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Antagonistic activities of *Pseudomonas fluorescens* and strain improvement of *Rhizobium* species

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Abstract

The *Pseudomonas fluoruescens* Migula is a well known soil borne and non pathogenic biological control agent. *P. fluorescens* has been found to show inhibition to the phytopathogens of *Rhizoctonia solani* Kuhn, *Bipolaris oryzae* Shoemaker, *Cochliobolus lunatus* Nelson, *Alternaria brassica* Berk, *Aspergillus niger* Van Tiegh, *Fusarium oxysporum* Schlechtendal and *Trichothecium roseum* Link. The antagonistic activity of the crude extract was studied by duel plate and poison plate assays. This results proved that the crude extract have antagonistic potential against fungal pathogens. The crude extract was purified by Thin Layer Chromatography (TLC) and showed the different coloured bands under Ultra Violet (UV). These crude antibiotic substance is also effective against the spores germination of phytopathogens that studied by spore germination assay. The strain improvement was also carried out on the nitrogen fixer *Rhizobium* species De Lajudie, using plasmid DNA transformation technique. The plasmid DNA from *P .fluorescens* was transformed to competent cells of *Rhizobium* species and the transformations was obtained and showed both biological control.

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Introduction

During the course of evaluation of agriculture, many empirical practices were evolved, and some probably involved biological control. Biological control means control of disease through any living microorganism. The early attempts at direct application of biological control of plant pathogens were made in 1920-1940. Hartley (1921) inoculated forest nursery soils with 13 antagonistic fungi in an attempt to control damping-off of pine seedlings. Henry (1931) found that eight cultures of actinomycetes¸ bacteria and fungi from soil, when inoculated in various combinations in sterilized soil, resulted in slightly less disease caused by *Helminthosporium sativum* on wheat. Following the laboratory tests of Henry (1931), Menzies (1959) showed in pot tests that the antagonistic microbiota of a soil suppressive to potato common scab also could be transferred to a condusive soil, making it suppressive. Alagesaboopathi (1990) reported the antagonistic potential of *Penicillium pinophilum* to the phytopathogenic fungus *Rhizoctonia solani.* There have been numerous reports of successful use of antagonistic fungi to control soil borne fungi (Wells *et al*., 1972; Harman *et al*., 1981; Elad *et al*., 1981; Nelson *et al.,* 1983; Anand *et al.,* 2010; Dev and Davande 2010; El-Mohamedy *et al.,* 2011).

Alagesaboopathi (1994) reported the biological control of damping off diseases of cotton seedling. Selvankumar *et al.,* (2007) reported that the seed bacterization and biocontrol activity of *Pseudomonas fluorescens*. *Pseudomonas* SP. is ubiquitous in agricultural soils, well adapted to growing in the rhizosphere. *Pseudomonas* possesses various kinds traits that make them well suited as biocontrol and growth-promoting agents (David, 2007). Activation of rice plant growth against *Rhizoctonia solani* using *Pseudomonas fluorescens, Trichoderma* and salicylic acid (Anitha and Das, 2011).

Numerous microorganisms in recent times have been a focus of attention as plant growth promotion or biocontrol agents against many phytopathogens. Among these *Pseudomonas fluorescens, Rhizobium* spp, *Azotobactor* spp, *Bacillus subtilis, Aspergillus* spp., *Trichoderma* spp., and mycorrhizae have been studied and used extensively. These biocontrol agents hold promise and tend to reduce the damage due to plant pathogens as well as dependence on the use of hazardous chemicals for plant disease management (Singh *et al.,* 2003). Among several biocontrol agents, fluorescent pseudomonads, equipped with multiple mechanisms for biocontrol of phytopathogens and plant growth promotion, are being used widely (Banasco *et al*., 1998; Pierson *et al*., 1994). They produce a wide variety of antibiotics, chitinolytic enzymes, growth promoting hormones, siderophores and catalase and can solubilize phosphorous (Kraus and Loper, 1995; Seong and Shin, 1996., Rodriguez and Fraga, 1999).

The antagonistic mechanism is also responsible for biological control of pathogens involving competition, enzymatic hydrolysis and antibiosis. Mainly Rhizosphere microorganisms are ideal for the use of biological control agents, since the rhizosphere provides the front line defense for root against attack by pathogens. The microganisms, especially plant growth promoting rhizobacteria, include *Pseudomonas, Arthrobacter, Agrobacterium, Cellulomons* and *Bacillus* species (Utkhede, 1986). The present research work is aimed at strain improvement of *Rhizobium* species from the fluorescent Pseudomonads, as biological control agents and to understand the mechanisms involved in the antagonistic interactions among fungal pathogens. Strain improvement through molecular biology techniques like horizontal gene transfer will hold a key in developing novel agents for successful application in fields and for commericialization.

Materials and methods

One gram of soil samples were collected and serially diluted in sterile saline solution. The diluents were

spread over the sterile King's media (King *et al.,* 1954). The plates were inverted and incubated at 37°C for 24 hours. After incubation the colony morphology and pigment production were observed and noted. The root nodules were collected from the groundnut plant and surface sterilized in 0.1% of mercuric chloride solution for 5 min and washed thrice in sterile distilled water. The surface sterilized root nodules were crushed by using mortar and pistle and the extract was collected. Then the loop full of root nodule extract was streaked on YEMA (Yeast Extract Mannifol Agar) medium with 0.1% of 2.5 ml/l congored. The plates were inverted and incubated at 37°C for 4-5 days. After incubation the colour of the colony and its morphology was observed and noted.

The antagonistic activity of *P. fluorescens* was studied against *Bipolaris oryzae, Rhizoctonia solani, Alternaria brassica, Fusarium oxysporum, Cochliobolus lunatus, Aspergillus niger* and *Trichothecium roseum* by duel culture technique. The potato dextrose agar plates were prepared and inoculated with 4mm disc of the fungal pathogen on the plates. The bacterial colony was inoculated at opposite side in the well. The plates were incubated at 28°C for 3 days and measured the antagonistic activity against the fungal species with diameter and noted.

The 4 mm discs shaped agar block cut off from the potato dextrose agar plates with fungal cultures and it was placed in periphery of the plate. The opposite points of the fungal cultures location make a 4mm well and added different concentration of crude microbial extract such as 50µl and 100µl. The plates were incubated at 28°C and observed the inhibition efficiency of the fungus after the 5th day of incubation. The King's A and King's B media were used as selective media for identification and fluoresin pigment production of *Pseudomonas* SP. So the culture was streaked on the King's A and on King's B plates and they were incubated at 28°C. The King's A plats were used for pigment production and the King's B plates were used to observe under UV light for fluorescence emissions of pigment in culture (King *et al.,* 1954).

Biological control activity

Antagonistic potential of *P. fluorescens*

The antagonistic activity of crude microbial extract was determined by the spores and sclerotia of the test pathogens were suspended in sterile saline (0.85% NaCl) and treated with 10% antibiotic solutions. Control was maintained in sterile saline. After 24 hours the germination of the spores in relation to the control was observed under high power objective (100X) and photographed. The organism was screened for antibiotic production by isolation of the antibiotic and its partial purification and its effect on various pathogens.

Isolation of the antibiotics

The antibiotic from the microbial culture was isolated by Toohey *et al.,* (1965) method. The 48 hours old bacterial culture in LB medium was centrifuged at 5,000 rpm for 5 minutes. The supernatant was acidified benzene with 0.1% acetic acid. The benzene extracts were evaporated in vaccum and the antibiotic crystals recovered. The antibiotic crystals were dissolved in benzene and spotted on 20cmX20cm Fluorescent indicator TLC plates (Sigma). The plates were developed with Benzene: acetic acid (95:5,v/v) solvent system and observed under UV(254nm) for fluorescing and quenching bands.

Strain improvement by Plasmid DNA Transformation to Rhizobium species

Isolation of Plasmid DNA

Plasmid DNA was isolated according to the method previously described (Casse *et al.,* 1979). Overnight bacterial culture was grown to mid-log phase and was centrifuged at 10,000 rpm for 10 minutes at 4°C. The cells were washed twice in TE Buffer (Tris 0.05 M, EDTA 0.02M, pH 8.0). The cells were weighed approximately 100mg by wet weight method and suspended in 0.5ml of TE buffer to which 9.5ml of lysis

buffer (TE buffer with 1% SDS, pH 12.45) was added. The mixture was stirred with a magnetic stirrer at 100 rpm for 90 seconds and incubated at 30°C for 20-25minutes. The pH of this mixture was lowered between 8,5 and 8.5 by adding 0.6µl of 2M Tris buffer (pH 7.0) and the mixture was stirred at 100 rpm for 2 min. The lysate was adjusted to 3% w/v NaCl. After 30 minutes, 10ml of TE saturated phenol (1:1 v/v) was added. The two phases were mixed and the mixture was then centrifuged at 10,000 rpm for 5 minutes at 4°C. The clear aqueous phase was collected and to it 0.1ml of 0.3M sodium acetate and 2 volumes of ice cold 95% ethanol was added to precipitate the DNA. It was incubated at - 20°C for 12 hours.

The precipitated DNA was recovered by centrifugation at 10,000 rpm for 10minutes at 4°C. The tubes were air dried to remove ethanol and the plasmid DNA pellet was dissolved in 100µl TES buffer (Tris-0.005M, EDTA-0.005M, NaCl - 0.05 M and pH 8.0).

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using 1% agarose gel in 1X TAE running buffer with. The gel was stained in ethidium bromide solution (0.5µg/ml) for 30 minutes and de-stained the gel. The gel was visualized under UV transilluminator and photographed using gel documentation.

Transformation

Preparation of competent cell and transformation of *Rhizobium* species with plasmid DNA was reported by Manniatis *et al,* (1982). A single colony of *Rhizobium* species was inoculated into yeast extract mannitol broth and incubated overnight on a rotary shaker (250 rpm) at 28°C. The cells were harvested by centrifugation at 10000 rpm for 10 minutes at 4°C and suspended in 10ml of ice cold 100mM $CaCl_2$ and incubated in ice for 60minutes, followed by a similar treatment in 100mM $MgCl_2$ for 60 minutes. The cells were centrifuged at 10,000 rpm for 10 minutes at 4°C and the pellet was resuspended in 3ml of ice cold $CaCl_2$ (100mM) and left overnight. Then 20µg of plasmid DNA was added to 200µl competent cell suspension, mixed gently and kept on ice for 30 minutes. A heat shock at 42°C was given for 2 minutes and the cells were immediately transferred to ice bath for 1 hour. To this 1.8ml of sterile LB broth was added and incubated at 28°C in shaker for 1 hour for the cells to recover. The cell suspension (100µl) was transformed on to YEMA plates amended with antibiotics ampicillin and benzylpenicillin at the concentration of 25µg/ml. The colonies that appeared after 24 hours were isolated and screened for the presence of plasmid.

Results and discussion

The bacterial strain with antagonistic activity was isolated from the soil sample. It has shown to exhibit antagonistic activity against plant pathogens such as *Bipolaris oryzae*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Alternaria brassica*, *Cochliobolus lunatus, Aspergillus niger and Trichothecium reseum* (Table 1) (Fig. 1). The morphology of fungal pathogen in potato dextrose agar is showing and the *P.fluorescens* was isolated and identified by biochemical tests.

Table 1. Inhibition of fungal pathogen by *Pseudomonas fluorescens* (100 µl) by dual plate assay.

Fungal Pathogen	Inhibition zone diameter (mm)
Rhizoctonia solani	7
Bipolaris oryzae	4
Alternaria brassica	43
Cochliobolus lunatus	30
Trichothecium roseum	33
Fusarium oxysporum	38
Aspergillus nigher	6

Difference growth between control (Minus bacterium inoculum) and treated (with bacterial inoculum).

The *P.fluorescens* strain was studied in the antagonistic activity and the activity was observed. The zone of inhibition was measured (Table 1). It showed

the presence of three spots from the ultra violet (UV) absorbing bands and one UV quenching spot (Fig. 2).

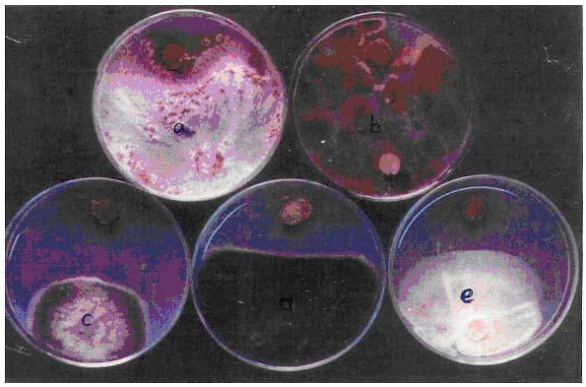


Fig. 1. Antifungal activity of *Pseudomonas fluorescens* against phytopathogens. a. *Rhizoctonia solani,* b. *Aspergillus nigher*, c. *Alternaria brassica,* d. *Cochliobolus lunatus*, e. *Trichotecium roseum*.

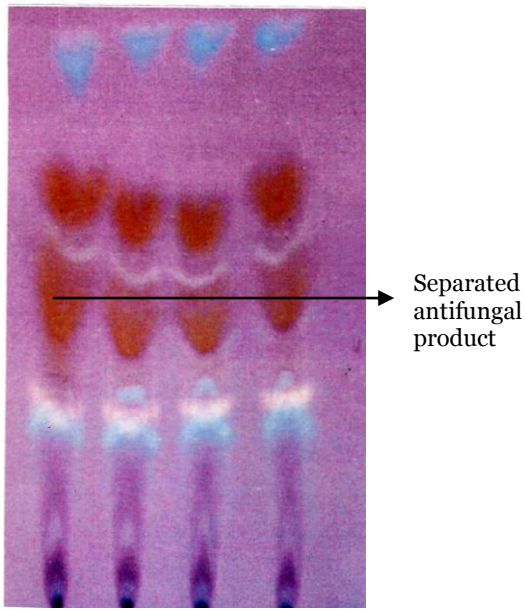
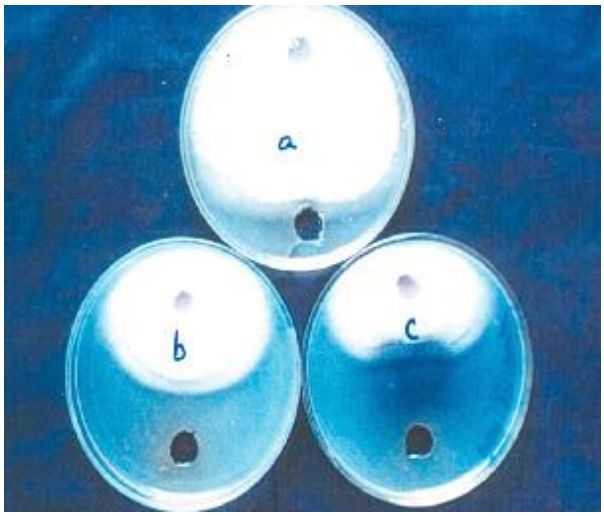


Fig. 2. Thin layer chromatogram of crude antibiotic extract.

The spores of the test pathogen were incubated with the antibiotic substances from the *P.fluorescens* for 24 hours. The 50 and 100µl of antibiotic substances and used for biocontrol assay against fungal pathogens (Fig. 3(a), 3(b)). The isolated *P.fluorescens* and *Rhizobium* species were studied its growth pattern on different antibiotic such as Benzyl penicillin, Chloramphenicol, and Ampicillin (25µ/ml) (Table - 2). The isolated *P.fluorescens* was studied with its plasmid profile. The plasmid DNA was isolated and its molecular weight (Kb) compared with λ DNA digest with Hind III enzyme. It showed approximately 4.3kb was observed on 1% of agarose gel (Fig. 4).

Fig. 3. Growth inhibition pattern of crude antibiotic against plant pathogen. A) *Fusarium oxysporum* - a. control, b. 50 µl of extract, c. 100 µl of extract.



B) *Biplaries oryzae* - a. control, b. 50 µl of extract, c. 100 µl of extract.

For strain improvement, the planed DNA from the *P. fluorescens* was transferred to *Rhizobium* species. Previously the *Rhizobium* species was studied with

antibiotic resistant sensitive pattern. The antibiotics were used as genetic marker for transformation. The transformants were initially selected as YEMA (Yeast Extract Mannitol Agar) plates amended with ampicillin (25µg/ml) and the replica plated as YEMA amended with chloramphenicol (25µg/ml) and benzyl penicillin (25µ/ml). The transforments were also screened with fungal pathogens for antagonistic activity (Fig. 5).

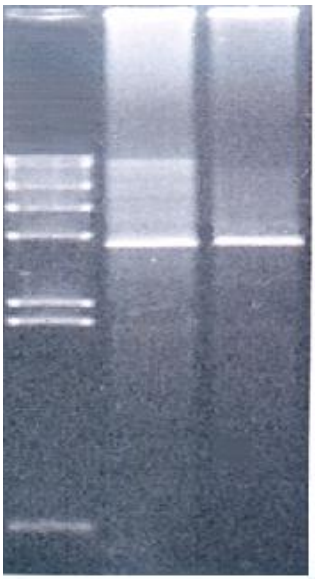


Fig. 4. isolation and visualization of plasmid DNA in Donor *(P.fluorescens*) and recipients (*Rhizobium* sp.). Lane 1 - λ DNA Digest I and Hind III, Lane 2 - Plasmid of transformants, Lane 3 - Plasmid of transformants.

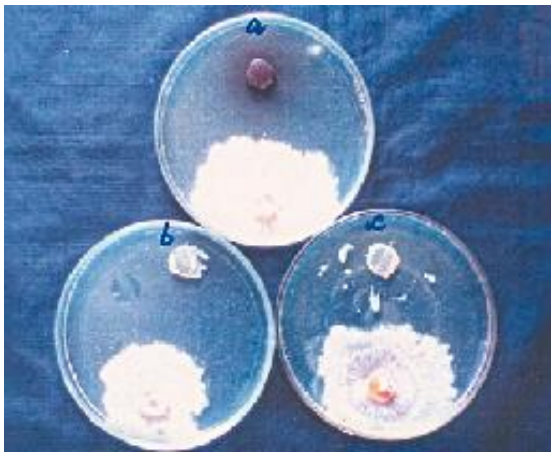


Fig. 5. Antagonistic potential *Rhizobium* transformants against plant pathogen. a. control, b and c transformed *Rhizobium* species**.**

Pseudomonas species especially the fluorescent have revolutionized in the field of biocontrol, as they emerge as the largest and potentially most promising group of bacteria that are well known as antagonists of many fungi (Kloepper *et al.*, 1981; Krishnamurthy and Gnanamanikam, 1998). Goud and Muralikrishnan (2009) have reported the biological control of three phytopathogenic fungi by *P. fluorescens* isolated from rhizosphere. Negi *et al.,* (2005) reported that the cold-tolerant fluorescent *Pseudomonas* isolates from Garhwal Himalayas as potential plant growth promoting and biocontrol agents in pea. Alagesaboopathi and Subramanian (2007) have reported the biological control of damping-off disease of *Gossypium hirsutum* L. seedling. The introduction of serviceable microorganisms for the biological control of soil borne plant pathogens has considerable potential in Agriculture (Weller *et al.,* 2007). Bacteria introduced on cotton, potato seed pieces, wheat and otgher species (Ganeshan and Manoj, 2005) have increased plant growth or decreased severity of root diseases. *P.fluorescens* is considered as biological control agent against several root diseases (Ursula *et al.,* 2000). Alagesaboopathi (1994) reported the describing antagonistic effects of *P. pinophilum* against plant pathogenic fungi. In another research. Integrated control of fruit rot and powdery mildew of chilli using the biocontrol agent *Pseudomonas fluorescent* and a chemical fungicide (Anand *et al.,* 2010). Dev and Dawande (2010) reported that the biocontrol of soil borne plant pathogen *Rhizoctonia solani* using *Trichoderma* spp. and *Pseudomonas fluorescens*. In another investigation, effect of using biocontrol agents on growth, yield, head quality and root rot control in *Broccoli* plants (El-Mohamedy *et al.,* 2011). They are logical candidates for biocontrol by competition for nutrients, especially against the slow growing pathogenic fungi (Weller, 1980). The present research work focuses the antagonistic effect of *P. fluorescens* as a novel broad - spectrum biocontrol agent, showing plasmid mediated antibiotic production.

Table 2. Sensitive and resistant pattern of isolated bacterial species against different antibiotics.

Antibiotic 25 mg/ml	*Pseudomonas fluorescens*	*Rhizobium* species
Benzyl pencillin	+	-
Kanamycin	-	-
Ciprofloxacin	-	-
Ampicillin	+	-
Amoxycilin	+	-
Rifampicin	-	-
Streptomycin	+	+
Chloramphenicol	+	-

+, Presence of growth

-, No activity

Pseudomonas population is the indigenous in the rhizosphere near the root base. The rhizosphere sheath is maximum probability of *Pseudomonas* due to nutrient availability and space availability for the microbes (Labeda *et al.*, 1976). It also highlights the antagonistic effects of fluorescent *Pseudomonas* used as biocontrol agent showing plasmid mediated antibiotic production. and it showed the successful biocontrol agent that requires broad spectrum of biocontrol activity against various pathogens.

The isolated soil borne *P. fluorescences* showed antagonistic activity of a wide range of paddy (*Rhizoctonia solani*, *Bipolaris oryzae*) and vegetable and fruit crop pathogens (*Curvularia* species, *Alternaria* species, *Fusarium oxysporum* and *Trichothecium roseum*). The inhibition of mycelial growth was transient but conidial inhibition was distinct and irreversible.

This study also reveals the antifungal metabolites isolated from the biocontrol agent *P. fluorescens*. The metabolites extracted and separated in thin layer chromatography, which showed different colour pattern under U.V. irradiation. The metabolites in *Pseudomonas* species evidenced by the work of Tombolini *et al.*, (1999), suggested that the antifungal metabolities including antibiotics chloroaphine, Oxychlororaphine and phenazine-1 carboxilic acid which gives green pigment and different peaks at 200, 360 and 420nm in adsorption spectrum analysis. The antibiotics are produced by *P. fluorescens in vitro* with pure cultures. The pure antibiotics directly inhibited spore germination and growth of the pathogen and suppressed disease.

Genetic engineering techniques to modify the kinds and amount of antibiotics produced by the strains and to introduce and express antibiotic biosynthetic genes in non producer organisms that have other desirable attributes will have immediate value as research tools and will represent a key to future generations of improved biocontrol agents.

Taking this into consideration and as the antibiotic production of the *P. fluorescens* was mediated by a plasmid DNA transformation experiments to transfer this biosynthetic gene to a non producer organism *Rhizobium* species. The transformants obtained and showed both biocontrol activity. Similar trails of mobilizing and expressing biosynthetic genes in non-producer organisms were carried out by Thomashow *et al.* (1990). The findings of the present study clearly indicate that *P. fluorescens* used here are potential candidate for biological control activities through strain improvement techniques.

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