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Efficacy of anti-microbial metabolites of *Pseudomonas fluorescens* (Trevisan) Migula against *Rhizoctonia solani* Kuhn. and *Pythium* sp.

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ABSTRACT: ***Pseudomonas fluorescens*** **isolate (1-12) were collected from the rhizosphere soil and tested for their efficacy against** ***Rhizoctonia solani*** **causing sheath blight of rice and** ***Pythium*** **sp. causing damping-off of chilli. Among the 12 isolates, *P. fluorescens* 3 and 4 were very effective in inhibiting the mycelial growth of *R. solani* and *Pythium* sp. All the 12 isolates of *P. fluorescens* were tested for the production of siderophore, salicylic acid and HCN. The isolates of *P. fluorescens* 3 and 4 alone showed higher production of siderophore, salicylic acid and hydrogen cyanide.**

KEY WORDS: Hydrogen cyanide, *Pseudomonas fluorescens*, *Pythium* sp., *Rhizoctonia solani*, salicylic acid, siderophore

INTRODUCTION

Fluorescent pseudomonad strains have been reported to control several diseases caused by soil borne pathogens (Vidhyasekaran and Muthamilan, 1995) and are known to survive in the rhizosphere. Biological control of plant diseases using antagonistic microorganisms offers a highly effective, economical and environmental friendly alternative to the use of synthetic pesticides (Emmert and Handelsman, 1999). The mode of action of the antagonistic organisms against various soil-borne plant pathogenic fungi, include biosynthesis of antibiotics, production of hydrolytic enzymes (Velazhahan *et al.*, 1999), production siderophore and competition for substrates. Successful bacterial antagonists often show a synergistic combination of mechanisms responsible for a successful antifungal interaction. The main objective of this study is to select an effective isolate of *P. fluorescens* for the management of *R. solani* and *Pythium* sp. Totally 12 isolates were collected from the rhizosphere soil and screened for their efficacy.

MATERIALS AND METHODS

Isolation of *Pseudomonas fluorescens* from rhizosphere soil

The experiments were conducted at Department of Plant Pathology, Agricultural College and Research Institute, Madurai during the year

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2003-2004. *P. fluorescens* isolates were collected from rhizosphere region of rice plants. After removing the loosely adhering soil from freshly excised roots, root segments (1g) were taken and suspended in 10 ml of sterile distilled water to get 10^{-1} dilution. Serial dilutions were made to get dilutions upto 10^{-5}. One ml of 10^{-2} and 10^{-3} dilution were pipetted out into sterile Petri-plates and 15 ml of King's B medium was added, and rotated clockwise and anticlockwise. The plates were incubated at room temperature (28±2°C) for 48 hours for the development of bacterial colonies. *P. fluorescens* isolates were identified according to Bergey's Manual of Systematic Bacteriology. These bacterial colonies were tested for their antagonistic activity against *R. solani* and *Pythium* sp. by dual culture technique.

In vitro screening of *Pseudomonas fluorescens* against *Rhizoctonia solani* and *Pythium* sp.

P. fluorescens isolates (1-12) were evaluated *in vitro* against *R. solani* and *Pythium* sp. by dual culture technique to select the most potent one. *P. fluorescens* isolates were streaked at one side of Petri - dish (1 cm away from the edge) containing PDA medium. A 9-mm mycelial disc from a seven days old PDA culture of *R. solani* or *Pythium* sp. were placed on the opposite side in the Petri-dish perpendicular to the bacterial streak and plates were incubated at room temperature (28 ± 2°C) for 2-5 days. At the end of incubation period, the zone of inhibition (cm) was recorded by measuring the distance between the edges of the fungal mycelium and the antagonistic bacterium. Three replications were maintained for each isolate.

Production of siderophore by *Pseudomonas fluorescens*

P. fluorescens isolates (1-12) were grown in King's B broth for three days at room temperature (28 ± 2°C) and centrifuged at 3000g for 10 minutes and the supernatants were collected. The pH of the supernatant was adjusted to 2.0 with diluted HCl and equal quantity of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. Five ml of ethyl acetate fraction was mixed with 5 ml of Hathway's reagent (1.0 ml of 0.1M $FeCl_3$ in 0.1 N HCl to 100 ml distilled water + 1.0 ml of potassium ferricyanide). The absorbance for dihydroxy phenol was read at 700 nm in spectrophotometer. A standard curve was prepared using dihydroxy benzoic acid. The quantity of siderophore synthesized was expressed as mmol benzoic acid ml^{-1} of culture filtrate. Three replications were maintained for each isolate.

Production of salicylic acid (SA) by *Pseudomonas fluorescens*

P. fluorescens isolates (1-12) were grown at room temperature (28 ± 2°C) for 48 hours on a rotary shaker in 250 ml conical flasks containing 50 ml of the succinate medium (Succinic acid – 4.0g; K_2HPO_4 – 6.0g; KH_2PO_4 – 3.0g; $(NH)_2SO_4$ – 1.0 g; $MgSO_4$ $7H_2O$ – 0.2g; distilled water – 1000 ml; pH – 7.0). Cells were then collected by centrifugation at 6000 g for five min and 4 ml of cell free culture filtrate was acidified to pH 2.0 with 1N HCl and SA was extracted in $CHCl_3$ (2x2ml). To the pooled $CHCl_3$ phases 4ml of distilled water and 5ml of 2M $FeCl_3$ were added. The absorbance of the purple iron SA complex, which was developed in the aqueous phase, was read at 527 nm in a spectrophotometer. A standard curve was prepared with SA dissolved in succinate medium. The quantity of SA in the culture filtrate was expressed as mg ml^{-1}. Three replications were maintained for each isolate.

Production of Hydrogen Cyanide (HCN) by *Pseudomonas fluorescens*

P. fluorescens isolates (1-12) were grown at room temperature (28 ± 2°C) on a rotary shaker in Tryptic Soy Broth (TSB). Filter paper (Whatman No.1) was cut into uniform strips of 10cm long and 0.5 cm wide, saturated with alkaline picrate solution and placed inside the conical flasks in a hanging position. After incubation at 28°C for 48 hours, the sodium picrate present in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The colour was eluted by placing the filter paper in a clean test tube containing 10 ml of distilled water and the absorbance was measured at 625 nm. Three replications were maintained for each isolate.

RESULTS AND DISCUSSION

Effect of *Pseudomonas fluorescens* isolates on *Rhizoctonia solani* and *Pythium* sp.

Among the 12 isolates of *P. fluorescens* tested, *P. fluorescens* 3 and 4 were very effective in inhibiting the mycelial growth of *R. solani* and *Pythium* sp. (13.6, 13.4mm) (Table 1) (Plate 1 and 2). Vidhyasekaran and Muthamilan (1995) identified the inhibitory action of *P. fluorescens* –1 isolate of *P. fluorescens* on fungi like *R. solani* and *Fusarium oxysporum*. This shows that various isolates of *P. fluorescens* produce antifungal compounds in different concentrations. Anitha and Tripathi (2001) reported that *P. fluorescens* inhibit a maximum of 69.82 per cent growth of *R. solani* and 88.5 per cent growth of *Pythium aphanidermatum* inciting seedling disease of okra when compared to control. Nielson and Sorensen (1999) demonstrated that isolates of *P. fluorescens* antagonistic to *R. solani* and *Pythium ultimum* produce endochitinase and chitobiosidase.

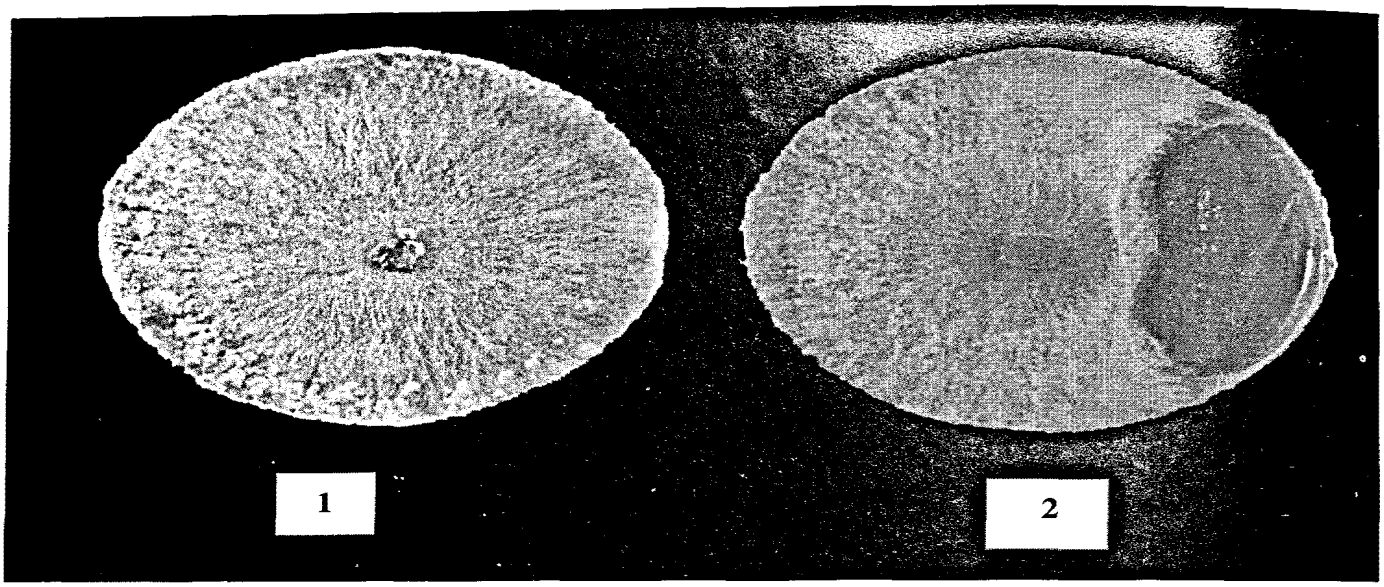
Production of siderophore by *Pseudomonas fluorescens*

The maximum siderophore production was recorded in *P. fluorescens* isolates 3 and 4. There was no relationship between the antagonistic (Table 2) potential of *P. fluorescens* isolates and their siderophore production capacity. Many *P. fluorescens* isolates are known to secrete fluorescent and yellow -green, water-soluble siderophores under iron-limiting conditions (O' Sullivan and O' Gara, 1992). These fluorescent siderophores, which have very high affinity for ferric iron, will form ferric-siderophores complex and make it unavailable to other organisms but the producing organisms can utilize these complexes via a specific receptor in their outer cell membrane (Buyer and Leong, 1986). Fluorescent pseudomonads produce several siderophores such as pyoverdine (Pseudobactin), pyochelin and salicylic acid (De Meyer and Hofte, 1997; Dave and Dube, 2000). Siderophores are also known to induce systemic resistance in plants. For example, the

Table 1. *In vitro* inhibition of mycelial growth of *R. solani* and *Pythium* sp. by *P. fluorescens*

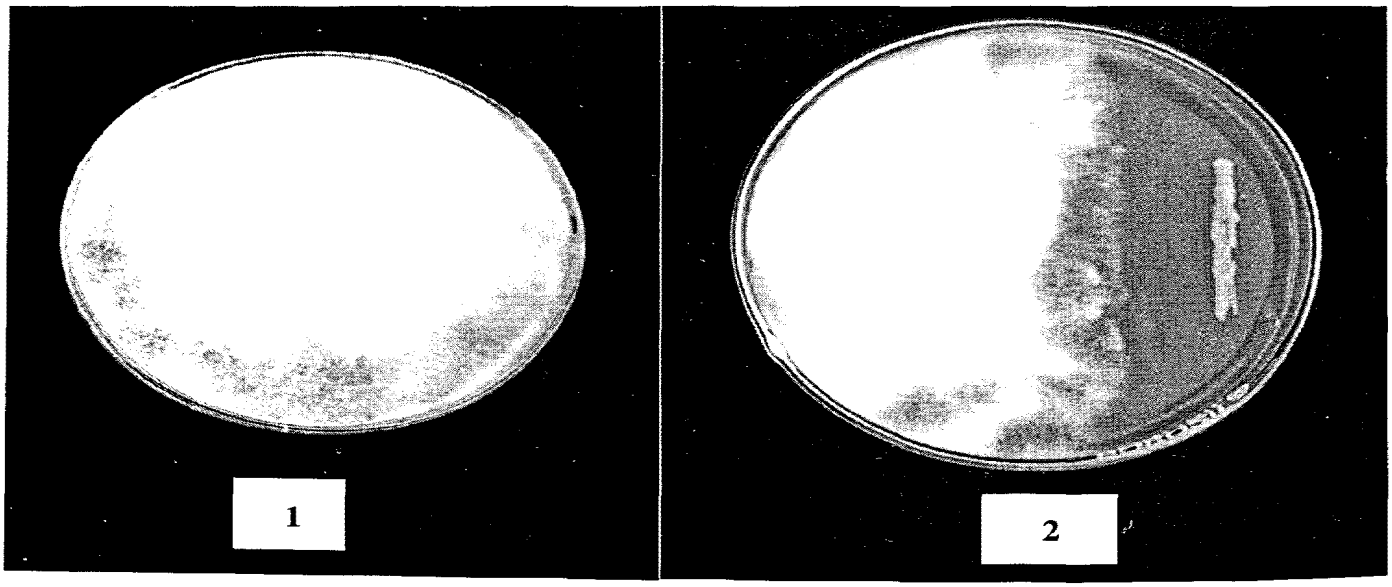
P. fluorescens isolates	Inhibition zone of *R. solani* (mm)	Inhibition zone of *Pythium* sp. (mm)
P.f.1	2.2^{i}	2.4^{gh}
P.f.2	5.4^{e}	5.2^{de}
P.f.3	13.6^{a}	13.4^{a}
P.F.4	12.2^{ab}	12.6^{ab}
P.f.5	3.6^{g}	3.4^{f}
P.f.6	8.4^{c}	9.6^{c}
P.f.7	9.2^{c}	2.0^{i}
P.f.8	2.0^{i}	8.8^{c}
P.f.9	5.2^{ef}	5.2^{de}
P.f.10	2.8^{gh}	2.8^{g}
P.f.11	6.4^{d}	6.2^{d}
P.f.12	3.0^{gh}	3.4^{f}

Means followed by the same letter in a column are not significantly different (P=0.05) by DMRT.



1. *R. solani* **2. *R. solani* with *P. fluorescens* isolate 3**

Plate 1. Inhibition of mycelial growth of *R. solani* by *P. fluorescens* isolate 3



1. *Pythium* sp. **2. *Pythium* sp. with *P. fluorescens* isolate 3**

Plate 2. Inhibition of mycelial growth of *Pythium* sp. by *P. fluorescens* isolate 3

purified pyoverdine from *P. fluorescens* WCS 374 induced resistance in radish against *Fusarium* wilt (Leeman *et al.*, 1996).

Production of Salicylic acid (SA) by *Pseudomonas fluorescens*

Among the 12 isolates of *P. fluorescens* tested for the salicylic acid production, the isolate *P. fluorescens* 3 and 4 (Table 2) produced more quantity of salicylic acid. Salicylic acid is another secondary metabolite produced by *P. fluorescens* isolates WCS 374 and WCS 417 g (Leeman *et al.*, 1996), and *Pseudomonas aeruginosa* 7 NSK2 (De Meyer and Hofte, 1997). Salicylic acid produced by

Table 2. Production of secondary metabolites by *P. fluorescens* isolates

Isolate	Salicylic acid production (μg ml^{-1})	Siderophore production (μ mol benzoic acid ml^{-1})	HCN production (Absorbance at 625nm)
P.f.1	5.0^{e}	3.7^{fg}	0.025^{fg}
P.f.2	1.7^{i}	4.6^{de}	0.04^{c}
P.f.3	13.2^{a}	11.2^{a}	0.09^{a}
P.f.4	11.5^{b}	12.1^{b}	0.08^{b}
P.f.5	10^{c}	9.3^{c}	0.06^{c}
P.f.6	6.5^{d}	3.5^{fg}	0.05^{d}
P.f.7	3.2^{gh}	4.3^{de}	0.04^{e}
P.f.8	1.7^{i}	5.5^{d}	0.03^{f}
P.f.9	1.4^{ij}	4.9^{de}	0.01^{h}
P.f.10	1.6^{ij}	3.2^{h}	0.03^{f}
P.f.11	3.5^{g}	4.5^{de}	0.02^{h}
P.f.12	4.6^{ef}	3.8^{f}	0.01^{h}

Means followed by the same letter in a column are not significantly different (P=0.05) by DMRT.

P. fluorescens in the rhizosphere is thought to be involved in ISR (Maurhofer *et al.*, 1998). Chen *et al.* (1999) reported that *Pseudomonas corrugata* strain 13 and *Pseudomonas aureofaciens* strains 63-28, varied in SA production *in vitro* induced the same level of resistance in cucumber against *Pythium* root rot.

Production of Hydrogen cyanide (HCN) by *Pseudomonas fluorescens*

Among the 12 isolates of *P. fluorescens* tested for the HCN production, the isolate *P. fluorescens* 3 and 4 (Table 2) alone showed higher production of HCN. The other isolates produced only negligible amount of HCN. Some fluorescent pseudomonads produce volatile hydrogen cyanide (HCN), which helps to suppress black root rot of tobacco (Voisard *et al.*, 1989) (Table 2).

Nagaraj kumar (2003) reported that two isolate of *P. fluorescens viz., P. fluorescens* MDU2 and *P. fluorescens* MDU 3 produced more HCN than other isolate tested. The increased production of HCN by the efficient isolate of *P. fluorescens* might have contributed to the effective inhibition of mycelial growth of *R. solani in vitro.*

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REFERENCES

Anitha, K. and Tripathi, N. N. 2001. Laboratory screening of fungal and bacterial antagonist against *Rhizoctonia solani* Kuhn. and *Pythium aphanidermatum* (Edson) Fitzp. inciting seedling diseases of Okra. *Indian Journal of Plant Pathology*, **29**: 146-148.

Buyer, J. S. and Leong, J. 1986. Iron transport-mediated antagonism between plant-growth promoting and plant deleterious *Pseudomonas* strains. *Journal of Biological Chemistry*, **261**: 791-794.

Chen, C., Belanger, R. R., Benhamou, N. and Paultiz, T. C. 1999. Role of salicylic acid in systemic resistance induced by *Pseudomonas* spp. against *Pythium aphanidermatum* in cucumber roots. *European Journal of Plant Pathology*, **105**: 477-486.

Dave B. P. and Dube, H. C. 2000. Detection and chemical characterization of siderophores of rhizobacterial fluorescent pseudomonads. *Indian Phytopathology*, **53**: 97-98.

De Meyer, G. and Hofte, M. 1997. Salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7 NSK2 induces resistance to leaf infection by *Botrytis cinerea* on bean. *Phytopathology*, **87**: 588-593.

Emmert, E. A. B. and Handelsman, J. 1999. Bio-control of Plant disease: a (Gram) positive perspeetive. *FEMS Microbiology letters*, **171**: 1-9.

Leeman, M., Den Ouden, F. M., Van Pelt, J. A., Dirkx, F. P. M., Stejil, H., Bakker, P. A. H. M. and Schippers, B. 1996. Iron availability affects induction of systemic resistance to *Fusarium* wilt of radish by *Pseudomonas fluorescens*. *Phytopathology*, **86**: 149-155.

Maurhofer, M., Reimmann, C., Schmidli – Sacherer, P., Heep S., Haas, D. and Defago, G. 1998. Salicyelic acid biosynthetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against tobacco necrosis virus. *Phytopathology*, **88**: 678-684.

Nagaraj Kumar, M. 2003. Biological control of rice sheath blight caused by *Rhizoctonia solani* Kuhn by using toxin – degrading *Pseudomonans fluorescens*. M. Sc. (Ag.) thesis, Tamil Nadu Agricultural University, Coimbatore.

Nielson, M. N. and Sorensen, J. 1999. Chitinolytic activity of *Pseudomonas fluorescens* isolates from barley and sugarbeet rhizosphere. *FEMS Microbial Ecology*, **30**: 217-227.

O' Sullivan, D. J. and O' Gara, F. 1992. Traits of fluorescent Pseudomonads involved in suppression of plant root pathogens. *Microbiological Review*, **56**: 662-676.

Velazhahan, R., Samiyappan, R. and Vidhyasekaran, P. 1999. Relationship between antagonistic activities of *Pseudomonas fluorescens* isolates against *Rhizoctonia solani* and their production of lytic enzymes. *Journal of Plant Disease Protection*, **106**: 244-250.

Vidhyasekaran, P. and Muthamilan, M. 1995. Development of formulations of *Pseudomonas fluorescens* for control of chickpea wilt. *Plant Disease*, **79**: 782-786.

Voisard, C., Keel, C., Hass, D. and Defago, G. 1989. Cyanide production by *Pseudomonas fluorescens* suppress black root rot of tobacco under gnotobiotic conditions. *EMBO Journal*, **8**: 351-358.

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