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Research article

Pseudomonas putida and *Bacillus amyloliquefaciens* alleviates the adverse effect of pesticides and poise soil enzymes activities in chickpea (*Cicer arietinum* L.) rhizosphere

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Abstract: Pesticide application for disease management is a major action for crop protection from last seven decades. The repeated application of pesticide is the most important cause of the reduction in microbial population. Soil microorganisms play an important role in efficient acquisition and transportation of nutrients to plant. Pesticides leached in soil disturb the activities of soil enzymes, such as β-glucosidase, dehydrogenase, phosphatases, protease and urease secreted by these microorganisms. This drastically reduces nutrient availability to the plants and soil fertility. *In vitro* experimental studies revealed that our PGPR (*Pseudomonas putida* and *Bacillus amyloliquefaciens*) have the ability to tolerate pesticides at concentrations such as Carbendazim (0.512%), Imidacloprid (3.27%) and Glyphosate (3.27%). We have observed an increase in PGP activities like IAA production, exopolysachchride production, biofilm synthesis, phosphate solubilization and siderophore production on the addition of pesticides at concentrations below there threshold values, on the contrary reduction in activities was noticed above these values. Soil enzymes activities from chickpea rhizosphere without PGPR inoculation showed variability on the application of pesticides whereas activities were found normal or increased with PGPR inoculation and pesticides application. Thus PGPR remains panacea for soils by managing adverse effects of pesticide application. Hence our results concluded that *P. putida* and *B. amyloliquefaciens* have the ability to reduce the negative impact of three pesticides and poise soil enzymes activities. Hence our PGPR acts as efficient biofertilizers to improve soil fertility and soil health.
Keywords: Carbendazim - Chickpea - Glyphosate - Imidacloprid - PGPR - Soil enzymes.

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most important legume crop. India produces nearly 75% of world's chickpea production. Often it is grown after cereal crops to interrupt onset of disease, weed control and improve soil nitrogen content. Seed is the main edible part of the plant and is a rich source of carbohydrate (48.2–67.6%), protein (12.4–31.5%) and fat (6%) especially for the vegetarian people (Rao 2010). Chickpea can fix atmospheric nitrogen through its symbiotic association with *Rhizobium* species thus helping in enhancing the soil quality. Pests involving insects, fungi and weeds are the major biotic factors which reduce crop yield by affecting the root-shoot growth. The key pest that limits pulses improvement in India comprises pod borers, foliar diseases and weeds. Pesticides uses remain minimal for pulses in India (Reddy & Reddy 2010). In a survey of pesticide use on pulse crop in four regions revealed that use of fungicides is 4–12%, herbicides 0–24%

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and insecticides 16–50% (IIPR 2010–11). To increase pulses production for the ever-increasing population application of chemical pesticides ensured high crop productivity.

Pesticides are substances or mixtures of substances proposed for destroying, preventing, mitigating or repelling pests (Grube *et al.* 2011). Pesticide application to the crop exposes soil and its microflora to its adverse effects. These includes pesticide interaction with soil enzymes (Gianfreda & Rao 2008), its binding with the active site of the soil enzymes affecting their catalytic activities (Tabatabai 1994) and/or pesticides utilized as a nutrient source by soil microbes resulting in biodiversity changes. A number of pesticides stimulate the growth of microorganisms, but other pesticides inhibit or have no effects on microorganisms when used at normal rates. We have included three pesticides *i.e.* Carbendazim, Imidacloprid and Glyphosate in our study. Glyphosate (N-(phosphonomethyl) glycine) a herbicide was commercialized in 1974. It has been demonstrated that the glyphosate can impact the rhizosphere community (Sheng *et al.* 2012) and rhizosphere processes (Ahemad & Kibret 2013). Among herbicides glyphosate [N-(phosphonomethyl) glycine] is the non-selective systemic herbicide most commonly used in agricultural practices on a global scale (Mijangos *et al.* 2009). Once glyphosate is absorbed by foliage, it is transported throughout the whole plant via the phloem to the growing tissues such as the shoot and root meristems. This mechanism results in glyphosate being exuded into rhizosphere soil (Neumann *et al.* 2006). Glyphosate [N-(phosphonomethyl) glycine] inhibits the enzymatic activity of 5-enolpyruvoyl-shikimate-3-phosphate synthase (EPSPS) in the shikimate pathway, thus preventing the synthesis of aromatic amino acids needed for plant growth and survival (Duke 2005). Carbendazim (methyl benzImizol-2-yl carbamate) is a systemic fungicide. It acts by interfering with microtubule formation during mitosis in fungal cells. The repeated use of pesticides into the crops may reach into the soil which disturbs local metabolism or enzymatic activities (Engelen *et al.* 1998, Liu *et al.* 2008, Hussain *et al.* 2009). Negative impacts of pesticides on soil enzymes such as dehydrogenase, oxidoreductases and hydrolase activities have been widely reported in the literature (Perucci & Scarponi 1994, Malkomes & Dietze 1998, Monkiedje & Spiteller 2002, Menon *et al.* 2005, Caceres *et al.* 2009). Imidacloprid (*N*-{1-[(6-Chloro-3-pyridyl) methyl]-4,5-dihydroImizol-2-yl}nitramide) is a systemic insecticide which works as insect neurotoxin of neonicotinoids class and acts on the central nervous system of insects. It is much low toxic for mammals also. Imidacloprid has become a popular insecticide worldwide due to systemic mode of action and low toxicity to humans and its use in field crops, vegetables and ornamentals (Ishaaya & Horowitz 1988, Matsuda *et al.* 2001, Nauen & Denholm 2005) Imidacloprid is registered in approximately 120 countries and is used on over 140 different agricultural crops (Buffin 2005) including chickpea.

Soil fertility is the result of the effective and cumulative role played by beneficial microbes in the soil. Pesticides uses have an adverse effect on useful microorganisms in rhizoshpere. Vice versa microbes have the ability to degrade pesticides and nullify influence of pesticides on microbial diversity in soil. The knowledge of these processes and extent of its ability to minimise losses by pesticides are still not well studied. To prevent the adverse effect of pesticides, rhizobial inoculants often used as bio-fertilizer and have the ability to maintains the nutrients and degrade the pesticides when applied to soil/seeds of legumes (Lo 2010). Since plant growth promoting rhizobacteria (PGPR) play positive role on plant health through a variety of mechanisms, including mineralization of nutrients, suppression of disease, improving plant stress tolerance and production of phytohormones (Berendsen *et al.* 2012, Figueiredo *et al.* 2011, Gupta *et al.* 2000). Soil enzyme activities are the direct reflection of the soil community to metabolic requirements and available nutrients. The interaction between soil components and pesticides influences the biochemical activity of bacteria. Telluric fungi (Hernandez-Rodriguez *et al.* 2006, Ronhede *et al.* 2007) and bacteria (Dong *et al.* 2005) are able to degrade or mineralize pesticides by enzymatic reactions. There is evidence that soil enzymes may provide valuable information on the transformation of pesticides in soils (Gianfreda & Baollag 1994, Kalam *et al.* 2004, Gil-Sotres *et al.* 2005, Hussain *et al.* 2009). The enzymes are active outside the cell where they catalyze reactions to break down the structure of the nutrient source to make it more accessible, but pesticides affect the soil enzyme and disturb the cycle. In India Maharashtra, Andhra Pradesh (including Telangana & Seemandhra), and Punjab are top three states which contribute about 45% consumption of pesticides. Andhra Pradesh is the highest pesticides consumer with 24% share. Soil enzymes are dehydrogenase, acid/alkaline phosphatase, β-glucosidase, urease, protease. Decrease urease activity in soil due to the application of pesticides reduces urea hydrolysis, which is generally beneficial, because it helps to maintain nitrogen availability to plants.

In the this paper we have reported that plant growth promoting traits of the bacteria such as biofilm

formation, mineral phosphate solubilisation, siderophore production, IAA production and EPS formation activity were estimated in presence of different concentrations of Carbendazim (fungicide), Imidacloprid (insecticide) and Glyphosate (herbicide) with the help of standard protocols routinely used in our laboratory. In our study, we have taken chickpea plant (BG-362) to evaluate the PGPR effect in presence of different pesticides on plant growth parameters. For this, we have used the *B. amyloliquefaciens* (SN13) and *P. putida* (RA) to see the effect of pesticides on them and Maximum Tolerance Level (MTL) was determined *in vitro*. *In vitro* tests were performed to find the interaction between the PGPR and Pesticides, while *in vivo* tests were performed to see the effects of pesticides on chickpea fortified with PGPR. We also did glasshouse experiments to see the effect of pesticide sprays on soil enzymatic activities of chickpea rhizosphere treated with PGPR.

MATERIALS AND METHODS

In vitro PGP traits evaluation of PGPR in presence of pesticides

Pseudomonas putida (RA) and *Bacillus amyloliquefaciens* (SN13) used in the study were procured from the lab depository, Division of plant-microbe interactions, CSIR-NBRI. Pesticides used in this study were Carbendazim (Car), Glyphosate (Gly) and Imidacloprid (Imi). Earlier we have performed *in vitro* PGP traits evaluation of both the bacterium and their synergistic effect for drought amelioration in chickpea (Kumar *et al.* 2016).

Assessment of bacterial strains for pesticides tolerance

The bacterial strains were tested for their pesticide tolerance onto agar plate 4/5 dilution method (Gupta *et al.* 1994) using nutrient agar medium. The freshly prepared agar plates were spreaded with 48 hrs grown *P. putida* and *B. amyloliquefaciens* cultures having 1 OD at 600 nm. These plates were spotted with 10 µl of different concentrations of Imi (10 to 2.097 %), Gly (10 to 0.156 %) and Car (1 to 0.156 %) based on recommended spraying concentration in field for disease management for chickpea crop in published literatures (Mishra *et al.* 2005, Andrabi *et al.* 2011, Yogeeswarudu & Venkata-Krishna 2014). Plates were incubated at 28°C for 48 hrs and the highest concentration of Car, Imi and Gly supporting bacterial growth were defined as the Maximum Tolerance Level (MTL) (Ahmed & Khan 2012).

Quantitative estimation of auxin, biofilm and exopolysachharide under pesticides treatments

Quantification of auxin production by bacterial strains was analysed using modified version (Brick *et al.* 1991) of method proposed by Gordon & Weber (1951) using Salkowski's reagent. For IAA production assay 5 ml of auxin medium was supplemented with varying concentrations *viz.* 2.09%, 3.2%, 4.09%, 5.14% Imi, 2.62%, 2.09%, 3.27%, 4.09% Gly and 0.32%, 0.4%, 0.51%, .6% Car respectively in culture vials in triplicates. Now a set of these culture vials were added with a single isolated colony of RA and a separate set with SN13. Cultures tubes were incubated in a rotatory shaker at 180 rpm at 28ºC. After 5 days, 5 ml culture from each treatment was centrifuged at 10,000 rpm for 15 min. 2 ml of supernatant was taken in fresh test tubes and 100 µl of 10 mM of Orthophosphoric acid followed by 4 ml of Salkowski's reagent was added. The mixture was incubated at 28˚C in dark for one hour. The absorbance of the samples resulting orange colour was measured at 530 nm in UV spectrophotometer (Thermo Fisher Scientific, USA). Reagent mixture without culture was taken as blank.

Biofilm formation assay was performed by inoculating bacterial strains in NB media. Take 5 ml of 48 hrs grown culture at 28ºC in test tubes supplemented with above-mentioned pesticides concentrations in triplicate. Now, 250 µl of this culture was transferred in a microtiter plate (Tarsons Pvt. Ltd., India) with the help of pipette and overnight incubated at 30°C for 24 hrs. Discard the culture and gently tapped on filter paper. Stain with 250 µl of 0.1% crystal violet then incubate at room temperature for 30 min. Crystal violet was removed by washing with distilled water. Finally absorbed crystal violet extracted with 250 µl of 95% ethyl alcohol for 1 hour. O.D was taken at 595 nm (Khan *et al.* 2012).

Exopolysachharide production assay was performed according to Dubois *et al.* (1956). Bacterial strains were grown in 50 ml Luria Bertani broth with 5% sucrose supplemented with different concentration of pesticides and incubated for 5 days at 28°C on a shaker at 100 rpm. Take 10 ml of this 5 days grown culture in 50 ml falcon tubes and add an equal volume of ethanol (absolute). Samples were overnight incubated at 4°C. Centrifuge the tubes at 10,000 rpm for 10 mins at 4°C, dry the pellet in hot air oven. Add 0.5 ml saline, 0.5 ml phenol (5%) and 1 ml H_2SO_4 (36%). Mix it and place it in a dark for 30–60 min. Absorbance was measured at 490 nm.

Qualitative estimation of phosphate solubilization and siderophore production under pesticides treatments

Phosphate solubilisation assay was performed using NBRIP medium (Nautiyal, 1999). A single colony of SN13 and RA was inoculated in each test tube containing 5 ml specially formulated medium NBRIP supplemented with varying concentration of three pesticides in triplicates. Inoculated tubes were incubated for 7 days at 28°C in a rotatory shaker at 180 rpm and daily observed for change in phosphate solubilizing activity visually. Tricalcium phosphate (TCP) solubilizing activity by SN13 and RA in the presence of three pesticides was observed for color change. Siderophore production was tested by CAS (Chrome azurol S) assay (Meyer & Abdallah 1978). RA and SN13 culture were inoculated in NB medium supplemented with different pesticides. CAS agar media plate was spot inoculated with 10 µl of the overnight grown bacterial culture of RA and SN13 containing pesticides. These plates were incubated at 28°C for 5 days. Siderophore production was detected by observing the yellow-orange halo zone.

Seed bacterization, sowing, growth parameter and pesticide spray

Chickpea seeds cultivar *viz.* BG362 was used for this experiment. Seeds bacterization was performed according to Nautiyal (1997). The overnight grown culture of RA and SN13 were used separately for seeds bacterization. Plastic pots (24 cm × 12 cm × 12cm) were filled with sterile soil composition (50% soil, 25% vermiculate, 25% coccopit, and 10% sand). Total 30 seeds for each treatment RA, SN13 and control were taken. Five seeds per pot were sown at 5 cm depth in 1.2 kg soil. Pots were kept in the glass house at temperature 24±2°C and 16: 8 light and dark conditions. The plants were irrigated with sterilized water. Plant growth parameters were recorded 30 days after sowing. RA and SN13 inoculated plants along with control were harvested in triplicates before pesticides spray for growth parameters like shoot, root length, fresh weight and dry weight. These plants were dried in hot air oven at 65°C for 5 days. After this shoot and root dry weight were recorded. Pesticides spray on chickpea plants was performed at different concentrations of Car, Imi and Gly choosen from MTL results (Ahmed and Khan 2012). For pesticides spray 1% Car (50% WP), 10% Gly (41% WP) and 10% Imi (17.8% S.L.) were sprayed on chickpea plants. The first spray was done after 30 days from sowing of chickpea seeds and second sprays after 25 days from the first spray.

Soil enzymes assay

Soil enzymes assay were performed after first and second spray of different pesticides from chickpea rhizosphere soil. To determine the positive or negative effect of pesticides on soil enzymes activity like dehydrogenase, acid/alkaline phosphate, urease activity, β-glucosidase activity and protease activity were analysed. This experiment was performed using three biological and three technical replicates. The data were statistically analyzed using SPSS 16.0 software (Statistical Package for the Social Sciences 16.0, SPSS Inc., USA, 1999) for DMRT (Duncan's Multiple Range Test).

The activity of Dehydrogenase enzyme was analysed according to Alef & Nannipieri (1995). Weigh 5 gm soil in 50ml tubes, containing 5 ml of 0.8% Triphenyl tetrazolium chlororide (TTC) solution. Tubes will be tightly sealed and incubated at 28˚C for 24 hrs in the dark on a shaker at 120 rpm. A control contains 5 ml Tris-HCl buffer (without TTC) will set up. After incubation, 40 ml acetone was added in each tube (intermediately shaken) and again incubated at RT for 2 hrs in the dark with manual shaking after every 10–15 min. Later, 3 ml from each tube was transferred falcon tubes, centrifuged and the absorbance of the supernatant was recorded at the 540 nm. Soil phosphatase activities were analysed according to Tabatabai & Bremner (1969) using p-nitrophenyl phosphate solution as the substrate. One gm of soil taken in 50 ml flask and add 0.25 ml toluene. Take 4 ml Modified Universal Buffere (MUB) (pH 6.5 for acid phosphates and pH 11 for the alkaline phosphates) and add 1 ml p-nitrophenyl phosphate (15 mM). Mix solution properly and incubate at 37˚C for 1 hr. After incubation, add 1 ml of $CaCl_2$ (0.5 M) and 4ml of NaOH (0.5 M). For control, add 1 ml of PNPP solution after the additions of $CaCl_2$ (0.5 M) and 4 ml of NaOH (0.5 M) immediately before filtration of the soil suspension. Mix the content properly and filter the soil suspension through whattman filter paper. Measure the absorbance at 400 nm.

β-glucosidase activity was examined by taking one gm soil in 50 ml Erlenmeyer flask, add 0.25ml of toluene, 4 ml of MUB solution and 1ml p-nitrophenyl-β-D glucoside (PNG) solution, stopper the flask and mixed properly and incubate at 37˚c for 1hr. After the incubation, add 1 ml of $CaCl_2$ solution, 4 ml tris-buffer (pH 12) swirl the flask and immediately take a filter through the Whatman filter paper (Eivazi & Tabatabai 1988). Measure the colour intensity at 400 nm (if intensity comes high then we dilute the filtrate with tris-buffer). For blank preparation, add substrate PNG before adding $CaCl_2$ and tris-buffer. The activity of urease

enzyme was assayed according to (Kandeler & Gerber 1988). Five gm of moist soil was taken in Erlenmeyer flask and 2.5 ml urea solution was added. Stopper the flask and incubate it, add 50 ml of KCl solution (74.6 g KCl and 10 ml of 1M HCl to make 1 litre solution) and shake the flask for 3 min. After the filtering the resulting suspension, filtrates were analyzed for the ammonium content. Take a 1ml filtrate in 50 ml flask, then add 9 ml DW, 5 ml of Na- salicylate/NaOH solution and 2 ml of the sodium dichloro isocyanurate solution and allow standing at RT for 30 min prior to measure O.D at 690 nm. For blank take 10 ml DW and add 2.5 ml urea solution, 5 ml of Na- salicylate/NaOH solution, and 2ml of sodium dichloro isocyanurate solution at the end of incubation and immediately before KCl addition. Protease activity was analysed by taking one gm of moist soil in a centrifuge tube, add 5 ml tris-buffer and 5 ml of Na-Caseinate solution stopper the tubes and mix properly, incubated at 50˚C for 2 hrs on the shaker bath. After incubation adds 5 ml 15% TCA solution and mix properly. Centrifuge the soil suspension at 10,000–12,000 rpm for 10 min. Take a 5 ml of the clear supernatant into the fresh tubes and add 7.5 ml alkaline reagent, incubate it at RT for 15 min. Finally add, 5 ml of folin's reagent. Filter the mixture through the filter paper and measure the absorbance at 700 nm. To prepare a control, add 5 ml of Na-Caseinate solution, incubate and finally adding the TCA solution (Ladd & Butler 1972).

Colony forming unit (CFU) assay

CFU assay of rhisophere soil was performed on 24, 48 and 72 hrs after the first and second pesticides spray. CFU was done by taking the rhizosphere soil samples of all treatments along with control to determine the effect of pesticides on viable microbial colonies. One gram soil sample of each treatment was taken in falcon and used to prepare a suspension of 10 ml using 0.85% NaCl saline, then vortex it and allow rotating at 28˚C on a rotator shaker at 180 rpm for one hour and performed spotting on NA plates. The numbers of colonies in different dilutions were determined by average CFU/ml values populations in triplicates per treatment and observe the effect of pesticides on colonies. CFU measurement was performed in biological triplicates and their technical triplicates.

Functional microbial diversity analysis using Biolog

Functional diversity of the soil microbial community was characterized using community level physiological profiles generated by carbon source utilisation pattern on Biolog EcoPlate (BIOLOG, Hayward, CA, USA). Microbial diversity analysis was performed for diversity indices like Shannon diversity, evenness, Simpson diversity and McIntosh diversity and evenness. Rhizosphere soil samples were used for biolog assay. One gram soil was dissolved in 10 ml of 0.85% of NaCl saline and incubated at 28ºC in rotator shaker for one hour at 200 rpm. From this, 125 microlitre suspension cultures was added to each well on Biolog Eco plate. Samples were loaded in triplicate and incubated at 28ºC in the incubator. Absorbance was taken at 590 nm after 24 hrs interval till 10 days.

RESULTS

Maximum tolerance level (MTL) of pesticides by bacterial strains

The highest concentration of Car (0.512%), Imi (3.27%) and Gly (3.27%) that supported the growth of RA and SN13 is referred as MTL (Table 1). These MTL values results were observed after 24 and 48 hrs from inoculation of pesticides on PGPR grown plates *in vitro*. There was similar size of hallo zone formation after 24 and 48 hrs of pesticide inoculation in case of both PGPR.

PGP traits evaluation of bacteria in presence of pesticides

Quantitative estimation of auxin, EPS and biofilm production by RA and SN13 in presence of different concentrations of pesticides has been performed. Auxin production by RA with 0.4% Car (52.17 $\mu g.ml^{-1}$), 3.27% Imi (56.09 $\mu g.ml^{-1}$) and 3.27% Gly (52.69 $\mu g.ml^{-1}$) were found maximum whereas SN13 produced maximum auxin at 0.4% Car (26.39 $\mu g.ml^{-1}$), 3.27% Imi (29.40 $\mu g.ml^{-1}$) and 2.62% Gly (26.95 $\mu g.ml^{-1}$) (Table 2). EPS production was measured in presence of pesticides showed its highest production by RA at 0.51% Car (306.80 $\mu g.ml^{-1}$), 3.2% Imi (330.20 $\mu g.ml^{-1}$) and at 3.27% Gly (287.52 $\mu g.ml^{-1}$) concentration in vitro. Similarly, EPS production by SN13 was maximum at 0.4% Car (306.80 $\mu g.ml^{-1}$), 3.2% Imi (359.67 $\mu g.ml^{-1}$) and at 3.27% Gly (309.62 $\mu g.ml^{-1}$). Biofilm production for both the bacteria was found highest at 0.4% Car, 3.2% Imi and 3.27% Gly concentrations. On further increasing concentrations of these pesticides, decrease in auxin, EPS and biofilm production was observed by RA and SN13.

Qualitative estimation of phosphate solubilisation and siderophore production by RA and SN13 in presence of pesticides was performed and found promising results. Phosphate solubilisation (Fig. 1A) and siderophore

production (Fig. 1B) was found the maximum in RA and SN13 samples containing Imi compared to control sample. Car treated RA and Gly treated SN13 produced lesser siderophore activity than other treatments.

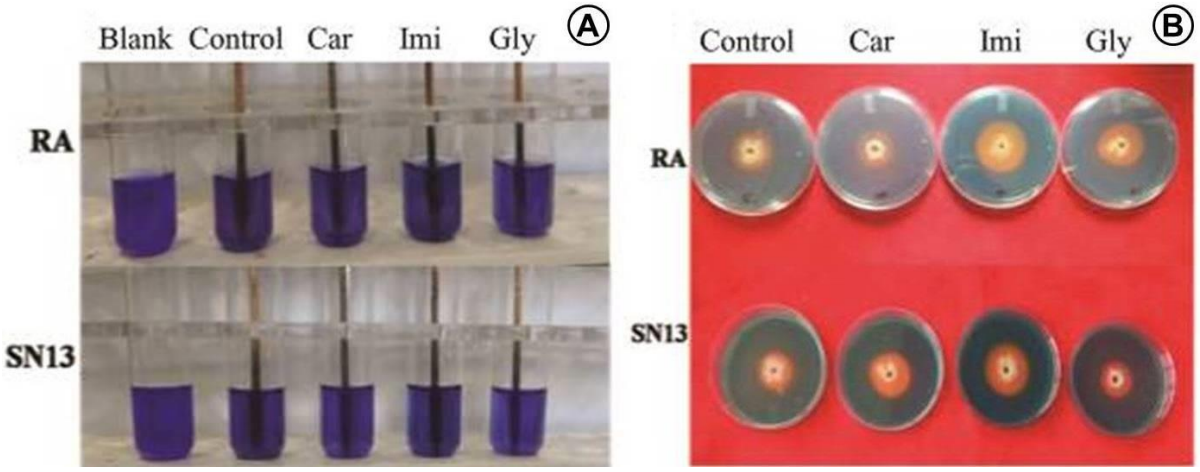

Figure 1. Qualitative estimation of *in vitro* PGP traits in presence of pesticides: **A,** Phosphate solubilisation by *Pseudomonas putida* (RA) and *Bacillus amyloliquefaciens* **(**SN13), Blank (without inoculation of PGPR), control (bacteria inoculated sample), Car (bacteria inoculated with carbendazim), Imi (bacteria inoculated with Imidacloprid), Gly (bacteria inoculated with glyphosate); **B,** Siderophore production by RA and SN13. [Control (bacteria inoculated sample), Car (bacteria inoculated with carbendazim), Imi (bacteria inoculated with Imidacloprid), Gly (bacteria inoculated with glyphosate)]

Table 1. Maximum tolerance level (MTL) values of three pesticides applied to 24 and 48 hrs grown *Pseudomonas putida* and *Bacillus amyloliquefaciens*.

S. No.	Pesticides	Concentration (%)	At 24 hrs		At 48 hrs	
			RA	SN13	RA	SN13
1	Carbendazim (0.2–1%)	1	-	-	-	-
		0.8	-	-	-	-
		0.64	-	-	-	-
		0.512	-	-	-	-
		0.409	+	+	+	+
		0.327	+	+	+	+
		0.209	+	+	+	+
		0.262	+	+	+	+
2	Glyphosate (2–10%)	10	-	-	-	-
		8	-	-	-	-
		6.4	-	-	-	-
		5.12	-	-	-	-
		4.096	-	-	-	-
		3.27	+	+	+	+
		2.62	+	+	+	+
		2.09	+	+	+	+
3	Imidacloprid (2–10%)	10	-	-	-	-
		8	-	-	-	-
		6.4	-	-	-	-
		5.12	-	-	-	-
		4.096	-	-	-	-
		3.27	+	+	+	+
		2.621	+	+	+	+
		2.097	+	+	+	+

Note: +, The tolerance of different pesticides by RA and SN13; -, The non-tolerance of different pesticides by RA and SN13 *in vitro*

Chickpea plant growth parameters

Chickpea cultivar (BG-362) was evaluated for plant growth promotion to see the effect of PGPR. Results (Table 3) clearly revealed that inoculation with RA and SN13 has significantly enhanced the plant biomass like root and shoot length, the fresh and dry weight of root and shoot of chickpea plant as compared to un-inoculated control.

Table 2. Quantitative PGP traits evaluation of RA and SN13 in presence of various concentrations of three pesticides Indole acetic acid (IAA), Exopolysachharide (EPS) and Biofilm production assay in presence of Carbendazim (0.32, 0.4, 0.51, 0.6), Imidacloprid (2.09, 3.2, 4.09, 5, 14) and Glyphosate (2.09, 2.62, 3.27, 4.09).

Pesticides used	**Carbendazim (%)**				**Imidacloprid (%)**				**Glyphosate (%)**				**Control**
Pesticides concentration	**0.32**	**0.4**	**0.51**	**0.6**	**2.09**	**3.2**	**4.09**	**5.14**	**2.09**	**2.62**	**3.27**	**4.09**	**0.00**
IAA production *Pseudomonas putida* ($\mu g.ml^{-1}$)	50.31 ±0.94	52.17 ±1.67	49.37 ±0.33	46.34 ±1.03	54.32 ±0.63	56.09 ±0.76	47.80 ±1.46	43.89 ±1.21	50.82 ±0.97	51.52 ±0.46	52.69 ±0.96	49.19 ±0.78	55.76 ±1.36
IAA production *Bacillus amyloliquefaciens* ($\mu g.ml^{-1}$)	23.12 ±1.15	26.39 ±0.51	22.89 ±1.17	21.65 ±0.68	26.37 ±0.90	29.40 ±0.61	24.73 ±1.17	23.10 ±0.89	23.92 ±0.93	26.95 ±1.21	25.08 ±0.77	22.94 ±1.71	27.79 ±2.14
EPS production *Pseudomonas putida* ($\mu g.ml^{-1}$)	252.63 ±8.30	261.30 ±10.32	306.80 ±9.76	220.35 ±8.87	298.57 ±16.01	330.20 ±9.58	289.90 ±5.55	251.77 ±16.07	266.28 ±13.89	282.32 ±18.77	287.52 ±17.10	251.33 ±15.82	313.73 ±6.84
EPS production *Bacillus amyloliquefaciens* ($\mu g.ml^{-1}$)	281.45 ±10.53	306.80 ±10.22	248.52 ±5.11	229.88 ±7.89	317.20 ±7.70	359.67 ±7.41	315.68 ±15.75	273.00 ±6.79	251.12± ±8.03	275.82± ±12.20	309.62± ±12.49	280.58± ±12.98	310.48 ±11.46
Biofilm production of *Pseudomonas putida* (OD@595nm)	0.57 ±0.05	0.72 ±0.03	0.46 ±0.04	0.28 ±0.01	0.65 ±0.05	0.84 ±0.03	0.62 ±0.01	0.56 ±0.01	0.24 ±0.04	0.38 ±0.04	0.57 ±0.02	0.38 ±0.01	0.74 ±0.01
Biofilm production *Bacillus amyloliquefaciens* (OD@595nm)	0.25 ±0.01	0.40 ±0.03	0.16 ±0.01	0.14 ±0.03	0.37 ±0.01	0.57 ±0.04	0.42 ±0.02	0.19 ±0.02	0.11 ±0.02	0.18 ±0.01	0.36 ±0.01	0.16 ±0.02	0.56 ±0.04

Table 3. Plant growth parameters in chickpea treated with *Pseudomonas putida* (RA) and *Bacillus amyloliquefaciens* (SN13) and under control condition measured 30 days after sowing.

Treatments	Root length (cm)	Shoot length (cm)	Fresh weight root (gm)	Fresh weight shoot (gm)	Dry weight root (gm)	Dry weight shoot (gm)	R/S DW ratio
Control	27.67±2.33	19.50±0.29	0.63±0.16	1.64±0.18	0.08±0.01	0.25±0.02	0.31
SN13	49.33±2.03	24.33±0.33	0.67±0.01	2.08±0.04	0.09±0.00	0.28±0.00	0.32
RA	30.00±2.08	23.67±1.33	0.66±0.03	1.91±0.18	0.08±0.01	0.25±0.02	0.34

Soil enzymes activity under pesticide treatments

Soil enzymes assay using chickpea rhizosphere soil was performed after first and second spray of pesticides. Dehydrogenase, alkaline/acid phosphatase, β-glucosidase, urease and protease activities were performed and found that RA and SN13 poise these activities in soil. Soil enzymes activities were found higher in individually RA and SN13 inoculated samples than control and other treatments. Dehydrogenase activity was inhibited in Car and Gly treated samples whereas Imi didn't affect this activity. Dehydrogenase activity was found induced in samples inoculated with RA and SN13 in presence of these pesticides (Fig. 2A). Acid phosphatase activity was enhanced in Car and Gly treated soil samples whereas activity was inhibited in Imi treated samples (Fig. 2B). RA and SN13 inoculated samples have higher acid phosphatase activity than other samples. RA and SN13 inoculated samples treated with Car, Gly and Imi resulted induced the activity of acid phosphatase. Alkaline

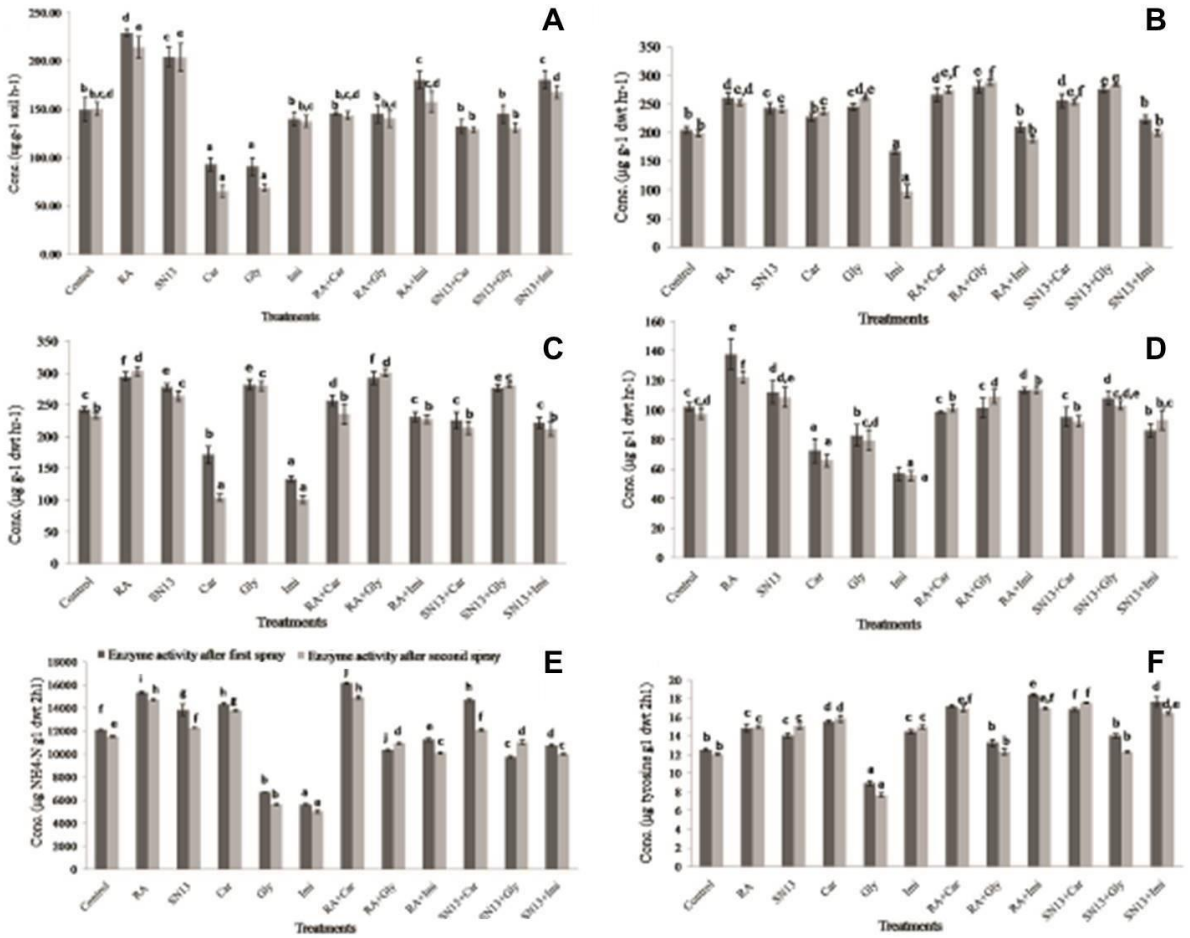

Figure 2. Soil enzymes activity in RA and SN13 inoculated soil samples after pesticides application: **A,** Dehydrogenase activity; **B,** Acid phosphatase activity; **C,** Alkaline phosphatase activity; **D,** β-glucosidase activity; **E,** Urease activity; **F,** Protease activity. [Control (without treatment), RA (samples inoculated with *Pseudomonas putida*), SN13 (samples inoculated with *Bacillus amyloliquefaciens*), Car (samples sprayed with Carbendazim), RA+ Car (samples inoculated with *P. putida* and sprayed with Carbendazim), Gly (samples sprayed with Glyphosate), RA+ Gly (samples inoculated with *P. putida* and sprayed with Glyphosate), Imi (samples sprayed with Imidacloprid), RA+ Imi (samples inoculated with *P. putida* and sprayed with Imidacloprid), SN13+Car (samples inoculated with *B. amyloliquifaciens* and sprayed with Carbendazim), SN13+ Gly (samples inoculated with *B. amyloliquifaciens* and sprayed with Glyphosate), SN13+Imi (samples inoculated with *B. amyloliquifaciens* and sprayed with Imidacloprid), bars represent the standard errors of the means (n = 3). Different letters within column represents significant difference at (P = 0.05) by using DMRT]

phosphatase activity was inhibited with Car and Imi but stimulated with Gly (Fig. 2C). RA inoculated sample individually as well as RA with Gly treated sample stimulated highest alkaline phosphatase activity. β-glucosidase activity was inhibited with all three pesticides. This activity was found higher in bacterized samples than control (Fig. 2D). Imi treated sample showing the highly inhibited activity of β-glucosidase after first and second spray. Sample inoculated with RA alone have highly stimulated this activity. Urease activity was found inhibited by Gly and Imi whereas stimulated with Car (Fig. 2E). RA and SN13 inoculated samples treated with Gly and Imi have enhanced urease activity than only Gly and Imi treated samples. Urease activity was highly induced in RA inoculated soil treated with Car after first and second spray. Protease activity was found stimulated in samples containing Car and Imi whereas highly reduced activity was found in Gly treated soil samples (Fig. 2F). Surprisingly, most of the soil enzymes activities found reduced which we have performed after second application of these three pesticides in comparison with the first application. These results proved that repeated application of pesticides affects soil enzyme activities. Results indicated that RA and SN13 have the ability to tolerate the pesticides and reduced its adverse effect in the soil at some point and play a beneficial role in maintaining the soil fertility. Figure 2 showing the soil enzyme activities in presence of RA and SN13 inoculated with and without pesticides spray. Both the PGPR play an important role in maintaining the soil enzyme activities and reduced the negative effect of pesticides in soil health.

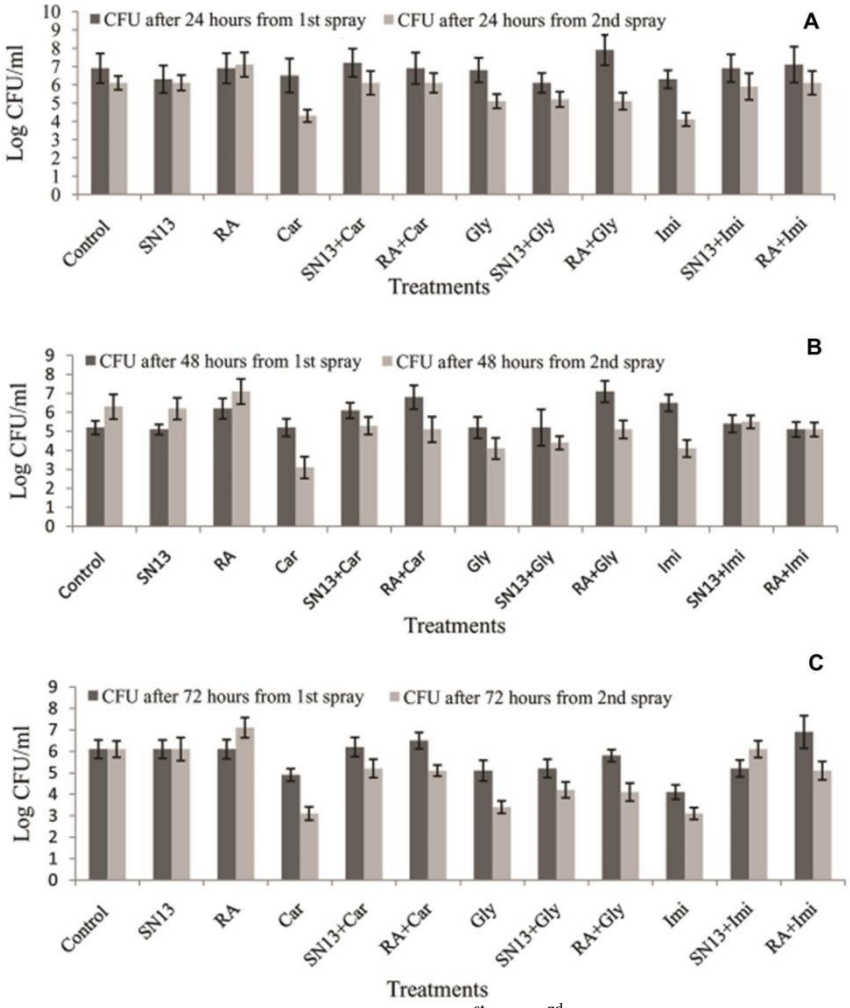

Figure 3. Colony forming unit assay: **A,** CFU after 24 hrs from 1st and 2nd spray of pesticides; **B,** CFU assay after 48 hrs from 1st and 2nd spray of pesticides; **C,** CFU after 72 hrs from 1st and 2nd spray of pesticides. [bars represent the standard errors of the means (n = 3)]

CFU assay in pesticide treated soil

CFU of rhizosphere soil was measured after 24, 48 and 72 hrs from 1st and 2nd spray of pesticides. The numbers of viable colonies were higher in RA inoculated than control soil samples with and without pesticides treatments. The decreased colonies were observed in Car, Imi and Gly treated soil samples after first and second spray whereas colonies were increased in RA and SN13 inoculated soil samples (Fig. 3). Pesticides treated

samples showed a reduction in CFU values after 72 hrs of 1st spray and after 48 hrs of 2nd spray. Samples with PGPR inoculations and with pesticides sprays showed relative less reduction in CFU values. These results (Fig. 3) revealed that the RA and SN13 reduced the adverse effect of pesticides and survival of bacteria is increased.

Biolog assay in pesticide-treated soil

Average well color development (AWCD) was calculated which showed the microbial activity in each well of the microplate. Microbial diversity indices like Shannon, McIntosh and Simpson functional diversity and related evenness have been determined (Table 4). These results revealed the bacterial population in the rhizosphere after pesticides spray. The Shannon and McIntosh diversity together with their evenness were reduced in Gly treated samples in presence of both bacterial strains. But in case of Car and Imi samples diversity indices were not changed with and without inoculation of RA and SN13.

Table 4. Functional diversities and evenness based on carbon source utilization pattern for chickpea rhizosphere treated with *Pseudomonas putida* (RA) and *Bacillus amyloliquefaciens* (SN13) with and without pesticides treatment.

S.No.	Sample	ShD	ShE	ShipD	McD	McE
1	Control	3.34±0.02	0.99±0.01	0.99±0.00	0.98±0.00	0.99±0.00
2	RA	3.33±0.02	0.98±0.00	0.99±0.00	0.98±0.00	0.99±0.00
3	SN13	3.31±0.03	0.97±0.00	0.99±0.00	0.98±0.01	0.98±0.00
4	Car	3.30±0.02	0.96±0.00	0.98±0.00	0.98±0.00	0.97±0.00
5	Gly	3.21±0.01	0.94±0.01	0.97±0.00	0.95±0.00	0.96±0.00
6	Imi	3.25±0.01	0.95±0.00	0.96±0.00	0.98±0.00	0.98±0.00
7	RA+Car	3.31±0.01	0.97±0.00	0.99±0.00	0.98±0.00	0.98±0.00
8	RA+Gly	3.22±0.02	0.95±0.01	0.99±0.00	0.97±0.00	0.97±0.01
9	RA+Imi	3.27±0.01	0.96±0.01	0.99±0.00	0.97±0.00	0.98±0.00
10	SN13+Car	3.33±0.01	0.98±0.01	0.99±0.00	0.98±0.00	0.99±0.00
11	SN13+Gly	3.18±0.03	0.95±0.01	0.99±0.00	0.96±0.00	0.97±0.01
12	SN13+Imi	3.30±0.01	0.96±0.00	0.99±0.00	0.98±0.00	0.98±0.00

Note: ShD, Shannon diversity; **ShE,** Shannon evenness; **SimpD,** Simpson diversity; **McD,** McIntosh diversity; **McE,** McIntosh evenness.

DISCUSSION

Pseudomonas putida and *Bacillus amyloliquefaciens* are well known for their PGP attributes. Production of IAA, EPS and biofilm by these bacteria were maximum at threshold concentrations of pesticides but above that production was reduced (Table 2). Pesticide uses may cause slight and temporary changes to microbial populations of soil and their activities even if applied at normal rates (Johnsen *et al.* 2001). In case of repeated application pesticides can interfere and disturb soil enzymatic activities and also affected local metabolism resulting a reduction in soil fertility. Soil dehydrogenases are the enzymes belong to oxidoreductase enzyme class and are its major representatives (Gu *et al.* 2009). Dehydrogenases are very important enzymes in the soil environment which are an indicator of the overall microbial activity of soil (Gu *et al.* 2009, Salazar *et al.* 2011). These occur intracellularly in all microbial cells (Yuan & Yue 2012). Dehydrogenase enzyme activity is repeatedly performed for measurement of any disturbance due to pesticides, or direct measure of soil microbial activity and trace elements or management practices to the soil. Our results revealed that dehydrogenase activity was inhibited in Car and Gly treated samples whereas Imi didn't affect this activity (Fig. 2). This enzyme activity was higher in the sample inoculated with RA and SN13 alone without pesticides than control. Phosphatases have five main groups of enzymes: phosphomonoesterases, phosphodiesterases, phosphotriesterases, phosphoamidases and pyrophosphatases. Among these five enzymes, phosphomonoesterases are most abundant in soils. This may be because of low substrate specificity of this group of enzymes (De Cesare *et al.* 2000). Phosphomonoesterases are further classified into two groups which are acid and alkaline phosphatase, on the basis of optimum pH for their activity. These two phosphatases are mainly found in animals and microorganisms. It was found by researchers that alkaline phosphatase activity gets inhibited when the fungicide is applied to soil (Rasool & Reshi 2010), while the activity of acid phosphatase increased. Acid phosphatase activity was observed highly inhibited immediately after Car addition compared to the control (Tortella *et al.* 2013). Researchers also found that other fungicides either had inhibited phosphatases activity or no effect (Bello *et al.* 2008, Yan *et al.* 2011). On the application of herbicides the activity of acid and alkaline phosphatase is either stimulated (imazethapyr) or unchanged (aurora 40 WG and rimsulfuron) (Perucci *et al.* 2000, Baćmaga *et al.* 2012). Phosphatases activities were found to be inhibited by herbicide application under different conditions of soil physicochemical properties and pesticides dose (Min *et al.* 2001, Tejada

2009). The acid and alkaline phosphatase activities may respond differently to application of insecticides. Sometimes the same insecticide may inhibit acid phosphatase and stimulate the activity of alkaline phosphatase, and vice versa (Cycon *et al.* 2010, Defo *et al.* 2011, Jastrzebska 2011). Overall it was found in earlier studies that pesticides play an inhibitory effect on the enzymatic activities involved in the phosphorus cycle. We found enhanced acid phosphatase activity in samples treated with Car and Gly whereas this activity was inhibited in by Imi. For bacterized samples treated with a recommended concentration of pesticides, an overall increase in acid phosphatase activity was observed for both the PGPR. β-glucosidase is an enzyme which plays important role in the decomposition/transformation of organic matter in the soil. Glucose is the final product which is an important source of carbon energy for soil microorganisms (Deng & Tabatabai 1994). In our results, β-glucosidase activity was found inhibited in samples treated with Car, Gly and Imi. This activity was found higher in RA and SN13 inoculated soil samples than control.

Hydrolysis of urea into carbon dioxide and ammonia is catalyzed by urease. Researchers have proven that herbicides and fungicides appear to have no effect (Cycon *et al.* 2010, Yan *et al.* 2011, Baćmaga *et al.* 2012) or reduced effect on the activity of urease (Sukul 2006, Caceres *et al.* 2009, Tejada 2011). Application of pesticides decreases the urease activity in the soil which is beneficial for plants by reducing the hydrolysis of urea because it helps to maintain nitrogen availability to plant (Antonious 2003). On the other hand, the fungicides validamycin and Car enhanced urease activity, respectively, up to 13–21% and to 70% (Qian *et al.* 2007, Yan *et al.* 2011). In our experiment, we found inhibited urease activity by Gly and Imi whereas stimulated with Car. Protease enzyme plays an important role in nitrogen mineralization which regulates the amount of available nitrogen to the plants. The protease activity was found to be higher after the application of endosulfan and chlorpyrifos at lower and medium concentrations. Pesticides applications had stimulated the activity of protease enzyme in comparison to control upto 21 days from incubation (Rasool & Reshi 2010). Protease activity in soil decreased on applications of insecticides on concentration more than 25 ppm. Our results revealed that protease activity was found stimulated in samples containing Car and Imi whereas reduced activity was found in Gly treated soil samples. In overall experiments, it was common observations results for second sprays were more drastic revealing that repeated use of chemicals leached to the soil and disturb the microbial population and changed the soil enzyme activities. PGPR (RA and SN13) inoculation has reduced the adverse effect by maintaining the microbial population and metabolic activities.

In biolog technique, the rate of colour development in wells provides information about the density and metabolic activity of bacterial cells in an inoculum, while the diversity of colour development in wells about microbial diversity in soil solution (Mondini & Insam 2003). We have concluded from the diversity study that with the use of pesticides a slight reduction in microbial diversity and evenness was observed but the sample inoculated with PGPR showed no such changes due to the survival of microbial population and poise of soil enzymes.

CONCLUSIONS

Our experimental evidences concluded that repeated application of pesticides disturb soil microbial dynamics. An increase in PGP trait activities like IAA production, exopolysachchride production, biofilm synthesis, phosphate solubilization and siderophore production on the addition of pesticides at concentrations below threshold values. Inoculation of *P. putida* and *B. amyloliquefaciens* maintain the equilibrium of microbial population in soil and also balance soil enzymatic activities. Thus the application of pesticides (Car, Imi and Gly) under PGPR (RA and SN13) inoculated soil would not reduce microbial population hence improve soil fertility.

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