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Antagonistic Activity of Pseudomonas Fluorescens Isolates Against Colletotrichum Capsici

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Abstract- The present studies were undertaken to investigate the effect of various isolates of Pseudomonas fluorescens in Tamil Nadu against chilli anthracnose. A total of Six isolates of P. fluorescens were isolated from rhizosphere soil of Major chilli growing areas in various part of Tamil Nadu. All the collected isolates of P. fluorescens were identified and purified using streak method. All the isolated showed results in gram staining (negative), gelatin liquefaction (positive), catalase test (positive), oxidase test (positive), starch hydrolysis (negative) and fluorescent pigmentation (positive) IAA production (positive). Among the various isolates, Pf_1 produced more quantity of IAA and siderophores production. Also, Pf_1 produced maximum reduction of mycelial growth C. capsici under dual culture and poisoned food techniques.

Keywords- Bio control agent, Colletotrichum capsici, Anthracnose, Pseudomonas

1. INTRODUCTION

Chilli (*Capsicum annum* L.) is one of the most important constituent of the cuisines of tropical and subtropical countries and the fourth major crop cultivated globally. Numerous varieties of chilli are grown for vegetables (green fruit), spices (ripe and dried form), condiments, sauces, and pickles occupying an indispensable position in Indian diet. Chilli crop is affected by several fungal, bacterial and viral diseases, of which chilli anthracnose causes considerable damage, inflicting severe quantitative and qualitative losses (Anand *et al*., 2010; Masoodi *et al*., 2012). The disease is caused by fungus *Colletotrichum capsici* that infect both unripe and ripe chilli fruits (Kraikruan *et al.,* 2008). The disease is both seed borne and air borne and affects seed germination and vigour to a great extent (Hemmannuvar *et al.,* 2001).

Several fungicides have been reported to be effective for management of fruit rot of chilli (Shovan *et al.,* 2008). However, indiscriminate use of chemicals led to the development of fungicidal resistance by the pathogen, environmental pollution, health hazards and high cost (Bajpai and Kang, 2010). Hence, a search for alternative methods of plant disease management is on increase. Recently, the role of plant growth promoting rhizobacteria (PGPR) *viz*., *Pseudomonas fluorescens* and *Bacillus subtilis* in biocontrol approaches for managing the pathogen in crop plants are well reported (Allu *et al.,* 2014; Sundaramoorthy and Balabaskar, 2012). Several *Pseudomonas* strains have been shown to activate ISR in plants against many fungal, bacterial and viral diseases (Chen *et al*., 2000). Recent investigations on mechanisms of biological control by plant growth promoting fluorescent pseudomonads revealed that several strains protect the plants from pathogen attack by strengthening the epidermal and cortical cell walls with deposition of newly formed barriers beyond infection sites including callose, lignin and phenolics (M'Piga *et al.,* 1997) and production of secondary metabolites such as siderophore, antibiotics, volatile compounds, HCN, enzymes and phytohormones (Nagarajkumar *et al.,* 2004). Hence, exploring the possibilities of identifying an effective native PGPR isolate is essential for the effective management of the disease. Therefore, the present studies were undertaken to investigate the effect of various isolates of *Pseudomonas fluorescens* against chilli anthracnose.

2. MATERIALS AND METHODS

2.1 Isolation and Morphological characterization of Pseudomonas fluorescens

Rhizoplane – colonizing *P. fluorescens* was isolated from fresh roots of chilli grown in six reigns of Tamil nadu. The soil particles loosely adhering to the roots were tested out and used for the isolation of *P. fluorescens*. A soil suspension was prepared from each rhizophere sample by shaking one g of soil sample in 10ml of sterile dist. water and serial dilutions were made. One ml of soil suspension from aliquot dilutions (10^{-5} to 10^{-8}) was aseptically added to sterile Petri dishes containing twenty ml of sterile King's medium and incubated at 28±2°C for 48 h. after incubation, well separated individual colonies with yellow green and blue white pigments were marked and detected by viewing under UV light. The individual colonies were picked up with sterile loop and transferred to fresh King's B slants and the pure cultures so obtained were stored in refrigerator at 4°C for further use.

Pure cultures of the selected isolates were streaked on King'B agar Petri dishes separately for colony development. The individual colonies were examined for shape and pigmentation of colonies.

Table 1. Isolation of various isolates of *P. fluorescens* from different locality

Antagonists	Locality	Source
Pf_1	Aduthurai	Rhizosphere Soil
Pf_2	Cuddalore	Soil
Pf_3	Kovilpatti	Soil
Pf_4	Sivapuri	Soil
Pf_5	Vallampadugai	Soil
Pf_6	Vadalore	Soil

2.2. Biochemical characterization

For the identification of *P. fluorescens*, certain biochemical test was conducted according to Bergey's Manual for Determinative Bacteriology (Breed *et al.*, 1989).

Gram staining

A loopful of bacterial culture was transferred on a clean slide and a smear was made which was air dried and heat fixed. The smear was flooded for one min. with ammonium oxylate crystal violet. Excess stain was poured off and the slide was washed in a gentle stream of water. Lugol's iodine solution was applied and allowed to remain for one min. Decolorized with 95 per cent ethyl alcohol. The smear was washed in gentle stream of water and counter stained with safranin for 30 seconds. The Gram negative cells appeared red in color and Gram positive cells appeared violet in color (Cyrabree and Hindshhill, 1975).

Starch hydrolysis

Filter paper was dipped in a day old culture suspension and was placed on Petri dishes containing starch agar medium and incubated for two days. The plates were than flooded with one per cent iodine solution. A colorless halo around the growth and blue color in rest of the plates showed utilization of starch by the microorganism (Stolpe and Godkeri, 1981).

Gelatin liquefaction

Filter paper discs were dipped in a day old culture suspension and were placed on Petri dishes containing gelatin nutrient agar medium. The Petri dishes were incubated at 30°C for two days and then flooded with 12.5 per cent $HgCl_2$ solution. The development of yellow halo around the growth indicated utilization of gelatin (Stolpe and Godkeri, 1981).

Fluorescent pigmentation

The test tubes containing sterile King's B medium, inoculated with the isolates of *Pseudomonas* sp. incubated for five days and observed. Yellowish green fluorescent pigment observed under UV light (365 nm) indicated positive results (King *et al*., 1954).

Estimation of IAA

Indole acetic acid (IAA) in the methanol fraction was determined by employing Salper reagent (Gordon and Paleg, 1975). To 1.5 ml of distilled water in a test tube 0.5 ml of methanol residue was mixed, four ml fresh Salper reagent was rapidly added, kept in complete darkness for one hour and read in spectrophotometer at 535 nm. From a standard curve prepared with known concentration of IAA, the quantity of IAA in the filtrate was calculated (1 division = 0.307 µg of IAA).

Extraction of siderophore from the medium

The spent culture fluid was separated from the cells by centrifugation at 7000 rpm for 15 min. The supernatant was concentrated to one fifth of the original volume by flash evaporation at 45°C. Catechol type phenolates were extracted with ethyl acetate from the culture supernatant twice with an equal volume of solvent at pH 2.0. the ethyl acetate layer was removed and evaporated to dryness and the residues were dissolved in a minimum quantity of dist. water, while hydroxamate types were measured from the untreated culture supernatant (Schwyn and Neilands, 1987).

Hydrogen Cyanide (HCN) production

Production of HCN was determined as per the method of Wei *et al*., (1996). Bacteria were grown on TSA supplemented with 4.4g/1 of glycine. White filter paper strips soaked in picric acid solution (2.5 g of Na_2CO_3 and 1 litre of water) were placed in the lid of each Petridish, sealed with parafilm and incubated for two to three days at 28±2°C. After incubation HCN production was indicated by the presence of a coloured zone around the bacteria.

2.3. Testing the antagonism of P. fluorescens against C. capsici

Dual culture technique

The antagonistic activity of *P. fluorescens* against *C. capsici* was tested by dual culture technique (Dennis and Webster, 1971). *P fluorescens* was streaked at one side of Petri dishes (one cm away from the edge) containing PDA. A 9 mm mycelial disc from seven days old PDA culture of *C. capsici* was placed at the opposite side of Petri dishes perpendicular to the bacterial streak and incubated at 28±2°C for 15 days. As *C. capsici* was slow growing in nature, inoculation of *P. fluorescens* was done after 72 h of inoculation of the pathogen. Petri dishes inoculated with fungal discs alone served as control. Three replications were maintained for each isolate. Observation on width of inhibition zone and mycelial growth of test pathogen was recorded and per cent inhibition of pathogen growth was calculated by using the formula proposed by Vincent (1927). $I = C - T / C \times 100$

Where,

I- Per cent inhibition

C-Mycelial growth of pathogen in control

T- Mycelial growth of pathogen in dual plate.

Poisoned food technique

The culture filtrate of the antagonists was separately incorporated into sterilized PDA medium at 5, 10, 15 and 20 per cent by adding the calculated quantity of the culture filtrates to the medium by means of a sterile pipette. The PDA medium without the culture filtrate served as control. The amended media were transferred to sterile Petri dishes separately @ 15 ml and allowed to solidify. Each plate was inoculated at the center with a fifteen day old (9mm) PDA culture disc of *C. capsici* and incubated at room temperature (28±2°C) for 15 days. Mancozeb 75% WP (0.25%) served as comparison and three replications were maintained for each treatment. The radial growth of the mycelium was measured after fifteen days of incubation. The results were expressed as per cent growth inhibition over control.

3. **RESULTS**

3.1. Isolation And Identification Of Biofungicides

A total of 6 isolates of *P. fluorescens* were isolated from rhizosphere soil of chilli in various part of Tamil Nadu. All the collected isolates of *P. fluorescens* were identified and purified using streak method. After

purification they were maintained as stock culture in king's B slant at 10^oC.

Morphological characteristics of *P. fluorescens* isolates

It was evident from the result presented in table 5 that, isolates of *P. fluorescens* produced round and irregular shaped colony. Pf_1, Pf_2, Pf_3, and Pf_5, produced colonies with round shapes, whereas Pf_4, and Pf_6, produced colonies with irregular shapes. All the isolates were found to have light greenish pigments (Pf_1, Pf_2, Pf_3 Pf_4, Pf_5 and Pf_6). Among the isolates four isolates showed *viz*., Pf_1, Pf_2, Pf_4, Pf_5, fluorescence under UV light, whereas others were non fluorescent (Table 2).

Biochemical characterization of *P. fluorescence* isolates

The results of the biochemical tests performed for the identification of the effective native isolates of *P. fluorescens* showed that all the isolates produced similar results with regard to gram staining (negative), gelatin liquefaction (positive), catalase test (positive), oxidase test (positive), starch hydrolysis (negative), and fluorescent pigmentation (positive). All the isolates showed positive results in IAA production. Among the isolates Pf_1 produced more quantity (4.1µg/ml) of IAA followed by Pf_6, Pf_4, Pf_3, Pf_2 and Pf_5 in the decreasing order of merit. The isolate Pf_1 recorded maximum (0.94 µg/ml) production of siderophores followed by Pf_6, Pf_4, Pf_3, Pf_2 and Pf_5 in the decreasing order of merit. All the isolates showed positive results with regard to hydrogen cyanide production. (Table 3).

3.2. In vitro* efficacy of *P. fluorescens* isolates against *C. capsici

Antagonistic activity of *P. fluorescens* isolates against *C. capsici* (Dual culture Technique)

The result of the dual culture technique indicated that all the isolates inhibited the growth of test fungus significantly (Table 4). Among the isolates, Pf_1 produced maximum reduction of mycelial growth (21.52mm) accounting for 76.08 per cent reduction over control. This was followed by the isolates Pf_6, Pf_4, Pf_3, Pf_2 and Pf_5 recording 73.48, 64.04, 67.70, 59.2 and 53.77 Percent respectively. The isolates Pf_5 was the least effective recording 53.77 per cent inhibition over control.

Effect of culture filtrates of *P. fluorescens* isolates at different concentration on mycelial growth of *C. capsici* (Poisoned food technique)

The results presented in table 5 revealed that all the isolates showed reduction on mycelial growth of *C. capsici.* Among the isolates, Pf_1 at concentration of 5, 10, 15 and 20 per cent concentration showed an increase in the inhibition of the mycelial growth recording 36.07, 22.87, 10.84 and 2.48 mm respectively. Also, a general increase in the concentration of the culture filtrate showed an increase in the inhibition of the mycelial growth of the test pathogen. Among the isolates the culture filtrate of Pf_1 at 20 per cent conc. was found to be effective in reducing the mycelial growth to the minimum (2.48mm) accounting for the highest per cent inhibition (97.24%) of the pathogen over control. This was followed by Pf_6 @ 20% conc recording (93.08% inhibition) and Pf_4 (88.11% inhibition). The least effect was found with isolate Pf_5 (69.46 per cent inhibition).

4. DISCUSSION

The present study clearly revealed that isolates of *P. fluorescens* produced round and irregular shaped colony. Pf_4 and Pf_6, produced colonies with irregular shape, Pf_1 Pf_2, Pf_3, and Pf_5, produced colonies with round shape. All the isolates had light greenish pigments. Among the isolates four isolates showed fluroescence under UV light whereas others were non fluorescent. According to Todar (2004), more than half of the *Pseudomonas* bacteria produced pyocyanin which is a blue-green pigment, while the non-pathogenic saprophyte *P. fluroescens* produced fluorescent pigment that is soluble and greenish. In the present study, all the four isolates produced light green fluorescence on King's B medium under ultraviolet light. The result of the biochemical tests performed for the identification of the effective native isolates of *P. fluorescens* showed that all the isolates produced similar result with regard to gram staining (negative), gelatin liquefaction (positive) catalase test (positive), oxidase test (positive), starch hydrolysis (negative), and fluorescent pigmentation (positive). All the isolates showed positive result in IAA production and HCN production. Further, the cultural, morphological and biochemical characters have been used to identify and confirm *P. fluroescens*. Similarly, in the present study also the cultural, morphological and biochemical characters confirmed the isolates to be *P. fluorescens* as reported by earlier workers (Meera and Balabaskar 2012; Meera *et al.,* 2013).

Also, In the present study, all the six isolates of *P. fluorescens* showed varying degrees of antagonism against *C. capsici*. Among the isolates, Pf_1 was the most antagonistic and formed the maximum inhibition zone and maximum per cent reduction on the mycelial growth of *C. capsici*. The mycoparasitic potential of *Pseudomonas* spp. is well documented (Whipps, 1997) and this phenomenon has often been used as means for *in vitro* screening of biocontrol agents (Elad *et al*., 1980). *P. fluroescens* isolates EBS 20 produced higher levels of extracellular metabolites like siderophore, salicylic acid and HCN when compared with other isolates which was highly effective in inhibiting the growth of *Pythium aphanidermatum* inciting chilli damping-off. (Muthukumar *et al*., 2010). Similarly, antifungal compounds such as pseudobactin, HCN, salicylic acid and 2-hydroxy phenazine produced by fluorescent pseudomonas suppressed plant pathogenic fungi (Pandey *et al*., 2006; Reddy *et al*., 2008). The antifungal metabolites produced by *P. fluorescens* might be attributed as the reason for the reduction in the growth of the pathogen and *P. fluorescens* were known to produce an array of low-molecular weight metabolites some of which were potential antifungal agents (O' Dowling and O' Gara, 1994). Earlier workers reported that *P. fluorescens* effectively reduced mycelial growth of other pathogens (Sundaramoorthy *et al*., 2013; Chacko and Gokulapalan, 2014).

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Table 2. Morphological characteristics of *P. fluorescens* isolates

Isolates	Cell shape	Colony type	Colony colour/ pigmentation	Reaction to UV light
Pf_1	Rod	Round	Light greenish	Fluorescent
Pf_2	Rod	Round	Light greenish	Fluorescent
Pf_3	Rod	Round	Light greenish	No reaction
Pf_4	Rod	Irregular	Light greenish	Fluorescent
Pf_5	Rod	Round	Light greenish	Fluorescent
Pf_6	Rod	Irregular	Light greenish	No reaction

Table 3. Bio chemical characterization of *P. fluorescence* isolates

Isolates	Parameters							
	Gram staining	Gelatin liquefaction	Catalase test	Oxidase test	Starch hydrolysis	Fluorescence pigment	*Estimation of IAA (ụg/ml)	*Siderophore production (Hydroxamate) (ụg/ml)	*Hydrogen cyanide production (ụg/ml)
Pf_1	Negative	Positive	Positive	Positive	Negative	Positive	4.1^{a}	0.94^{a}	8.82^{a}
Pf_2	Negative	Positive	Positive	Positive	Negative	Positive	2.6^{e}	0.65^{e}	6.88^{f}
Pf_3	Negative	Positive	Positive	Positive	Negative	Positive	3.1^{d}	0.88^{c}	8.28^{d}
Pf_4	Negative	Positive	Positive	Positive	Negative	Positive	3.6^{c}	0.86^{d}	8.41^{c}
Pf_5	Negative	Positive	Positive	Positive	Negative	Positive	2.5^{f}	0.62^{f}	6.97^{e}
Pf_6	Negative	Positive	Positive	Positive	Negative	Positive	3.8^{b}	0.90^{b}	8.54^{b}

*Values in the column followed by same letters not differ significantly by DMRT (P=0.05)

Table 4. Antagonistic activity of *P. fluorescens* isolates against *C. capsici* (Dual culture technique)

S. No.	Native isolates	*Mycelial growth the pathogen (mm)	Per cent inhibition over control (%)
1.	Pf_1	21.52^{a}	76.08
2.	Pf_2	36.71^{e}	59.21
3.	Pf_3	29.07^{d}	67.70
4.	Pf_4	32.36^{c}	64.04
5.	Pf_5	41.60^{f}	53.77
6.	Pf_6	23.86^{b}	73.48
7.	Control	90.00^{g}	00.00

Values in the column followed by common letters do not differ significantly by DMRT (P=0.05)

Table 5. Effect of culture filtrates of *P. fluorescens* isolates at different concentrations on mycelial growth of *C. capsici*
(Poisoned food technique)

S. No.	Isolates	*Mycelial growth the pathogen (mm)				Per cent inhibition over control			
		5 %	10 %	15 %	20 %	5%	10%	15%	20%
1.	Pf_1	36.07^a	22.87^a	10.84^a	2.48^a	59.92	74.58	87.95	97.24
2.	Pf_2	56.79^e	44.74^e	31.30^e	22.33^e	36.90	50.28	65.22	75.18
3.	Pf_3	51.82^d	39.18^d	26.45^d	15.99^d	42.42	56.46	70.61	82.23
4.	Pf_4	46.61^c	33.11^c	20.82^c	10.70^c	48.21	63.21	76.86	88.11
5.	Pf_5	61.77^f	50.76^f	36.17^f	27.48^f	31.36	43.60	59.81	69.46
6.	Pf_6	41.29^b	27.81^b	15.78^b	6.22^b	54.12	69.00	82.46	93.08
7.	Mancozeb 75% WP (0.25% conc.)	0.00^a	0.00^a	0.00^a	0.00^a	100	100	100	100
8.	Control	90.00^g	90.00^g	90.00^g	90.00^g	0.00	0.00	0.00	0.00

*Values in the column followed by same letters not differ significantly by DMRT (P=0.05)