academicJournals

Vol. 7(39), pp. 4689-4700, 27 September, 2013
DOI: 10.5897/AJMR2013.5809
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Siderophore mediated antagonism of fluorescent Pseudomonads against soil borne plant pathogenic fungi in West Bengal, India

A. Adhikari[1,2#], K. Sarker[2,3*#], M. De Roy[4], I. Bhattacharya[2], T. Mandal[1], P. K. Dasmohapatra[3] and S. Dutta[2*]

[1]Department of Biotechnology, O. I. S. T.,Vidyasagar University, Midnapore, India.
[2]Department of Plant Pathology, BCKV, Mohanpur, Nadia, India.
[3]Department of Microbiology, Vidyasagar University, Midnapore, India.
[4]ADA (Polba block), Hoogly, W. B.; Present add: STL, PORS, Berhampore, West Bengal, India.

Accepted 8 September, 2013

The rhizobacterial isolates from different agro ecological region of West Bengal, used in this study, were identified as fluorescent pseudomonads based on biochemical and molecular characterization. Six effective antagonist isolates were selected for further studies of secondary metabolite (siderophore) production with respect to bacterial growth at different time intervals (6 to 96 h), the nature of siderophore production (hydroxamate and/or catecholate type), effect of iron supplementation at its different concentration on siderophore production and antagonistic potentiality. Observations of higher level of growth and siderophore production, for most of the isolates, were recorded after 72 h of incubation. The maximum zone of inhibition and siderophore production was recorded at 0.5 µM and the least at 10 µM iron concentration. With the gradual increase in iron supplementation in king's B medium, the siderophore production as well as *in vitro* antagonistic potentiality decreased in terms of enhancement of diameter of fungal mycelium and reduction of percent inhibition of pathogen by fluorescent pseudomonads in dual culture assay method.

Key words: Fluorescent pseudomonads, siderophore, ferric ion, *Sclerotium rolfsii, Rhizoctonia solani*.

INTRODUCTION

Many genera of plant root-inhabiting bacteria have potential as biological control agents (Weller, 1988). The genus *Pseudomonas* belongs to the γ-subclass of the Proteobacteria and includes mostly fluorescent Pseudomonads as well as a few non-fluorescent species. Particularly fluorescent *Pseudomonas* spp. has received much attention because of their suggested involvement in natural disease suppressiveness of certain soils (Weller et al., 2002). Iron-regulated metabolites are mainly referred to as siderophores. The siderophores are high-affinity iron chelators in bacteria, enabling uptake of Fe (Braun, 2001). Iron is essential for the growth of almost all organisms but, in many environments, the amount of free iron is below 10^{-7} M, which is the required concentration by most bacteria for growth (Ratledge and Dover, 2000). Bacteria have developed several strategies for the acquisition, solubilization and transportation of iron (Guérinot, 1994). The most efficient mechanism of iron acquisition is the secretion of high affinity iron-chelating compounds, so-called siderophores. The siderophores chelate iron in the extracellular environment and the resulting ferric siderophore complex is recognized by

*Corresponding authors. E-mail: subrata_mithu@yahoo.co.in, kanish.sarker@gmail.com.

#Authors contributed equally to this work.

Table 1. Origins and different crop rhizospheres of the isolates.

Fluorescent pseudomonad isolates	Location	Region	Crop rhizosphere
MFP9	Coochbehar	Terai	Maize
CHFP23	Nadia	Gangetic alluvial	Chilli
TFP16	West Midnapore	Lateritic	Tomato
FPK7	Bagdaha, North 24Pgs	Gangetic alluvial	Pointed gourd
FPK8	Bagdaha, North 24Pgs	Gangetic alluvial	Pointed gourd
FPK10	Habra, North 24 Pgs	Gangetic alluvial	Cucumber

siderophore-specific membrane receptors, enabling uptake of Fe by the bacterial cells (Braun, 2001). Many bacteria produce more than one type of siderophore, as shown for *Enterobacter cloacae*, *Mycobacterium smegmatis* and *Pseudomonas* spp. (Adilakshmi et al., 2000; Mercado-Blanco et al., 2001). There are several reports where the effective role of siderophore on antagonistic potentiality under *in vitro* and *in vivo* cinditions, has clearly been discussed (Pal and Gokarn, 2010; Adhikari et al., 2013; Subashri et al., 2013). Although considerable structural variation exists among the several dozen siderophores chemically characterized at the present time, most can be classified as hydroxamates or catechols (Neiland, 1981). The present study on siderophore producing fluorescent pseudomonads was concentrated on the screening for their siderophoregenesis, siderotyping and siderophore mediated antibiosis under *in-vitro* conditions against pathogenic fungi with different concentration of iron supplement.

MATERIALS AND METHODS

Isolation of bacteria

Soil samples were collected from different crop rhizospheres from different agro-ecological region of West Bengal (Table 1). Roots were shaken vigorously to remove loosely adhering rhizospheric soil. To isolate rhizospheric bacteria, 10 g of rhizospheric soil were shaken in 90 ml sterile phosphate buffer saline (PBS) and were four fold diluted and plated on King's B (KB) medium. A total of 100 bacterial isolates were isolated and fluorescent colonies, screened through UV light (k = 365 nm), with different morphological characteristics were subcultured by repeated streaking on KB medium. On the basis of siderophore production property five highly and one moderately siderophore producing isolates were selected for further studies.

Characterization of siderophore producing bacteria

Bacterial identification was done by biochemical analysis on the basis of the tests of oxidase, arginine dihydrolase, denitrificacation, gelatine hydrolysis, levan production, and acid production from trehalose, utilization of tryptophane and L-tartrate coupled with amplification of the *Pseudomonas* genus specific 618 bp fragment of 16S rDNA genes by PCR using the primers PA-GS-F and PA-GS-R corresponding to the 5' (5'-GACGGGTGAGTAATGCCTA-3') end and 3' end (5'-CACTGGTGTTCCTTCCTATA-3') of the 16S rDNA gene (Spilker et al., 2004). Amplification was confirmed by analyzing 5uL of each PCR reaction mixture on a 1% agarose gel (Sambrook et al., 1989).

Screening of Siderophore producing bacteria

Qualitative assay

Siderophore production by all the isolates was tested qualitatively by Chrome Azural S (CAS) plate assay (Schwyn and Neilands, 1987). The isolates were spotted over cetrimide agar and incubated for 48 h at 28°C. After incubation, a thin layer of CAS reagent in 0.7% agar was spread on the bacterial growth and plates were again incubated for 24 h at 28°C, formation of yellow orange color zone around the colonies in plate assay indicated the siderophore production.

Quantitative assay

All the isolates were grown at 28±2°C on a rotary shaker in King's B broth for three days and centrifuged at 10,000 rpm for 10 min and the supernatant was collected. The pH of the supernatant was adjusted to 2.0 with 1 N HCl and equal quantity of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. 5 mL of ethyl acetate fraction was mixed with 5 mL of Hathway's reagent (The reagent was prepared by adding 1 mL of 0.1 M ferric chloride in 0.1 N HCl to 100 mL of distilled water, and to this 1 mL of 0.1 M potassium ferricyanide was added). The absorbance for dihydroxy phenols was read at 700 nm in a Spectrophotometer (Reeves et al*.,* 1983). A standard curve was prepared using different concentration of dihydroxy benzoic acid at 700 nm. The quantity of siderophore synthesized was expressed as µ mol benzoic acid mL^{-1} of culture filtrate.

Siderophore typing

Hydroxamate type of siderophore

Csaky's test: Hydroxamate type of siderophore was determined by hydrolyzing 1 ml supernatant of overnight grown culture with 1 ml of 6 N H_2SO_4 in boiling water bath for 6 h or 130°C for 30 min. Further, this hydrolyzed sample was buffered by adding 3 ml of sodium acetate solution. To this, 0.5 ml iodine was added and allowed to react for 3-5 min. After completion of reaction, the excess iodine was destroyed with 1 ml of sodium arsenate solution. Finally 1 ml of α - Naphthylamine solution was added and allowed to develop the colour. Wine red colour formation indicates production of hydroxamate type of siderophore (Gillan et al., 1981).

Neilands spectrophotometric assay

The hydroxamate nature of siderophore was detected by Neilands spectrophotometric assay (Jalal and Vander Helm, 1990; (Neilands, 1981) where a peak between 420-450 nm on addition of 2% aqueous solution of $FeCl_3$ to 1 mL of supernatant indicated presence of Ferrate hydroxamate.

Catecholate type of siderophore

Arnow's Test

To 1 ml of cell-free supernatant, 1 ml of nitrite-molybdate reagent with 1 ml NaoH solution was added. Finally, 1 ml of 0.5 N HCl was added and allowed to develop colour. Yellow colour formation indicates production of catecholate type siderophore (Arnow, 1937).

Spectrophotometric assay

Catecholate nature of siderophore was detected by the method of Jalal and Vander Helm (1990) using spectrophotometric assay where a peak at 495 nm on addition of 2% aqueous solution of $FeCl_3$ to 1 mL of supernatant indicated the presence of siderophores of catecholate nature.

Temporal variation of bacterial growth and siderophore production

All the six fluorescent pseudomonads were grown in king's B medium by submerged culture method in 50 mL conical flasks and incubated at 28±2°C on a rotary shaker at 150 rpm. Samples were withdrawn in duplicate at definite time intervals from 6 h up to 96 h. One set was used for growth measurement at 600 nm and the other for estimation of siderophore at 700 nm (Reeves et al., 1983).

Influence of iron on production of siderophore

In order to determine the concentration of iron at which siderophore biosynthesis is minimized in fluorescent pseudomonads under study, all the cultures were grown in king's B media, externally supplemented with different concentrations of iron ($FeCl_3$. $6H_2O$) viz., 0.5, 1, 1.5, 2.5, 5 and 10 µM. Following the incubation at 28±2°C for 72 h at 120 rpm, siderophore contents were estimated at 700 nm. To remove traces of iron, glasswares were cleaned with 6 M HCl and with double distilled water.

In vitro antagonistic effect on mycelial growth of phytopathogen under different iron limiting conditions

An agar plug (7 mm diameter) taken from actively growing fungal culture such as *Rhizoctonia solani* and *Sclerotium rolfsii* were placed on the surface of the plate-enhancing medium supplemented with $FeCl_3$ at different concentration that is 0.5, 1, 1.5, 2.5, 5, 10 µM. Simultaneously, fluorescent pseudomonas isolates were streaked 3 cm away from the agar plug at both sides towards the edge of Petri plates (Kumar et al*.,* 2000). Plates inoculated with fungal agar plugs alone were used as control. The plates were incubated at 28±2°C until fungal mycelia completely covered the agar surface in control plate. Observations on mycelial growth of test pathogens were recorded and percent inhibition of pathogen growth were calculated:

$$\%I = 100(C-T)/C$$

Where, I = inhibition of mycelial growth, C = growth of pathogen in the control plate (cm) and T = growth of pathogen in dual cultures (cm).

Statistical analysis

All the isolates were clustered with respect to their siderophoregenesis at different ferric ion concentration, zone of inhibition, inhibition percentage, temporal growth measurement and siderophore production at different time intervals by critical difference at 5% probability level using DMRT by SPSS v.10.

RESULTS AND DISCUSSION

Characterization of siderophore producing bacteria

Biochemical characterization

Among the bacterial isolates, five highly and one moderately siderophore producing isolates were confirmed as fluorescent pseudomonads based on biochemical tests such as gram reaction, oxidase, arginine dihydrolase, denitrificacation, gelatine hydrolysis, levan production, and acid production from trehalose, utilization of tryptophane and L-tartrate. Microscopic observation showed that all the six isolates were rod shaped, motile, gram negative, and fluorescent in the presence of UV light (Rao et al*.,* 1999; Srivastava et al*.,* 1999). All the isolates exhibited positive reaction in catalase, oxidase, arginine di hydrolase and negative in TABAC hypersensitivity test (Bossis et al*.,* 2000). All the five isolates except FPK 10 were positive in gelatin liquefaction test; isolates MFP9 and FPK7 showed positive in trehalose test; MFP9 was positive in levan production and FPK7, FPK8 showed denitrification activity; whereas, the isolate TFP16 exhibited positive reaction in all the cases except TABAC hypersensitivity (Table 2).

Molecular characterization

PCR assays employing genus specific primer pair PA-GS-F/PA-GS-R produced 618 bp DNA fragments of the predicted size for all the six isolates (Figure 1). With the presence of the 618 bp amplicon the six putative siderophore producing isolates were confirmed as *Pseudomonas sp*. (Spilker et al*.,* 2004). All the isolates produced diffusible fluorescent pigment which was confirmed in the presence of UV light, which indicated that all the isolates belonged to fluorescent pseudo-monads.

Qualitative assay for siderophore production

All the six isolates were subjected to screening for side-

Table 2. Different biochemical tests performed to identify the isolates.

Fluorescent pseudomonad isolates	Fluorescence	Arginine	Oxidase	catalase	Tabac	Gelatine	Trehalose	Levan	Denitrification	L-ara	L(+)tart
MFP9	+	+	+	+	-	+	+	+	-	NA	NA
CHFP23	+	+	+	+	-	+	-	-	-	NA	NA
TFP16	+	+	+	+	-	+	+	+	+	+	+
FPK7	+	+	+	+	-	+	+	-	+	NA	NA
FPK8	+	+	+	+	-	+	-	-	+	NA	NA
FPK10	+	+	+	+	-	-	-	NA	NA	NA	NA

'+' indicates positive; '-' indicates negative; 'NA' indicates not applicable; 'L-ara' indicates L- arabinose; 'L (+) tart' indicates L-tartarate.

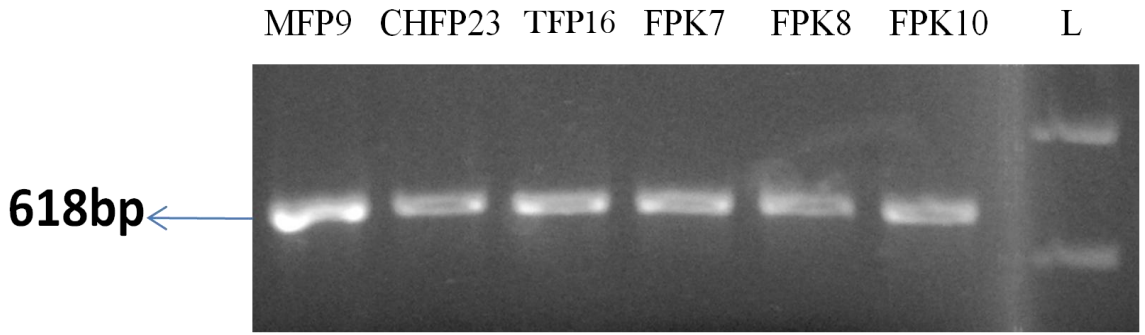

Figure 1. Agarose gel electrophoresis of PCR amplified genus specific 618 bp 16S rDNA amplicons; Lanes are named with respective isolates; L: 100 bp molecular marker.

rophore production. Isolates CHFP23, FPK8, FPK10 produced a strong reaction with the CAS reagent (Table 3). After incubating for a period of 24 h in the dark at 28°C, a change in color from blue to orange was observed in the agar plate (Figure 2). The color change from blue to orange resulted by siderophoretic removal of Fe from the dye.

Quantitative assay for siderophore production

All the six isolates were subjected to screening for siderophore production. Three isolates, viz., FPK8, FPK10, CHFP23, were found to be highly siderophore producer such as 15.20 (± 0.33), 13.9 (± 0.30) and 11.55 (± 0.21), µM/ml respectively, whereas, moderately siderophore production was obtained from the isolates TFP16 and FPK7 and the least siderophore production was observed in MFP9 (Table 4).

The siderophores have very high affinity for ferric iron and form ferric-siderophores complex making it unavailable to other organisms but the producer organism can utilize these complexes via a specific receptor in their outer cell membrane (Buyer and Leong, 1986). Fluorescent pseudomonads produce several siderophores such as pyoverdine, pyochelin (Dave and Dube, 2000) and are important to agriculture because they can reduce crop yield losses caused by bacteria and fungi in the root environment. The protective activity is related to the production and excretion of siderophores which efficiently chelate the iron in the root environment. This iron deficiency leads to an impaired growth of the deleterious micro-organisms.

Table 3. Sideropore production under *in vitro* condition by six different fluorescent pseudomonads.

Fluorescent pseudomonad isolates	Siderophore production in CAS agar plate assay
MFP9	+
CHFP23	+++
TFP16	++
FPK7	++
FPK8	+++
FPK10	+++

'+' indicates weak producer; '++' indicates medium producer and '+++' indicates good producer.

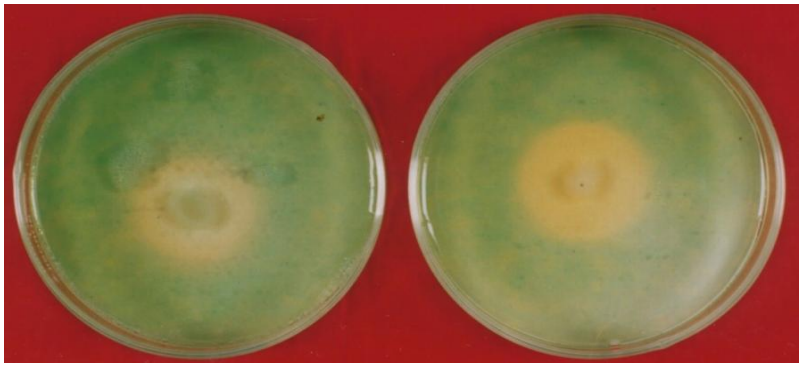

Figure 2. Siderophore production by bacterial isolates in CAS plate assay.

Table 4. Sideropore production under *in vitro* condition by six different fluorescent pseudomonads.

Fluorescent pseudomonad isolates	Siderophore production (µM benzoic acid/mL) Mean ± SD
MFP9	2.04 ± 0.13
CHFP23	11.55 ± 0.21
TFP16	9.87 ± 0.25
FPK7	6.98 ± 0.23
FPK8	15.20 ± 0.33
FPK10	13.9 ± 0.30

Average mean ± SD of three replicates, $P < 0.05$.

Assay for siderophore typing

The type of siderophore was determined by Csaky and Arnow assay, where isolates TFP16 and FPK8 has shown hydroxamate type of siderophore production that is formation of wine red colour in supernatant. Further spectrophotometric analysis of the culture, TFP16 and FPK8 in King's B medium showed a sharp peak between 420 to 450 nm (Figure 3a) and clearly indicated the presence of siderophores of ferrate hydroxamate nature.

Catecholate type of siderophore was determined by Arnow's test where the formation of yellow colour was observed in the supernatant of the isolates MFP9, CHFP23, FPK7, FPK10. A peak seen between 490 to 505 nm confirmed the presence of siderophores of catecholate nature (Figure 3b and 3c) (Ali and Vidhale, 2011). Evidence for the production of catecholate or hydroxamate siderophores by different bacterial species has been shown previously (Hohlneicher et al., 1995; Howard et al., 2000; Gull and Hafeez, 2012).

Siderophores usually form a stable, hexadentate, octahedral complex with Fe^{3+} preferentially compared to other naturally occurring abundant metal ions. Siderophores are usually classified by the ligands used to

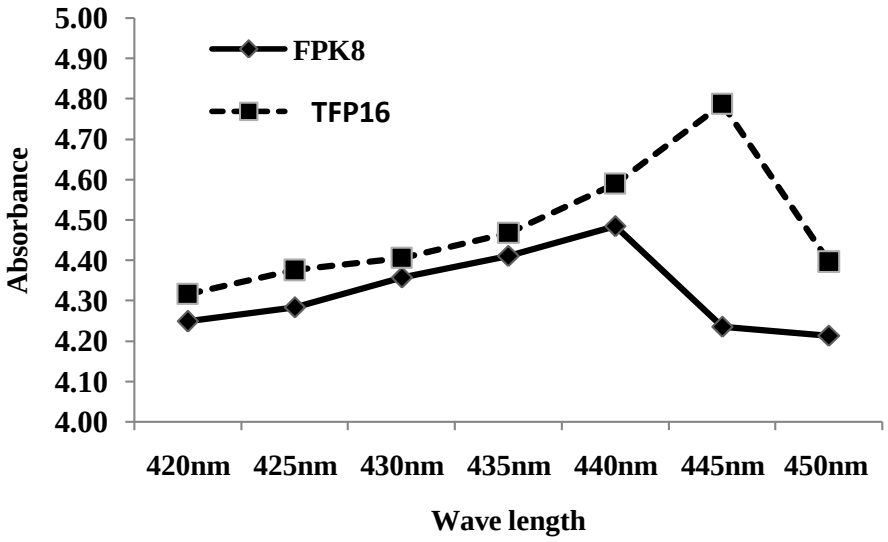

Figure 3a. Absorption characteristics FPK8 and TFP16 extract confirming hydroxamate nature of siderophores. Scanning of absorption characteristics were between 400-550 nm; a sharp peak between 420 and 450 nm confirmed hydroxymate nature of siderophore of FPK8 and TFP16.

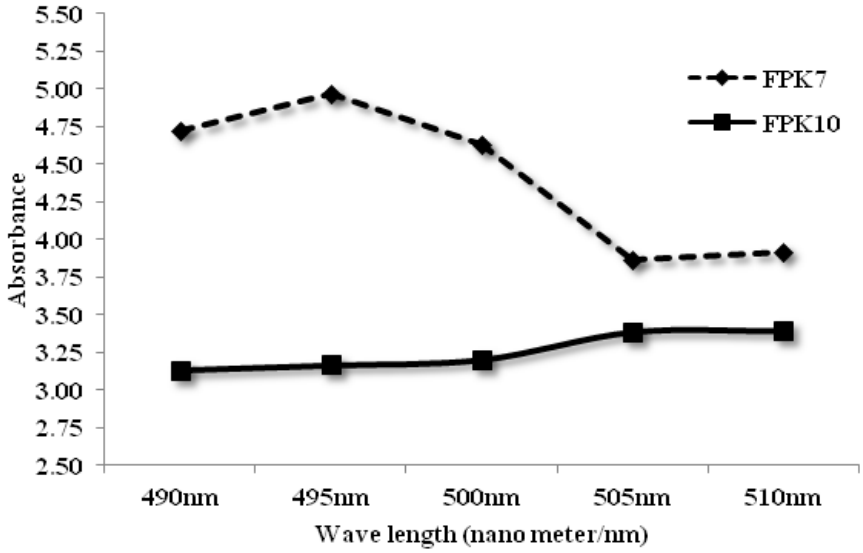

Figure 3b. Absorption characteristics FPK7 and FPK10 extract confirming catecholate nature of siderophores. Scanning of absorption characteristics were between 400-550 nm; a sharp peak between 490 and 510 nm confirmed catecholate nature of siderophore of FPK7 and FPK10.

chelate the ferric iron. The majors groups of siderophores include the catecholates (phenolates), hydroxamates and carboxylates (ederivatives of citric acid) (Hofte, 1993).

Temporal variation of bacterial growth and siderophoregenesis

In the growth and siderophore production, as depicted in Figure 4a to 4f, an exponential phase from 6 to 48 h was observed regarding the isolates MFP9, CHFP23, TFP16 and FPK8; stationary phase was initiated after 48 h. In the case of the isolates FPK7 and FPK10, growth was ceased after 72 h. Threshold level of siderophore production was observed after 12 h of incubation. Syeed et al. (2005) also found similar result that siderophore production started after 12 h of incubation, while working

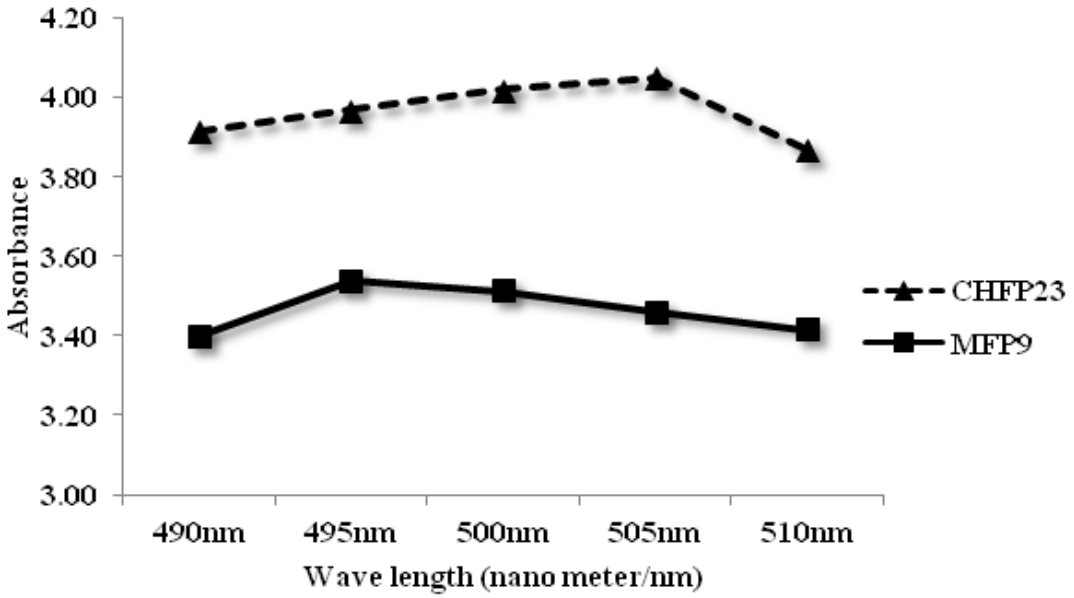

Figure 3c. Absorption characteristics MFP9 and CHFP23 extract confirming catecholate nature of siderophores. Scanning of absorption characteristics were between 400-550 nm; a sharp peak between 490 and 510 nm confirmed catecholate nature of siderophore of MFP9 and CHFP23.

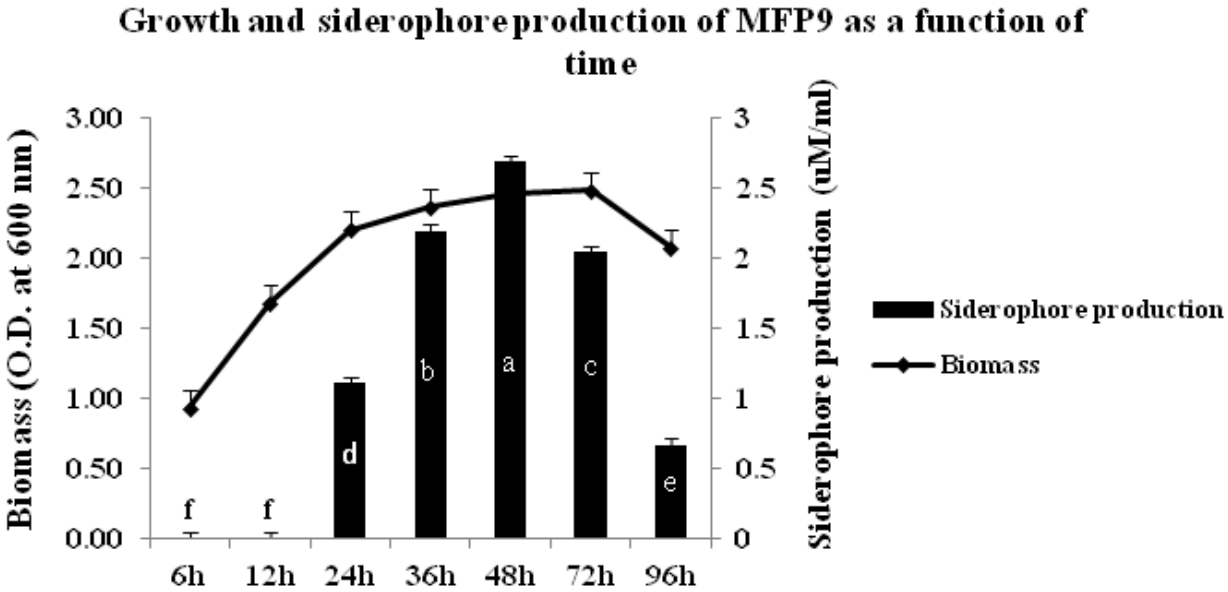

Figure 4a. Temporal growth measurement and siderophore production of MFP9 as a function of time. Vertical bar shows critical difference values.Values are mean of three replications. A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

with two fluorescent pseudomonads. For all the five isolates except MFP9, production of siderophore was increased up to 72 h and declined thereafter, for MFP9 it was after 48 h. Thus, maximum siderophore yield was observed at the end of log phase growth (Sharma and Johri, 2003).

Influence of iron on siderophoregenesis

In order to assess the influence of iron concentration on siderophore release of six different isolates, King's B media supplemented with different concentration of ferric ion was used. After 72 h of incubation, maximum side-

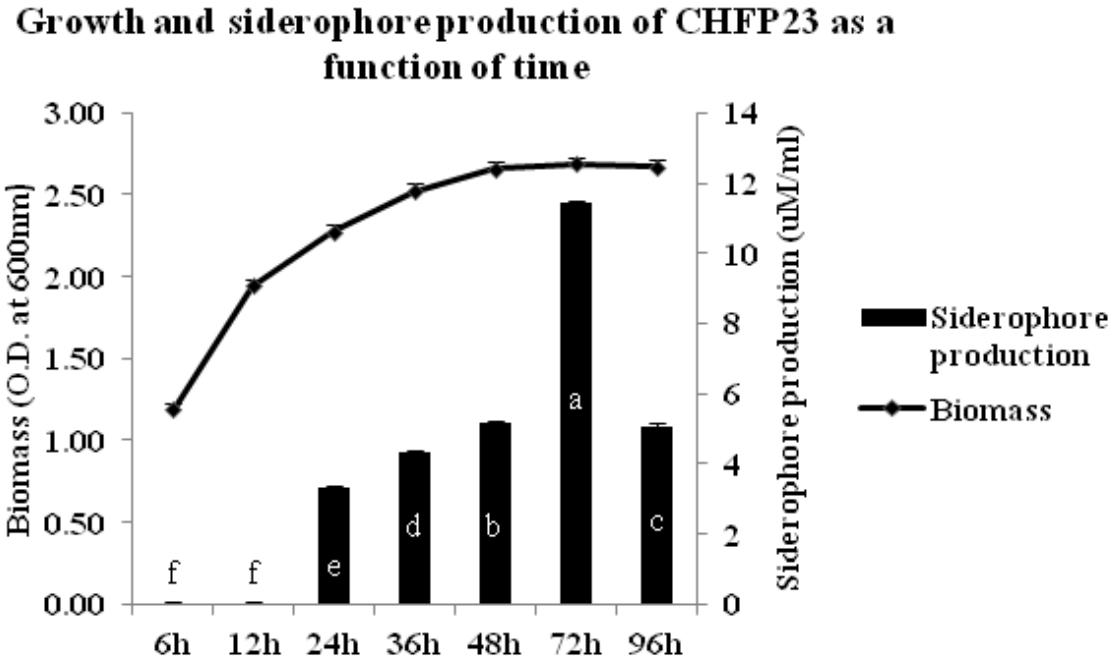

Figure 4b. Temporal growth measurement and siderophore production of CHFP23 as a function of time. Vertical bar shows critical difference values. Values are mean of three replications .A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

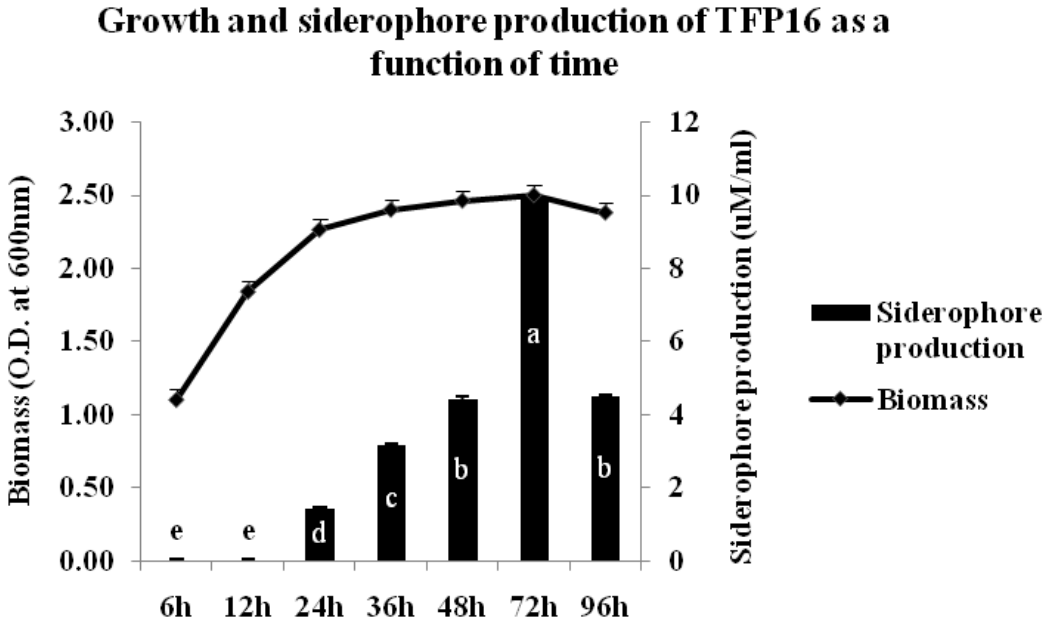

Figure 4c. Temporal growth measurement and siderophore production of TFP16 as a function of time. Vertical bar shows critical difference values. Values are mean of three replications .A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

rophore productions for all the isolates under study were obtainable at 0.5 µM Fe^{3+} concentration and the lowest siderophore level was recorded at 10.0 µM Fe^{3+} (Table 5). Siderophores are iron-specific compounds which are synthesized and secreted under iron stress condition and for each isolate under study siderophore production was

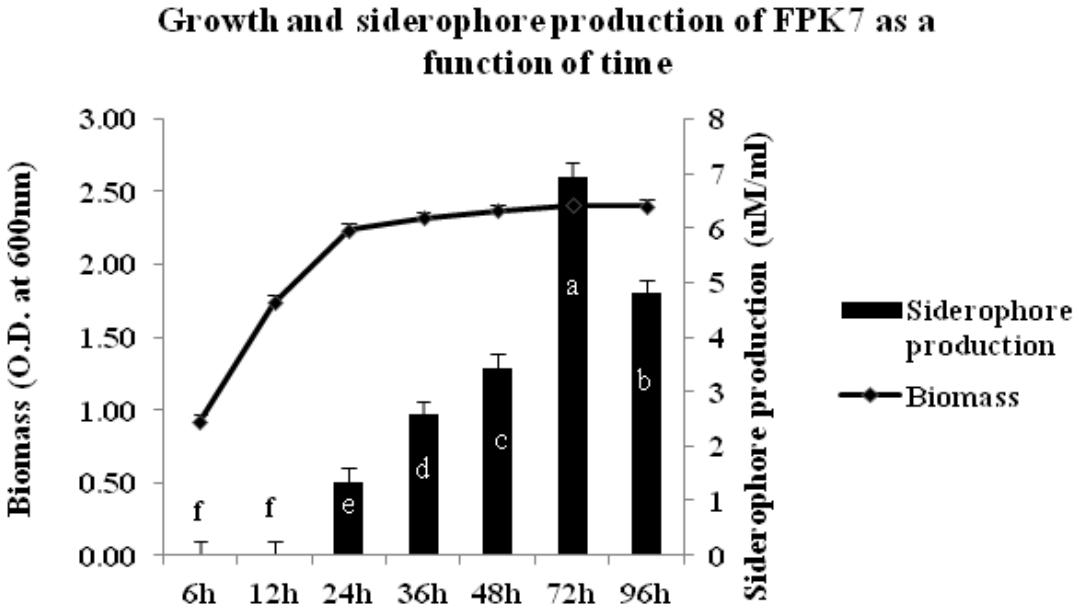

Figure 4d. Temporal growth measurement and siderophore production of FPK7 as a function of time. Vertical bar shows critical difference values. Values are mean of three replications .A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

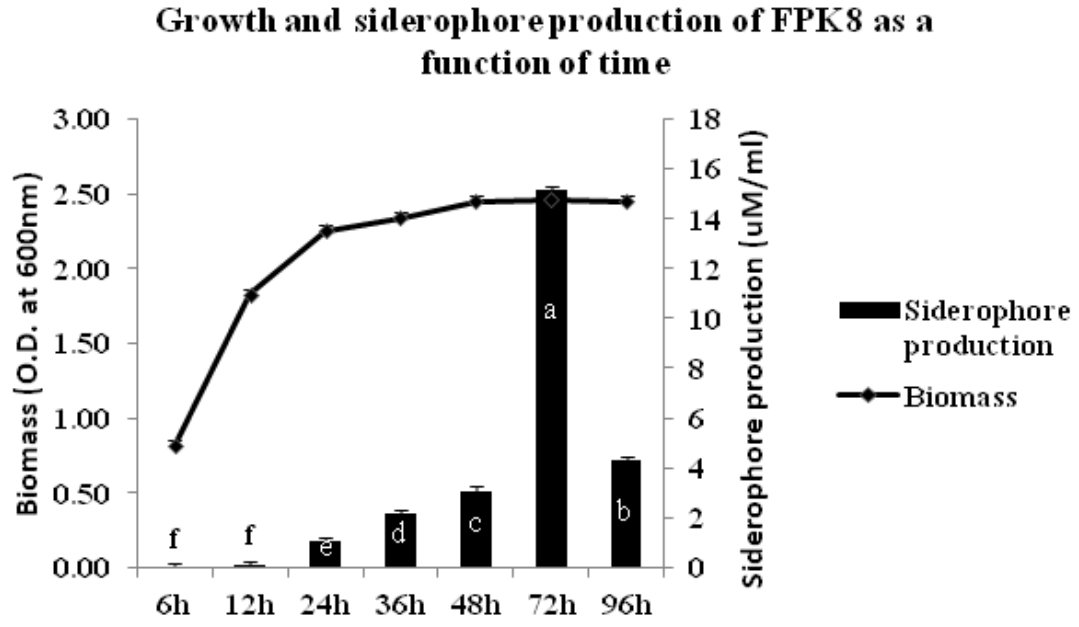

Figure 4e. Temporal growth measurement and siderophore production of FPK8 as a function of time. Vertical bar shows critical difference values. Values are mean of three replications .A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

gradually repressed with the increasing concentration of iron (0.5, 1, 1.5, 2.5, 5, 10µM $FeCl_3$) (Budzikiewicz, 1993; Rachid and Ahmed, 2005; Sayyed et al., 2005).

The universal repressor 'Fur' acts together with iron under iron rich conditions. When the intra-cellular environment is iron rich, the Fe^{2+}-Fur complex bind to promoter containing a 'Fur box' and represses transcription of the genes involving in iron uptake. Moreover,

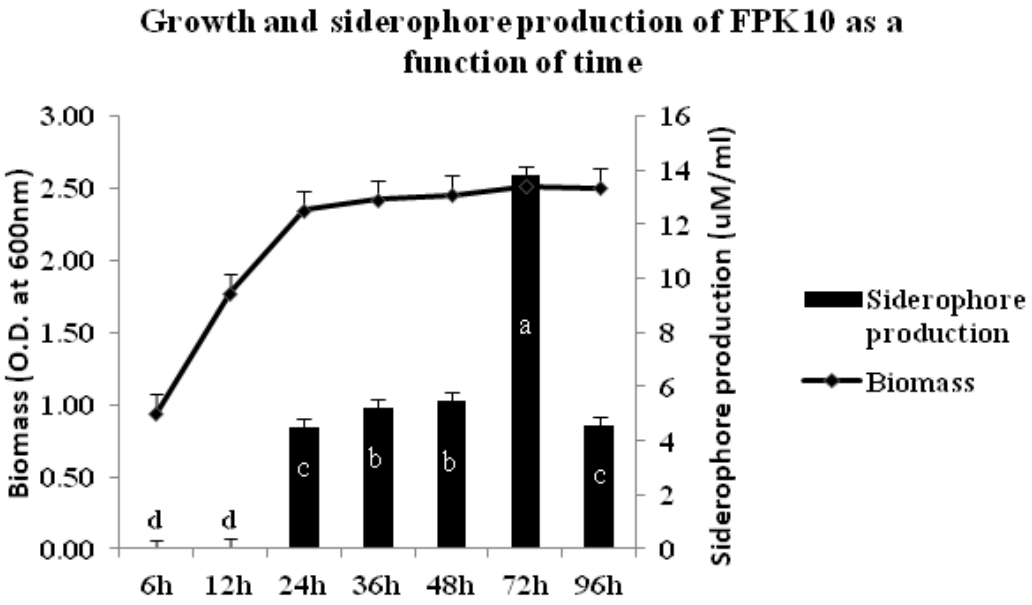

Figure 4f. Temporal growth measurement and siderophore production of FPK10 as a function of time. Vertical bar shows critical difference values.Values are mean of three replications .A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

Table 5. Influence of different ferric ion concentration on siderophoregenesis (µM benzoic acid/mL).

Fluorescent pseudomonad isolates	Siderophoregenesis (µM benzoic acid/mL)				
	0.5 µM $FeCl_3$	1.5 µM $FeCl_3$	2.5 µM $FeCl_3$	5 µM $FeCl_3$	10 µM $FeCl_3$
MFP9	0.53^{e}	0.20^{c}	0.00^{e}	0.00^{e}	0.00^{e}
CHFP23	6.81^{b}	5.92^{a}	5.71^{a}	4.48^{a}	2.62^{a}
TFP16	5.84^{c}	5.62^{a}	5.16^{b}	3.04^{c}	1.95^{b}
FPK7	4.76^{d}	4.49^{b}	3.73^{d}	3.02^{c}	1.60^{c}
FPK8	11.33^{a}	4.46^{b}	4.25^{c}	3.53^{b}	2.45^{a}
FPK10	5.18^{cd}	4.46^{b}	3.53^{d}	2.17^{d}	1.15^{d}
SEM (±)	0.216	0.198	0.104	0.115	0.080
CD (p = 0.05)	0.683	0.624	0.329	0.364	0.255

Values are mean of three replications. A common letter (on the superscript of each value) means they are not significantly different (p= 0.05) by DMRT.

there are several reports of *Pseudomonas aeruginosa* and *Vibrio anguillarum* regarding positive regulatory mechanisms of siderophore producing genes and iron uptake systems by a combination of positive regulatory proteins and cognate siderophore but the details are under study (Chen et al., 1996; Crosa, 1997; Heinrichs and Poole, 1996; Brandel et al*.,* 2012).

Invitro antagonistic effect on mycelial growth of phytopathogen under different iron limiting conditions

All the isolates were tested for *in vitro* siderophore mediated antagonism at different iron (Fe^{3+}) concentration against *R. solani* and *S. rolfsii*. All the six isolates showed percent inhibition, to some extent, at all the different iron concentrations. Although, the present study argued that percent inhibition and or zone of inhibition decreased with increasing iron concentration when all the other parameters such as growth temperature, incubation period, defined media compositions are constant (Kumar et al*.,* 2002; Sharma and Johri, 2003).

At 0.5 µM iron concentration, the percent inhibition of mycelial growth of *S .rolfsii* and *R. solani* for FPK8 were 71.1 and 73.3% respectively, which are the maximum among all the isolates, whereas, the same was 52.2% at 10 µM concentration of iron. The similar phenomenon is true with regard to the other isolates (Table 6a and b). It is apparent from the data that the siderophore mediated

Table 6a. Siderophore mediated *in vitro* antagonism against *S. rolfsii* at different iron concentration.

Fluorescent pseudomonad isolates	Different concentration of ferric ion									
	0.5 (µM)		1.5 (µM)		2.5 (µM)		5 (µM)		10 (µM)	
	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibiti (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)
MFP9	1^{c}	63.3^{d}	0.6^{b}	58.9^{c}	0.2^{d}	52.2^{e}	0.0^{d}	44.4^{e}	0.0^{b}	38.9^{e}
CHFP23	1.5^{a}	67.8^{c}	0.9^{a}	60.0^{c}	0.7^{a}	55.6^{c}	0.5^{a}	51.1^{c}	0.2^{a}	43.3^{c}
TFP16	1.2^{b}	57.8^{e}	1.0^{a}	55.6^{d}	0.4^{c}	53.3^{d}	0.2^{c}	46.7^{d}	0.0^{b}	41.1^{d}
FPK7	0.7^{d}	71.1^{a}	0.5^{b}	68.9^{a}	0.2^{d}	64.4^{a}	0.0^{d}	58.9^{a}	0.0^{b}	52.2^{a}
FPK8	0.6^{d}	68.9^{b}	0.5^{b}	66.7^{b}	0.5^{b}	61.1^{b}	0.3^{b}	56.7^{b}	0.2^{a}	50.0^{b}
FPK10	0.3^{e}	53.3^{f}	0.2^{c}	47.8^{e}	0.0^{e}	44.4^{f}	0.0^{d}	37.8^{f}	0.0^{b}	33.3^{f}
SEM (±)	0.057	0.254	0.054	0.409	0.019	0.043	0.011	0.136	0.017	0.107
CD (p = 0.05)	0.181	0.803	0.172	1.288	0.060	0.136	0.037	0.430	0.054	0.338

Values are mean of three replications .A common letter (on the superscript of each value) means they are not significantly different (p= 0.05) by DMRT.

Table 6b. Siderophore mediated *in vitro* antagonism against *R. solani* at different iron concentration.

Fluorescent pseudomonad isolates	Different concentration of ferric ion									
	0.5 (µM)		1.5 (µM)		2.5 (µM)		5 (µM)		10 (µM)	
	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)
MFP9	1.3^{b}	64.4^{e}	1.2^{b}	61.1^{e}	1.0^{a}	58.9^{e}	0.7^{b}	56.7^{d}	0.4^{b}	47.8^{c}
CHFP23	1.6^{a}	75.6^{a}	1.3^{a}	73.3^{a}	$+0.9^{a}$	71.1^{a}	0.7^{b}	67.8^{a}	0.5^{a}	52.2^{a}
TFP16	1.3^{b}	67.8^{d}	1.2^{b}	64.4^{d}	1.0^{a}	62.2^{d}	0.8^{a}	58.9^{c}	0.4^{b}	50.0^{b}
FPK8	0.8^{c}	73.3^{b}	0.6^{d}	72.2^{b}	0.4^{b}	65.6^{b}	0.2^{d}	60.0^{b}	0.0^{d}	52.2^{a}
FPK7	0.8^{c}	72.2^{c}	0.7^{c}	67.8^{c}	0.5^{b}	63.3^{c}	0.4^{c}	58.9^{c}	0.2^{c}	52.2^{a}
FPK10	0.4^{d}	55.6^{f}	0.2^{e}	54.4^{f}	0.0^{c}	51.1^{f}	0.0^{e}	43.3^{e}	0.0^{d}	34.4^{d}
SEM (±)	0.012	0.176	0.013	0.136	0.045	0.133	0.021	0.170	0.021	0.047
CD (p = 0.05)	0.039	0.556	0.042	0.430	0.141	0.421	0.067	0.536	0.068	0.148

Values are mean of three replications. A common letter (on the superscript of each value) means they are not significantly different (p= 0.05) by DMRT.

antagonism may be one of the important characters for the fluorescent pseudomonas under study.

Conclusion

Based on these results, it can be concluded that the production of different and potent antifungal hydroxymate and catecholate siderophore by fluorescent pseudomonads against *R. solani* and *S. rolfsii* are being reported and could be effective bio-control agent against these phytopathogens. Reduction of antagonism by the addition of iron indicated that siderophores were inhibitory to fungal growth and showed a significant effect. Though different mechanisms might be responsible for the inhibition of *R. solani* and *S. rolfsii* but siderophore production might be the main bio-control mechanism associated with the antagonistic potentiality of the rhizobacterial isolates of West Bengal.

ACKNOWLEDGEMENT

We, hereby, express our sincere thanks to AICRP on Vegetable Crops, Directorate of Research, B.C.K.V., Kalyani, West Bengal, India.

REFERENCES

Adhikari A, Dutta S, Nandi S, Bhattacharya I, DeRoy M, Sarkar G, Mandal T (2013). Antagonistic potentiality of native rhizobacterial isolates against root rot disease of okra, incited by *Rhizoctonia solani*. Afr. J. Agric. Res. 8(4):405-412.

Adilakshmi T, Ayling PD, Ratledge C (2000). Mutational analysis of a role for salicylic acid in iron metabolism of *Mycobacterium smegmatis*. J. Bacteriol. 182:264-271.
Ali SS, Vidhale NN (2011). Evaluation of Siderophore produced by Different clinical Isolate *Pseudomona aeruginosa*. Int. J. Microbiol. Res. 3(3):131-135.
Arnow LE (1937). Colorimetric determination of the components of 3,4-dihydroxyphenylalanine tyrosine mixtures. J. Biol. Chem. 118:531-537.
Bossis E, Lemanceau P, Latour X, Gardan L (2000). The taxonomy of *Pseudomonas fluorescens* and *Pseudomonas putida*: current status and need for revision. Agronomie 20:51-63.
Brandel J, Humbert N, Elhabiri M, Schalk IJ, Mislin GLA, Gary AMA (2012). Pyochelin, a siderophore of *Pseudomonas aeruginosa*: Physiochemical characterization of the iron(III) copper(II) and zinc (II) complexes. Dalton. Trans. 41:2820-2834.
Braun V (2001). Iron uptake mechanisms and their regulation in pathogenic bacteria. J. Med. Microbiol. 291:67-79.
Budzikiewicz H (1993). Secondary metabolites from fluorescent *Pseudomonas*. FEMS. Microbiol. Rev. 104:209-228.
Buyer JS, Leong J (1986). Iron transport mediated antagonism between plant growth promoting and plant deleterious *Pseudomonas* strains. J. Biol. Chem. 261:791-794.
Chen Q, Wertheimer AM, Tolmasky ME, Crosa JH (1996). The AngR protein and the siderophore anguibactin positively regulate the expression of iron-transport genes in *Vibrio anguillarum*. Mol. Microbiol. 22:127-134.
Crosa JH (1997). Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. Microbiol. Mol. Biol. Rev. 67:319-336.
Dave BP, Dube H (2000). Detection and chemical characterisation of siderophores of rhizobacterial fluorescent pseudomonads. Ind. Phytopathol. 53:97-98.
Gillan AH, Lewis AG, Andersen RS (1981). Quantitative determination of hydroxamic acids. Anal. Chem. 53:841-844.
Guerinot ML (1994). Microbial iron transport. Annu. Rev. Microbiol. 48:743-772.
Gull M, Hafeez FY (2012). Characterization of siderophore producing bacterial strain *Pseudomonas fluorescens* Mst 8.2 as plant growth promoting and biocontrol agent in wheat. Afr. J. Microbiol. Res. 6(33):6308-6318.
Heinrichs DE, Poole K (1996). PchR, a regulator of ferripyochelin receptor gene (*fptA*) expression in *Pseudomonas aeruginosa*, functions both as an activator and as a repressor. J. Bacteriol. 178:2586-2592.
Hofte M (1993). Iron chelation in plants and soil microorganisms. (Barton, L.L. and Hemming, B.C. (eds.): Academic Press, San Diego.
Hohlneicher U, Hartmann R, Taraz K, Budzikiewicz H (1995). Pyoverdin, ferribactin, azotobactin-a new triade of siderophores from *Pseudomonas chlororaphis* ATCC 9446 and its relation to *Pseudomonas fluorescens* ATCC 13525. Zeitschrift fur Naturforschung C. 50:337-344.
Howard D, Rafie HR, Tiwari A, Faull KF (2000). Hydroxamate siderophores of *Histoplasma capsulatum*. Infect. Immun. 68:2338-2343.
Jalal MAF, Vander Helm D (1990). Handbook of microbial iron chelates: Isolation and spectroscopic identification of fungal siderophores: Winklemann, G. (ed.), Pegamon Press Oxford.
Kumar G, Sharma RC, Rai SN (2000). Biocontrol of banded leaf and sheath blight of maize by peat based *Pseudomonas fluorescens* formulation. Ind. Phytopathol. 53:190-192.
Kumar RN, Arasu VT, Gunasekaran P (2002). Genotyping of antifungal compounds producing plant growthpromoting rhizobacteria,
Mercado-Blanco J, Van der Drift KMGM, Olsson PE, Thomas-Oates JE, Van Loon LC, Bakker PAHM (2001). Analysis of the *pmsCEAB* gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. J. Bacteriol. 183:1909-1920.
Pseudomonas fluorescens. Curr Sci 82:1463-1466.
Neilands JB (1981). In: Development of iron chelator for clinical use Martell AE, Anderson WJ, Badman DG (eds.), Elsevier: Microbial iron transport compounds (siderophores) as chelating agents: North Holland:Amsterdam.
Pal RB, Gokarn K (2010). Siderophores and Pathogenecity of Microorganisms. J. Biosci. Tech. 1 (3):127-134.
Rachid D, Ahmed B (2005). Effect of iron and growth inhibitors on siderophores production by *Pseudomonas fluorescens*. Afr. J. Biotechnol. 4(7):697-702.
Rao ChVS, Sachan IP, Johri BN (1999). Influence of fluorescent pseudomonads on growth and nodulation of lentil *(Lens esculentus)* in *Fusarium* infested soil. Ind. J. Microbiol. 39:23-28.
Ratledge C, Dover LG (2000). Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. 54:881-941.
Reeves M, Pine L, Neilands JB, Bullows A (1983). Absence of siderophore activity in *Legionella* spp. grown in iron deficient media. J. Bacteriol. 154:324-329.
Sambrook J, Fritsh EF, Maniatis T (1989). Molecular cloning: Laboratory manual.2nd ed. Cold Spring Harbor laboratory press. Cold Spring Harbor: NY
Sayyed RZ, Badguar MD, Sonawane HM., Mhaske MM, Chincholkar SB (2005). Production of microbial iron chelators (siderophores) by fluorescent pseudomonads. Ind. J. Biotechnol. 4:448-490
Schwyn B, Neilands JB (1987). Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160:47-56.
Sharma A, Johri BN (2003). Growth promoting influence of siderophore-producing *Pseudomonas* strains GRP3A and PRS9 in maize (*Zea mays* L.) under iron limiting conditions. Microbiol. Res. 158:243-248.
Spilker T, Coenye T, Vandamme P, LiPuma JJ (2004). PCR-Based Assay for Differentiation of *Pseudomonas aeruginosa* from Other Pseudomonas Species Recovered from Cystic Fibrosis Patients. J. Clin. Microbial. 2074-2079.
Srivastava R, Johri BN, Sharma A (1999). Colonization of wheat (*Triticum aestivum* L.) root by fluorescent pseudomonas GRP3 and PRS9. Ind. J. Microbiol. 39:205-210.
Subashri R, Raman G, Natarajan S (2013). Biological control of pathogens and plant growth promotion potential of fluorescent Pseudomonads. Bact Agrobiol: Disease Management. 77-110.
Weller D, Raaijmakers JM, McSpadden Gardener BB, Thomashow LS (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu. Rev. Phytopathol. 40:309-348.
Weller DM (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26:379-407.