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Effects of organophosphate pesticides on siderophore producing soils microorganisms

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ABSTRACT

The aim of the current study was to evaluate the effects of four organophosphate pesticides on the siderophores producing soil microorganisms or plant growth promoting rhizobacteria (PGPR). Individual effects of acephate, glyphosate, monocrotophos and phorate as well as in combinations of two were tested against the five siderophore producing soils microorganisms viz, *Pseudomonas fluorescens* (NCIM-5096), *Rhizobium leguminosarum* (NCIM-2749), *Bacillus brevis* (NCIM-2532), *Azotobacter vinelandii* (NCIM-2821), and *Salmonella typhimurium* (NCIM-2501). The dose dependent effect was observed for the siderophore production assay. The effects of combinations were found to be more significant as compared to single pesticide(s). The overall sequence of adverse effect of the four pesticides on the siderophore production observed was: phorate > acephate > monocrotophos > glyphosate which was linear with the toxicity of pesticides. Further, it was noticed that PGPR strain *Pseudomonas fluorescens* (13–66%) was the least affected by the pesticides under study and *Salmonella typhimurium* (20–75%) was the most affected PGPR strain. The overall order of adverse effect of pesticides on the PGPR strains was as: *Bacillus brevis* (19–80%) > *Salmonella typhimurium* (20–75%) > *Rhizobium leguminosarum* (21–72%) > *Azotobacter vinelandii* (22–81%) > *Pseudomonas fluorescens* (13–66%). In combination, applications of Acpt + Phor have shown more adverse effects as compared to other combinations. Combination of Gly + Mono has no or least adverse effects on the PGPR strains. Present study may contribute to understand the role of siderophores and siderophores producing microorganisms in the metal ion uptake and risk associated with the applications of pesticides.

1. Introduction

Most of the applied chemicals or pesticides, even if sprayed on weeds and foliage of crop, directly leach into the soil and affect the growth and development of plant growth promoting bacteria (PGPR) or soil microbial communities (Ahemad and Khan, 2012; Crowley and Kraemer, 2007; Georgieva et al., 2018; Gopalakrishnan et al., 2017). PGPR including siderophores and siderophores producing microflora are an important biological component of the soil ecosystem and have a vital role in soil fertility through their role in organic matter decomposition and nutrient cycling (Hider and Kong, 2010; Neilands, 1952, 1995). Maximum siderophores grow under the supply of metal ions, but that growth is inhibited if the metal ions deprived by other chelating groups (chemicals and microorganism) (Rajkumar et al., 2010; Renshaw et al., 2002). Siderophores ("metal ions carrier") are low-molecular-weight chelating agents (200–2000 Da), having high affinity iron chelating agents secreted by microbial communities such as bacteria, fungi and other microflora (Butler and Theisen, 2010; Khan et al., 2009; Schmidt, 1999). In both Gram-positive and Gram-negative rhizobacteria, M^{m+} siderophore complex on bacterial membrane is reduced to $M^{(m-1)+}$ which is further released into the cell from the siderophore via a gating mechanism linking the outer and inner membranes (where M = metal

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ion) (Teintze et al., 1981). Unlikely, during the reduction process, the siderophore compounds may be recycled/destroyed (Crowley and Kraemer, 2007; Vansuyt et al., 2007). Thus, siderophores act as solubilizing agent for metal ions from organic compounds or minerals under various stress conditions of metal ions. Usually, siderophores form strong stable complexes with metals that are of environmental concern, such as Al, Cu, Fe, Ga, and Zn. Binding of the siderophore ligand or acid to a metal increases the uptake of soluble metal concentration (Butler and Theisen, 2010; Khan et al., 2009; Schmidt, 1999).

Numerous studies (*in-vitro* and *in-vivo*) has been reported on decomposition of organophosphate pesticides (OPs) in the presence of microorganisms including plant growth promoting rhizobacteria (PGPR) (Ahemad and Khan, 2011; Digrak and Özçelik, 1998; Kumar et al., 2015b; Lo, 2010; Meena et al., 2015; Patel et al., 2017; Sidhu et al., 2019). As, PGPR are classified based on their functional activities as (i) bio-fertilizers (enhancing nutrient availability), (ii) phyto-stimulators (production of phytohormones), (iii) rhizo-remediators (degrading contaminants) and (iv) bio-pesticides (production of secondary metabolites with anti-fungal and anti-bacterial properties) (Ahemad and Khan, 2011 & 2012; Bhattacharyya and Jha, 2012; Kumar et al., 2015b). If we search about the effect of pesticides on PGPR, maximum investigations highlighting the dose dependent effects of pesticides on PGPR, i.e. with the increase of dose adverse effect of pesticides on PGPR increases (Ahemad and Khan, 2011; Digrak and Özçelik, 1998; Lo, 2010). The hazardous effect of pesticides on PGPR is directly proportional to the persistence and dose level. Repeated use of pesticides having high persistency level in the soils may further increase the threat (Singh et al, 2015, 2016, 2017). As per above siderophore are also part of PGPR. In this context, the chemistry of siderophore and siderophore producing microorganisms is intriguing as a potential metal chelator (Kizhakedathil and Devi, 2018; Kumari et al., 2018a and b).

Moreover, Acephate (Acpt), glyphosate (Gly), monocrotophos (Mono) and phorate (Phor) are widely used organophosphate (OP) pesticides for agricultural applications worldwide (Grube et al., 2011; Kumar et al., 2014; Kumar et al., 2015a & 2015b; Kumar et al., 2016; Kumar et al., 2017; Kaur et al., 2017; Kumar and Singh, 2018; Kumar et al., 2019; Singh et al., 2019; Sidhu et al., 2019). OPs inhibit the reversible hydrolysis of acetylcholine, and therefore the persistence of OPs for longer time in the environment is a problem. In literature, there is no study reported on the Gly, Acpt, Mono, and Phor interactions with siderophore producing microorganism or siderophore production. Sub-lethal or excess dose level applications of Acpt, Gly, Mono and Phor in combinations may adversely effects soils microorganisms including PGPRs. More significantly, no study is reported for combinations or under "cocktailed effect" of Acpt, Gly, Mono and Phor with each other. Consequently, the main objectives of the current study were (1) to determine the effects of Acpt, Gly, Mono and Phor on the siderophore producing microorganism or siderophore production as single and in combinations or "cocktailed effect", (2) to determine the above mention effect quantitatively through UV–visible spectrometric technique.

2. Materials and methods

2.1. Chemicals and reagents

Technical grade (98% pure) Gly, Acpt, Mono & Phor was supplied by the Gautmi Ltd, Hyderabad (India). All other chemicals including NaOH, HCl, CH_3COO and metal salts (iron) were of analytical (AR) grade and were purchased from Loba Chemie Mumbai (India). All the standard solutions were prepared in volumetric flasks and kept at 5 °C during the whole experimentation to avoid the decomposition risk at high temperatures.

2.2. Strain selection

Plant growth promoting bacterial (PGPR) strains were purchased from National Chemical Laboratory, Pune-India, with NCIM accession numbers. The strains were cultured as per standard specifications or protocols supplied by NCL, Pune. The strains with NCIM accession numbers are as follows: *Pseudomonas fluorescens* (NCIM-5096), *Bacillus brevis* (NCIM-2532), *Rhizobium leguminosarum* (NCIM-2749), *Azotobacter vinelandii* (NCIM-2821), and *Salmonella typhimurium* (NCIM-2501). The selected strains can secrets siderophores (chelating molecules), those facilitate the PGPR activities like nitrogen fixation through chelating mechanism. These strains are reported to cause nodule formation in pea plants, fixes atmospheric nitrogen and reported to produce tyrocidin and gramicidin which is a potent antimicrobial agent. Such characteristics of microbes improve plant health and/or growth by diverse mechanisms. Selected strains were further tested for their PGPR activities by assessing the their indole acetic acid production, Phosphate solubilization ability, and sidherophore production as mentioned below.

2.3. Bioassays for plant growth promoting traits of selected strains

2.3.1. Indole acetic acid production

Production of indole acetic acid (IAA) in the selected strains were determined by inoculation of culture into Luria Bertani (LB) broth amended with 50 µg per millilitre L-tryptophan The cultures were kept at 30 °C for 2–3 days and then centrifuged at cooling centrifuge at 10,000 g for 10 min. The supernatant were further used and the concentration was determined using Salkowski reagent at 530 nm (Colo et al. 2014).

A standard curve was prepared by taking different concentrations of standard IAA solution (0.1%–1%) and the final volume was ended to 2 mL with distilled water. After that 4 mL of Salkowski reagent was added to the solution and incubated for 30 min at room temperature and absorbance was calculated at 530 nm and values were calculated by plotting a standard curve by using standard Indole acetic acid at 530 nm.

2.3.2. Phosphate solubilization assay

For qualitative assay of Phosphate solubilization activity, the isolates were grown in Pikovskaya medium. Bacterial cultures were inoculated as spots and remain incubated at 30 °C for 2–3 days. The appearance of the halo zone nearby the inoculated spot confirms that the isolate has the capability to solubilize the phosphates (Colo et al., 2014). All the experiments will be carried out in triplicates.

The values solubilizing efficiency was calculated using following formula:

$$Solubilizing\ \ effciency = \frac{Z - C}{C} \times 100$$

Z = zone of Solubilization (mm); C = Diameter of colony (mm).

For quantitative estimation, the isolated strains were assessed by evaluation of solubilization of insoluble calcium phosphate into soluble form in PVK broth under agitated conditions. One mL of inoculums from the test tubes with O.D. 0.5 (λ 600) was added to 100 mL Erlenmeyer flasks containing 25 mL of Pikovskaya's broth & further incubated at 30 °C till maximum solubilization was observed. Uninoculated flasks were taken as controls. The samples were centrifuged at 15,000 rpm for 10 min and supernatant was collected taken as a sample for a further test for phosphorus solubilised as given by John (1970).

2.3.3. Siderophore production

For qualitative analysis, same ingredients of King's B media; Protease peptone - 3.00 g, Di-potassium hydrogen phosphate – 0.23 g, Magnesium sulphate hepta-hydrate – 0.23 g and Agar - 3.00 g were taken into four flasks of 250 mL capacity and dissolved in 150 mL distilled water. In addition to ingredients of King's B media, 0.007 g $FeCl_3$ was added to each flask. First flask was treated as control. In the second flask 3.75 mg pesticide was added i.e. 25 mg/L. In the third flask 27.00 mg pesticide was added i.e. 100 mg/L. In the fourth flask 54.00 mg pesticide was added i.e. 200 mg/L. All four flasks were allowed for autoclaving. The

autoclaved media of each flask (10 mL/plate) was poured on autoclaved petri plates in a laminar flow. Petri plates under the laminar flow were allowed to solidification. After solidification, 100 μL/petri-plates of cultures, of above mentioned siderophore producing PGPR bacterial strains were spread on the media. All the plates were capped gently, sealed well using the paraffin film, and incubated at 28 ± 2 °C for 72 h. The fluorescent pigments of the bacterial colonies were assessed using an ultraviolet lamp. Fluorescent pigments formed were considered as an indication of siderophore production (Ahemad and Khan, 2011 & 2012; Kumar et al., 2015b). All the experiments were performed in triplicates.

2.3.4. Effect of pesticides on siderophore production

2.3.4.1. Qualitative analysis. For qualitative analysis, same ingredients of King's B media; Protease peptone - 3.00 g, Di-potassium hydrogen phosphate – 0.23 g, Magnesium sulphate hepta-hydrate – 0.23 g and Agar - 3.00 g were taken into four flasks of 250 mL capacity and dissolved in 150 mL distilled water. In addition to ingredients of King's B media, 0.007 g $FeCl_3$ was added to each flask. First flask was treated as control. In the second flask 3.75 mg pesticide was added i.e. 25 mg/L. In the third flask 27.00 mg pesticide was added i.e. 100 mg/L. In the fourth flask 54.00 mg pesticide was added i.e. 200 mg/L. All four flasks were allowed for autoclaving. The autoclaved media of each flask (10 mL/plate) was poured on autoclaved petri plates in a laminar flow. Petri plates under the laminar flow were allowed to solidification. After solidification, 100 μL/petri-plates of cultures, of above mentioned siderophore producing PGPR bacterial strains were spread on the media. All the plates were capped gently, sealed well using the paraffin film, and incubated at 28 ± 2 °C for 72 h. The fluorescent pigments of the bacterial colonies were assessed using an ultraviolet lamp. Fluorescent pigments formed were considered as an indication of siderophore production (Ahemad and Khan, 2011 & 2012; Kumar et al., 2015b).

2.3.4.2. Quantitative analysis. A well established method described by the Castaneda et al. (2005) was used for the quantitative analysis. Briefly, for the quantitative analysis, same ingredients of King's B media; Protease peptone - 3.00 g, Di-potassium hydrogen phosphate – 0.23 g, and Magnesium sulphate hepta-hydrate – 0.23 g, were taken into three flasks of 250 mL capacity and dissolved in 150 mL distilled water. In addition to ingredients of King's B media, 0.007 g $FeCl_3$ was added to each flask. First flask was treated as control. In the second flask 3.75 mg pesticide was added i.e. 25 mg/L. In the third flask 54.00 mg pesticide was added i.e. 200 mg/L. All three flasks were allowed for autoclaving. The autoclaved media of each flask (10 mL/test tube) was poured in autoclaved test tubes under laminar flow. Test tubes under the laminar flow were allowed to cool. After cooling, 100 μL/test tube of cultures of above mentioned siderophore producing PGPR bacterial strains were added. All the test tubes were capped using foil and sealed by using the paraffin film and incubated at 28 ± 2 °C for 72 h. The material of test tubes were transferred into sterilized centrifuged tubes under laminar flow and allowed for centrifugation at 5000 rpm for 15 min. The clear supernatants were utilized for analysis. The absorbance spectra were recorded using a double beam spectrophotometer (Shimadzu 1800) in 1.0 cm cells, against distilled water blank.

Similarly, the above mentioned procedure was also used to determine the effect of Acpt, Mono & Phor and combinations of Gly, Acpt, Mono & Phor. Different concentrations of Acpt, Gly, Mono & Phor were prepared separately followed by mixing of equal volumes to prepare the mixture of pesticides. For example, to prepare 25 mg/L of Acpt + Gly, 5 mL of Acpt having concentration 25 mg/L was mixed with 5 mL of Gly having concentration 25 mg/L and named as 25 mg/L of Acpt + Gly.

To determine the % changes in siderophore production at 25 and 200 mg/L of pesticide following formula was used; % change = (A-B)/A*100, where A = absorbance of different strains at 0 mg/L of pesticide; B = absorbance of different strains at 25, 50, 100 and 200 mg/L of pesticide.

2.4. Statistical analysis

Origin software 6.0 and 8.0 were used for the ANOVA statistical analysis. Use of statistical analysis was done for quantitative analysis of siderophore production, metal ion content variations observed in wheat growth experiments and to compare the degradation or stability differences that were observed in the thermal analysis experiments.

3. Results

3.1. Indole acetic acid production

The indole acid production by the five selected strains varies considerably. Highest production of IAA was observed in *Pseudomonas fluorescens* (NCIM-5096), *Rhizobium leguminosarum* (NCIM-2749), *Bacillus brevis* (NCIM-2532), and *Azotobacter vinelandii* (NCIM-2821), respectively, with production concentration up to 83, 74.5, 59 and 48.10 μg/mL. In *Salmonella typhimurium* (NCIM-2501), the concentrations of IAA production is quite low (20 μg/mL) as compared to other strains. Usually indole acetic acid acts as signal molecules. The production of Indole acetic acid was influenced by the availability of substrates, culture conditions, etc. and it also varies from species to species.

3.2. Phosphate solubilization

All the five selected strains (*Pseudomonas fluorescens* (NCIM-5096), *Bacillus brevis* (NCIM-2532), *Rhizobium leguminosarum* (NCIM-2749), *Azotobacter vinelandii* (NCIM-2821), and *Salmonella typhimurium* (NCIM-2501) were able to form clear zone on Pikovskaya's agar plates after 7 days of incubation. When quantified spectrophotometrically, maximum phosphate was solubilised by putative *Bacillus brevis* (NCIM-2532), by forming clear zone of 3 mm diameter on Pikovskaya's agar plate. The range of P-solubilization varied from 2 to 16 μg/mL. Most of the phosphate solubilizing strains increase the bio-availability of phosphorus to plant by solubilizing organic phosphorus compounds & convert inorganic phosphorus into readily available form which is considered a positive indicator of utilizing the microbes as biofertilizers for crop production and beneficial for sustainable agriculture.

3.3. Qualitative analysis for the effects of pesticides on the siderophore production

Qualitative analysis has revealed that, all the strains produced siderophores at good rates in the absence of pesticides (Gly, Acpt, Mono & Phor). The reduction in the yellow green fluorescence pigment production was observed with the addition of pesticides at different doses (25, 50, 100 and 200 mg/L). The production of yellow green fluorescence pigment was found to be dose dependent, which decreased with the increase in dose of pesticides. Qualitative analysis depicted that when pesticide(s) was (were) applied at 25, 50, 100 and 200 mg/L, the sharp decrease was observed in yellow green fluorescence production (Supplementary Figs. S1–S4). Qualitatively, it was observed that with the addition of pesticides, the siderophore production or yellow green fluorescence production decreased as compared to control (Supplementary Figs. S1–S4).

3.4. Quantitative analysis on the effects of pesticides on the siderophore production

Quantitatively analysis of siderophore production was performed by using the double beam UV–visible spectrophotometer over a range of 300–420 nm. The changes in the siderophore production with the addition of pesticides on *Pseudomonas fluorescens* is exhibited in Figs. 1 and 2. Here, the negative scale in Fig. 2 is representing the negative

effect (in percentage) of pesticide(s) at two different dose levels (25 and 200 mg/L). In Fig. 2, the percentage changes in the siderophore production at 25 mg/L and 200 mg/L are represented with squared box and rounded circle. In, qualitative analysis, all strains have shown dose dependent effect on siderophore production. With the application of various concentrations (from 25 to 200 mg/L) of pesticides the significant changes were noticed.

Fig. 2 (a) shows the order of adverse effect of Gly on siderophore production as *Azotobacter vinelandii* (23%) ~ *Rhizobium leguminosarum* (23%) > *Salmonella typhimurium* (21%) > *Bacillus brevis* (19%) >> *Pseudomonas fluorescens* (13%) with the addition of 25 mg/L of Gly. With the addition of 200 mg/L of Gly, the order of siderophore production was *Azotobacter vinelandii* (66%) > *Bacillus brevis* (63%) > *Salmonella typhimurium* (58%) > *Rhizobium leguminosarum* (50%) >> *Pseudomonas fluorescens* (43%). It was observed that the siderophore production was significantly decreased with the increase of concentration of Gly at significant level $p < 0.05$. In case of Gly, Fig. 2 (a) exhibits that, with the addition of 25 and 200 mg/L of Gly, maximum decrease in siderophore production occures on the *Azotobacter vinelandii* strain while there was minimum change observed on the *Pseudomonas fluorescens* strains (at significant level $p < 0.05$).

Acpt has shown higher adverse effects as compare to that of Gly Fig. 2 (b). Here, with the addition of 25 mg/L of Acpt, the order of adverse effect of Acpt on siderophore production was; *Azotobacter vinelandii* (37%) > *Salmonella typhimurium* (33%) > *Rhizobium leguminosarum* (27%) >> *Bacillus brevis* (26%) >> *Pseudomonas fluorescens* (20%). With the addition of 200 mg/L of Acpt, the decreasing order of siderophore production was; *Azotobacter vinelandii* (81%) > *Bacillus brevis* (79%) > *Salmonella typhimurium* (75%) > *Rhizobium leguminosarum* (72%) >> *Pseudomonas fluorescens* (62%). It was observed that, the siderophore production was significantly decreased with the increase of concentration levels of Acpt at significant level $p < 0.05$. Fig. 2 (b) exhibits that, with the addition of 25 and 200 mg/L Acpt, maximum decrease in siderophore production was observed on *Azotobacter vinelandii* and minimum decrease was observed on *Pseudomonas fluorescens* at significant level $p < 0.05$.

Mono has shown more adverse effects on PGPR strains as compared to that of Gly, but least as compared to Acpt Fig. 2 (c). At 25 mg/L, the observed order of adverse effect on different strains was: *Azotobacter vinelandii* (28%) > *Salmonella typhimurium* (25%) ~ *Rhizobium*

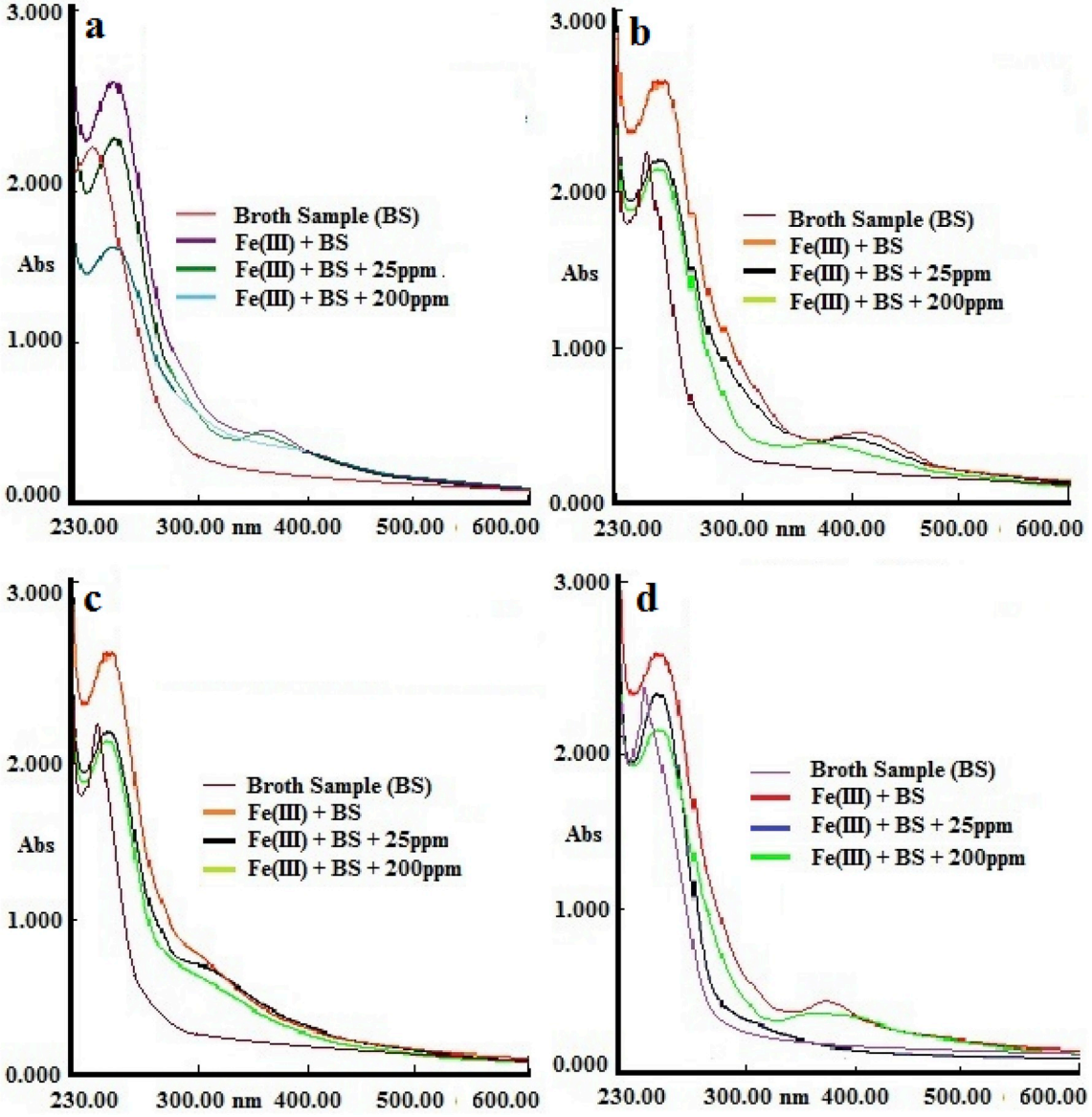

Fig. 1. UV–visible analysis on the effect of pesticides on siderophore production of *Pseudomonas fluorescens* strain at 25 and 200 ppm dose level of pesticides. (**a**) analysis for acephate (**b**) analysis for glyphosate (**c**) analysis for monocrotophos and (**d**) analysis for phorate.

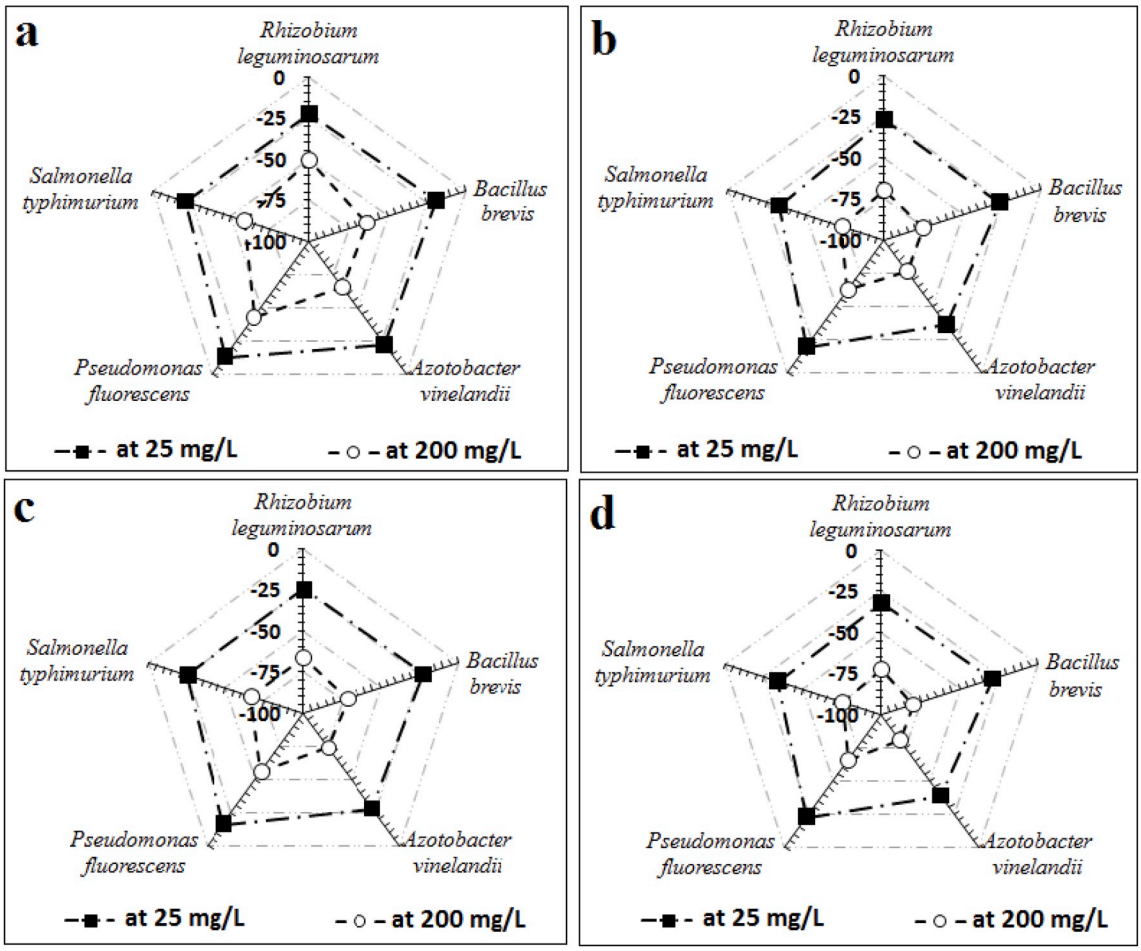

Fig. 2. Quantitative effect of Gly (**a**), Acpt (**b**), Mono (**c**), and Phor (**d**) at 0, 25 and 200 mg/L on the siderophore production by five PGPR strains w. r. t. the % decrease in siderophore production of different PGPR strains at significant level $p < 0.05$.

leguminosarum (24%) > *Bacillus brevis* (23%) >> *Pseudomonas fluorescens* (16%). With the addition of 200 mg/L of Mono, the decreasing order of siderophore production was; *Azotobacter vinelandii* (74%) > *Bacillus brevis* (71%) > *Salmonella typhimurium* (66%) ~ *Rhizobium leguminosarum* (66%) >> *Pseudomonas fluorescens* (55%). It was observed that, the siderophore production was significantly decreased with the increase of concentration levels of Mono at significant level $p < 0.05$. Fig. 2 (c) exhibits that with the addition of 25 and 200 mg/L Mono, maximum decrease in siderophore production was observed on *Azotobacter vinelandii* and minimum decrease was observed on *Pseudomonas fluorescens* at significant level $p < 0.05$.

Fig. 2 (d) depicted that Phor has affected the siderophore production at significant extent as compare to other three pesticides. At 25 mg/L of Phor, the order of adverse effect of Phor on siderophore production was; *Azotobacter vinelandii* (39%) > *Salmonella typhimurium* (34%) > *Rhizobium leguminosarum* (32%) > *Bacillus brevis* (29%) >> *Pseudomonas fluorescens* (23%) (Fig. 2(d)). With the addition of 200 mg/L of Phor, the decreasing order of siderophore production was; *Azotobacter vinelandii* (81%) > *Bacillus brevis* (80%) > *Rhizobium leguminosarum* (76%) > *Salmonella typhimurium* (75%) >> *Pseudomonas fluorescens* (66%) (Fig. 2 (d)). It was observed that, the siderophore production was significantly decreased with the increase of concentration levels of Phor at significant level $p < 0.05$. Fig. 2 (d) exhibits that, with the addition of 25 and 200 mg/L Phor, maximum decrease in siderophore production was observed on *Azotobacter vinelandii* and minimum decrease was observed on *Pseudomonas fluorescens* at significant level $p < 0.05$.

3.5. Effect of pesticides in combination or cocktailed effect on siderophore production

Qualitative analysis under the cocktailed effect revealed the decrease in siderophore production of all PGPR strains (Supplementary Fig. S5). The effects of combinations of pesticides were found to be more as compared to single or pure dose of pesticide. Production of yellow green fluorescence pigment was reduced with the addition of Acpt, Gly, Mono & Phor in combinations. Siderophore inhibition was found to be dose dependent which decreases with the increase in dose.

Quantitative analysis of siderophore production was performed by using the double beam UV–visible spectrophotometer. The percentage changes in the siderophore production on addition of 25 and 200 mg/L of Acpt + Gly is shown in Fig. 3. With the addition of 25 mg/L of Acpt + Gly, the decreasing order of siderophore production was; *Azotobacter vinelandii* (42%) > *Salmonella typhimurium* (37%) ~ *Rhizobium leguminosarum* (33%) > *Pseudomonas fluorescens* (27%) > *Bacillus brevis* (24%). Similarly, on addition of 200 mg/L of Acpt + Gly, the decreasing order of siderophore production was; *Azotobacter vinelandii* (89%) > *Pseudomonas fluorescens* (84%) > *Rhizobium leguminosarum* (75%) > *Salmonella typhimurium* (72%) > > *Bacillus brevis* (68%). Fig. 3 has exhibits that, siderophore production was significantly decreased with the increase of dose levels of different combinations at significant level ($p < 0.05$). At the dose level of 200 mg/L of Acpt + Phor in combination, the observed percentage decrease in siderophore production with different strains was; *Azotobacter vinelandii* (67%), *Rhizobium leguminosarum* (75%), *Salmonella typhimurium* (80%), *Pseudomonas fluorescens* (80%) and *Bacillus brevis* (64%).

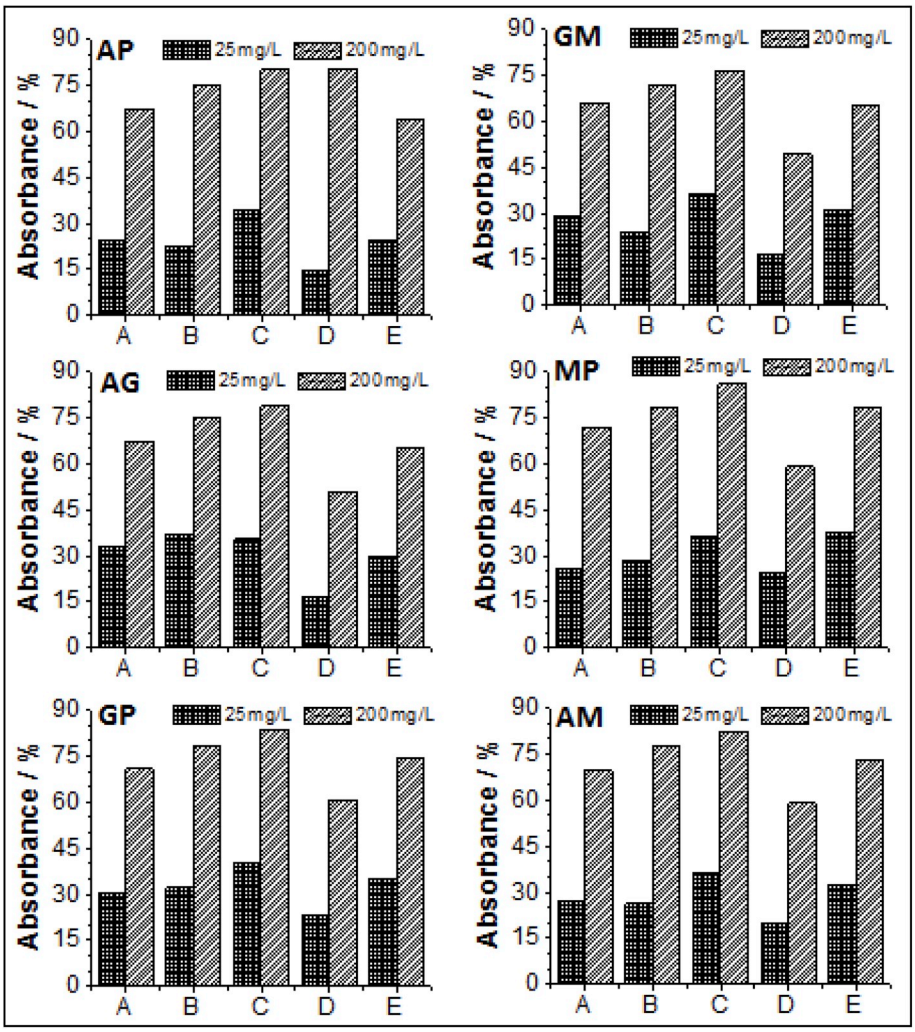

Fig. 3. Mixture or cocktailed effect of pesticides at their dose level of 25 mg/L, and 200 mg/L on siderophore production with (A) *Azotobacter vinelandii*, (B) *Rhizobium leguminosarum* (C) *Salmonella typhimurium,* (D) *Pseudomonas fluorescens,* and (E) *Bacillus brevis*, at significant level $p < 0.05$. *Here, A = Acpt, G = Gly, M = Mono and P = Phor.*

At 200 mg/L of Acpt + Gly, the observed percentage decrease in siderophore production with different strains was; *Azotobacter vinelandii* (67%), *Rhizobium leguminosarum* (75%), *Salmonella typhimurium* (79%), *Pseudomonas fluorescens* (51%) and (E) *Bacillus brevis* (66%). At 200 mg/L of Mono + Gly, the observed percentage decrease in siderophore production with different strains was; *Azotobacter vinelandii* (66%), *Rhizobium leguminosarum* (71%), *Salmonella typhimurium* (76%), *Pseudomonas fluorescens* (49%) and (E) *Bacillus brevis* (65%).

At 200 mg/L of Gly + Phor, the observed percentage decrease in siderophore production with different strains was; *Azotobacter vinelandii* (72%), *Rhizobium leguminosarum* (79%), *Salmonella typhimurium* (86%), *Pseudomonas fluorescens* (59%) and (E) *Bacillus brevis* (78%). At 200 mg/L of Mono + Phor, the observed percentage decrease in siderophore production with different strains was; *Azotobacter vinelandii* (71%), *Rhizobium leguminosarum* (79%), *Salmonella typhimurium* (84%), *Pseudomonas fluorescens* (61%) and (E) *Bacillus brevis* (74%). At 200 mg/L of Acpt + Mono, the observed percentage decrease in siderophore production with different strains was; *Azotobacter vinelandii* (70%), *Rhizobium leguminosarum* (77%), *Salmonella typhimurium* (82%), *Pseudomonas fluorescens* (59%) and (E) *Bacillus brevis* (73%).

It was observed that, the effect on the siderophore production was dose dependent similarly as for individual pesticide, i.e. siderophore production decreased with increase in dose. In current study, the observed order of pesticide in combination or cocktailed on the siderophore production was: Acpt + Phor > Mono + Phor > Gly + Phor > Acpt + Mono > Acpt + Gly > Gly + Mono. It was observed that, pesticides as individual or in combination have low effect on *Pseudomonas fluorescens* and greater adverse effect on *Rhizobium leguminosarum* strain.

4. Discussions

Among PGPR group, *Pseudomonas*, *Rhizobium*, *Bacillus* and *Azotobacter* are one of the important generas studied over the decades because of their nitrogen fixation, used as a biofertilizer and produces array of growth-promoting substances and helps to replace chemical fertilizer for the sustainable agriculture production (Carlos et al., 2019; McRose et al., 2019; Sayyed et al., 2005). These species are known for siderophore production, indole acetic acid production, and phosphate solubilization (Carlos et al., 2019). Selected strains have ability to secret chelating molecules or siderohpores those aid the iron uptake which facilitates the PGPR activities mainly siderophore production. *Salmonella typhimurium* is known for the phosphate solubilization through the positive regulation by cyclic adenosine monophosphate (cAMP) and the cAMP receptor protein (CRP) for two enzymes produced by *Salmonella typhimurium*, an acid hexose phosphatase and a cyclic phosphodiesterase (Kier et al., 1977 & 1979).

Since, plant growth promoting rhizobacteria (PGPR) are the diverse

class of bacterial strains found near the roots of the plant or rhizosphere. They induce plant growth by the indirect or direct methods (Ahemad and Khan, 2011; Digrak and Özçelik, 1998; Lo, 2010). PGPR regulate plant growth through by producing various hormones like producing siderophores, indole acetic acid, hydrogen cyanide, nitrate and enhancing phosphate solubilization etc (Ahemad and Khan, 2011 & 2012; Duke et al., 2012; Kumar et al., 2015b). Pesticides can inhibit the siderophore production of soil microorganisms by two ways i.e. direct and indirect effects. Through the direct effects where the toxic chemicals kill these microorganisms before the siderophore production or other plant growth promoting activities (HCN production, ammonia and nitrate production, indole acetic acid production, etc.) (Mohamed et al., 2019; Prasad et al., 2018). Through the indirect effects where the chelating chemicals inhibit the siderophore production or other plant growth promoting activities by competing with siderophore produced by these microorganisms (Mohamed et al., 2019; Prasad et al., 2018; Verma et al., 2018; Winkelmann, 2017; Zaidi et al., 2017). Most drastically, pesticides can inhibit the metal ions uptake through both ways. In recent study, effect of various pesticides were tested against the PGPR strains where glyphosate and monocrotophos were found toxic at 900 and 2100 μg/ml (Shahid et al., 2019). Various authors have shown that Gly can adversely affect PGPRs through excess production of ammonia and through the excess dose applications of Gly (Ahemad and Khan, 2011 & 2012; Duke et al., 2012). In case of Gly, the results presented in current study were same as like of Ahemad and Khan (2011) & 2012 where dose dependant results were found and Gly has shown minimum adverse effects on PGPRS as compared to other pesticides.

The dose of pesticides could inhibit the production of siderophore was proved in the current study. Study on the Acpt, Mono, Phor and cocktailed effect was performed first for the time with interesting and significant result findings. It is reported that Gly application may cause significant difference on the soils enzyme activities includes respiration, β-glucosidase, β-glucosaminidase activity, and dehydrogenase activity (Duke et al., 2012). Gly applications reduced the counts of Mn solubilizing PGPRs, consequently, uptake of Mn reduced in soil and plants (Bernards et al., 2005). Researches claimed that this happened due to decrease in production siderophore production and indole acetic acid (Ahemad and Khan, 2011 & 2012). Metal solubilizing capacity of the microorganisms can increase with the increase in the siderophore production (Duke et al., 2012). In present study, all pesticides including Gly have decreased the siderophore production, and conditions were more drastic under high dose level. Effect of Gly on N-fixation and nodulation has been analyzed by various authors (Ahemad and Khan, 2011 & 2012; Zobiole et al., 2010), depends upon the applications of Gly, formulation of Gly and time of applications of Gly, little variation in above factors may cause severe damage of soil community. In current study, dose dependent effect of Gly on *Rhizobium leguminosarum* was observed. In present study it was found that remaining three pesticides (Acpt, Mono and Phor) has more adverse effects on the PGPR strains and their siderophore production capacity. The conditions were more drastic under the cocktailed effects. Lo, 2010 have explained that mixed form of pesticides can cause more adverse effects on the soil microorganisms through the inhibitions of the plant growth promoting activities (like producing siderophores, indole acetic acid, hydrogen cyanide, nitrate and enhancing phosphate solubilization) of the strains. It was noticed that PGPR and other beneficial soil microorganisms acts as per the dose (concentration or individual or mixed) and toxicity level of the chemicals including the pesticides (Mohamed et al., 2019; Prasad et al., 2018; Verma et al., 2018; Winkelmann, 2017; Zaidi et al., 2017).

5. Conclusion

It was concluded that pesticides under study (Gly/Acpt/Mono/Phor) can inhibit the growth of soil microorganisms i.e. PGPR. In qualitative and quantitative assay, the he siderophore production was decreased with the applications of pesticides under study. The overall order of adverse effect of pesticides under study on the siderophore production ability of four PGPR microorganisms was *Bacillus brevis* > *Salmonella typhimurium* > *Rhizobium leguminosarum* > *Azotobacter vinelandii* > *Pseudomonas fluorescens*. Among the four pesticides, at different dose levels, the order of adverse effect on siderophore production was phorate > acephate > monocrotophos > glyphosate which is linear with toxicity of pesticides. Lesser the adverse effects of pesticide on microorganism, greater will be the utility of pesticide as a source of nitrogen carbon and phosphate by microorganism. Results of this study may help the environmental researchers to use the selected PGPR for the bioremediation of pesticides under study as per their adverse effects.

Safety

Acephate, glyphosate, monocrotophos and phorate are organophosphate that inhibits the activity of cholinesterase. Their direct contact should be avoided. Work with these pesticides should be performed in a fume hood using gloves and eye protection.

Declaration of competing interest

There is no statement on conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101359.

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