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Antagonistic Activity of Fluorescent *Pseudomonads* against a Polyphagous Soil Born Plant Pathogen – *Sclerotium Rolfsii*

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Abstract

A total of 35 fluorescent *Pseudomonad* strains were isolated from forest litter of seshachalam hill range. Out of 35 bacterial isolates 19 were found to be antagonistic to *Sclerotium rolfsii* in *in vitro* conditions. The growth inhibition zone of *Sclerotium* by fluorescent *Pseudomonads* on potato dextrose agar varied from 4-9 mm with an average of 6.2 mm. Among the isolated fluorescent *Pseudomonads PSTPT13* found to be most potential. All the 19 isolates were characterized morphologically, biochemically and functionally. The mechanism of fungal toxicity was also observed by the production of HCN, siderophore and cell wall degrading enzymes. In this study we observed fungistatic activity is mainly due to the production of HCN, Siderophore and protease enzyme. This is the first report on fluorescent *Pseudomonads* isolated from forest litter of Seshachalam hill range the first ever biodiversity reserve of India with antagonistic activity against *Sclerotium*.

Keywords: Polyphagous; Antagonism; *Fluorescent pseudomonads; Sclerotium rolfsii*

Introduction

Sclerotium rolfsii is a polyphagous soil borne pathogen infecting over 500 plant species worldwide causing huge losses. Though the fungus is seed and soil borne; soil borne inoculum is more important in causing infection and disease development. For the soil borne pathogens, use of fungicides is not practical due to exorbitant cost and environmental hazards involved. Hence integrated management of the disease using biocontrol agents and chemicals is the best. The pathogen is distributed in tropical and subtropical regions of the world where high temperature prevails. The fungus has a wide host range of 500 species in about 100 families including vegetables, flowers, cereals, forage plants and weeds. Some of the common hosts include Legumes, Crusifers, Tomato, Chrysanthemum, Peanuts and Tobacco in which the pathogen causes a great economic loss. In ground nut, it caused 25% of seedling mortality in the cultivar JL- 24 at parbhani [1]. Thiribhuvanamal et al. [2] observed that 30% of crop loss in tomato was due to *S. rolfsii*. Harinath Naidu [3] reported that *S. rolfsii* caused 40.05% mortality in *Crossandra* in Chittoor district of Andhra Pradesh.

Several chemical pesticides are used to manage this disease [4-6]. Indiscriminate use of chemical pesticides in modern agriculture has resulted in the development of several problems such as pesticide resistance in pest resurgence of target and non-target pests, destruction of beneficial organisms like honey bees and chemical residues in food, feed and fodder. More over fungicidal application as seed or soil treatment however has been found to be ineffective against these pathogens as the propagules are capriciously distributed in the soil and often beyond the reach of chemicals [7]. Biological control therefore holds a promise as a strategy for disease management and it is environment friendly too. Antagonistic bacteria especially fluorescent pseudomonads have been widely used against a number of phyto-pathogens [8,9]. For successful functioning of introduced microbial bio-inoculants and their influence on soil, health efforts have been made to explore soil microbial diversity of indigenous community their distribution and behavior in soil habitats [10]. Seshachalam hills that forms part of the Eastern Ghats which spreads in parts of Chittor and Kadapa districts in Indian state of Andhra Pradesh. The hill range has been declared as first ever biodiversity reserve in Andhra Pradesh by Union Ministry of Environment and forests recently in 2010. Keeping this in view and the growing importance of biological control agents, the present study was carried out. The main objective was to evaluate the biocontrol efficiency of indigenous fluorescent psuedomonads against *Sclerotium rolfsii*. There appears to be no report on the antagonistic effect of fluorescent pseudomonads isolates from seshachalam hills of Chittor district.

Materials and Methods

Collection of soil samples

Soil samples were collected from forest litter of Seshachalam hills, Andhra Pradesh. Randomized block design was employed to collect the samples. Collected soils were sealed in sterile polyethylene bags.

Isolation of bacteria

One gram soil from different sampling sites was placed in 9 ml of saline solution and incubated for 2 hours in an orbital shaking incubator at 180 rpm. Later a loop of the resulting bacterial suspension was spread plated on King's B Agar medium [11] and incubated at 37°C. After 2 days the colonies were screened for fluorescence under UV light (366 nm).

Selection of antagonistic bacteria

A total of 35 bacterial isolates obtained from litter were screened for antagonism against *Sclerotium rolfsii*. Antagonism of bacteria against *Sclerotium* was examined using a modified method of Montealerge et al. [12]. A loopful of culture from each purified bacterial isolate was inoculated into 50 ml of King's B agar broth and incubated for 48 hours

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at 37°C. Subsequently, 100 µl of bacterial suspension of each isolate was placed on different 10 mm diameter sterile paper discs (Whatman, UK) four different discs were spaced around a central 10 mm plug of 2 day old *Sclerotium* on potato dextrose agar. The plate was incubated for 7 days at 30°C and the size of the inhibition zone of hyphal growth was determined. Bacteria which showed no suppression of fungal growth were discussed. The inhibition test was replicated three times. The active bacterial isolates were preserved in 20% glycerol at -20°C.

19 bacterial isolates showed antagonistic activity in the pre evaluation test were subjected to further confirmation by the standard co-inoculation PDA technique [13]. The bacterial plugs (6 mm diameter) were removed from a 48 hrs grow culture on king's B plate. The bacterial plugs were transferred to the surface of PDA plates, which had been inoculated with fungal spore suspension (or) mycelial plug. After the plates were incubated at 28°C for 3 days radial growth percentage of the test fungi was measured using following formula:

RI(%)= Rc-Ri/Rc *100

where,

RI=Radial growth inhibition

Rc= Radial growth in control plates

Ri= Radial growth in incubated plates

Phenotypic and biochemical characteristics of antagonistic bacteria

Phenotypic characters like grams reaction, levan production, optimum growth temperature, fluorescence, Gelatin hydrolysis, citrate utilization test, Oxidase, β-galactosidase activity, Catalase test, Indole production were conducted for 19 bacterial isolates which showed antagonistic activity.

Assimilation of carbohydrates

Substrate utilization profiles were tested using Hi carbohydrates (Himedia, Mumbai, India) Cell suspension was established in sterile saline using 24 hours grown culture. The density of the suspension was made to 0.5 O.D at 620 nm. An aliquot of 50 µl of this suspension was inoculated at 30°C for 48 hrs and on to the substrates such as lactose, xylose, fructose, Galactose, glycerol, Trehalose, Mannitol, Sucrose, Ribose, Glucose and incubated at 30°C for 48 hrs.

Cell wall degrading enzymes

Cell wall degrading enzymes like protease, cellulase and pectinase were also studied [13,14].

Plant growth promoting traits

Phosphate solubilizing enzyme production was assessed by using pikovskaya medium [15]. For all the isolates siderophore production was also observed.

Volatile toxicity of antagonistic fluorescent *Pseudomonads*

In order to identify volatile toxicity in antagonistic strains HCN production test was conducted by using filter paper pre soaked in picric acid solution. The productions of volatile toxic compounds were assured by taking the following criteria.

No colour change	- No HCN production	-
Brownish coloration	- Weak HCN Production	- Weak HCN production
Brownish to Orange	- Moderate HCN production	- Moderate HCN production
Complete Orange	- Strong HCN production	- Strong HCN production

Numerical taxonomy

A binary code matrix of each strain was constructed linearly composing presence (1) /absence (0) of data derived from the biochemical tests of antagonistic fluorescent *Pseudomonads*. SPSS.16 version was used to compute similarities or dissimilarities in the form of an average taxonomic distance which was used to perform hierarchical, agglomerative and neighbor joining clustering. Dendrogram was constructed from the similarity matrix obtained in the SPSS.16 program.

Results

A total of 35 strains of fluorescent *Pseudomonads* were isolated from forest litter. Most of the isolated bacteria developed pale green to dark green pigmentation on king's B agar and released a sweet grape like odour and pyocyanine pigment. This was an indication that isolated bacteria were pseudomonads. By exposing the plates to UV light fluorescent pseudomonads were picked up.

Among them a total of 19 isolates were found to inhibit mycelial

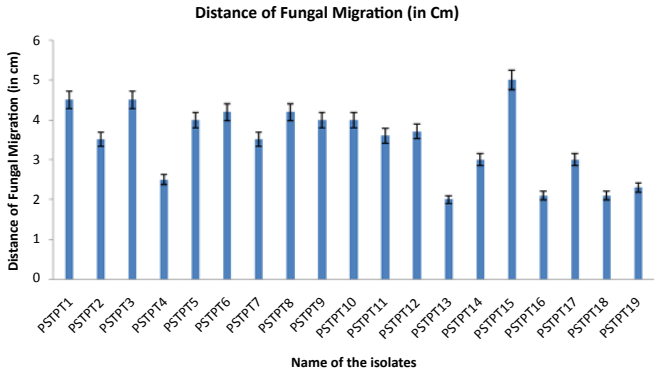

Figure 1: *Sclerocium* mycelial migration by antagonistic fluorescent *Pseudomonads*.

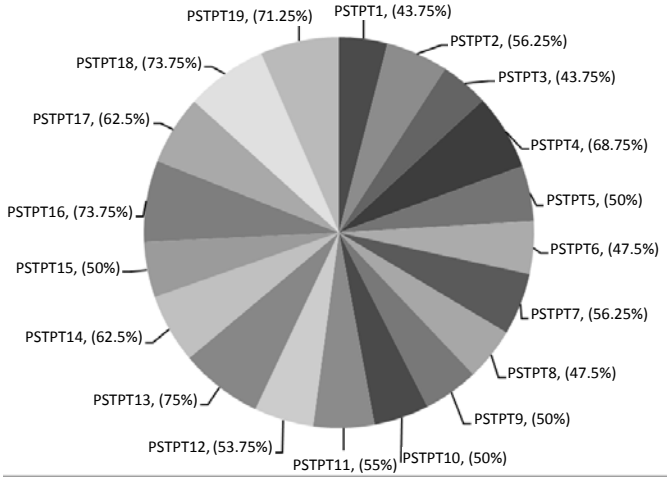

Figure 2: Radial growth inhibition percentage against *Sclerotium rolfsii* by antagonistic fluorescent *Pseudomonads*.

Phenotypic characters	Isolates(PSTPT)																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Levan production	-	-	+	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+
Optimum growth temperature	25°C	25°C	25°C	25°C	25°C	25°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C	40°C	40°C	40°C	40°C	0°C	0°C
Gelatin hydrolysis	+	+	-	-	-	-	-	+	+	+	+	+	+	+	-	-	+	+	-
Citrate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ONPG	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	-
Glycerol	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	-	+	+	+	+	-	+	+	-	-	+	-	-	+
Mannitol	+	-	+	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+
Sucrose	+	+	-	-	+	+	-	+	+	+	+	+	-	-	-	+	+	+	+
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 1: Phenotypic and Biochemical characteristics of antagonistic fluorescent *Pseudomonads*.

Name of the Isolate	Pectin	Cellulase	Protease
PSTPT1	+-	-	+
PSTPT2	+	-	+
PSTPT3	+	-	+
PSTPT4	+	-	+
PSTPT5	+	-	+
PSTPT6	+	-	+
PSTPT7	+	-	+
PSTPT8	+	-	+
PSTPT9	+	-	+
PSTPT10	+	-	+
PSTPT11	+	-	+
PSTPT12	+	-	+
PSTPT13	+	-	+
PSTPT14	+	-	+
PSTPT15	+	-	+
PSTPT16	+	-	+
PSTPT17	+	-	+
PSTPT18	+	-	+
PSTPT19	+	-	+

Table 2: Production of cell wall degrading enzymes from antagonistic Fluorescent *Pseudomonads*.

growth on PDA plates in a triplicate assay. All antagonistic isolates produced an inhibition zone varied from 2.0 cm to 4.5 cm (Figure 1) and radial growth inhibition percentage from 43.75% to 73.75%. However isolates PSTPT16 and PSTPT18, were found to be potential antagonists against *Sclerotium* with almost 73.75% of radial growth inhibition (Figure 2) percentage.

P**henotypic Characterization of fluorescent Pseudomonads:** All bacteria showed positive results for fluorescence, oxidase and catalase. A total of 11 isolates were positive for levan production and 8 isolates were negative .Most of the isolates were able to grow at optimum temperature from 25°C-30°C. However isolates PSTPT 15, 16 and 17 were grown at 40°C. Only two isolates showed optimum temperature at 0°C. About 57% of the isolates were positive for gelatin liquefaction and 43% were negative. All the isolates were able to utilize citrate except isolate PSTPT 16. Further Isolate17 was the only positive for ONPG production. All strains are able to utilize glucose but exhibited varying degrees of utilization towards other carbon sources such as lactose, mannose, galactose, ribose etc. (Table 1).

Production of Cell wall degrading enzymes

The functional characterization demonstrated the diversity and fungitoxic ability of antagonistic fluorescent Pseudomonads. These strains did not produce cellulase. But all the strains are able to produce pectin and protease (Table 2).

Mechanism of biological control

In vitro HCN by the antagonistic strains was tested by the picric acid assay method and 78.9% isolates were found to produce HCN. However isolate PSTPT 5, 6 and 19 were strong HCN producers which turned the colour of the filter paper in to complete orange. Three isolates were moderate HCN producer and 7 were weak producer of HCN (Table 3).

Name of the Isolate	Volatile toxic substance (HCN) Production	Extent of HCN production
PSTPT1	Brownish to orange coloration	Moderate
PSTPT2	Brownish coloration	Weak
PSTPT3	Brownish to orange coloration	Moderate
PSTPT4	Brownish to orange coloration	Moderate
PSTPT5	Complete orange coloration	Strong
PSTPT6	complete orange coloration	Strong
PSTPT7	No coloration	No
PSTPT8	No coloration	Weak
PSTPT9	No coloration	Weak
PSTPT10	Brownish	Weak
PSTPT11	No coloration	No
PSTPT12	Brownish	Weak
PSTPT13	No coloration	No
PSTPT14	Brownish	Weak
PSTPT15	Brownish	Weak
PSTPT16	Brownish	Weak
PSTPT17	Brownish	Weak
PSTPT18	No coloration	No
PSTPT19	Complete Orange	Strong

Table 3: Volatile toxicity of antagonistic Fluorescent *Pseudomonads*.

Case	PSTPT1	PSTPT2	PSTPT3	PSTPT4	PSTPT5	PSTPT6	PSTPT7	PSTPT8	PSTPT9	PSTPT10	PSTPT11	PSTPT12	PSTPT13	PSTPT14	PSTPT15	PSTPT16	PSTPT17	PSTPT18	PSTPT19
PSTPT1	1.000	.549	.222	.405	.545	.632	.314	.627	.424	.671	.738	.249	.198	.813	.497	.497	.588	.545	.839
PSTPT2	.549	1.000	.471	.506	.372	.588	.424	.198	.513	.738	.588	.222	.753	.663	1.000	.588	.863	.839	.471
PSTPT3	.222	.471	1.000	.152	.314	.405	.055	.549	.198	.706	.663	.458	.392	.738	.671	.405	.506	.458	.663
PSTPT4	.405	.506	.152	1.000	.497	.279	.249	.588	.222	.753	.458	.346	.424	.671	.718	.094	.671	.497	.588
PSTPT5	.545	.372	.314	.497	1.000	.835	.545	.458	.627	.753	.588	.497	.663	.671	.941	.718	.888	.632	.458
PSTPT6	.632	.588	.405	.279	.835	1.000	.176	.405	.152	.718	.545	.588	.627	.632	.680	.000	.497	.443	.671
PSTPT7	.314	.424	.055	.249	.545	.176	1.000	.372	.131	.888	.738	.405	.588	.706	.753	.176	.588	.545	.627
PSTPT8	.627	.198	.549	.588	.458	.405	.372	1.000	.198	.706	.549	.458	.624	.506	.888	.405	.627	.588	.424
PSTPT9	.424	.513	.198	.222	.627	.152	.131	.198	1.000	.627	.471	.506	.314	.424	.706	.152	.285	.222	.588
PSTPT10	.671	.738	.706	.753	.753	.718	.888	.706	.627	1.000	.458	.863	.424	.671	.588	.835	.671	.632	.706
PSTPT11	.738	.588	.663	.458	.588	.545	.738	.549	.471	.458	1.000	.706	.624	.372	.888	.545	.738	.458	.663
PSTPT12	.249	.222	.458	.346	.497	.588	.405	.458	.506	.863	.706	1.000	.549	.888	.835	.443	.784	.753	.588
PSTPT13	.198	.753	.392	.424	.663	.627	.588	.624	.314	.424	.624	.549	1.000	.696	.372	.506	.471	.424	.725
PSTPT14	.813	.663	.738	.671	.671	.632	.706	.506	.424	.671	.372	.888	.696	1.000	.753	.632	.458	.405	.738
PSTPT15	.497	1.000	.671	.718	.941	.680	.753	.888	.706	.588	.888	.835	.372	.753	1.000	.680	.497	.718	.888
PSTPT16	.497	.588	.405	.094	.718	.000	.176	.405	.152	.835	.545	.443	.506	.632	.680	1.000	.632	.443	.545
PSTPT17	.588	.863	.506	.671	.888	.497	.588	.627	.285	.671	.738	.784	.471	.458	.497	.632	1.000	.249	.839
PSTPT18	.545	.839	.458	.497	.632	.443	.545	.588	.222	.632	.458	.753	.424	.405	.718	.443	.249	1.000	.706
PSTPT19	.839	.471	.663	.588	.458	.671	.627	.424	.588	.706	.663	.588	.725	.738	.888	.545	.839	.706	1.000

Table 4: Similarity matrix for biochemical characteristics of antagonistic fluorescent *pseudomonads*.

Numerical analysis

Numerical analysis of phenotypic characteristics revealed a high degree of polymorphism. All the strains were grouped in to two different phenons (Figure 2). The similarity range among antagonistic strains was 0.37-0.94 (Table 4). The first phenon consists of six strains (PSTPT 6, 16, 9, 4, 3, 7) and second phenon consists of a total of 13 strains (Figure 3).

Production of plant growth promoting traits

All the strains were able to produce plant growth promoting traits like phosphatase and siderophore (Table 5).

Discussion

Soil is considered as a store house of microbial activity. These functions of soil microorganisms are central to the decomposition process and nutrient cycling. They play an important role in soil processes that determine plant productivity. The hill chain of Eastern Ghats is recently recognized as Biodiversity reserve. In spite of this there is no report on the diversity of fluorescent Pseudomonads in this region. Among the 35 isolates 19 isolates exhibited antagonistic activity against *Sclerotium rolfsii*. The biological control of soil borne pathogens with antagonistic bacteria particularly *Pseudomonas sp.*, belonging to plant growth promoting Rhizobacteria has received prominent attention because of the dual role of these bacteria in plant growth promotion and diseases control [16]. Fluoroscent pseudomonad strains found to be effective against *Sclerotium rolfsii* [17]. Cook [9] reported that certain plant associated bacteria particularly fluorescent pseudomonads have been exploited for suppression of crop diseases. Similar results on the effectiveness of fluorescent pseudomonads against plant pathogenic fungi like *Fusarium, Rhizoctonia, Sclerotium, Pythium* [18-24] and bacteria like *Ralstonia solanacearum* and *Xanthomonas campestris* have been reported earlier .The effectiveness of fluorescent pseudomonads against multiple pathogens are also known.

Our work demonstrates the ability of fluorescent pseudomonads to produce fungistatic metabolites such as siderophores, HCN and protease by the bacteria. *Pseudomonas sp.* are known to produce volatile compounds. One such metabolite is HCN. Afsharmanesh

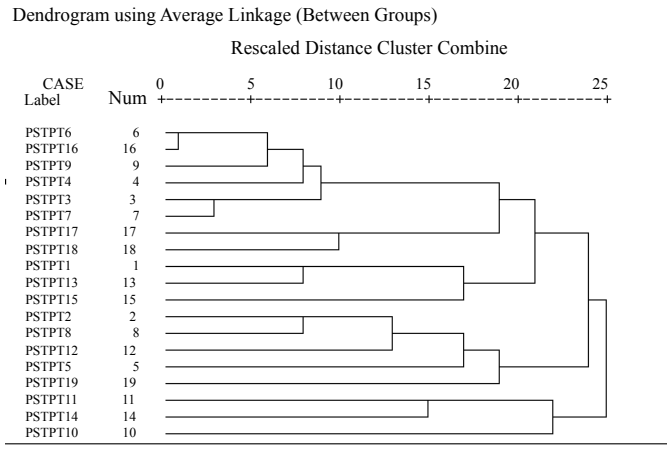

Figure 3: Dendogram using Average Linkage (Between Groups).

Name of the Isolate	Phosphate solubilizing enzyme	Siderophore production
PSTPT1	_	+
PSTPT2	-+	+
PSTPT3	+	+
PSTPT4	_	+
PSTPT5	_	+
PSTPT6	+	=
PSTPT7	+	+
PSTPT8	+	+
PSTPT9	+	=
PSTPT10	+	+
PSTPT11	_	+
PSTPT12	_	+
PSTPT13	_	+
PSTPT14	_	_
PSTPT15	_	+
PSTPT16	_	_
PSTPT17	+	+
PSTPT18	+	+
PSTPT19	+	+

Table 5: Growth promoting traits of antagonistic fluorescent *pseudomonads*.

et al. [23] suggested that fungal growth is mainly inhibited by HCN production and siderophore production. Apart from the biocontrol potential, fluorescent pseudomonads possess other functional properties like, mineral phosphate solubilisation, production of plant growth promoting substances and enzyme activity. Besides testing the fluorescent pseudomonads for beneficial functions like Phosphate solubilisation, PGPS production and biocontrol potential, their ability to produce commercially important enzymes like protease and chitinase was also examined. Out of the 19 antagonistic isolates, all the isolates are able to produce protease but none of the isolates produced Chitinase and cellulase. The results of present investigation indicated a high degree of functional diversity among antagonistic fluorescent pseudomonads isolated from forest litter of Eastern Ghats.

Conclusion

Strains reported in this study suppress Sclerotium effectively by single or multiple modes of action. Results also revealed that the antifungal activities and other plant beneficial traits appear to be the general and genetically dispersed traits of fluorescent pseudomonads. Knowledge on phenotypic and functional traits of antagonistic bacteria will help to determine their fitness for successful bio-fertilization and biological control. This study reveals for the first time the presence of bacteria with antagonistic activity against *Sclerotium rolfsii* in Eastern Ghats forest litter an untapped resource. It also provides essential information to develop broad spectrum biocontrol agent.

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