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ORIGINAL ARTICLE

Production, characterization and iron binding affinity of hydroxamate siderophores from rhizosphere associated fluorescent *Pseudomonas*

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Abstract

Fluorescent *Pseudomonas* (FP) is a major group of plant growth promoting rhizobacteria and a well-known synthesizer of siderophores, which imparts a selective advantage on rhizosphere competence and their biocontrol traits. The present study was aimed at examining the factors affecting the production of siderophores and their potential biocontrol traits. Sixteen FP isolates were shortlisted based on their siderophore-producing ability in chrome azural S medium. The isolates were checked for variations in siderophore production under varying incubation times, temperatures, pH, iron (Fe^{3+}) concentrations and mutagens. In addition, the iron binding affinity of siderophores, mycelial inhibition assay and plant growth promotion traits were assessed. Results showed that the siderophore production was highly influenced by the time of incubation, changes in pH, temperature and iron concentration. Chemical characterization showed that the produced siderophores were hydroxamates. Maximum siderophore production was observed at pH 7 whereas UV and EtBr exposure invariably suppressed siderophore production drastically in all isolates. All FPs from maize rhizosphere showed excellent siderophore production which could be due to the competence in strategy-II of the plant rhizosphere and significant growth inhibition on *Fusarium oxysporum*. Our results suggest the inclination of siderophores to iron, in terms of various criteria affecting production and the possible role of environmental mutations that affect the natural iron harvesting mechanism.

Key words: siderophore, hydroxamate, fluorescent *Pseudomonas*, succinate medium, iron-binding, mutation

Introduction

Iron is an essential element of all living microorganisms by serving as a cofactor for various enzymes, with the possible exceptions of *Lactobacilli* and *Borrelia burgdorferi* (Masaki *et al.* 2013; Sah and Singh 2015). Although plentiful, iron does not normally occur in its biologically relevant Fe^{2+} form under aerobic conditions since it is unstable in nature (Krewulak and Vogel 2008; Sah and Singh 2015). Iron availability for microbial assimilation in environments such as the rhizosphere, is limiting (Mishra *et al.* 2008). Microorganisms overcome iron scarcity through two mechanisms: i) iron acquisition by cognate receptors using low molecular weight iron chelators (siderophores), ii) receptor-mediated iron acquisition from host proteins. Siderophores are non-protein, non-porphyrin compounds synthesised by microbes, where its synthesis is repressed when iron is abundant in the environment (Jenifer *et al.* 2013). The major difference between these two mechanisms is that the siderophores can be taken up by the bacterial cell as intact molecules, whereas prior to transport, iron must be extracted from the host carrier proteins such as transferrin or lactoferrin (Krewulak and Vogel 2008). The mode of action of siderophores in the biocontrol of plant pathogens

appears to be through the induction of systemic resistance, which depends on iron-regulated metabolites (Helmy *et al.* 2008).

More than 500 different siderophores have been identified. Essentially they have the same basic structure, which consists of a ligand that binds to iron and a peptide backbone that interacts with the receptor of bacterial membrane (Lee *et al.* 2011). They are diverse, highly specific and biosynthesized from amino acids by nonribosomal peptide synthetases (NRPS) or from dicarboxylic and diamine compounds in NRPS independent manner (Miethke and Marahiel 2007). Structurally, siderophores are classified as catacholates (phenolates), hydroxamates and carboxylates (Murugappan *et al.* 2011). Secondary siderophores are weakly expressed or not expressed under iron limited conditions (Miethke and Marahiel 2007). Most aerobic microorganisms produce at least one siderophore, and in some cases, a single bacterial strain can produce two or more (Helmy *et al.* 2008).

Extreme environmental conditions are quite common on the earth and the microbial diversity in such areas is of particular interest as microorganisms inhabiting these places are well adapted to the prevailing atmospheric and edaphic conditions (Mishra *et al.* 2008). The secondary alleochemicals produced by plant growth promoting rhizobacteria (PGPR), which include siderophores, antibiotics, mycolytic enzymes, volatile organic metabolites, systemic fungicides, etc. enable them to combat the adverse environmental circumstances (Saraf *et al.* 2014; Khan *et al.* 2016). In the present study an attempt was made to study the various parameters that affect siderophore production in rhizosphere isolated fluorescent *Pseudomonas* (FP) and their binding affinity towards iron under *in vitro* conditions.

Materials and Methods

Isolation, screening and identification of isolates for siderophore production

Plants with their root region soil were carefully removed, placed in sterile plastic bags and gently transferred to the laboratory. The soil adhering to roots was carefully removed, serially diluted and colonies were isolated using King's B medium with 8-HQ supplementation (120 ppm) as per Fallahzadeh-Mamaghani *et al.* (2009).

Pseudomonas colonies which were fluoresced under ultraviolet light (λ_{max} = 365 nm) were isolated, biochemically characterized, purified and stored at 4°C. Qualitative siderophore production of FPs was analysed using universal chrome azural S (CAS) medium (Alexander and Zuberer 1991).

In order to identify the genus of the shortlisted FPs, the genomic DNA was amplified using *Pseudomonas*-specific 16S primer pair Psmn 289-5'-GGTCGA GAGGATGATCAGT-3' and Psmn 1258 5'-TTAGC TCCACCTCGCGGC-3' (Widmer *et al.* 1998). The polymerase chain reaction (PCR) conditions were optimized according to Varsha and Kumudini (2016).

Chemical characterization of siderophores

CAS-positive isolates were tested for the quantitative siderophore production in succinate medium and quantified at 24 h intervals for 96 h (Gupta *et al.* 2008; Ali and Vidhale 2011). Then, extracted cell free culture supernatants were analysed for hydroxamates by spectrophotometry (Shimadzu UV-1800, Japan). Characterization of siderophore was carried out chemically as per Neilands assay (1981), confirmed by Tetrazolium test (1954) and Arnow's assay (1937).

Effect of incubation time, pH, temperature, Fe^{3+} concentration and mutagenic agents (UV and EtBr) on siderophore production

The culture density of the overnight FP cultures of the isolates was adjusted to ~1 × 10^8 CFU · ml^{-1} (OD_{600} = 0.3). 20 µl of this was inoculated into succinate medium and incubated at 37°C. Cultures were centrifuged at 10,000 rpm for 10 min. Cell free supernatant was diluted with 0.5 M phosphate buffer (pH 7) and absorbance was read at 400 nm.

To evaluate the ability of FPs to produce siderophores at varying pH, isolates were allowed to grow in succinic acid medium for 72 h at different pH (5–9). Siderophore production was analysed every 24 h. To study the influence of different incubation temperatures, isolates were inoculated into succinic acid medium and incubated at four different temperatures (16, 25, 37 and 50 ± 2°C). To determine the effect of iron concentration in siderophore production, isolates were allowed to grow in succinate medium (37°C) supplemented with Fe^{3+} (ferric ammonium citrate, Merck, India) at different concentrations (1, 10 and 50 µM). Siderophore production was recorded at 24 h intervals.

To study the role of mutagens on siderophore production, 24 h old cultures of FPs in Luria Bertani medium (LB) were pelleted by centrifugation (3,000 rpm for 15 min). Fluorescent *Pseudomonas* were treated with three different concentrations of ethidium bromide (EtBr; 0.05, 0.1 and 1.5 g · l^{-1}) as per Kamal *et al.* (2003) at a cell concentration of ~1 × 10^8 CFU · ml^{-1}. They were incubated for 30 min and washed until the remnants of EtBr were completely removed and re-inoculated into succinate medium, incubated for 72 h and checked for siderophore production. Physical

mutagenesis was carried out using ultraviolet (UV) as per Gawel *et al*. (2012) with minor modifications. Bacterial cells were pelleted from overnight LB culture broth and exposed to UV-light (λ_{max} = 365 nm) for 30 and 60 s. The pellets were re-inoculated into succinate medium and analysed for siderophore production.

Iron binding affinity of siderophores

72 h old FP cultures were centrifuged and the supernatant was transferred to a separating funnel. An equal volume of chloroform containing 3% (w/v) 8-hydroxy quinolone (8-HQ, Himedia, India) was added and the chloroform layer was separated. Three different concentrations (10, 25 and 50 µM) of Fe^{3+} (ferric ammonium citrate – Merck, India) were added to the supernatant. Optical density was read (λ_{max} = 450 nm) after of 5, 15, 30, 60, 90 and 120 min (Ali and Vidhale 2011).

In vitro antifungal activity and seed germination studies

All the FPs were tested for their *in vitro* antifungal activity by well diffusion assay (Zongzheng *et al*. 2009) against the fungal pathogen *Fusarium oxysporum* (MTCC-1755) and the zone of inhibition was recorded after 7 days.

Tomato seeds (PKM 1 variety) were soaked in 72 h old FP suspension (washed in saline) for 2 h, blot dried and plated on to blotter discs. They were incubated in a growth chamber for 15 days along with distilled water control. Root length and shoot length were measured and percentage germination was calculated.

Statistical analysis

All the experimental analyses were carried out in triplicate. Means were compared using ANOVA with Graph Pad Prism 6.01 version and IBM SPSS statistics 20.

Results and Discussion

Siderophores represent one important class of molecules which solubilize Fe^{3+} from the surrounding environment. In the present study, the siderophore producing ability of a set of shortlisted FPs was carried out. Additionally, the ability of FPs to respond to the common mutagens and their phytoaugmentation traits were analysed.

Isolation, shortlisting and molecular identification of FPs

Comprehensive screening of siderophore showed orange halos around bacterial colonies on CAS agar plates, indicating siderophore production. Based on the qualitative analysis, 16 FPs were shortlisted for further studies (Table 1). It was noticeable that six out of 16 shortlisted isolates were from maize rhizosphere and were excellent producers of siderophores. Previously many authors reported siderophore-producing FPs as biocontrol agents to combat plant pathogens (Beneduzi *et al*. 2012; Gawel *et al*. 2012). Lemanceau *et al*. (2009) reported that in plants with the strategy-II iron-acquisition system, only indirect mechanisms may account for improved plant nutrition by pyoverdines due to their higher affinity towards iron, than phytosiderophores. Moreover, a plant's iron status affects the composition of rhizosphere micro-flora, which is probably due to secretion of phenolics, eventually resulting in elevated siderophore synthesis (Jin *et al*. 2010).

Table 1. Shortlisted CAS positive fluorescent *Pseudomonas* isolates and their site of collection

Pseudomonas isolates	Site of collection
P88	*Nyctanthes arbour-tristis*, Assam
T125, T126	*Solanum lycopersicum*, Karnataka
M131, M132, M136, M138, M139, M140	*Zea mays*, Karnataka
R40, R44, R45, R61	*Oryza sativum,* Bihar
C43	*Capsicum annum*, Karnataka
J49	*Jasminum sambac*, Bihar
A55	*Aloe vera,* Bihar

Partial amplification of 16S rRNA of the 16 listed isolates using *Pseudomonas* specific primers showed a 957 bp amplified product in all the isolates including control strains. The molecular weight of the amplified product, confirmed by 1 kb ladder, showed that all isolates belong to genus *Pseudomonas*.

Chemical characterization of siderophores

Succinate medium, following incubation, turns green to yellow as is characteristic of pyoverdins. The presence of siderophores is seen when, with the addition of ferric chloride, it turns red or purple. Spectrophotometric assay results showed a λ_{max} at 408 nm, which was absent in a de-ferrated condition (Fig. 1). An absorption peak between 360 and 420 nm is characteristic of pyoverdins (Bhattacharya 2010), confirming the presence of pyoverdins. Ferric chloride assay did not show any peak at 495 nm and in 190–280 nm, indicating the absence of catacholates and carboxylates (Baakza *et al*. 2004). Positive results for the tetrazolium salt test confirmed the hydroxamate nature of siderophores. Siderotyping is a very useful analysis which correlates well-defined species and also detects

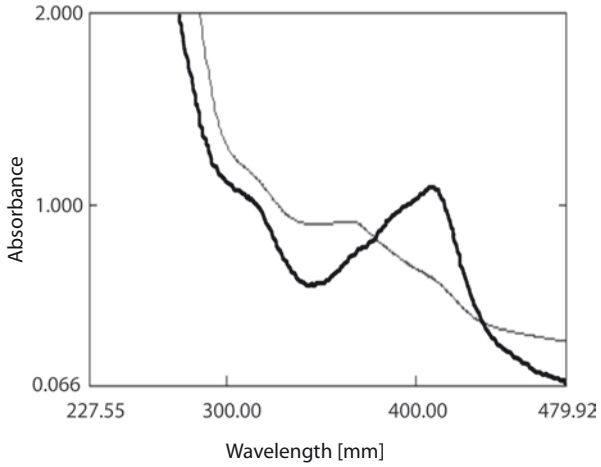

Fig. 1. Representative depiction of UV-visible spectrum of elutedsiderophores by fluorescent *Pseudomonas* isolates. Peak at 408 nm indicates the presence of ferric Fe^{3+}

misclassified individual strains among fluorescent/ non-fluorescent *Pseudomonas.* This explains the role of pyoverdine as a powerful taxonomic marker (Meyer *et al*. 2002). The latest study on the peptide side chains of pyoverdin siderophores from *Pseudomonas putida* showed that the structure is unique among all pyoverdins. This shows the strength of the peptide structure for phylogenic analysis (Ye *et al*. 2014).

A time course study on siderophore production was carried out up to 96 h. Maximum siderophore production was observed after 72 h by M139 (182.03 ± 9.79 μg · ml^{-1}), followed by T125 (165.71 ± 5.26 μg · ml^{-1}) as shown in Figure 2. Siderophore production gradually increased till 72 h, followed by a steady decline in all the isolates. Therefore, further experiments were restricted to 72 h incubation.

Several studies on strain-to-strain variations in the production of siderophores have been reported

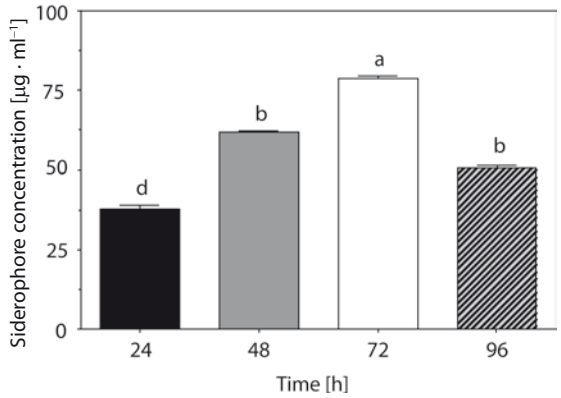

Fig. 2. A time course assay on production of siderophores by fluorescent *Pseudomonas* isolates in succinic acid medium. Data of the qualitative analyses are represented as mean ± standard error where n = 3. Means were compared between treatments by Duncan multiple-ranges test. Different letters indicate significant differences among treatments ($p \leq 0.05$)

previously (Tailor and Joshi 2012). Nagata *et al.* (2013) reported that tomato plants supplemented with siderophore from *P. fluorescens* ATCC13525 showed increased iron absorption efficiency in comparison to direct iron supplementation. Although pyoverdine-mediated antagonism gives a competitive advantage to the FP (Lemanceau *et al*. 2009), this requires a specific outer membrane receptor, which is advantageous to the organism under iron stress (Nagata *et al.* 2013). Previous reports clearly indicate that *Pseudomonas* can produce siderophores with different chemical natures. This may be due to evolutionary pressures lodged on microbes to produce structurally different siderophores which cannot be transported by other microbial transport systems (Tripati *et al.* 2005). Synthesis of more than one siderophore and the production of multiple siderophore uptake systems by a single bacterial species are common. The selective advantages conferred by the multiplicity of siderophore synthesis remains unclear. Recent evidence suggests that siderophores may have other physiological roles besides their involvement in iron acquisition (Adler *et al*. 2012).

The role of physical parameters in siderophore production

Studies on the effect of temperature showed reduced siderophore production at 16°C and 50°C. The optimum temperature for siderophore production was 37°C, but seven isolates (R40, C43, R44, R45, J49, A55 and R61) showed high siderophore production, 70.90 ± 0.77, 73.90 ± 1.39, 97.24 ± 4.57, 59.81 ± 1.31, 108.93 ± 0.76, 96.54 ± 0.80 and 75.39 ± 1.06, at 28°C after 72 h incubation (Fig. 3A). This result indicates the specificity of isolates to temperature. Other isolates had moderate siderophore production at room temperature indicating that they are more versatile in the context of siderophore production. Isolates P88, T125, M132, M136, M138 and M139 showed the highest siderophore production at 37 ± 2°C instead of room temperature (107.57 ± 4.65, 165.71 ± 5.26, 135.03 ± 4.68, 147.70 ± 1.38, 141.57 ± 2.50 and 182.03 ± 7.97, respectively), which may be due to the adaptation of FPs to the environmental temperature. It was observed that with a change in pH, there was a marginal difference in siderophore production. The highest siderophore production was observed at pH 7. But production drastically declined as pH diverged towards acidic and alkaline ranges (Fig. 3B). The effect of Fe^{3+} on siderophore production showed that 1 μM iron concentration resulted in the highest siderophore production, 53.86 ± 0.58 (by isolate A55) followed by 53.68 ± 0.16 and 51.77 ± 0.26 (by the isolates M138 and M132, respectively) (Fig. 4). In the present study an increased siderophore production with reduced Fe^{3+} concentration was observed.

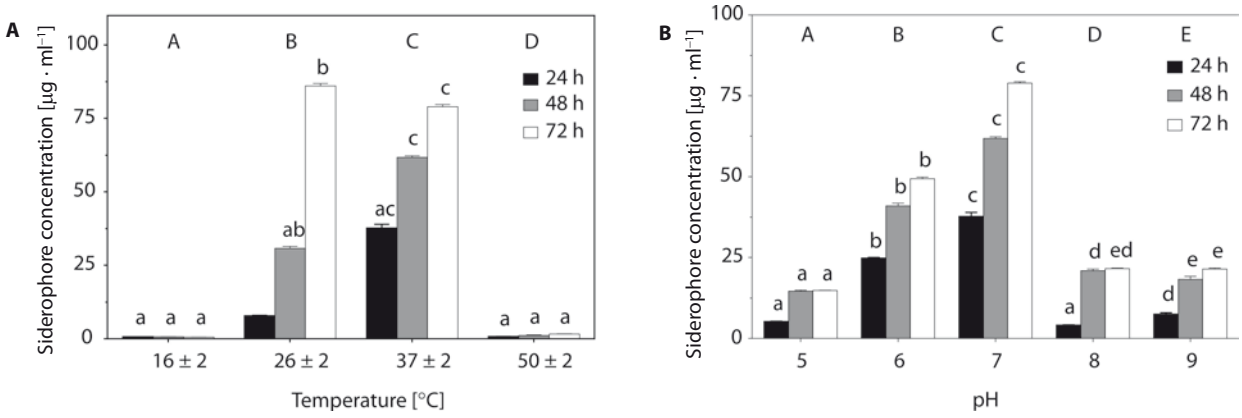

Fig. 3. Impact of (A) temperature and (B) different pH on hydroxamate siderophore production by fluorescent *Pseudomonas* isolates. Data presented are mean ± standard error of three independent replicate experiments. Means were compared between treatments using two-way ANOVA. Same letters are not significantly different according to Tukey's HSD-test ($p \leq 0.0001$) between treatments. Capital letters indicate significant mean difference between variables and small letters indicate significance between time

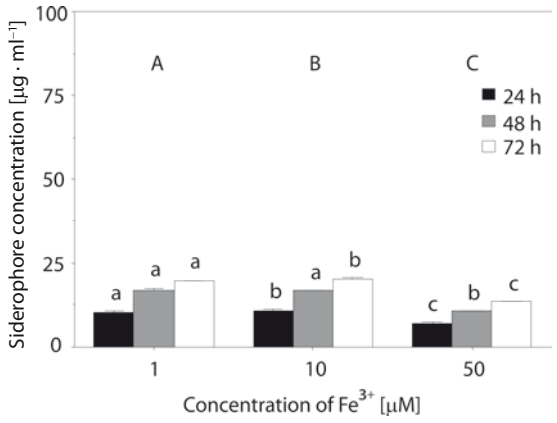

Fig. 4. Production of hydroxamate siderophores by fluorescent *Pseudomonas* isolates in the presence of varying concentrations of Fe^{3+} in succinic acid medium. Data presented are mean ± standard error of three independent replicate experiments. Means were compared between treatments using two-way ANOVA. Same letters are not significantly different according to Tukey's HSD-test ($p \leq 0.0001$) between treatments. Capital letters indicate significant mean differences between concentrations and small letters indicate significance between time

The specificity of pH in siderophore production indicates that the soil pH is a critical factor for iron acquisition mechanisms. In addition to pH, heavy metal concentrations in the bacterial environment also influence siderophore biosynthesis (Rajkumar *et al*. 2009). Biosynthesis and secretion of siderophore by microbial systems are known to be related to the requirement of iron for the metabolism of specific growth substrates. In the environment, the presence of aromatic compounds along with easily metabolizable co-substrates supports siderophore production (Gaonkar *et al*. 2012). Environmental factors modulating siderophore synthesis include pH, the ionic state of iron, the presence of other trace elements and an adequate supply of carbon, nitrogen and phosphorus (Duffy and Défago 1999). Bacterial growth as well as siderophore production is stimulated by ammonium sulphate and amino acids. However, the optimum siderophore yield was obtained with urea (Sayyed *et al*. 2005). Statistical analysis using two-way ANOVA in the analysed parameters revealed a significant difference in p value except in siderophore production at 16°C, where p value was 0.78.

Iron-binding affinity of siderophores

The binding affinity experiments showed that hydroxamates have an expeditious affinity with Fe^{3+}, whereas there was a change in affinity with respect to the surrounding iron concentration. A drastic absorption of Fe^{3+} by siderophores was observed within 5 min, followed by non-significant increments in absorption (Fig. 5). Further increments in affinity were less, which were evident in all three concentrations of Fe^{3+}, as free siderophore ligands left in the sample were extremely limited. Different concentrations of Fe^{3+} (i.e., 10, 25 and 50 µM) did not show much variation in affinity. Binding affinity with Fe^{3+} showed well advanced fondness with the crucial metal ion. The siderophore-iron binding is completed during the initial 5 min and the affinity progress against time is uniform even at higher iron intensity.

The role of mutation in siderophoregenesis

From the mutation study results with UV and EtBr (Figs. 6A and B) it can be concluded that the resulting mutation suppressed siderophore production to a great extent. UV at 60 s and EtBr at higher concentrations (0.5 and 1.0 µM) further suppressed hydroxamate biosynthesis. Ferric uptake regulator (*Fur*) is a widespread bacterial protein that regulates the expression of iron acquisition and storage systems in

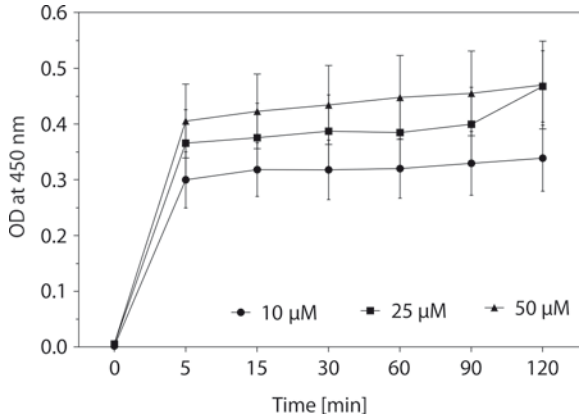

Fig. 5. Time course assay of *in vitro* iron binding affinity of siderophores produced by fluorescent *Pseudomons* isolates, with varying ferric ion (Fe^{3+}) concentration. Data represented are the mean ± standard error of three replicates. Vertical bars indicate standard error. Two-way ANOVA analysis showed a non-significant p value ($p < 0.0001$)

response to intracellular iron. *Fur* is a key regulator of iron metabolism, acting as a classical iron-dependent repressor of protein-coding genes (Lewin *et al*. 2002). Transcriptional studies on *P. fluorescens* Pf-5, showed that among the up-regulated pyoverdine biosynthesis gene clusters, the highly expressed gene was *pvdS* which encoded for the extra-cytoplasmic function sigma factor, a transcriptional regulator of pyoverdine biosynthesis genes (Lim *et al*. 2012). Bacterial evolution demands mutations to permit adaptation through sequence variation by still preserving genome integrity. UV, the customary non-ionizing radiation, can induce adjacent pyrimidine bases in a DNA strand to become a covalent dimer causing oxidative damage to DNA (Kozmin *et al*. 2008). Both of the mutations could not stimulate higher siderophore production in bacterial isolates. It was observed that mutation in bacterial genome resulted in suppression of ferric homeostasis, reported by Schwyn and Neilands (1987). Genomic mutation on FP, major rhizosphere group bacteria, which affects siderophore-iron binding efficiency, has to be analysed in detail.

Mycelial inhibition traits and phytoaugmentation traits

All the isolates were checked for their ability to inhibit the pathogenic *F. oxysporum* strain and the results are represented in Table 2. It is worth pointing out that all the high siderophore producers inhibited the *F. oxysporum*, where M140 demonstrated maximum inhibition (55.0 ± 2.6 mm). Hence, it can be speculated that production of siderophores, possibly in combination with antimicrobial agents, produced by FPs, leads to *F. oxysporum* inhibition. Plant protection through siderophore producing rhizobacteria has emerged as a possibility in sustainable crop management (Karimi *et al*. 2012; Solanki *et al*. 2014). The rhizobacterially synthesized siderophores are excellent competitors for Fe, thus reducing the Fe availability to the phytopathogens (Beneduzi *et al*. 2012). This is a very common and significantly useful action of rhizobacteria in sustainable agriculture.

Growth promotion study results showed that the FPs are capable of inducing root and shoot growth with higher germination percentages than in control plants. Bacterial seed treatment resulted in improved physical parameters, where M140 showed the longest shoot length 53.8 ± 02.4 mm and root length 209.6 ± 05.9 mm, against control.

Plant growth promotion effect exerted by siderophore producing rhizobacteria, especially FPs has been reported previously in different plant systems (Sharma and Johri 2003; Ahmed and Holmström 2014). A report by Anitha and Kumudini (2014) clearly demonstrated the impact of secondary metabolites, especially siderophores, in the growth promotion of tomato plants, using FPs.

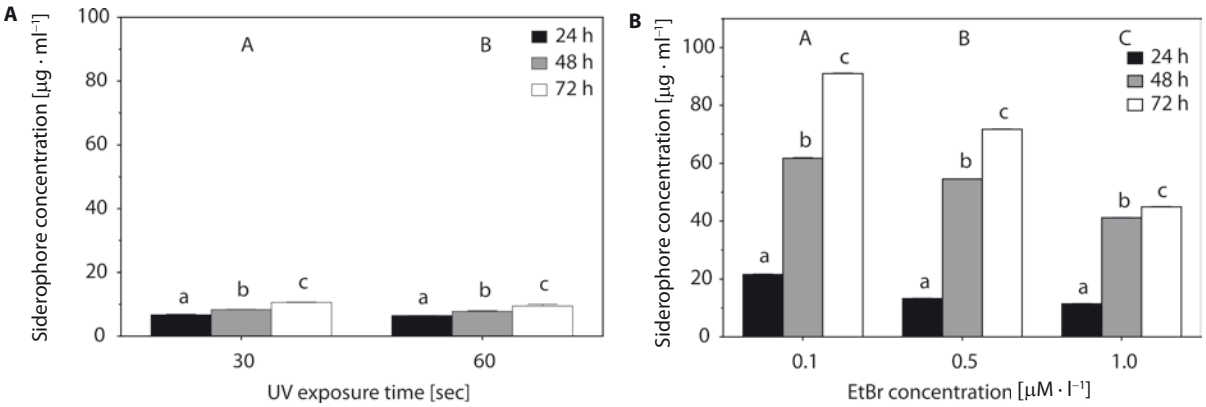

Fig. 6. Effect of (A) UV mutagenesis and (B) EtBr on time course production of hydroxamate siderophores by fluorescent *Pseudomonas* isolates. Data presented are mean ± standard error of three independent replicate experiments. Means were compared between treatments using two-way ANOVA. Same letters are not significantly different according to Tukey's HSD-test ($p \leq 0.0001$) between treatments. Capital letters indicate significant mean difference between concentration and small letters indicate significance between time

Table 2. *In vitro* antifungal activity against *Fusarium oxysporum* and phytoaugmentation traits of fluorescent *Pseudomonas* isolates

Pseudomonas isolates	Zone of inhibition [mm]	Shoot length [mm]	Root length [mm]
P88	0	53.9 ± 03.5	160.4 ± 10.6
T125	0	64.8 ± 03.6	170.4 ± 05.6
T106	0	52.2 ± 04.5	154.2 ± 09.2
M131	24.3 ± 2.8	38.1 ± 02.3	86.0 ± 08.7
M132	27.2 ± 2.3	51.2 ± 03.7	161.8 ± 06.5
M136	40.3 ± 0.9	34.4 ± 03.3	184.4 ± 07.6
M138	26.3 ± 2.7	48.3 ± 02.5	202.4 ± 06.9
M139	43.0 ± 2.6	44.9 ± 02.8	201.2 ± 05.5
M140	55.0 ± 2.6	53.8 ± 02.4	209.6 ± 05.9
R40	0	34.1 ± 06.9	124.0 ± 08.5
R44	0	38.7 ± 03.8	113.7 ± 08.2
R45	0	46.4 ±16.2	113.3 ± 34.6
R61	0	37.5 ± 03.0	113.0 ± 06.8
C43	0	32.4 ± 02.1	155.7 ± 11.3
J49	0	41.6 ± 03.4	92.6 ± 08.3
A55	0	39.6 ± 03.5	102.4 ± 07.3
Control*	—	29.0 ± 02.3	97.4 ± 04.7

*distilled water treated control

Conclusions

There are various reports that pesticides, designed to target specific organisms, also affect non-target microbial flora also. Fungicides and insecticides are reported to be capable of exerting an adverse impact on natural soil microflora (Cycoń and Piotrowska-Seget 2007). However, there are very few studies on the impact of siderophore production, on various soil factors in the terrestrial habitat. The present study undertook a detailed analysis of siderophore characterization, and of the common factors which affect their production. It is known that maize plants (Graminaceous family) produce phytosiderophores which offer a competence to the bacterial siderophores in rhizosphere terrain. This could be the reason for its high siderophore production nature. The difference in the quantities of siderophores produced by different organisms is logical. As the natural iron 'collectors' in the soil rhizosphere the chemical agents which are used need further study since they possibly spoil the natural biocontrol agents, and can have a long-term impact on nature.

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