

Biological control of three phytopathogenic fungi by *Pseudomonas fluorescens* isolated from rhizosphere.

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Citation

M Goud, V Muralikrishnan. *Biological control of three phytopathogenic fungi by Pseudomonas fluorescens isolated from rhizosphere.*. The Internet Journal of Microbiology. 2008 Volume 7 Number 2.

Abstract

Plant protection is an important area which needs attention since most of the hazardous inputs added into the agricultural system are in the form of plant protection chemicals. *Pseudomonas fluorescens* possess a variety of promising properties which make it a better biocontrol agent. The objectives of the present study were to isolate *P. fluorescens* from soil, to check its antagonistic activity and effect of its secondary metabolites on three fungal plant pathogens by in vitro techniques. *P. fluorescens* was isolated from rhizosphere soil on King's B medium and its antagonistic effect on three fungal plant pathogens was studied in vitro. Its antagonistic activity was checked by co-inoculation with the fungal isolates. In pour plate method, *P. fluorescens* on coinoculation with fungal pathogens decreased their growth rate. Maximum inhibition was observed in *Pythium ultimum* (80%) followed by *Macrophomina phaseolina* (70%) and *Pyricularia oryzae* (50%). Effect of the separated secondary metabolites on the fungal growth by broth dilution technique and antifungal activity by agar well diffusion technique was studied. *P. fluorescens* produces a broad-spectrum antifungal compound, which inhibits a variety of plant Pathogenic fungi and inhibits *Pythium ultimum* more when compared to other plant pathogens in the present study. Further investigations on the type of antifungal components and in vivo experiments will make *P. fluorescens* as one of the most suitable biocontrol agent in suppressing the phytopathogenic fungi and replace chemical fungicides.

INTRODUCTION

Research on a more sustainable and environmental friendly agriculture system is the need of the hour, as there is a growing concern on the deteriorating quality of the environment as a result of the intensive agriculture. Despite the many achievements of modern agriculture, certain cultural practices have actually enhanced the destructive potential of diseases. It is true that a huge number of fungal diseases plague the crop plants throughout the year when a farmer fails to take proper preventative measures. Plant disease control, therefore has become heavily dependent on fungicides to combat the wide variety of fungal diseases.

A land mark study published by the us environment protection agency indicates that in the US alone 3000-6000 cancer cases are induced annually by pesticide residues on foods and another 50-150 by exposure to pesticides during application (Goud, 2004). This type of findings have made the governments of many countries increasingly aware of the drawbacks of many chemical pesticides in terms of their effect on the environment, as well as on the grower and consumer of agriculture products.

Plant protection is an important area, which needs attention since most of the hazardous inputs added into the agricultural system are in the form of plant protection chemicals. Studies aimed at replacing pesticides with environmentally safer methods are currently being conducted at many research centers.

Biological control of plant diseases assumes a greater importance at this juncture. The efficient use of rhizosphere microorganisms to control plant pathogens has been reported worldwide in different plants. Soil pseudomonads possess a variety of promising properties, which make them better biocontrol agents (Cook, 1993). In the last two decades endophytic bacteria especially *Pseudomonas fluorescens* have received considerable attention as potential biocontrol agent of a number of soil borne pathogens. Unfortunately, the seemingly inherent variable performance of most biocontrol strains between field locations and cropping seasons has hampered commercial development, and relatively few biological agents are registered for use in production agriculture (Cook, 1996).

The objectives of the present study were to isolate *P. fluorescens* from soil, to check its antagonistic activity and

effect of its secondary metabolites on three fungal plant pathogens by in vitro techniques.

MATERIAL AND METHODS

BACTERIAL CULTURE

Rhizosphere soil suspension was prepared and 10⁻⁶ and 10⁻⁷ dilutions were spread on King's B medium (James, 1990). After 2 days incubation the colony which initially colourless and later produced fluorescent pigment when observed under U.V light were subcultured in slants. *Pseudomonas fluorescens* is characterized based upon its biochemical tests (Table 1)

FUNGAL CULTURES

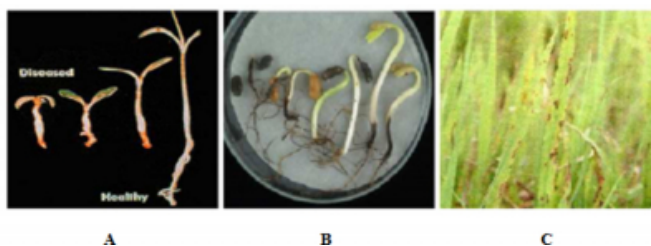
Fungal plant pathogens namely *Phythium ultimum* (damping off) (fig 1A), and *Macrophomina phaseolina* (root rot) (fig 1B), *Pyricularia oryzae* (blast disease) (fig 1C) were obtained from the Department of Plant Pathology, Faculty of Agriculture, Annamalai University, India. They are grown on potato dextrose agar (PDA) at 23-25°C.

Figure 1- A: *Phythium ultimum* exhibiting damping off disease.

B: *Macrophomina phaseolina* exhibiting rot disease.

C: *Pyricularia oryzae* exhibiting blast disease.

Figure 1



SCREENING FOR ANTAGONISTIC ACTIVITY

CROSS STREAK ASSAY

A heavy inoculum from an actively growing *P. fluorescens* was applied as a streak by a loop on the edge of a PDA plate and cross-streaked with cultures of *Phythium ultimum*, *Macrophomina phaseolina*, *Pyricularia oryzae* and incubated in inverted position at 28°C for 3 days. Antagonistic activity was observed by inhibition of the growth of fungal cultures.

POUR PLATE METHOD

A loopful of *P. fluorescens* culture and respective test fungal culture was added simultaneously into 5ml of molten agar

mixed the suspension by shaking the tube and poured in the already prepared PDA plates. For each test fungal plate a control plate was also maintained by adding only test fungal culture. The plates were incubated for 3 days at 28°C. Antagonistic activity was checked by percentage of fungus inhibited by comparing with the control plate.

EXTRACTION OF ANTIFUNGAL COMPOUND.

A loop full of actively growing culture of *P. fluorescens* was inoculated into 1 liter flask containing 250 ml king's B broth and incubated for 3 days at 30°C. The broth was centrifuged at 6000 rpm for 25min. The supernatant was treated with ammonium sulphate and the resultant precipitate was used as crude antifungal compound.

ANTIFUNGAL ASSAY

Effect of crude antifungal compound on fungal biomass 50ml PD broth was inoculated with 4mm test fungal mat. 1ml of crude compound with a concentration of 50mcg/ml, 100mcg/ml and 150mcg/ml in methanol was added and incubated for 5 days at 28°C. Control flasks were maintained with out the compound. Dry weight of fungus was taken and compared with control.

AGAR WELL DIFFUSION METHOD

Seeded agar plates of each test fungal organism were prepared. With a sterile borer, 6mm wells were prepared in which 70 micro liters of crude compound with a concentration of 50mcg/ml, 100mcg/ml and 150mcg/ml of methanol was added. These plates were then kept for incubation for 3 days at 28°C. Control well was maintained by adding only methanol. Antifungal efficiency was calculated by measuring the zone of inhibition.

REPRODUCIBILITY OF THE RESULTS

All data are the mean of at least three independent experiments showing consistent results.

RESULTS AND DISCUSSION

Fluorescent pseudomonads are of great importance in biotechnology because of the ability of several strains to degrade xenobiotics, control plant pathogens, and act as human pathogens. The ability of some strains to colonize the plant rhizosphere allows its use in rhizoremediation (Brazil et al.,1995; Karlson,1998). The isolation and enumeration of bacteria belonging to the genus *pseudomonas* from various habitats are often necessary due to their importance in a diverse range of microbiological phenomena. *P. fluorescens*

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is considered as biological control agent against various root diseases (Ursula et al., 2000).

The currently accepted diagnostic medium for the detection of fluorescence is king's B medium (Djibaoui and Bensoltane, 2005). In the present study, the organism is isolated on King's B medium (fig 2 A&B) and based on the biochemical tests the organism isolated was authenticated as *P. fluorescens* (Table 1).

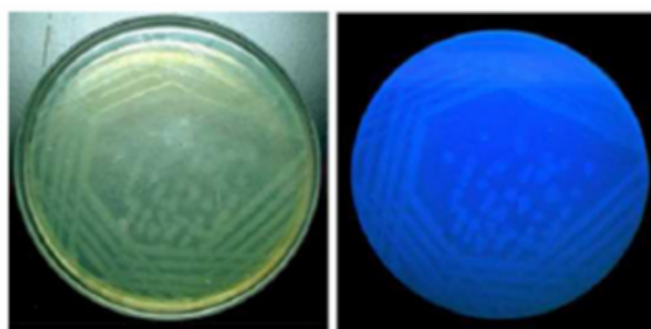
Figure 2

Table 1: Biochemical characteristics of

Character	Reaction
Colony character	White -yellow
Grams nature	negative
shape	rod
motility	motile
Fluorescent pigment	positive
Gelatin liquefaction	positive
Starch hydrolysis	negative
Growth at 4°C	positive
Utilization of nitrate	positive

Figure 3

Figure 2- A: Pure culture of on Kings B medium under normal light B: Pure culture of on Kings B medium under U.V light



A

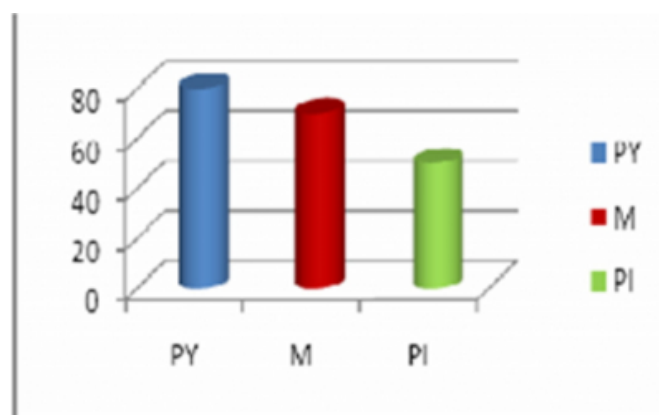
B

Cross streak assay and pour plate method are done to test the antagonistic activity of *P. fluorescens* on phytopathogenic fungi. *P. fluorescens* on cross streaking with the fungal pathogens revealed that it has been producing some inhibitory compound that suppresses the fungal growth. Similar results were obtained for *P. fluorescens* inhibiting the growth of plant pathogens (Brion and Genevieve., 1999).

In pour plate method, *P. fluorescens* on co-inoculation with fungal pathogens decreased the growth rate (fig 3). Maximum inhibition was observed in *P. ultimum* (80%) followed by *M. phaseolina* (70%) and *P. oryzae* (50). *P. ultimum*, a pathogen on cotton causing damping off is suppressed by *P. fluorescens* by releasing of phenazine 1- carboxylic acid (Wilson et al., 2000). *P. oryzae* causing rice blast was inhibited by *P. fluorescens* in vitro and this inhibitory compound was also developed into talc based power (Vidhyasekaran et al., 1997).

Figure 4

Figure 3: Percentage of inhibition exhibited by *p. fluorescens* as observed in pour plate method; PY- , M- M., PI-



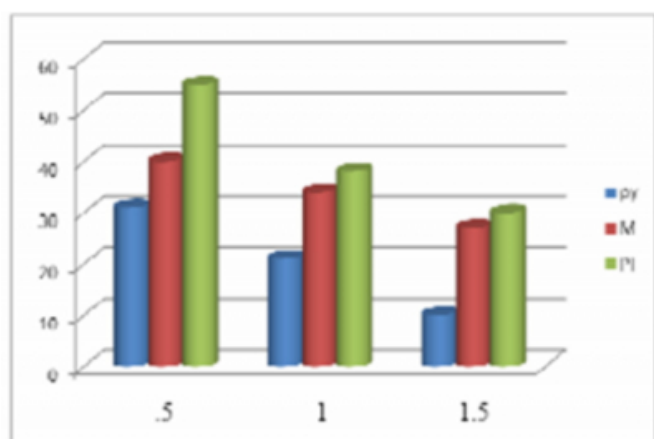
Cross streak assay and pour plate method can be used to observe the interaction between the organisms in natural environments. Results from the above methods indicate that *P. fluorescens* is exhibiting antagonistic property towards all the three fungal pathogens in the present study. The introduction of beneficial microorganisms for the biological control of soil borne plant pathogens has considerable potential in agriculture (Weller et al., 2007). Bacteria introduced on potato seed pieces, cotton, wheat and other species (Ganeshan and Manoj, 2005) have increased plant growth or reduced severity of root diseases.

Dry weight of fungal growth under different concentrations of crude antibiotic was taken as a measure of effect of crude antibiotic on phytopathogenic fungi. In *Pythium ultimum* the

dry weight reduced from 0.40mg to 0.31mg (50mcg/ml), 0.21(100mcg/ml) and 0.10(150mcg/ml). As the concentration of crude antibiotic was increased the dry weight of fungus decreased. It could be reasoned out that the antifungal compounds present in the culture filtrate have inhibited the growth of the fungi. Similar trend of results were obtained with *M.phaseolina* and *P.oryzae* (fig 4). Purified compounds showed inhibitory activity towards *P.ultimum* (Zhengyu et al., 2004).

Figure 5

Figure 3: Measurement of fungal biomass (expressed in mg) as observed in flask culture method; PY- , M- M., PI-

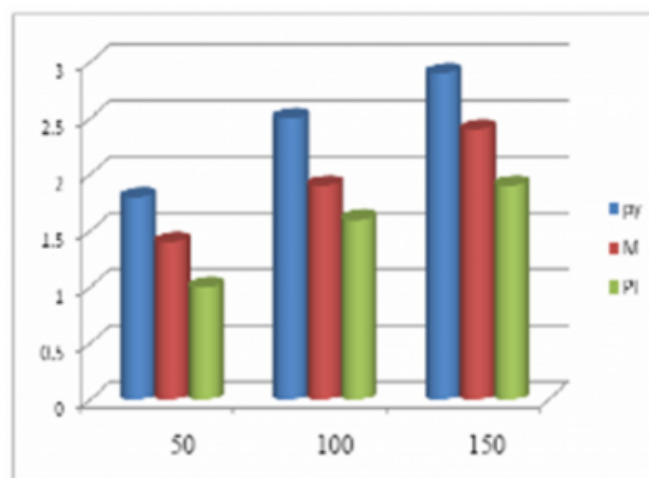


P.fluorescens produces a variety of antibiotics such as phenazine 1- carboxylic acid (Zhengyu et al., 2004), 2,4-diacetyl phloroglucinol(Weller et al., 2007; Ramesh et al., 2002), oomycin A (Ursula, 1995) etc. These antibiotics alone or in combination with some siderophores inhibit the growth of phytopathogenic fungi.

Growing the pathogenic fungi in the vicinity of compounds on PDA medium can test the efficacy of antagonistic activity of *P.fluorescens* (Nielsen and Sorensen, 2002). Concentrated culture broth of *P.fluorescens* when applied in PDA plates with fungus showed inhibition of growth of fungus. All the 3 fungal pathogens were inhibited by the action of crude antibiotic as evidenced by the zone of clearance. Effect of concentration of crude antibiotic was measured by measuring the zone of inhibition on PDA plates (fig 5). The efficacies for *P.ultimum* were 18mm, 25mm and 29mm, for *M.phaseolina* 14mm, 19mm and 24mm and for *P.oryzae* were 10mm, 16mm, 19mm. The inhibitory activity depended on the concentration of the compound used for the assay.

Figure 6

Figure 3: Measurement of inhibition zones (expressed in mm) as observed in agar well diffusion assay method; PY- , M- M., PI-



As the concentration of the compound increased the zone of clearance increased. Pure compounds like pyrrolnitrin decreased the incidence of damping off (Lambert, 1866) and phenazine 1-carboxylic acid controlled take all disease (Zhengyu et al., 2004). This suggests that *P.fluorescens* produces a broad spectrum antifungal compound, which inhibits a variety of plant Pathogenic fungi and inhibits *P.ultimum* more when compared to *M.phaseolina* and *P.oryzae*. Its wide antagonistic activity against several phytopathogens in vitro shows its potential to be used as a broad spectrum biocontrol agent (Fuente et al., 2004).

CONCLUSION

As agricultural practices become more sustainable, there is an increasing need for ecologically sound methods of disease control. Biological control, which exploits the natural antagonistic activity of certain root-colonizing bacteria against fungal pathogens, is one such approach. Biological control agents often perform inadequately under field conditions, however, and this has impeded acceptance of the technology as an alternative to chemical pesticides. Soil pseudomonads possess a variety of promising properties which make them better biocontrol agents. Although the present study is not an initiative but helps in better understanding and utilization of *P.fluorescens* as biocontrol agent. Further investigations on the type of antimicrobial components and in-vivo experiments will make *P.fluorescens* as one of the most suitable candidate biocontrol agent in suppressing the phytopathogenic fungi and replace chemical fungicides.

ACKNOWLEDGEMENTS

One of the authors (M.J.P.G) is thankful to M. Deiveekasundaram, Head, Department of Soil and Agriculture Microbiology, Annamalai University for providing the necessary facilities and awarding the degree "Master of Philosophy in Microbiology".

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