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Antagonistic Effect of *Pseudomonas fluorescens* Isolated From Soil of Doon Valley (Dehradun–India) on Certain Phyto-Pathogenic Fungi

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ABSTRACT

In line with the major biological control measures to control the soil borne fungal infection in crop plants were worked out in District Dehradun (Uttarakhand) India. *Pseudomonas fluorescens* was isolated from soil samples collected from various places of Dehradun district and was investigated for antagonistic activity against native fungal phytopathogens viz., *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Erysiphe cruciferarum*, *Fusarium oxysporum* by dual culture method. Maximum antagonistic effect of *P. fluorescens* was observed against *F. oxysporum* (73.24 ±2.40 %) followed by *A. alternata* (51.20 ± 0.12 %), *A. flavus* (37.85 ± 0.83%), *E. cruciferarum* (37.12 ± 0.66%) and least by *A. niger* (32.13 ± 1.77 %). Relative percentage of germination of spores of phyto-pathogen in culture filtrate of *P. fluorescens* was also investigated against these phyto-pathogens and it was found correlated with the antagonistic effect. Present study shows the prevalence of natural biological control on the phyto-pathogenic fungi.

INTRODUCTION

Sustainable agricultural practice all over the world is of utter importance. Several modern approaches such as use of agrochemicals, hybrid variety, genetically modified crops, fertilizers and organic farming have made agriculture more sustainable. Nevertheless, microbial infections especially the fungal plant pathogens, damage a huge clutch of the crop in the entire world every year. Diseases such as stem canker and leaf blight caused by *Alternaria altanata* (Gilchrist and Grogan, 1975; Akhtar et al., 2004), damping off, vine canker, bunch rot of grapes, black rot of onions and garlic and seedling rots caused by *Aspergillus niger* are some of the major fungal diseases of the crops common in various parts of the world, *Fusarium oxysporum* cause vascular wilt or cortical rot diseases in many agricultural crops (Nelson et al., 1981). *Aspergillus flavus* is associated with cob rot in maize under hot and humid conditions. *Erysiphe cruciferarum* is the major causal agent of powdery mildew in crucifers (Koch and Slusarenko, 1990) especially in above ground plant parts (Saharan et al., 2005). Although, the use of chemical pesticide is being discouraged all over the world, however, the fungicide application to protect the crops from fungal infections is quite common especially in developing countries creating environmental hazards responsible for complicated diseases in human and other creatures. The alternative methods to control most of the fungal infection of plants include the use of resistant or genetically modified variety of crop plants or the use of soil borne, non-pathogenic microbes (bacteria) capable of inhibiting the growth of fungal phyto-pathogen commonly called as biological control agents (BCA) and plant growth promoting rhizobacteria (PGPR). Among these BCA are more advantageous to control phytopathogens (Nautiyal, 2001) especially by soil borne strains of fluorescent pseudomonads at roots (Cook et al., 1995; Péchy-Tarr et al., 2005) because of their capacity to colonize plant roots and the production of antifungal metabolites (Haas and Keel, 2003). Much emphasis has been given to the use of *Pseudomonas fluorescens* as a bio-control agent (Walsh et al., 2001) and to the molecular mechanism of their action (Delany et al., 2000). The present case study of district Dehradun of Uttrakhand, India was designed to ascertain the antifungal ability of isolated strains of *Pseudomonas spp*. from the various places of the district.

MATERIALS AND METHODS

Biological material

Pure cultures of *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Erysiphe cruciferarum*, *Fusarium oxysporum* were obtained from Forest Research Institute (FRI), Dehradun. These cultures were grown aerobically on potato dextrose agar (PDA) at 25±1°C for 3 days and maintained at 4±1°C for further use.

Sample collection

Around 80-100 g soil were randomly collected from the rhizosphere of individual tea plant from various locations in Dehradun district at an altitude of 1000 feet MSL and screened for the presence of *Pseudomonas sp*.

Isolation of bacteria

Ten grams of each soil sample was transferred in 250 mL of conical flask containing 100 mL of sterilized distilled water and then was kept in incubating shaker at 28±1°C for 24 h. Then 1 mL of this suspension was made into serial dilution up to 10-3 as per the standard method (Nakayama, 1981). An aliquot of 0.1 mL of diluted suspensions was spread over the solidified surface of sterilized nutrient agar in Petri plates. The plates were incubated for 24 h at 28±1°C. Plate count method was performed to enumerate the bacteria present in the sample. Pure cultures were then prepared by sub culturing isolated colonies of unique morphology.

Screening tests

Among the bacterial isolates *Pseudomoans fluorescens* was screened on the basis of morphological and biochemical characters as per Bergey's Manual of Determinative Bacteriology by Rapid kit method for Enterobacteriaceae (KB001, HiMedia) which includes Indole, Methyl red, Voges Proskuer's, Citrate utilization, Glucose, Adonitol, Arabinose, Lactose, Sorbitol, Mannitol, Rhamnose and Sucrose tests. Test results of all isolates and results of standard culture of *P. fluorescens* (Rhodes, 1959) were used to categorized isolates in relatively homogeneous groups by cluster analysis (using single linkage method) by computer aided software (SPSS-22.0, 2014).

Tests for production of secondary metabolites

Indole acetic acid (IAA) production

Production of IAA by screened Pseudomonad was determined by the methods of Gordon and Weber (1951). The bacterial isolates were grown on Luria Bertani (LB) Broth and incubated at 28±1oC for 24 h in shaker at 120 rpm. Exponentially grown culture (10^8 cells per cell) was centrifuged at 10000 rpm for 15 min at 4°C to collect supernatant. Two drops of orthophosphoric acid (Sigma Aldrich) was added to 2 ml of supernatant). Appearance of pink colour confirmed the production of IAA.

Hydrogen cyanide (HCN) production

Production of HCN by screened isolates was determined by the method of Paul et al. (2005). Each bacterial isolates was spread on King's B medium supplemented with 4.4 g/L of glycine in separate Petri plates. Filter paper strips soaked in picric acid solution (2.5 g picric acid + 12.5 g Na_2CO_3 in 1L of water) were placed on the lid of plate. The Petri plate were sealed with parafilm and incubated at 28±1°C for 72 h. Production of HCN was indicated by the change in colour of the paper strips from yellow to brown. The intensity of colour was recorded visually.

Siderophores production

The ability to produce siderophore by screened bacterial isolates was determined by Paez et al. (2005). Acidic ferric chloride

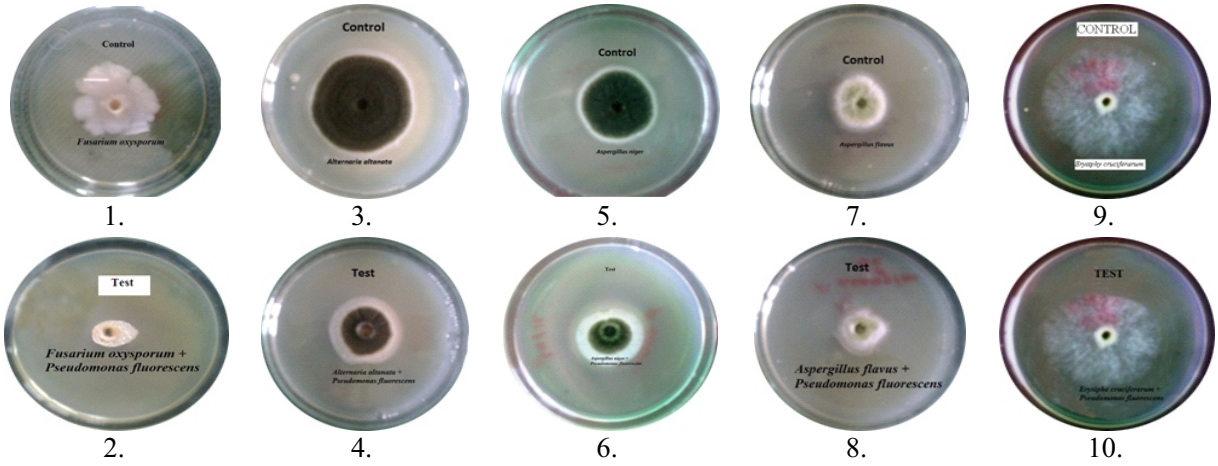

Fig. 1: *Fusarium oxysporum*, *Alternaria alternate*, *Aspergillus flavus*, *A. niger* and *E. cruciferarum* – control plates in first row (1, 3, 5, 7 and 9) and dual culture plates with *P. fluorescens* second row (2, 4, 6, 8 and 10).

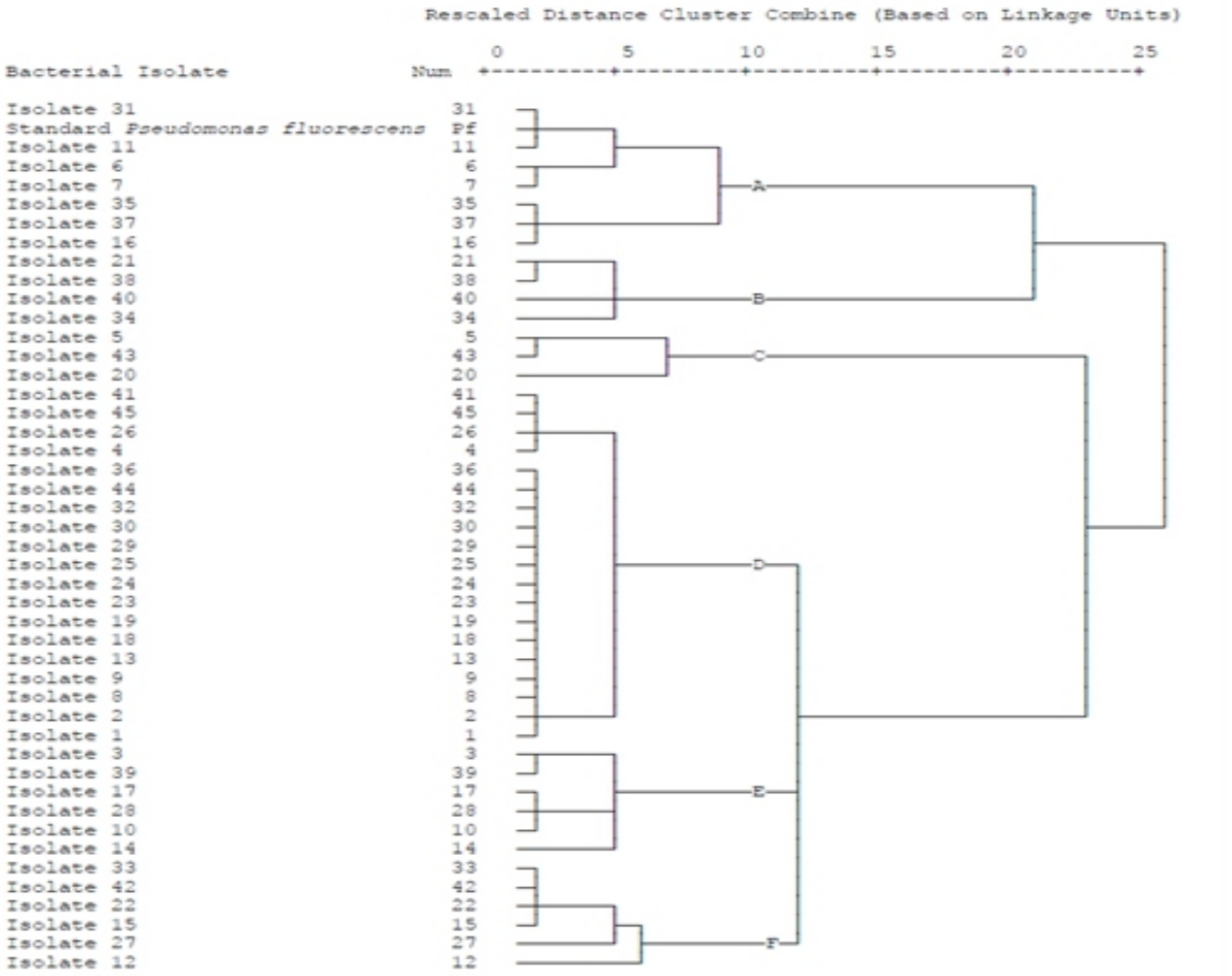

Fig 2: Dendrogram of 45 bacterial isolates with standard test results of *Pseudomonas fluorescens (Pf)*.

prepared by dissolving 1.62 mg $FeCl_3$ in 8.5 µl concentrated HCl and mixed thoroughly in CTAB solution. This mixture was then mixed with sterilized and molten King's B agar containing chrome azurol and poured in Petri plate. The bacterial isolates were spot inoculated on medium surface and incubated at 28±1°C for 24 h. The yellow zone indicates the positive result.

Antagonistic activity

Antagonistic activity of best *Pseudomonas* isolate of was determined by the dual culture method of Skimdore and Dickinson (1976) with slight modification. A well of 10 mm diameter was made on center of the PDA plate with a sterilized cork borer and was inoculated with the 0.1 ml of broth cultures (10^8 cells per mL) of selected bacterium. After 30 minutes test fungus was spot inoculated in same well. Plates were incubated aerobically at 28±1°C for 5 days. Only test fungus inoculated in Petri plate was considered as control. Followed by incubation radial growth of the fungus was measured and percent antagonistic effect (PAE) of was calculated by using formula: % PAE= [(C-T)/C] × 100; where, C = area of fungal growth in control plate; T = area of fungal growth in test plate.

In vitro assay for volatile metabolite

The center of the PDA plate was inoculated with 8 mm disc of individual phyto-pathogenic test fungus. The plate was placed upside down over another plate containing overnight culture of best *Pseudomonas* isolate in sterilized King's B broth (25 mL). The both plates were sealed airtight with parafilm and were incubated at 28±1°C.

Table 1. Biochemical characters and production of metabolites by screened bacterial isolates.

S. No.	Characteristics	Rhizospheric *Pseudomonas* isolates							Standard result
		Isolate-6	Isolate-7	Isolate-11	Isolate-16	Isolate-31	Isolate-35	Isolate-37	
1.	Shape	Uniformly bacilli	Uniformly bacilli	Uniformly bacilli	Uniformly bacilli	Uniformly bacilli	Uniformly bacilli	Uniformly bacilli	Uniformly bacilli
2.	Gram staining	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
3.	Motility	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile
4.	Growth on Pseudomonas Fluorescein Agar	Yellow Pigment	Yellow Pigment	Yellow Pigment	Yellow Pigment	Yellow Pigment	Yellow Pigment	Yellow Pigment	Yellow Pigment
5.	Indole test	-	-	-	-	-	-	-	-
6.	Methyl red test	-	-	-	-	-	-	-	-
7.	Voges Proskuer's test	-	-	-	-	-	-	-	-
8.	Citrate utilization test	+	+	+	+	+	+	+	+
9.	Glucose fermentation	-	-	-	-	-	-	-	-
10.	Adonitol test	+	+	+	+	±	+	±	+
11.	Arabinose test	+	+	±	+	±	+	+	*
12.	Lactose test	+	+	+	+	+	+	+	+
13.	Sorbitol test	+	+	+	+	+	+	+	+
14.	Mannitol test	+	+	+	+	±	+	+	+
15.	Rhamnose test	+	+	+	+	±	+	+	+
16.	Sucrose test	±	+	+	+	+	+	±	+
17.	Nitrate Reduction	+	+	±	+	+	±	+	+**
18.	Starch Hydrolysis	-	-	-	-	-	-	-	-
19.	Catalase test	+	+	+	+	+	+	+	+
20.	Oxidase test	+	+	+	+	+	+	+	+
21.	Growth on Kings B Medium	+	+	+	+	+	+	+	+
22.	Starch Hydrolysis	+	+	+	+	+	+	+	+
23.	IAA production	+	+	+	+	+	+	+	+
24.	Siderophore production	+	++	+	+	+	+	+	+
25.	HCN production	+	+++	+	+	+	+	+	+

+ Positive; - Negative; ± Not clear; ++ Good; +++ Very good; * no standard result
** confirmed by unbaised reaction with zinc (after reduction of nitrite into N_2)

Table 2. Effect of *P. fluorescens* (isolates 11) on fungal growth.

S. No.	Phyto-pathogenic fungi	Antagonistic effects (%)	Radial growth inhibition due to volatile metabolites (%)	Spore germination inhibition due to culture filtrate (%)
1.	*Fusarium. oxysporum*	73.24 ± 2.40	36.25±0.74	63.17±1.78
2.	*Aspergillus niger*	32.13 ± 1.77	31.55±1.11	42.28±2.51
3.	*Alternaria alternata*	51.20 ± 0.12	24.37±0.83	34.38±4.37
4.	*Aspergillus flavus*	37.85 ± 0.83	19.34±0.65	25.74±1.72
5.	*Erysiphe cruciferarum*	37.12 ± 0.66	11.58±1.46	21.37±1.73

Values are expressed as Mean±SD ($p < 0.05$)

Another set of plate having sterilized broth in lower plate was considered as control. The radial growth of fungi after 72 h of infection were measured and compared with control. Percent radial growth inhibition was calculated similarly by the formula given in previous test method.

Spore germination inhibition test

Germination inhibition of fungal spores was evaluated by standard method of Dhingra and Sinclair (1993) with slight modifications. The active culture of best isolate was prepared by inoculation in nutrient broth at 28±1°C for 15 to 18 h and then filtered

through membrane filter (Millipore). The pH of filtrate was adjusted to 6.5 and 100 µl of filtrate was transferred on separate cavity slides. Few spores form fresh culture of test fungi were inoculated in each cavity slide aseptically using inoculating wire loop. All cavity slides were kept in BOD incubator (in dark) at 28±1˚C. Slides were investigated under light microscope for germination of spores after every 6 hrs. The germination percentage of fungal spores in culture filtrate was calculated by formula (% germination of fungal spore = (Ng/Nt) × 100); Where, Nt = total number of fungal spores and Ng = number of germinated fungal spore). Percent germination of fungal spores in sterilize NB was taken as control. The actual germination inhibition percentage (%GI) of fungal spores affected by bacterial filtrate was determined by formula:

$$\%GI = \left[100 - \left(\frac{\text{Percent germination of fungal spores in culture filtrate}}{\text{Percent germination of fungal spores in control}} \times 100\right)\right]$$

RESULTS AND DISCUSSION

A total of 45 bacterial isolates were obtained from the rhizospheric soil of tea plants. The biochemical analysis exhibit the presence of *Pseudomonas fluorescens* in some samples (Table 1). A dendrogram based on binary data generated from microscopic, culture and biochemical tests results of all isolates and standard results of *P. fluorescens* (abbreviated as Pf') was constructed by Statistical software SPSS 16 and presented in Fig 1. The accessions are clearly divided into 6 major clusters, A, B, C, D, E and F. Within cluster 'A' three sub clusters of isolate 11, 31 and Pf; isolate 6 and 7 and isolate 16, 35 and 37, respectively, linked at distance of zero unit advocated for their resemblance with *P. fluorescens*. Syntheses of secondary metabolites by these isolates were confirmed by positive results of IAA, siderophore and HCN production tests. Moreover, isolate 11 showed potential intensity of colour change in siderophore and HCN production test, respectively, hence was considered as best and selected for antagonistic test against phytopathogenic fungi.

All the fungi selected in present study showed variable degree of reduction in their radial growth (Fig 2) in the presence of *P. fluorescens* (isolate 11) either in antagonistic test or in-vitro assay for volatile metabolite (Table 2). Maximum antagonistic effect of *P. fluorescens* was showed against F. oxysporum (73.24 ± 2.40 %) followed by *A. alternata* (51.20 ± 0.12 %), A. flavus (37.85 ± 0.82%), *E. cruciferarum* (37.12 ±0.65%) and *A. niger* (32.13 ± 1.76 %). Similar results were found in in-vitro assay for volatile metabolite i.e., 36.25±0.74, 31.55±1.11, 24.37±0.83, 19.34±0.65 and 11.58±1.46, respectively (Table 2). These results supported the bio control potential of *P. fluorescens* previously studied by many investigators (Weller, 1998; Trivedi et al., 2008; Mushtaq et al., 2010; Dewangan et al., 2014).

It was observed that metabolites in culture filtrate of isolate 11 caused comparable inhibitory effects on spore germination of F. oxysporum (63.17±1.78) followed by A. alternata (42.28±2.51), *A. flavus* (34.38±4.37), *E. cruciferarum* (25.74±1.72) and *A. niger* (21.37±1.73) (Table 2). Studies suggest that *Pseudomonas spp*. produce diffusible metabolites like pyoluteorin (PLT), phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol (DAPG) (Bangera and Thomashow, 1996) and 2-4-de-epoxy-2-3-didehydro-rhizoxin (Whright et al., 1999) in many cases were believed associated with antimicrobial effects (Haas et al., 2000). The anti-fingal activity of these and other metabolites like siderophore, HCN, ammonia, lipase and chitinase have also been attributed in various literatures (Thomashow and Weller 1990; Dowling and O'Gara, 1994; Haas et al., 2000; Haas and Keel, 2003; Péchy-Tarr et al., 2005).

Research has repeatedly point the side effects of synthetic fungicides and screening of rhizospheric microorganisms from various plants that have been evaluating for their antagonistic effects against wide range of phyto-pathogens. *P. fluorescens* has efficient root colonizing property and it provides nutrition to the host plants and hence is placed in PGPR group (Nelson, 2004; Ganeshan and Kumar, 2005). It is rarely pathogenic to human (Gunasekran et al., 2002) making it an effective biocontrol for treating crops.

CONCLUSION

The soil borne phyto-pathogenic fungi viz., *Aspergillus flavus*, *A. niger*, *Alternaria alternata*, *F. oxysporum*, and *E. cruciferarum* were investigated to be controlled by the biological agents in line of sustainable agricultural technology development. *Pseudomonas fluorescens* was isolated from the soil of Dehradun Valley found to have substantial impact on the fungi indicating the potential of microbial cells for controlling the fungal diseases that otherwise damage the crop production to a massive scale. However; the effective application of these biological controlling agents is yet to investigate especially in the hilly areas.

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