	21±1.2ef	+	+
	200	10±2ab	26±1.3d	18±1.1d	28±1.6cd	24±2.3bc	+	+
	300	10±1ab	24±1.2f	16±1.5f	26±1.7e	26±2.4ab	+	+
Thiamethoxam	25	11±2a	30±1.1ab	21±1.3ab	31±2.3ab	20±2.3f	+	+
	50	11±1a	28±1.2b	18±1.7d	30±2.3ab	21±2.2ef	+	+
	75	11±1a	23±1.6g	16±1.2f	28±1.5cd	23±1.1cd	+	+
Hexaconazole	40	11±2a	26±1.3d	14±1.5h	28±2.4cd	22±1.2de	+	+
	80	10±1ab	23±1.5g	12±1.3i	25±1.6f	24±2.5bc	+	+
	120	9±1b	20±1.1h	10±1.5j	21±1.4h	25±1.6bc	+	+
Metalaxyl	1500	11±1a	28±1.5b	19±1.2cd	28±1.7cd	22±1.9de	+	+
	3000	11±2a	27±1.6cd	18±1.5d	26±2.3e	23±2.2cd	+	+
	4500	10±1ab	25±1.2ef	16±1.3f	23±1.9g	25±2.2bc	+	+
Kitazin	96	11±1a	29±1.7b	20±1.2b	30±1.8ab	20±1.5fg	+	+
	192	11±1a	28±1.3b	19±1.5cd	27±1.6de	21±2.2ef	+	+
	288	11±1a	26±1.0d	17±1.5ef	25±2.1f	23±1.8cd	+	+
Control (without pesticide)		11±1a	32±1.5a	22±1.1a	32±1.6a	20±1.3f	+	+
LSD ($p \leq 0.05$)		0.67	2.41	1.35	2.31	1.42	-	-
F value (treatment)		74.2	238.4	155.9	278.6	384.7	-	-

Values indicate mean of three replicates. Mean values (±S.D.) followed by different letters are significantly different within a row or column at $p \leq 0.05$ according to Tukey test. aChrome azurol S agar; bSalicylic acid; c2,3 Dihydroxy benzoic acid; dIndole acetic acid; eTryptophan concentration (µg ml-1); fExopolysaccharides; gHydrogen cyanide; + indicates positive reaction

Discussion

Pesticide-tolerance

In our study, *Rhizobium* sp. strain MRP1 depicted the abnormally high tolerance to an array of the selected pesticides of various chemical families. The MTL values of pesticides ranged from 2200 µg ml^{-1} to 3000 µg ml^{-1}. Tolerance or resistance in microorganisms against pesticides is a complex process which is regulated both at physiological/genetic level of microorganism. And hence, the microorganisms that developed resistance to pesticides are frequently capable of biodegrading them (Kumar et al., 1996; Ortiz-Hernández and Sánchez-Salinas, 2010). The temporary resistance (tolerance) against pesticides in general, is attributed 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 pesticide (Bellinaso et al., 2003). Permanent resistance, on the other hand, depends upon genetic modifications, inherited by the subsequent generation of microbes (Johnsen et al., 2001; Herman et al., 2005).

Siderophore production

In the present study, *Rhizobium* sp. strain MRP1 exhibited plant growth promoting traits like production of siderophores, phytohormone and exo-polysaccharides in substantial amount in both the absence and presence of pesticide-stress. 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 (Miethke and Marahiel, 2007; Khan et al., 2010).

Indole acetic acid production

In our study, *Rhizobium* sp. strain MRP1 produced IAA in substantial amount both in the presence and the absence of pesticide-stress. Plant hormones play an important role for the growth and development of plants. Auxins like indole-3-acetic acid (IAA) are among the most studied growth regulators and are believed to be essential for plants because no plants are known to be unable to synthesize it (Callis, 2005). It is the most common auxin in plants. Other compounds with auxin-like activity, such as indole-3-butyric acid, phenyl acetic acid, and 4-chloro-IAA, also reported, but little is known about their physiological roles (Kende and Zeevaart, 1997). Although the ability to produce phytohormones is primarily attributed to the plant kingdom, they are also widespread among soil and plant associated microbes such as bacteria including *Rhizobium* sp. (Costacurta and Vanderleyden, 1995). Phytohormones produced by microbes in the rhizosphere are a means for their interaction with plants (Christiansen-Weniger, 1998). By the excretion of IAA synthesized from transamination and decarboxylation of tryptophan, these microbes locally change the endogenous hormone balance of the host, thereby promoting plant cell division, growth, and nutrient release, and supporting their own growth (Glick et al., 1999; Khan et al., 2010). Moreover, 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 inhibit primary root growth (Ma et al., 2009).

Production of exo-polysaccharides, HCN and ammonia

Rhizobium sp. strain MRP1 produced other PGP substances like EPS, HCN and ammonia. The EPS helps to protect bacteria against desiccation, phagocytosis and phage attack besides supporting N_2 fixation by preventing high oxygen tension (Tank and Saraf, 2003). Moreover, EPS play a very important role in legume-*Rhizobium* symbiosis, as acidic EPS produced by rhizobia are essentially required for nodule invasion and, consequently, for effective nitrogen-fixing symbiosis with many legumes in nodule formation (Ahemad and Khan, 2012).

Rhizobacteria protect the growing plants from pathogen attack by directly killing parasites by producing HCN (Devi et al., 2007). The ammonia released by the rhizobacterial strain plays a signaling role in the interaction between rhizobacteria and plants and also increase the glutamine synthetase activity (Chitra et al., 2002).

Decline of PGP substances released by Rhizobium exposed to pesticide-stress

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 (Ahemad and Khan, 2011a). Pesticides adversely affect protein synthesis and the metabolic enzymes (Kapoor and Arora, 1996; Boldt and Jacobsen, 1998). Therefore, it seems probable that pesticides employed in this study might have inhibited the functioning of the enzymes participating in different metabolic pathways of PGP traits in *Rhizobium* sp. strain MRP1. Additionally, pesticides 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 exposed to high concentration of pesticides (Kumar et al., 2010; Ahemad and Khan, 2011b).

Conclusions

Overall, this study implies that pesticides not only affect the growth of pea-specific rhizobia but also have an adverse impact on their PGP activities. This study suggested that screening of pesticides on the basis of degree of *in vitro* toxicity to PGP functions of beneficial rhizobacteria would result into eco-friendly pest management as well as sustainability of soil fertility. These results imply further research on toxicological effects of pesticides on PGP activities of soil microflora at molecular level to fortify the effective implementation of this approach to protect the soil ecosystem from pesticide hazard.

Acknowledgments

The authors thank Dr. N.A. Naqvi, Parijat Agrochemicals, New Delhi, India, for providing technical grade pesticides. Financial assistance from University Grants Commission (UGC), New Delhi, India is also gratefully acknowledged.

References

Aamil, M., A. Zaidi and M. S. Khan. 2005. Biotoxic effects of organophosphorus insecticides on agronomically important

microbial communities in soil. Pollut. Res. 24:487-491.

Ahemad, M. and M. S. Khan. 2010a. Ameliorative effects of *Mesorhizobium* sp. MRC4 on chickpea yield and yield components under different doses of herbicide stress. Pestic. Biochem. Physiol. 98:183-190.

Ahemad, M. and M. S. Khan. 2010b. Phosphate-solubilizing and plant-growth-promoting *Pseudomonas aeruginosa* PS1 improves greengram performance in quizalafop-p-ethyl and clodinafop amended soil. Arch. Environ. Contam. Toxicol. 58:361-372.

Ahemad, M. and M. S. Khan. 2011a. Functional aspects of plant growth promoting rhizobacteria: recent advancements. Insight Microbiol. 1:39-54.

Ahemad, M. and M. S. Khan. 2011b. Assessment of pesticide-tolerance and functional diversity of bacterial strains isolated from rhizospheres of different crops. Insight Microbiol. 1:8-19.

Ahemad, M. and M. S. Khan 2012. Productivity of greengram in tebuconazole-stressed soil, by using a tolerant and plant growth-promoting *Bradyrhizobium* sp. MRM6 strain. Acta Physiol. Plant. 34:245-254.

Alexander, D. B. and D. A. Zuberer. 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biol. Fertil. Soils 12:39-45.

Ayansina, A. D. V. 2009. Pesticide use in agriculture and microorganisms, In: Khan, M.S., A. Zaidi and J. Musarrat (Eds). pp. 261-284. Microbes in Sustainable Agriculture. Nova Science Publishers, New York.

Bakker, A. W. and B. Schipper. 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp mediated plant growth stimulation. Soil Biol. Biochem. 19:451-457.

Bellinaso, M. L., C. W. Greer, M. C. Peralba, J. A. Henriques and C. C. Gaylarde. 2003. Biodegradation of the herbicide trifluralin by bacteria isolated from soil. FEMS Microbial Ecol. 43:191-194.

Boldt, T. S. and C. S. Jacobsen 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, J. M., R. M. Bostock and S. E. Silversone. 1991. Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. Appl. Environ. Microbiol. 57:535-538.

Callis, J. 2005. Plant biology: auxin action. Nature 435:436-437.

Chitra, R. S., V. C. Sumitra and D. S. Yash. 2002. Effect of different nitrogen sources and plant growth regulators on glutamine synthetase and glutamate synthase activities of radish cotyledons. Bulg. J. Plant Physiol. 28:46-56.

Christiansen-Weniger, C. 1998. Endophytic establishment of diazotrophic bacteria in auxin-induced tumors of cereal crops. Critic. Rev. Plant Sci. 17:55-76.

Costacurta, A. and J. Vanderleyden. 1995. Synthesis of phytohormones by plant-associated bacteria. Critic. Rev. Microbiol. 21:1-18.

de Oliveira, T. A., B. Ronchi-Teles, C. R. V. da Fonseca, S. L. R. da Silva, P. A. Santos and C. V. Nunez. 2012. Insecticidal activity of *Vitex cymosa* (Lamiaceae) and *Eschweilera pedicellata* (Lecythidaceae) extracts against *Sitophilus zeamais* adults (Curculionidae). Emir. J. Food Agric. 24:49-56.

Devi, K. K., N. Seth, S. Kothamasi and D. Kothamasi. 2007. Hydrogen cyanide-producing rhizobacteria kill subterranean termite *Odontotermes obesus* rambur. by cyanide poisoning under *in vitro* conditions. Curr. Microbiol. 54:74-78.

Dye, D. W. 1962. The inadequacy of the usual determinative tests for the identification of *xanthomonas* spp. Nat. Sci. 5:393-416.

Glick, B. R., C. L. Patten, G. Holguim and D. M. Penrose. 1999. Biochemical and Genetic Mechanisms used by Plant Growth Promoting Bacteria. Imperial College Press, London, River Edge, NJ.

González-Rodríguez, R. M., R. Rial-Otero, B. Cancho-Grande, C. Gonzalez-Barreiro, J. Simal-Gándara. 2011. A review on the fate of pesticides during the processes within the food-production chain. Crit. Rev. Food Sci. Nutr. 51:99-114.

González-Rodríguez, R. M., R. Rial-Otero, B. Cancho-Grande, J. Simal-Gándara. 2008. Occurrence of fungicide and insecticide residues in trade samples of leafy vegetables. Food Chem. 107:1342-1347.

Gordon, S. and R. P. Weber. 1951. The colorimetric estimation of IAA. Plant Physiol. 26:192-195.

Herman P. L., M. Behrens, S. Chakraborty, B. M. Crastil, J. Barycki and D. P. Weeks. 2005. A three component dicamba O-demethylase from *Pseudomonas maltiphilia* strain DI-6: Gene isolation, characterization and heterologous expression. J. Biol. Chem. 280:24759-24767.

Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley and S. T. Willams. 1994. Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, USA.

Johnsen, K., C. S. Jacobsen, V. Torsvik and J. Sorensen. 2001. Pesticide effects on bacterial diversity in agricultural soils-a review. Biol. Fertil. Soils 33:443-453.

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

Kende, H. and J. Zeevaart. 1997. The five "classical" plant hormones. Plant Cell 9:1197-1210.

Khan, M. S., A. Zaidi, M. Ahemad, M. Oves and P. A. Wani. 2010. Plant growth promotion by phosphate solubilizing fungi-current perspective. Arch. Agron. Soil Sci. 56:73-98.

Kumar, N., J. I. Anubhuti Bora and M. K. Amb. 2010. Chronic toxicity of the triazole fungicide tebuconazole on a heterocystous, nitrogen-fixing rice paddy field cyanobacterium, *Westiellopsis prolifica* Janet. J Microbiol. Biotechnol. 20:1134-1139.

Kumar, S., K. G. Mukerji and R. Lal. 1996. Molecular aspects of pesticide degradation by microorganisms. Critic. Rev. Microbiol. 22:1-26.

López-Pérez, G. C., M. Arias-Estévez, E. López-Periago, B. Soto-Gonzalez, B. Cancho-Grande and J. Simal-Gandara. 2006. Dynamics of pesticides in potato crops. J. Agric. Food Chem. 54:1797-1803.

Ma, Y., M. Rajkumar and H. Freitas. 2009. Improvement of plant growth and nickel uptake by nickel resistant-plant-growth promoting bacteria. J. Hazard. Mate. 166:1154-1161.

Miethke, M. and M. A. Marahiel. 2007. Siderophore-based iron acquisition and pathogen control. Microbiol. Mole. Biol. Rev. 71:413-451.

Mody, B. R., M. O. Bindra and V. V. Modi. 1989. Extracellular polysaccharides of cowpea rhizobia: compositional and functional studies. Arch. Microbiol. 1:2-5.

Ortiz-Hernández, M. L. and E. Sánchez-Salinas. 2010. Biodegradation of the organophosphate pesticide tetrachlorvinphos by bacteria isolated from agricultural soils in México. Rev. Int. Contam. Ambient 26:27-38.

Pham, C. H., J. Min and M. B. Gu. 2004. Pesticide induced toxicity and stress response in bacterial cells. Bull. Environ. Contam. Toxicol. 72:380-386.

Reeves, M. W., L. Pine, J. B. Neilands and A. Balows. 1983. Absence of siderophore activity in *Legionella species* grown in iron-deficient media. J Bacteriol. 154:324-329.

Singh, G. and D. Wright. 2002. *In vitro* studies on the effects of herbicides on the growth of rhizobia. Lett. Appl. Microbiol. 35:12-16.

Somasegaran, P. and H. J. Hoben. 1994. Handbook for Rhizobia: Methods in Legume *Rhizobium* Technology. Springer, New York.

Tank, N. and M. Saraf. 2003. Phosphate solubilization, exopolysaccharide production and indole acetic acid secretion by rhizobacteria isolated from *Trigonella foenum-graecum*. Ind. J. Microbiol. 43:37-40.

Van Eerd, L. L., R. E. Hoagland and J. C. Hall. 2003. Pesticide metabolism in plants and microorganisms. Weed Sci. 51:472–495.

Vincent, J. M. 1970. A Manual for the Practical Study of Root Nodule Bacteria. Blackwell Scientific Publications, Oxford, UK.