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Research Article

In vitro antibiosis of Fluorescent pseudomonads against *Fusarium oxysporum* and *Rhizoctonia bataticola* and effect of the antifungal metabolite on fungal biomass

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Abstract: In recent years, biological control has become a promising alternative to chemical control in the management of soil borne disease A group of root-associated bacteria, plant growth promoting rhizobacteria (PGPR), intimately interact with the plant roots and consequently influence plant health and soil fertility. Among these PGPR, fluorescent psuedomonads occur commonly in the rhizosphere of plants and help suppress disease establishment and spread by secretion of antibiotics. The present study focuses on the extraction and identification of the antifungal compound secreted by *Psueudomonas fluorescens*. Considering the global significance of antifungal metabolites in disease suppression and consequent applicability of psuedomonads in biological control strategies, the bio control potential of fluorescent pseudomonads against *F. oxysporum* and *R. bataticola* have been reported. These compounds were identified as phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine (2-OH-PHZ).

Keywords: Antifungal compounds, phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine (2-OH-PHZ), *Pseudomonas fluorescens* Os25*, Fusarium oxysporum* and *Rhizoctonia bataticola*.

Introduction

The treatment of non-pathogenic, plant growth promoting rhizobacteria (PGPR) has been suggested as an alternative strategy for the control of plant diseases. Fluorescent pseudomonads have been widely tested for bio control against fungal pathogens because of their rapid growth rate and their ability to colonize rhizosphere to a large extent besides their ability to suppress the soil borne pathogens. They are known to suppress soil-borne plant pathogens through the production of secondary metabolites including antibiotics.

The antibiotics synthesized by biocontrol fluorescent pseudomonads include, agrocin 84, agrocin 434, herbicolin, oomycin, phenazines, pyoluteorin, pyrrolnitrin, acetylphloroglucinols and cyanides. Currently, over 50 naturally occurring Phenazine compounds have been described and mixtures of different Phenazine derivatives can occur simultaneously in one organism. Almost all Phenazines exhibit broadspectrum activity against bacteria and fungi. In addition to inhibiting fungal pathogenesis, Phenazines play an important role in microbial competition in rhizosphere, including survival and competence.

Materials and Methods

In vitro antibiosis against Phytopahtogens:

The plating medium (Potato dextrose agar) was divided into two sets and one set was amended with FeCl3 (100 µg ml-1). The bacterial isolates were streaked at one end of Petri dish (1 cm away from the edge of the plate) containing PDA medium. The mycelial disc (8 mm dia) of five days-old culture of phytopathogens, *Fusarium oxysporum* and *Rhizoctonia solani*, was placed on the opposite end of the Petri dish perpendicular to the bacterial streak. Three replicates were maintained for each isolate. The plates were incubated at room temperature for 7 days. The zone of inhibition of radial growth of the pathogens was measured in mm scale. Fungal radial growth inhibition (a clear zone between the edges of fungal mycelia and bacterial colonies) was calculated after 5-7 days of incubation by the procedure suggested by Fokkema, 1973.

Extraction of Antibiotic Compound from the culture:

Extraction of antibiotic from fluorescent Pseudomonas was done following the method described by Howell and Stipanovic (1980) with slight modifications. The bacterial lawn was prepared by growing the culture on King's B agar plates for 7 days. The culture was extracted with 80% aqueous acetone (20ml per plate), filtered through cheese cloth and the filtrate was centrifuged at 12000×g for 10 min. The supernatant was evaporated to remove acetone. To the residue, NaCl (5% w/v) solution was added and centrifuged at 12000×g for 10 min. The supernatant was extracted twice with diethyl ether and dried over anhydrous calcium chloride. The compound in crystalline form was used for infrared spectral studies, TLC, UV and mass spectroscopy for identification the antibiotic compound.

Effect of antibiotic compound on fungal biomass:

The Potato dextrose broth of 50 ml, containing different concentrations of antibiotic compound (25 µg ml-1, 50 µg ml-1 and 100 µg ml-1), was inoculated with mycelial disc (8 mm) of five

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days old culture of phytopathogens, and were incubated for 7 days. After the incubation period the fungal biomass was estimated. Control set was maintained without addition of antibiotic compound.

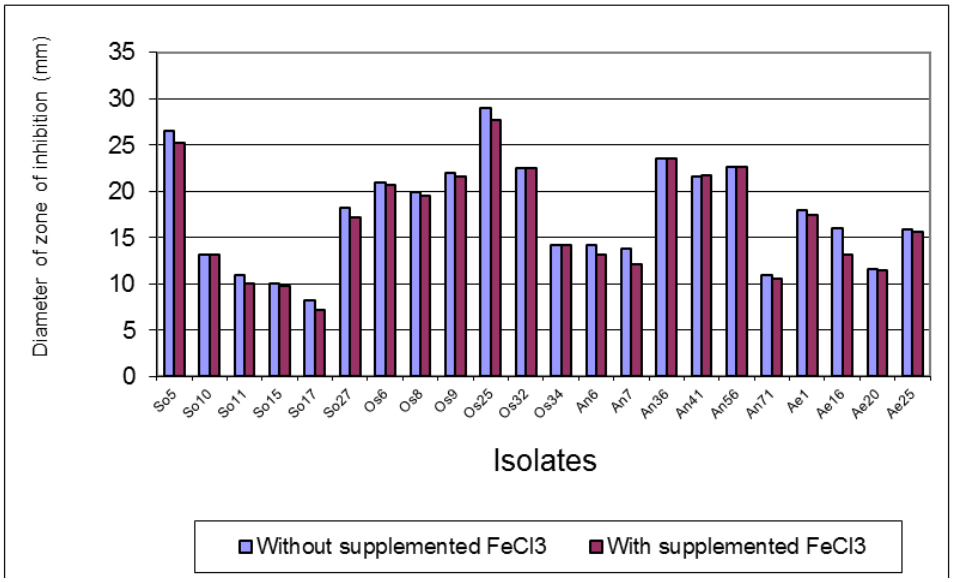
Results and Discussion

***In vitro* bio control potential of the isolates of fluorescent pseudomonads against phytopathogens:** In the present study, the bio control potential of the isolates against fungal pathogens *Fusarium oxysporum* and *Rhizoctonia bataticola* were evaluated.

Table.1: *In vitro* bio control potential of the isolates of fluorescent pseudomonads against phytopahtogen, *Fusarium oxysporum*

Isolates	Diameter of zone of inhibition (mm)	
	Without supplemented $FeCl_3$	With supplemented $FeCl_3$
So5	26.46	25.15
So10	13.12	13.17
So11	10.96	10.05
So15	10.01	9.69
So17	8.14	7.16
So27	18.16	17.14
Os6	20.97	20.66
Os8	19.94	19.45
Os9	22.01	21.61
Os25	28.95	27.69
Os32	22.41	22.45
Os34	14.17	14.15
An6	14.16	13.16
An7	13.76	12.14
An36	23.45	23.46
An41	21.57	21.64
An56	22.61	22.59
An71	10.96	10.51
Ae1	17.96	17.46
Ae20	11.56	11.46
Ae25	15.86	15.61

Fig.1:



The isolates of fluorescent Pseudomonas were tested for their ability to antagonize the phytopathogen *Fusarium oxysporum* both in the iron deficient as well as iron supplemented media. The isolates exhibited antibiosis against *F. oxysporum* both in the iron deficient as well as iron supplemented conditions. Although, these 22 isolates exhibited antagonistic activity against F. *oxysporum* in both the iron supplemented and iron deficient conditions, differences were always found among the isolates with respect to the extent of antagonism against *F. oxysporum* (Fig 1). The isolate *P. fluorescens* Os25 showed a highest degree of antagonism (inhibition zone of 28.95 mm) which was followed by the isolate *P. fluorescens* So5 (inhibition zone of 26.46 mm) under iron deficient condition. The least degree of antagonism was found with isolate *P. aeruginosa* So17 (inhibition zone of 8.14 mm) under iron deficient condition (Table 1)

Under iron supplemented conditions, the antagonistic ability of all the isolates was reduced slightly. The strains showed antagonistic activity against *Fusarium oxysporum* with inhibition zones ranging between 27.69 mm and 7.16 mm. The isolate *P. fluorescens* Os25 exhibited higher level of antagonism (27.69 mm inhibition) and was followed by the isolates *P. fluorescens* So5 and

P. fluorescens Ah36 with inhibition zones 25.15 mm and 23.46 mm respectively under the iron supplemented conditions.

Similar observations were made by Rini and Sulochana (2007). They found that among the 56 *Pseudomonas fluorescens* isolates infecting tomato; P20 and P28 were the most inhibitory isolates against *F. oxysporum* with inhibition zones 11.25 and 10.75 mm respectively in King's B medium and 7.5 and 7.25 mm respectively in PDA. Hebbar *et al.,* (1992) indicated that the nutrient source or its concentration in the medium might affect the production of antifungal compounds which are responsible for the antagonistic activity of fluorescent pseudomonads in different media.

In vitro assessment in dual plate assay resulted in 8-10.8 mm inhibition zone after 3 days of inoculation of *P. fluorescens* isolate Pf4-99 against *Macrophomina phaseolina* which suggested the extracellular secretion of antifungals by this pseudomonad (Vinod kumar *et al.,* 2007).

Yogesh kumar *et al.,* (2005) reported four rhizospheric strains of *Pseudomonas fluorescens* (Pf), viz. Pf-102, Pf-103, Pf-110 and Pf-173 which were antagonistic against *Fusarium solani* f. sp. pisi (causal agent of root rot in pea) and the mode of inhibition of *F. solani* f. sp. pisi by Pf-102 and Pf-103 was fungistatic, while Pf-110 and Pf-173 were lytic in their action.

The isolates were tested for their antagonistic potential against the phytopathogen *Rhizoctonia bataticola* in iron-free and iron-supplemented medium. The isolates showed antagonistic activity against *R.bataticola* both in the iron-deficient and iron-supplemented conditions. Their antagonistic activity was slightly less in iron supplemented conditions.

In iron-deficient condition, strains *P. fluorescens* Os 25 and *P. fluorescens* So5 were the most inhibitory isolates against *R.bataticola* with inhibition zones 29.46 mm and 29.46 mm respectively. The isolate *P. aeruginosa* So17 exhibited least antagonism inhibition zone 9.86 mm.

Under iron-supplemented condition the strain *P. fluorescens* Os25 showed antagonistic activity against *R. bataticola* with inhibition zones ranging between 27.16 mm and 8.01 mm. The strain *P. fluorescens* So5 exhibited slightly higher antagonism with inhibition zone 27.16 mm, followed by strain *P. fluorescens* Os25 (inhibition zone 26.96 mm) and *P. fluorescens* Os32 (inhibition 23.00 mm). The data is represented in table 2.

Similar results were observed by Rini and Sulochana (2007). They reported that in tomato, *Pseudomonas fluorescens* isolates P28 and P51 exerted the maximum inhibitory effect against Rhizoctonia solani as evidenced by the widest inhibition zones of14.25 and 14 mm respectively for P28 and P51 in KMB and 7.5 mm for both in PDA.

When the fluorescent pseudomonads strains were tested for their in-vitro antagonistic activity against *R. solani* on King's B media with and without FeCl3, the results showed that the inhibition of test pathogen, *Rhizoctonia solani* by the strains of were in the range of 25 to 36% in the presence of FeCl3 whereas the inhibition ranged from 66 to 85% in the absence of $FeCl_3$ and the mechanism of siderophore mediated antibiosis was evidenced (Prasanna reddy *et al.,*2010).

Howell and Stipanovic (1979) reported that cotton seedling survival increased from 30% to 79% when planted into *R. solani*-infested soil by pre-plant treatment of seeds with the strain of *P. fluorescens* or pyrrolnitrin. A plant growth-promoting isolate of a fluorescent Pseudomonas spp. EM85 was found strongly antagonistic to *Rhizoctonia solani*, a causal agent of damping-off of cotton and the isolate produced HCN, siderophores, fluorescent pigments and antifungal antibiotics (Pal *et al.,* 2000).

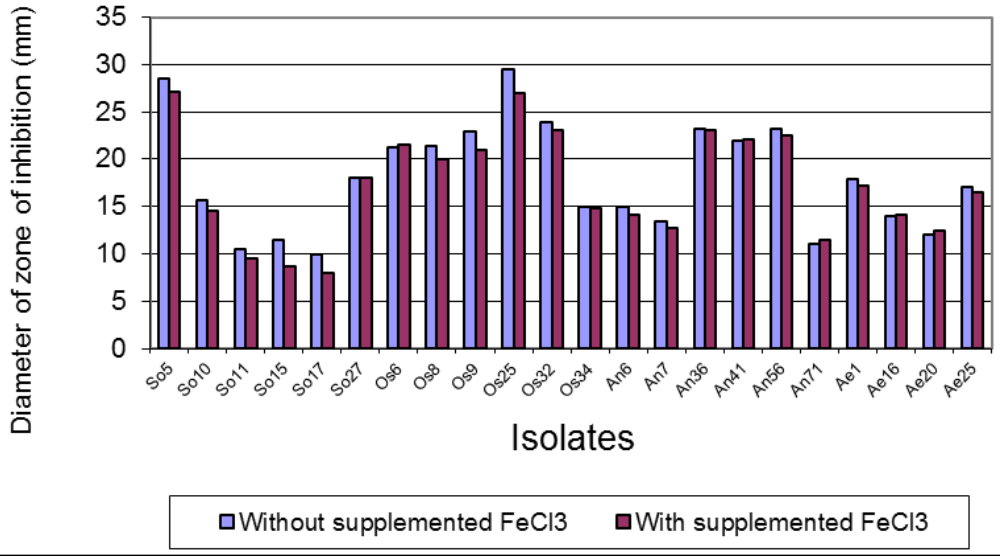
The results on biocontrol potential of the isolates against *F. oxysporum* and *R. bataticola* showed that isolates *P. fluorescens* Os25 and *P. fluorescens* So5 exhibited greater antagonistic activity against the pathogenic fungi. The isolates showed antagonistic activity against *F. oxysporum* and *R. bataticola* both in the iron-deficient and iron-supplemented conditions. Their antagonistic activity was slightly less in iron supplemented conditions (Fig. 1 and 2). The reported higher biocontrol potential of the isolates against *F. oxysporum* and *R. bataticola* in iron-deficient conditions is related to the siderophore production by the isolates in iron-deficient conditions and this explains the mechanism of siderophore mediated antibiosis.

Similarly, Dileep Kumar (1998) reported a reduction in antagonism when iron was included in the growth medium. Furthermore, fluorescent Pseudomonas strains had no antagonistic activity against *Pythium ultimum* in the presence of 100 µM FeCl3 (Gill and Warren, 1988). The results presented here clearly showed that iron has a role on inhibition of *F. oxysporum* and *R. bataticola* by fluorescent pseudomonads

Table.2:

Isolates	Diameter of zone of inhibition (mm)	
	Without supplemented $FeCl_3$	With supplemented $FeCl_3$
So5	28.54	27.16
So10	15.65	14.59
So11	10.51	9.46
So15	11.46	8.71
So17	9.86	8.01
So27	18.00	18.01
Os6	21.22	21.46
Os8	21.44	20.01
Os9	22.96	21.01
Os25	29.46	26.96
Os32	23.96	23.00
Os34	15.00	14.75
An6	14.96	14.15
An7	13.45	12.76
An36	23.15	23.00
An41	22.00	22.01
An56	23.15	22.46
An71	11.01	11.46
Ae1	17.95	17.23
Ae16	14.00	14.15
Ae20	12.00	12.40
Ae25	17.00	16.51

Fig.2: *In vitro* biocontrol potential of the isolates of fluorescent *Pseudomonas* against phytopahtogen, *Rhizoctonia bataticola*



Similar results were observed by Rini and Sulochana (2007). They reported that in tomato, *Pseudomonas fluorescens* isolates P28 and P51 exerted the maximum inhibitory effect against *Rhizoctonia solani* as evidenced by the widest inhibition zones of14.25 and 14 mm respectively for P28 and P51 in KMB and 7.5 mm for both in PDA.

Significant damage to plant growth, nodulation and yield of *Vigna mungo* due to pathogenic infection by *R.bataticola* even at low inoculation level indicate that the crop is a good host for the pathogen (Fazal *et al.,* 1998). When the fluorescent pseudomonads strains were tested for their in-vitro antagonistic activity against *R. solani* on King's B media with and without FeCl3, the results showed that the inhibition of test pathogen, *Rhizoctonia solani* by the strains of were in the range of 25 to 36% in the absence of FeCl3 whereas the inhibition ranged from 66 to 85% in presence of FeCl3 and the mechanism of siderophore mediated antibiosis was evidenced (Prasanna reddy *et al.,* 2010).

Howell and Stipanovic (1979) reported that cotton seedling survival increased from 30% to 79% when planted into *R. solani*-infested soil by pre-plant treatment of seeds with the strain of *P. fluorescens* or pyrrolnitrin. A plant growth-promoting isolate of a fluorescent Pseudomonas spp. EM85 was found strongly antagonistic to *Rhizoctonia solani*, a causal agent of damping-off of cotton and the isolate

produced HCN, siderophores, fluorescent pigments and antifungal antibiotics (Pal *et al.,* 2000).

P. fluorescens strain C7R12 is a spontaneous rifampicin-resistant mutant of a wild-type strain C7 previously isolated from the rhizosphere of flax cultivated in a soil that suppresses fusarium wilts caused by nonpathogenic *Fusarium oxysporum* (Lemanceau *et al.,* 1988).

The results on biocontrol potential of the isolates against *F. oxysporum* and *R. bataticola* showed that isolates isolate P. fluorescens Os25 and So5 exhibited greater antagonistic activity against the pathogenic fungi. The antagonistic activity of most of the isolates was more or less similar both under iron-deficient and iron-supplemented conditions. These results are in agreement with that of Dileep Kumar and Bezbaruah (1997) who reported the antibiotic potential of fluorescent Pseudomonas strain RRLJ 181 against *F. oxysporum, F. udam, F. solani, F. monoliformae, F. semitectum, Fomes lamoensis* and *Ustulina zonata.* Similar fluorescent pseudomonads-induced inhibition of *Macrophomina phaseolina* (Bhatia *et al.,* 2003), *Sclerotium rolfsii* and *S. sclerotiorum* and *F. oxysporum* (Cattelan *et al.,* 1999; Tripathi and Johri, 2002) have been found. These reports indicate that iron in growth medium has little effect on inhibition of pathogenic fungi.

However, Dileep Kumar (1998) reported a reduction in antagonism when iron was included in the growth medium. Furthermore, fluorescent Pseudomonas strains had no antagonistic activity against *Pythium ultimum* in the presence of 100 µM FeCl3 (Gill and Warren, 1988). The results presented here clearly showed that iron has no role on inhibition of *F. oxysporum* and *R. bataticola* by fluorescent Pseudomonas.

Determination of Antifungal Compound:

Antifungal activity was observed against *F. oxysporum,* and *R. bataticola*. In this report, major secondary metabolites of newly isolated strain, Os25 of *P. fluorescens* were characterized by TLC, infra-red, UV and mass spectroscopy. The compounds were identified as phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine (2-OH-PHZ).

Effect of antifungal compound on fungal biomass:

The active compound, PCA from the isolate, *P. fluorescens* Os25 was tested for its ability to inhibit the growth of the phytopathogens, *Fusarium oxysporum* and *Rhizoctonia bataticola* and biomass production of *F. oxysporum* and *R. bataticola*. PCA inhibited the growth of the phytopathogens and progressively decreased the biomass production of the phytopathogens with increasing concentrations in growth medium. The results are depicted in Fig. 3 and 4.

The antibiotic metabolite, PCA of 25 µg ml-1 concentration exhibited 15% inhibition and PCA of 100µg ml-1 exhibited 37% inhibition against *F. oxysporum*. Similar results were also observed with *R. bataticola*. PCA of 25µg ml-1, 50µg ml-1 and 100 µg ml-1 concentrations exhibited 20%, 29% and 40% inhibitions respectively against *R. bataticola*.

As the concentration of crude antibiotic increased, the biomass of phytopathogens decreased. The antifungal metabolite, PCA of 25 µg ml-1, 50 µg ml-1, 100 µg ml-1 concentrations, decreased the biomass of *F. oxysporum* from 600 mg to 520 mg, 480 mg and 390 mg respectively. Similar results were also observed with *R. bataticola*. While initially, the biomass of *R. bataticola* was 560 mg, it decreased to 450 mg at 25 µg ml-1 concentration of PCA and the biomass further decreased to 390 mg at 50 µg ml-1 concentration. The biomass of *R. bataticola* was reduced to 320 mg at 100 µg ml-1 concentration of PCA.

Fig.3: Effect of antifungal compound on biomass production of *Fusarium oxysporum*

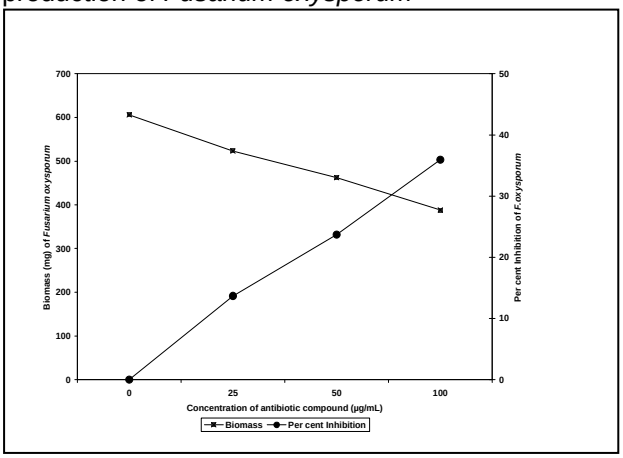
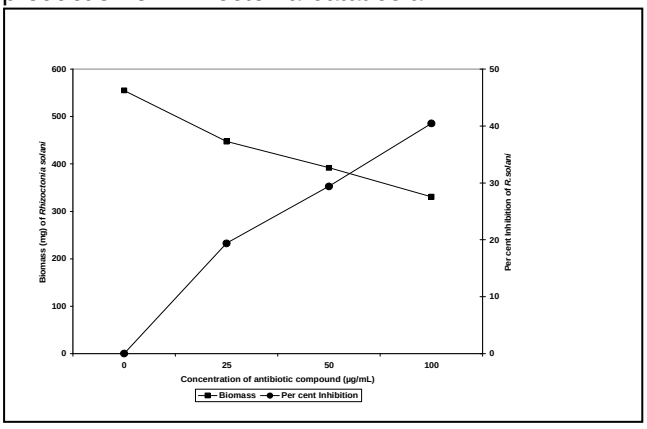


Fig.4: Effect of antifungal compound on biomass production of *Rhizoctonia bataticola*



All of these data suggest that isolate P. fluorescens Os25 could be used as a potential effective biocontrol agent and a biofertilizer, to decrease the

incidence of plant diseases and promote the plant growth.

Similar results were reported by Goud and Muralikrishnan (2009) in the antibiotic assay of fluorescent pseudomonads on fungal biomass. They added crude antifungal compound of concentrations 50mcg/ml, 100mcg/ml and 150mcg/ml in methanol to 4mm *Pythium ultimum* fungal mat and found that the dry weight of test fungal mat reduced from 0.40mg to 0.31mg, 0.21 mg and 0.10 mg respectively.

Several reports have shown that the phenazine-producing *Pseudomonas fluorescens* strains were able to suppress the plant pathogens both *In vitro* and in vivo (Chin-A-Woeng *et al.,* 1998; Timms-Wilson *et al.,* 2000). Mahajan-Miklos *et al.,* (1999) reported that Phenazines, a large class of low molecular weight compounds produced by a variety of pseudomonads, are known to cause fast killing of *Caenorhabditis elegans*, a common soil inhabiting nematode.

The broad-spectrum activity exhibited by Phenazine compounds against fungi and other bacteria is not well understood. However, it is believed by Deepti. Dwivedi and Johri, (2003) that Phenazines can accept electrons, yielding a relatively stable anion radical that readily undergoes redox cycle. It includes biosynthesis of Mn-containing superoxide dismutase (MnSOD) which causes enhanced production of $O2^-$ (superoxide radical).

Ran *et al.,*(2003) reported that the phenazines, which are analogues of flavin coenzymes, inhibit electron transport and have various pharmacological effects on animal cell. Britigan (1992) observed that in the presence of ferripyochelin, phenazines catalyse the formation of hydroxyl radicals, which damage lipids and other macromolecules. Interestingly, reduced phenazine-1-carboxamide can release soluble Fe2+ ions from insoluble Fe (OH) 3 at neutral pH, which raises the possibility that phenazines might contribute to iron mobilization in soils (Hernandez *et al.,* 2004).

The foregoing discussion clearly indicates the involvement of phenazine antibiotic in biological control of plant pathogens.

Fluorescent pseudomonads produce highly potent broad spectrum antifungal molecules against various phytopathogens, thus acting as effective bio control agents. They could serve as promising bio-inoculants for agricultural system to increase productivity since the action of such bacteria is highly specific, ecofriendly and cost-effective. The use of bio-inoculants based on fluorescent pseudomonads appears as a worthwhile approach for exploring disease management.

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