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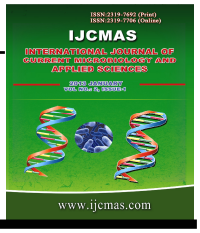

Original Research Article

Isolation of siderophore producing bacteria from rhizosphere soil and their antagonistic activity against selected fungal plant pathogens

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A B S T R A C T

A total of 11 isolates were isolated from the soil samples, among which 5 isolates were screened from tomato rhizosphere soil sample and 6 isolates from paddy rhizosphere soil sample. Eleven isolates were tested for the production of siderophore using blue agar medium. Out of the 11 isolated cultures, 4 cultures namely C2, C3, C8, C11 were found to produce siderophore. Based on morphology, cultural characteristics and biochemical tests the siderophore producing isolates were identified. Based upon the results C2 was identified as Non-fluorescent *Pseudomonas* sps, C3 as *Bacillus* sps, C8 as Fluorescent *Pseudomonas* sps and C11 as *Azotobacter* sps. The eight fungal isolates were isolated from Sabaroud's chloramphenicol agar. The isolates were named as F1, F2, F3, F4, F5, F6, F7, and F8. The bacterial cultures namely C2, C3, C8, C11 were tested for their antifungal activity against the fungal isolates F3, F4, F7, F9. Non fluorescent *Pseudomonas* showed 83.5% activity against *Fusarium sps*. *Bacillus sps* exhibited 85.8% inhibition against *Sclerotium sps*, 90.5% inhibition against *Fusarium sps*, 41.8% inhibition for *Alternaria sps*. Fluorescent *Pseudomonas* showed 92.9% activity against *Sclerotium sps*, 83.5% inhibition for *Fusarium sps*, 38.1% inhibition against *Alternaria sps*. *Azotobacter sps* had antagonistic activity of 92.9% against *Sclerotium sps*, 45.4% antagonism for *Alternaria sps*. Maximum activity was observed for *Alternaria sps* by *Bacillus sps* (C3)32±0.5mm, Fluorescent *Pseudomonas sps* (C8) 34±0.1mm and *Azotobacter sps* (C11) 30±0.4mm.

K e y w o r d s

Siderophore;
Pseudomonas sps;
Bacillus sps;
Azotobacter sps,;
Fusarium;
Alternaria.

Introduction

Bacteria are generally the predominant initial inhabitants of newly expanded leaves, while yeast and filamentous Fungi dominate later in the growing season (Srivastava and Shalini, 2008). The extraordinary use of chemical fungicides resulted in environmental pollution and ill health to biotic community as a whole. Therefore biological method of plant disease management seems to be a better alternative to chemical fungicides in managing the

disease. Control of phytopathogens by biological means was environmentally advantageous in comparison to chemical control methods which had many risks on human health and environment (Parani and Saha, 2009). Biological control is thus being considered as an alternative way of reducing the use of chemicals in agriculture. Many species of soil microbes namely *Azotobacter, Pseudomonas, Bacillus, Streptomyces* etc produce sideropore. Among these microbes *Pseudomonas* species is the active siderophore producer.

Pyoverdines (PVDs) are the main siderophores synthesized by fluorescent pseudomonads under iron-limiting growth conditions. These molecules are essential for virulence of *Pseudomonas aeruginosa* in human diseases and are one of the mechanisms involved in the suppression of fungal soil-borne plant diseases (Jacques *et al.,* 2003). Interest in the *Pseudomonads* has increased recently because of the possible use of siderophores as biopesticides and the possible use of *pseudomonads* in detoxifying chemical wastes through a wide range of enzymatic metabolic activities. Iron metabolism has also been studied because of the potential role of microbial siderophores in facilitating uptake of heavy metals and their metabolism under certain growth conditions (Rachid and Ahmed, 2005). There were more than 80 heterocyclic nitrogen-containing natural products of phenazines synthesized by florescent *Pseudomonas spp* (Hassanein *et al.,* 2009). Several antibiotic-like substance have been identified, including bacteriocins and phenazine antibiotics, but one of the most important mechanism responsible for the suppression of plant pathogens for *Pseudomonas spp*. is siderophore-mediated competitions for iron. Secondary metabolites produced by certain species of *Bacillus* show antifungal activity against phytopathogenic microorganisms (Feio *et al.,* 2004). Some authors have suggested that the use of such strains or species, or their metabolites, may be an alternative to agrochemical plant protection.

The present study is focused on antifungal activity of siderophore producing soil bacteria against few plant fungal pathogens. In the present study soil samples were screened for siderophore producing bacteria by spread plate technique. The isolates were characterized by the routine microbiological techniques. Siderophore production was screened using blue agar. Antifugal property of the isolated bacteria was tested against the selected plant fungal pathogen.

Materials and Methods

Rhizosphere soil contains a variety of microbes which includes plant pathogens and symbiotic microbes. Rhizosphere soil samples were collected from the garden, Mohammed Sathak College, Sholinganallur and from a paddy field in Porur. From the collected soil sample was serially diluted and plated on nutrient agar plates and on Ashby's glucose agar medium for isolating *Azotobacter* sps. The plates were incubated at 27^{o}C for 24 hours. Ashby's medium plates were incubated at room temperature for 3-5 days. The isolated bacterial strains were tested for the production of siderophore using blue agar medium. Blue agar was prepared and the ph was adjusted to 7.0±0.2. The test organism was streaked on blue agar plates. The plates were incubated at 27^{o}C for 48 hours. The isolated screened bacteria were identified using microbiological tests. Garden rhizosphere soil was collected from the campus of Mohammed Sathak College and from the paddy field in Porur. The soil samples were subjected to serial dilution and the dilutions were plated on sabouraud chloramphenicol

agar medium. After incubation at room temperature for 5 days developed fungal colony was subcultured for further identification (Aneja, 2003) .The isolated fungal cultures were subjected to Lactophenol cotton blue staining technique and the fungal pathogens namely *Fusarium* sps, *Aspergillus niger*, *Sclerotium* Sps and *Alternaria* sps were identified.

Antifungal compound was extracted using nutrient broth. In 500ml flask, 200ml growth medium was prepared, autoclaved and cooled. It was then inoculated with 10 ml bacterial suspension (8 hours old). The ph 7±0.2 was maintained. The flask was incubated in shaker at 30^{o}C for 48 hours. The incubated culture was centrifuged at 5000g for 20 minutes. This procedure was repeated twice. Bacterial free supernatant was obtained (Hassanein *et al.,* 2009). All the five bacterial isolates were tested for their inhibitory activity against the four isolated pathogens. Each fungal pathogen was grown on PDA plate till it covered the whole surface of the agar. With the help of a sterile borer, a disc of fungal pathogen was placed at the centre of a fresh PDA plate. Twenty four hour old culture of each bacterial strain was then streaked parallely on either side of the fungal disc 3cm away from the disc. The plates were kept for incubation at 30^{o}C for 96 hours. Visual observations on the inhibition of fungal pathogens were recorded after 96 hours of incubation in comparision with the PDA plate simultaneously inoculated with only the fungal pathogen (Parani and Saha, 2009). Zone between the bacteria and fungus was used as an indication for the extent of antagonism. Percentage of inhibition was calculated as:

$$\frac{\text{Colony growth diameter in checked plates-Colony growth diameter in each treatment.}}{\text{Colony growth diameter in checked plates}} \times 100$$

Results

A total number of 11 isolates were isolated from the soil samples, among which 5 isolates were screened from tomato rhizosphere soil sample and 6 isolates from paddy rhizosphere soil sample. The isolated pure cultures of bacteria namely C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11 were preserved in nutrient broth containing glycerol at -70^{o}C. Eleven isolates were tested for the production of siderophore using blue agar medium. Out of the 11 isolated cultures, 4 cultures namely C2, C3, C8, C11 were found to produce yellowish-orange colonies which is positive for siderophore production. Based on morphology, cultural characteristics and biochemical tests the siderophore producing isolates were identified. Based upon the results C2 was identified as Non-fluorescent *Pseudomonas* sps, C3 as *Bacillus* sps, C8 as Fluorescent *Pseudomonas* sps and C11 as *Azotobacter* sps(Table.1).

The eight fungal isolates were isolated from Sabaroud's chloramphenicol agar. The isolates were named as F1, F2, F3, F4, F5, F6, F7, and F8. The cultures were preserved in PDA slants. The isolated fungal cultures were identified by colony morphology and microscopic observation by Lactophenol cotton blue (LPCB) staining technique.

Based upon the results F3 was identified as *Sclerotium* sps, F4 as *Fusarium* sps, F6 as *Aspergillus niger* and F8 as *Alternaria* sps. Fluorescent *Pseudomonas* was mass cultivated in nutrient broth. The broth was centrifuged twice to collect the extracts. The extracted product was collected and stored separately at 4^{o}C. The bacterial cultures namely C2, C3, C8, C11 were tested for their antifungal activity against the fungal

isolates F3, F4, F7, F9. The zone of inhibition was measured and presented.

A zone of inhibition of 14±0.2mm was produced against *Fusarium sps* by non fluorescent *Pseudomonas sps* (C2). But *A.niger, Sclerotium sps* and *Alternaria sps* were not inhibited by non fluorescent *Pseudomonas sps*.

Table.1 Antifungal activity of siderophore producing bacterial isolates

Test Fungal Cultures	C2	C3	C8	C11
Sclerotium sps.	-	12±0.4mm (85.8%)	16±0.1mm (81.1%)	6±0.2mm (92.9%)
Fusarium sps.	14±0.2mm (83.5%)	8±0.3mm (90.5%)	14±0.4mm (83.5%)	-
A.niger	-	-	-	-
Alternaria sps.	-	32±0.5mm (41.8%)	34±0.1mm (38.1%)	30±0.4mm (45.4%)

(-) = no inhibition

Note: Mean values of triplicates ± S.D, values given in parentheses indicate % maximum activity.

C2- Non-fluorescent *Pseudomonas.*

C3- *Bacillus sps.*

C8- Fluorescent *Pseudomonas.*

C11- *Azotobacter sps.*

Bacillus sps (C3) inhibited *Sclerotium sps, Fusarium sps* and *Alternaria sps* by about 12±0.4mm, 8±0.3mm, 32±0.5mm respectively. But *A.niger* was not inhibited by *Bacillus sps* (C3).

Fluorescent *Pseudomonas* (C8) showed activity against *Sclerotium sps, Fusarium sps* and *Alternaria sps* by about 16±0.1mm, 14±0.4mm, and 34±0.1mm respectively. But there was no inhibition for *A.niger*.

Azotobacter sps (C11) inhibited *Sclerotium sps* and *Alternaria sps*. by a diameter of about 6±0.2mm and 30±0.4mm respectively. But *Fusarium sps* and *A.niger* was not inhibited by *Azotobacter sps* (C11).

Maximum activity was observed for *Alternaria sps* by *Bacillus sps* (C3)32±0.5mm, Fluorescent *Pseudomonas sps* (C8) 34±0.1mm and *Azotobacter sps* (C11) 30±0.4mm.

The plates were observed and the growth of pathogens in the treated plate was measured. Non fluorescent *Pseudomonas* showed 83.5% activity against *Fusarium sps*. *Bacillus sps* exhibited 85.8% inhibition against *Sclerotium sps,* 90.5% inhibition against *Fusarium sps*, 41.8% inhibition for *Alternaria sps*. Fluorescent *Pseudomonas* showed 92.9% activity against *Sclerotium sps*, 83.5% inhibition for *Fusarium sps*, 38.1% inhibition against *Alternaria sps*. *Azotobacter sps* had antagonistic activity of 92.9% against *Sclerotium sps*, 45.4% antagonism for *Alternaria sps*.

Discussion

Several soil microorganisms are known to improve the plant growth directly through nutrient mobilization and production of plant hormones and indirectly through suppression of plant pathogens or by inducing systemic resistance in plants. The use of chemical fertilizers has been necessitated due to cultivation of high yielding varieties. This has resulted in degradation of soil health. Biological methods offer an excellent alternate strategy for effective control of various diseases and plant growth promotional activity. While organic manures like FYM, compost, vermicompost, green manure etc. are satisfactory sources for the supply of plant nutrients, we are yet to find suitable alternatives to pesticides for the control of insect pests and diseases of crop plants. Improvement in agricultural sustainability requires optimal use and management of soil

fertility and soil physical properties both of which rely on soil biological processes and soil biodiversity. Biological methods offer an excellent alternate strategy for effective control of various diseases and plant growth promotional activity.

In the present study soil samples were collected from the rhizosphere region to isolate bacteria. The fluorescent *Pseudomonas* was isolated from rhizosphere soil of rice field.In the current report siderophore production was observed in non fluorescent *Pseudomonas*, *Bacillus sps*, fluorescent *Pseudomonas*, *Azotobacter sps* in blue agar plates. This is in accordance with the study report of Ahmad *et al.,* 2008 in which siderophore production was exhibited by free living rhizospheric isolates of *Azotobacter* (16.22%), fluorescent *Pseudomonas* (11.11%) and *Bacillus sps* (10%). *Bacillus sps* have been reported to produce catechol-siderophore by Park *et al.,* 2005 and Garner *et al.,* 2004. This is similar to the present study where we have found out the production of siderophore by *Bacillus sps*. *Azotobacter sps* has been reported to produce plant growth promotion, suppressed plant disease and siderophore production by Saharan and Nehra, 2011.

In the current study Fluorescent *Pseudomonas* inhibited *Fusarium sps* by a diameter of 14±0.4mm, inhibition percentage was 83.5%. This is in accordance with the study report of Rini and Sulochana, 2007 that fluorescent *Pseudomonas* inhibited *Fusarium* by more than 60%. Inhibition of *Sclerotium sps* by Fluorescent *Pseudomonas* was about a diameter of 16±0.1mm which is about 81.1%. In the similar way *Pseudomonas cf. monteilii* inhibited *Sclerotium rolfsii* upto 94% in terms of dry weight in an experiment carried out by Rakh *et al.,* 2011. It was proved that *Pseudomonas fluorescens* showed antifungal activity against *Sclerotium rolfsii* and *Fusarium udum*. This also correlates with the study report of Shivakumar, 2007 where he has revealed fluorescent *Pseudomonas* inhibited *Sclerotium sps* and *Fusarium sps*. In another report by Rachana and shalini, 2010 *Pseudomonas* shared antifungal activity against *Alternaria cajani*, *Curvalaria lunata*, *Fusarium sps* and *Bipolaris sps.*

In the present investigation *Bacillus sps* inhibited *Fusarium sps* by a diameter of 8±0.3mm which is similar to the study report of Ekundayo *et al.,*, 2010 in which *Bacillus subtilis* has least inhibitory activity on *Fusarium oxysporum*. Jamil *et al.,* 2011 studied that *Pseudomonas sps* increases soil fertility. In the present study, two isolates of siderophore producing *Pseudomonas* namely non fluorescent *Pseudomonas* and fluorescent *Pseudomonas* were isolated from rhizosphere soil also serve as antifungal agent beyond soil fertility. Sagahón *et al.,* 2011 stated that among the soil isolates, *Bacillus subtilis*, *Pseudomonas fluorescens* showed antifungal activity against phytopathogens. The present study fluorescent *Pseudomonas* exhibited biocontrol potential against fungi that induce red-rot disease of sugarcane.

In the present study *Bacillus sps* isolated from rhizosphere soil sample showed antifungal activity against *Sclerotium sps* (12±0.4mm), *Fusarium sps* (8±0.3mm) and *Alternaria sps* (32±0.5mm). This result coincides with the antagonism of *Bacillus sps* isolated from marine biofilms against terrestrial phytopathogenic fungi by Morales *et al.,* 2009 and Leclere *et al.,* 2005. According to Carissimi *et al.,* 2009 *Bacillus sps* exhibited antifungal activity against *Bipolaris sorokiniana* which supports the present investigation. *Azotobacter chroococcum* exhibited activity against

Aspergillus flavus, Aspergillus terrus and Fusarium oxysporum. This report was correlated with the present study where s*Azotobacter sps* exhibited antifungal activity against *Sclerotium sps* (6±0.2mm) and *Alternaria sps* (30±0.4mm).

Most of the developing countries do not have the industrial development, which helps to subsidize high input agriculture in developed countries. So scientists are exploring new ways of meeting the nutritional needs of crop plant aiming at high productivity. Soil microorganisms like bacteria and fungi have a particular important role in exploration of these new approaches. One of the beneficial activities of the organism is the production of siderophores. So, siderophore producing organisms will be making the soil fertile and they also have antifungal activity against phytopathogens (Girish *et al.,* 2010).

Siderophores are non-porphyrin, non-protein compounds that bind iron and their synthesis is repressed when this element is abundant. Most strains of bacteria, actinomycetes and fungi produce siderophores under iron limitation conditions. Siderophores chelate, the ferric ions with a high specific activity and serve as vehicles for the transport of irons (Fe^{3+}) into the cell. Most of the siderophores have hydroxamate, catechol or carboxylate ligands.

The economic stability of India is dependant on the agricultural yield. Great deal of research carried out in the last 50 years by agricultural scientists has its major thrust on increasing crop productivity. Microbial metabolite production and their applicability in controlling plant diseases is gaining momentum in agriculture. The scope of developing these microbial metabolites for commercial pesticides as an alternative to chemical fungicides is gaining importance due to increased concerns on environmental pollution, pathogen resistance and high plant protection costs. Siderophores enable bacteria to take up iron under conditions of limited availability of the element in the environment. They are responsible for the dissolution, chelation and transport of iron (III) into the cell. Siderophore-mediated and antibiotic-mediated suppression of soil-borne plant diseases are the two most studied mechanisms involved in biocontrol by *Pseudomonas*. A further study can be carried out based on different factors. Thus the present studies serve as a good indication of siderophore producing bacteria as a biocontrol agent. A further study in this field can bring about a solution for the problems faced in the field of agriculture.

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