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FRIENDLY BACTERIA PROPPING UP LEGUMES DEVELOPMENT IN PESTICIDE CONTAMINATED SOIL

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ABSTRACT

The plant growth promoting rhizobacteria (PGPR) are having a crucial role in the soil fertility. However, the accumulation of various pesticides into the soil is harmful for their existence in the rhizosphere and also for their activities. The present investigation was aimed to study the friendly nature of *Pseudomonas* sp. in the growth promotion of some leguminous plants by its special abilities like production of siderophore and its resistance for the organophosphate pesticides. *Pseudomonas aeruginosa* NCIM 2036 was observed to produce pyoverdine even in presence of monocrotophos. The highest level of pyoverdine was detected after 72 hrs of growth at 25°C in iron-free succinate medium supplemented with monocrotophos. The addition of iron (III) to the growth medium strongly repressed the synthesis of siderophore. The *Pseudomonas* sp. was further tested for its plant growth promotional effects in presence of monocrotophos using *Vigna radiata* (L. Wilzeck) as a standard legume plant and it is found to be significantly effective in plant growth promotion under pot culture conditions.

KEY WORDS: **Rhizosphere,*Pseudomonas*, Pyoverdine, Monocrotophos, Growth promotion**

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INTRODUCTION

Soil is a complex ecosystem containing heterogeneous microbial flora and functional activities of this flora have a crucial role in soil fertility. A group of bacteria termed PGPR (plant-growth-promoting rhizobacteria), facilitate the plant growth by their functional activities like N2 fixation, phosphate solubilisation and production of siderophores and phytohormones etc[11]. Iron is essential for almost all forms of life, which is used at the active site of many important redox enzymes dealing with cellular respiration involving oxidation and reduction[19]. Iron plays crucial role in development and growth of plants as in chlorophyll synthesis and chloroplast development and if plants are deprived of iron they may become prone to chlorosis[8] which further affect the overall growth of the plant. Although iron accounts for about 4% of the total content of minerals in the earth's crust, under aerobic conditions or in alkaline or neutral environment it occurs in the form of complexes that are refractory to solubilization, which is the reason of its limited availability[9]. In order to solubilize and sequester insoluble ferric iron, many organisms have evolved efficient and high affinity iron acquisition system. They produce an extra cellular, low molecular weight (500-1000 D) iron chelating compound termed as siderophore[16]. Siderophore scavenges iron from insoluble mineral phases to soluble ones that can be transported to plants or microbial cells by energy dependant membrane transport system[17]. The synthesis of siderophores by bacteria is also one of the main factors inhibiting the growth and development of bacterial and fungal pathogens[3,14].

Current agricultural practices are heavily dependent on various pesticides for the larger productivity. Organophosphorus pesticides alone make up for 70 percent of the pesticides used world wide[6]. However, accumulation of such pesticides into soils beyond certain threshold levels is detrimental to rhizospheric microorganisms and their activities[18].So it has become a thirst to uncover certain efficient microbes which can overcome the problems by showing resistance against such pesticides as well as promoting the plant development. So this exploration attempts to study the production, detection and estimation of the siderophore from *Pseudomonas aeruginosa* NCIM 2036 in the presence of monocrotophos under different cultural conditions and its gracious effects on the growth of *Vigna radiata* (L. Wilzeck) in the presence of monocrotophos.

MATERIALS AND METHODS

Culture of *Pseudomonas aeruginosa* NCIM 2036 was obtained from NCIM Pune, (M.S.) INDIA and maintained on the nutrient agar medium[1]. All the laboratory glasswares used in the experiments were left for 24 hours in a 6M hydrochloric acid in order to remove all ions of iron and then washed few times with deionized water with conductivity less than 0.1 micro siemens[16]. All non iron containing media components were of "analytical reagent" grade and obtained from local suppliers.

- ***Extraction of pure pesticides***

Extraction of pure pesticide from commercially available formulations was carried out as they contain other ingredients like emulsifiers and stabilizers. The extraction of monocrotophos was carried out from Phoskill (36% E.C). 1ml of Phoskill pesticide was taken in 10ml acetone and shaken well; then it was mixed with equal amount of chloroform and 5 mL of distilled water and again shaken vigorously and allowed to stand for 20 min. The chloroform layer was carefully removed and passed through anhydrous sodium sulfate to remove traces of water. It was then evaporated at 35°C to get fine crystals or amorphous powders of the monocrotophos pesticide. The residue obtained was washed again with chloroform-water mixture to remove all impurities[4].

- ***Detection of siderophores***

Siderophore production by *Pseudomonas aeruginosa* NCIM 2036 was tested qualitatively

by using the universal Chrome Azurol Sulphonate (CAS) liquid as well as plate assay[16].The study organism was inoculated on CAS-Cetrimide agar and incubated for 24 hours at room temperature; formation of orange color zone around the colonies in plate assay indicated the siderophore production. In liquid assay, periodically removed cell free supernatant from production medium was mixed with CAS reagent; color changes from blue to orange due to scavenging of iron complexed with CAS reagent by produced siderophore[15].

- ***Production and estimation of siderophore in presence of monocrotophos***

For production of siderophore, 24 hours old culture of *Pseudomonas aeruginosa* NCIM 2036 was inoculated at constant rate of 1% v/v in slightly modified iron free succinate medium containing K_2HPO_4 0.6%; KH_2PO_4 0.03%; $(NH_4)_2SO_4$ 0.01%; $MgSO_4$, $7H_2O$ 0.02%, succinic acid 0.4% and monocrotophos 0.01%, pH 7.0 and incubated at room temperature on a rotary shaker at 120 rpm[13]. During incubation, aliquots of media were removed after 24 hours, growth was monitored at 550 nm further it was centrifuged at 8000 rpm at 4 ºC for 10 min and cell free supernatant was subjected to record absorbance spectrophotometrically at 400 nm for determination of pyoverdine[13]. The production of pyoverdine was determined in terms of the ratio of A_{400}/A_{550}[9].

- ***Effect of variable carbon source on siderophore production***

For production of siderophore, the basal salt medium containing monocrotophos was supplemented with different carbon sources like succinic acid, glycerol and mannitol at 0.4% w/v, which was then inoculated with *Pseudomonas aeruginosa* NCIM 2036 and incubated at room temperature for 24 hours. The siderophore content was quantified as described earlier.

- ***Effect of incubation period on siderophore production***

Another factor determining the production of siderophore was the incubation period. In this study, succinate medium containing monocrotophos was inoculated with *Pseudomonas aeruginosa* NCIM 2036 and incubated at room temperature at 120 rpm; the amount of siderophore produced was recorded after every 24 hours.

- ***Effect of temperature of incubation***

It is well known that *Pseudomonas aeruginosa* could grow in temperature as high as 42^{o}C. However, the effect of different incubation temperatures, ranging from 15^{0}C to 35^{0}C on siderophore production was studied, in which the succinate medium containing monocrotophos was inoculated with study organism and incubated for 72 hours at 120 rpm for various incubation temperatures.

- ***Effect of pH on siderophore production***

The effect of pH on siderophore production was studied, in which standard succinate medium containing monocrotophos was adjusted to different pH values ranging from 5.0 to 9.0 and then inoculated with *Pseudomonas aeruginosa* NCIM 2036 and incubated for 72 hours at 25^{0}C at 120 rpm.

- ***Iron regulation***

To study the effect of iron on siderophore production, succinate medium containing monocrotophos was supplemented with various concentrations of iron ($FeCl_3$) ranging from 0 to 100 µM and inoculated with study organism keeping all other conditions optimal.

- ***Plant growth promotion study by pot culture technique***

The *Vigna radiata* (L. Wilzeck) seeds were surface sterilized by 0.1% mercuric chloride for 2 min and rinsed four times with sterile distilled water. The surface sterilized seeds were inoculated with broth cultures of *Pseudomonas aeruginosa* NCIM 2036 for 30 min. Uninoculated seeds treated with sterile nutrient broth were used as controls, then seeds were removed and allowed to dry. 1 kg dry weight of the sterilized and non-sterilized soil was taken

into separate sterile pots. Both soil samples were thoroughly mixed with sterile deionized water containing 100 ppm of monocrotophos per Kg^{-1} of soil and then seeds were sowed in the soil as a test (inoculated seeds) and control (uninoculated seeds). Sterile water was added in the pots daily and observed for root length and shoot length after 10 days.

- ***Detection of siderophores***

Pseudomonas aeruginosa NCIM 2036 was able to produce siderophore in iron free succinate medium containing monocrotophos which was detected by color change of CAS (Chrome Azurol Sulphonate) from blue to orange by using plate as well as liquid CAS assay, which confirmed presence of siderophores (Figure-1 and 2).

RESULTS

CAS plate assay

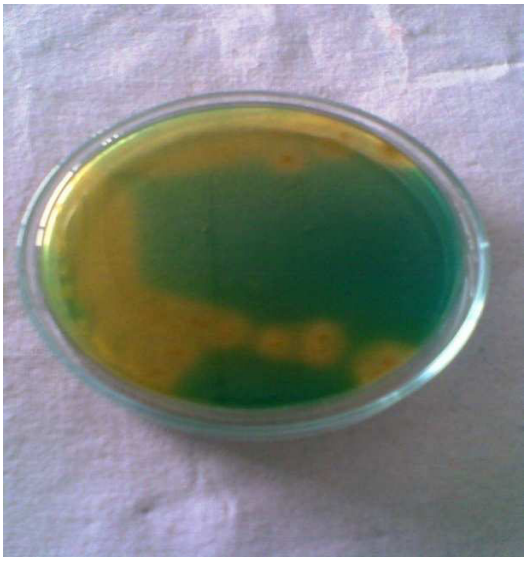


Figure1
Formation of orange zonearound the colonies indicates siderophore production

Liquid CAS assay

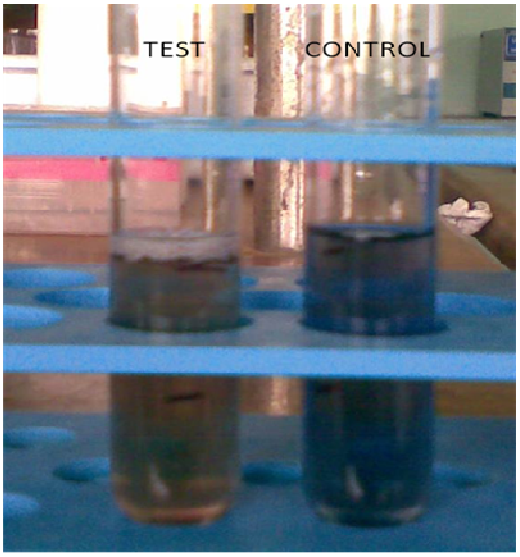


Figure 2
The change in color of the blue dye- chrome azurolsulphonate (CAS)assay solution to orange indicating the presence of siderophore

- ***Effect of different carbon sources on siderophore production***

Among the carbon sources tested succinate showed maximum production than glycerol and mannitol. It can be seen that succinate showed comparatively more productivity of siderophore which is about 2 times more than glycerol and 4 times more than mannitol (Figure-3).

Effect of carbon sources on the production of siderophore

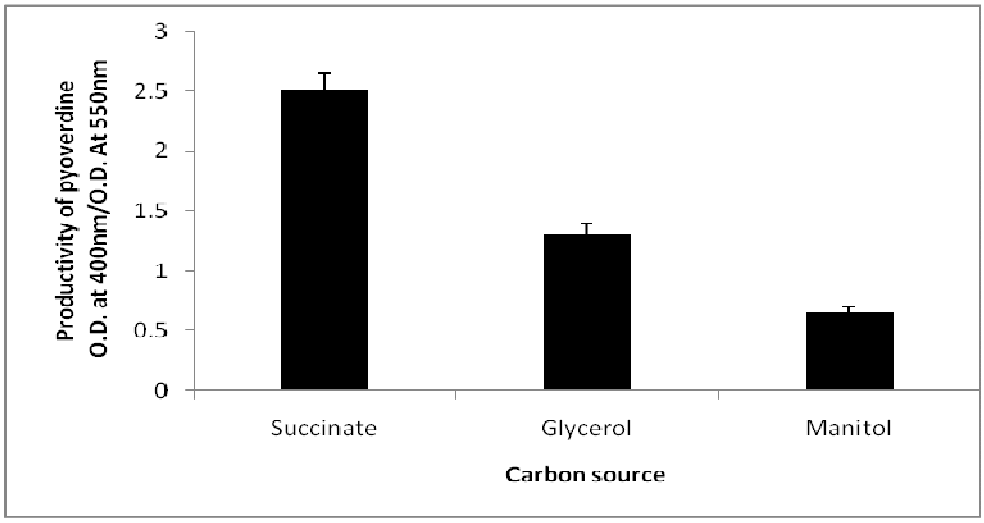


Figure 3
Mean error bars in the graph represent the mean ± standard error

- ***Production of siderophore with respect to time***

Study organism showed a varied level of siderophore production with respect to incubation period. Intensive production of pyoverdine was observed on the third day of incubation, followed by a slight decline thereafter (Figure-4).

Effect of cultivation period on the production of siderophore

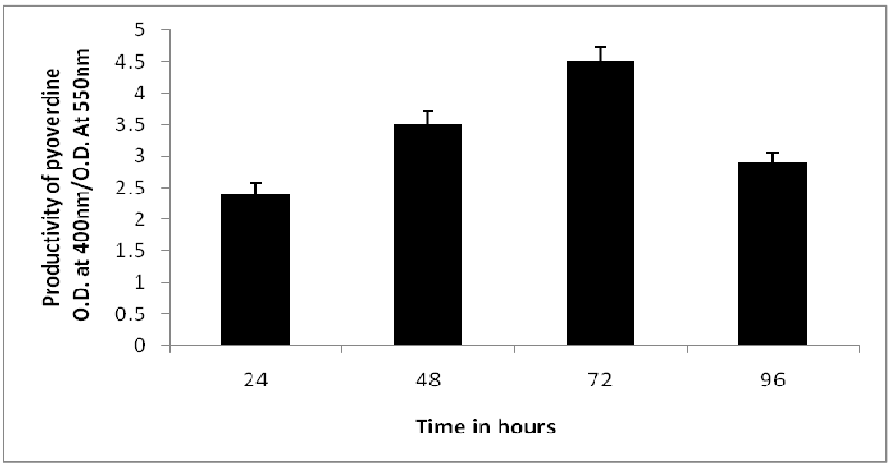


Figure 4
Mean error bars in the graph represent the mean ± standard error

- ***Influence of temperature on siderophore production***

It has been observed that the highest amount of pyoverdine was synthesized at 25^{o}C temperature. Inspite of higher biomass, the organism showed declined production of pyoverdine at 35°C temperature. However, in contrast at 15°C temperature although there was low biomass it shown lower productivity. (Figure 5)

Effect of temperature on the production of siderophore

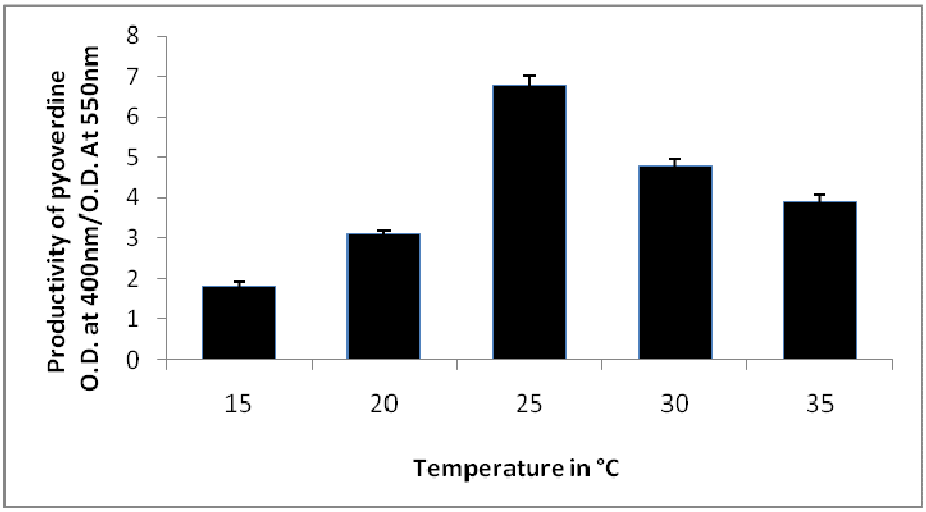
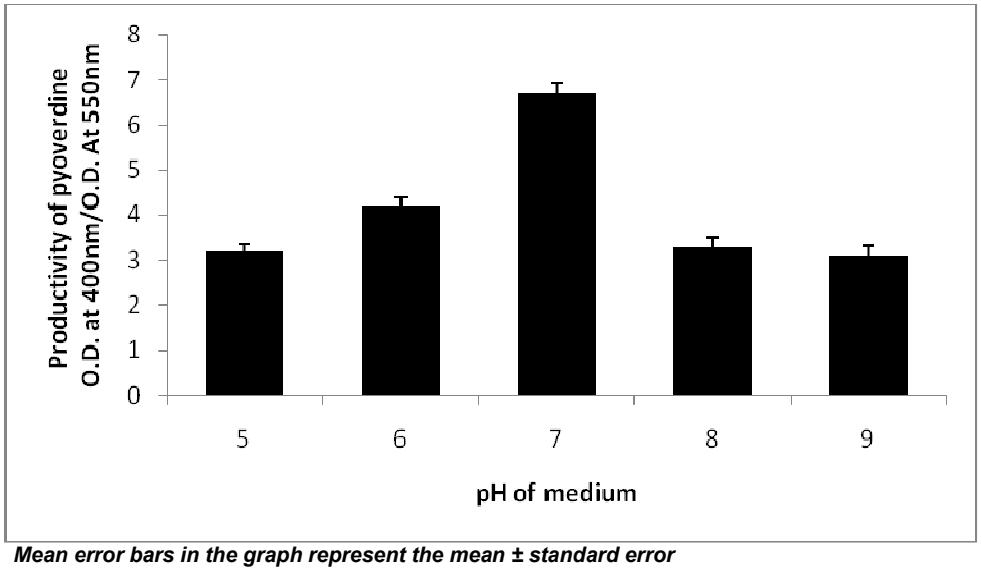


Figure 5
Mean error bars in the graph represent the mean ± standard error

- ***Effect of pH on production of siderophore***

Highest level of pyoverdine in media was achieved at pH 7.0 with highest biomass keeping other conditions optimal; both the acidic and alkaline environments are unfavorable for the pyoverdine production, while the alkaline environment has shown dramatically decreased productivity. (Figure 6)

Figure 6
Effect of pH on the production of siderophore

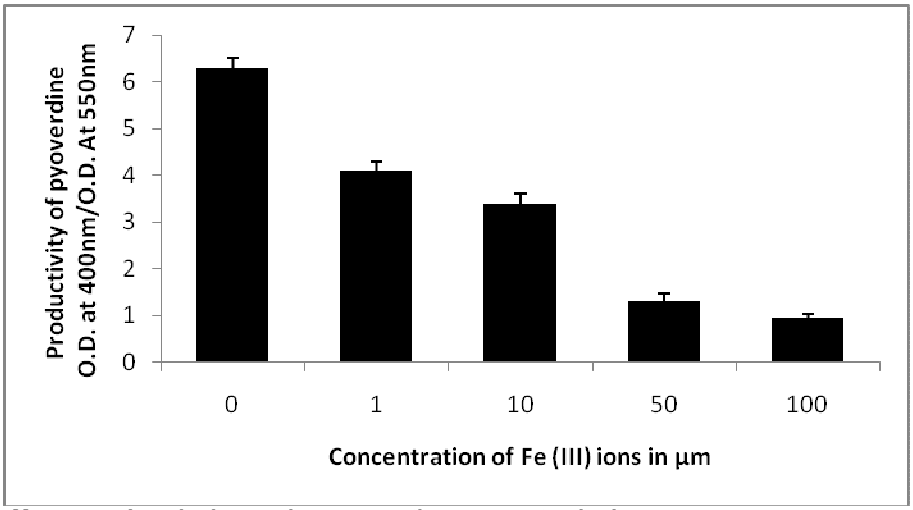


Mean error bars in the graph represent the mean ± standard error

- ***Effect of iron concentration on siderophore production***

Iron concentration (Fe^{3+}) is a crucial determining factor in siderophore production. The concentration of pyoverdine gradually decreased with the increase in the concentration of Fe^{3+} ions. In the experiment, addition of only 1µM of iron in the medium caused about 40% reduction in the siderophore production. (Figure 7)

Figure 7
Effect of Fe^{3+} ions on the production of siderophore



Mean error bars in the graph represent the mean ± standard error

- ***Effect of siderophores on plant growth***

Influence of the *Pseudomonas aeruginosa* NCIM 2036 on the growth promotion of *Vigna radiata* (L. Wilzeck) is summarized in Table-1. Test organism shown a positive stimulatory effect on the plant growth in both of the experimental set up using sterile soil and non sterile soil.

Table 1
Growth promotion of Vigna radiata by Pseudomonas aeruginosa NCIM 2036

		Stem		Root	
		Average length in cm	% Increase	Average length in cm	% Increase
Sterile soil	Control plant	10.8±0.4	-	6.7±0.2	-
	Test plant	14.5±0.5	+34.3%	9.1±0.4	+35.8%
Non sterile soil	Control plant	11.2±0.2	-	7.0±0.2	-
	Test plant	14.7±0.4	+31.3%	9.2±0.3	+31.4%

DISCUSSION

The formed siderophores were detected by CAS assay in which siderophore scavenge the iron complexed with CAS reagent, so there is color change of the reagent from blue to orange, which confirms the presence of siderophore[15]. In the next experiment, effect of different carbon source tested on the productivity of siderophores. It has been reported earlier that succinate is regarded as a factor that stimulates the synthesis of this siderophore[7, 12, 13]. The 3-amino moiety of the chromophore in pyoverdine is substituted with a group derived from succinate[10], so media containing succinate shows maximum production of siderophore. The maximal production of siderophore was observed on the third day of incubation, since pyoverdine is secondary metabolite, its synthesis starts during the late log phase and in stationary phase[9]. Presence of pesticide in the medium might be a reason of delayed production of siderophore. In the study of optimum temperature, maximum siderophore production was observed at 25ºC. This is because the soil temperature usually does not exceed 25ºC[9]. The reduced level of pyoverdine in the media with pH 8.0 and onwards may be due to instability of the

structure of the siderophore in alkaline solutions[12, 13].

Addition of iron in the media affects the siderophore production. This is because siderophores are iron-specific compounds which are secreted under low iron stress[5]. The standard succinate medium without added iron permitted the synthesis of pyoverdine[13]. In the last experiment the plant growth was promoted in sterile soil as well as non sterile soil samples. It is also observed in current experiments that the leaves of test plants were free from certain fungal infections while some of the controls are observed to be susceptible for the same. It might be because of the siderophoral activity which is well documented in the control of various plant diseases[2]. The main aspect of the friendliness of the studied *Pseudomonas* sp. is that it is able to promote the growth of leguminous plants in the presence of considerable amounts of organophosphate pesticides. This is a very interesting phenomenon which will be helpful nowadays because the excessive use of pesticides hampers the growth and activity of plant growth promoting rhizobacteria which results in the decreased productivity. To the best of our knowledge this is the first report which shows that in the presence of the pesticide the organism is able to produce siderophores, and as this organism is also known for its plant growth promoting activities so it can be potentially used as a bioinoculant in the agro industries to overcome the problems of pesticides contaminations in soil.

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