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Full Length Research Paper

Efficacy of *Pseudomonas fluorescens* on control of chilli fruit rot caused by *Colletotrichum capsici*

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The fungitoxic effects of 10 isolates of *Pseudomonas fluorescens* from various parts of Tamil nadu were evaluated under *in vitro* conditions on growth of *Colletotrichum capsici*, the causal agent of chilli fruit rot. The identity of *P. fluorescens* was confirmed by DNA sequence analysis of the isolates. *P. fluorescens* PI-1 was the most effective showing 75.6% inhibition of colony growth with minimum mean mycelial growth (4.7 cm) of the pathogen. The aim of this research work was to study the use of biocontrol agents as an alternative to fungicides in the control of fruit rot of chilli.

Key words: Polymerase chain reaction (PCR) analysis, disease management, plant growth promotion.

INTRODUCTION

Chilli (*Capsicum annuum* L.) is an important spice crop of India. It is affected by several fungal, bacterial and viral diseases, of which chilli anthracnose causes considerable damage, inflicting severe quantitative and qualitative losses. The estimated loss due to this disease ranged from 8 - 60% in different parts of India. The fungus C*olletotrichum capsici* infects both unripe (green) and ripe (red) chilli fruits and survives on seed as acervuli and micro sclerotia (Suthin Raj and Christopher, 2009; Suthin Raj et al., 2013). Infection of *C. capsici* is higher at the mature fruit stage than in the early fruit stage. The fungus pathogen is both seed borne and air borne and affects seed germination and vigour to a greater extent. Several fungicides have been reported to be effective in the management of fruit rot of chilli (Gopinath et al., 2006; Shovan et al., 2008). However, the indiscriminate use of fungicides leads to toxic residues on chilli products, development of fungicide resistance and also serves as a cause for environmental pollution (Suthin Raj et al., 2012). Therefore, under intensive chilli cultivation, there is an urgent need to develop alternative disease control measures. The present investigation screened various *P. fluorescens* isolates (confirmed by PCR analysis) against *C. capsici in vitro* and in a field trial on their efficacy to control *C. capsici*

MATERIALS AND METHODS

Collection of seed materials

Fresh seed samples of *C. capsici* were collected from the Department of Agronomy, Annamalai University, Chidambaram, Tamil Nadu.

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Isolation, maintenance and identification of *C. capsici*

Diseased chilli fruits showing typical symptom of anthracnose disease were collected fresh from 20 conventional chilli growing areas of Tamilnadu. The pathogens isolated from each of these localities formed one isolate of *C. capsici.* The pathogen was isolated onto potato dextrose agar (PDA) medium from diseased specimens showing typical symptoms. The infected portion of the fruit was cut into small pieces, surface sterilized in 0.1% mercuric chloride solution for 30 s and then washed in repeated changes of sterile distilled water and plated onto sterile PDA medium in 9 cm Petri dishes. The plates were incubated at room temperature (28 ± 2°C) for five days and then observed for fungal growth. Pure cultures were obtained using the single spore isolation technique (Rangaswami, 1958). Identification of the isolates was confirmed by comparing them with the pure culture obtained from ITCC, IARI, New Delhi and the purified isolates were maintained on PDA slants.

Evaluation of bacterial antagonism against *C. capsici*

Isolation of bacterial antagonist

Ten strains of *P. fluorescens* were isolated from the rhizospheric soil of healthy chilli cultivating fields. A sample of 10 g soil was suspended in 100 ml of sterile physiological water and shaken vigorously at 28°C for 30 min. Serial dilutions were plated on pneumococcal cell wall (PCW) isolation agar medium, and each dilution was incubated at 30°C until colonies were observed. Bacterial colonies that exhibited fluorescence at 365 nm (King et al., 1954) were selected and purified for further studies.

In vitro antagonistic activity

Dual culture technique (Dennis and Webster, 1971)

P. fluorescens was multiplied on King's 'B' medium (20 g pblightease peptone, 1.5 g magnesium sulphate, 1.5 g dipotassium hydrogen phosphate, 15 g agar agar, 10 ml glycerol, 1000 ml distilled water and pH adjusted to 7.2). An 8 mm disc of the pathogen from an actively growing PDA culture was placed onto PDA plates 1.5 cm away from the edge of the plate, and incubated at room temperature (28 ± 2°C). After 48 h, actively growing cultures of the respective bacterial isolates were separately streaked onto the medium on the opposite side of the plate, 1.5 cm away from the edge of the plate. The inoculated plates were re-incubated at room temperature (28 ± 2°C). Three replications were maintained for each bacterial isolate. Control PDA plates were inoculated with the pathogen alone. The radial growth of the pathogen was measured after 48 h. The results were expressed as percent growth inhibition over control.

Mycelial dry weight

Potato dextrose broth was prepared in 250 ml Erlenmeyer flasks and autoclaved. Aliquots of 5, 10, 15 and 20 ml of culture filtrates of *P. fluorescens* taken from 48 h were added to 45, 40, 35 and 30 ml broth in flasks to give a final concentration of 10, 20, 30 and 40% of the culture filtrate in the broth. All the flasks were inoculated with 8 mm culture discs of *C. capsici* and incubated at 28 ± 1°C for 10 days. Flasks containing broth without any culture filtrate served as controls. Each treatment was replicated three times. After 15 days of incubation, the mycelial mat was harvested on a previously weighed filter paper and dried at 105°C for 12 h in a hot air oven, cooled in a desiccator and the mycelial weight was recorded and expressed as mg/50 ml broth.

The most effective isolates of *P. fluorescens* ($P.I_1$) were used for further study.

Identification *P. fluorescens* by PCR

DNA extraction

Template DNA was prepared by boiling 200 µl of bacterial suspension in milliQ (OD_{600}= 0.6) in safe lock Eppendorf tubes for 10 min. The tubes were immediately cooled on ice and centrifuged (20,000 g x 10 min, 5°C). The supernatants were subsequently kept on ice or at -20°C. A microlitre of template DNA suspension was used for each reaction.

PCR amplification

All the PCR amplification were performed in a volume of 50 µl containing ≈50 to 100 ng of bacterial genomic DNA solution, 5 µl of 10 Ml PCR buffer, 200 µM of each dNTP; 2 mM of $MgCl_2$; 0.5 µM of each primer and 0.5 U of taq polymerase (obtained from Genei, Bangalore). DNA 16S specific region for *P. fluorescens* amplification was performed using the primer set 16SPSEfluF and 16SPSER (16SPSEfluF 5'-TGCATTCAAA-ACTGACTG-3'; 16SPSER 5'-AATCACACCGTG-GTAACCG-3'). The forward primer is species specific, while the reverse is family specific. The primers were developed and compared with partial regions 16S of *P. fluorescens* belonging to group 1 (NCBI, National Center for Biotechnology Information) by software DNA sis 2.0 and the following thermal profile: 2 min at 94°C; 5 cycles consisting of 94°C for 45 s, 55°C for 1 min, 72°C for 2 min; 35 cycles consisting of 92°C for 45 s, 60°C for 45 s, 72°C for 2 min; final extension of 72°C for 2 min; and final cooling at 4°C. The amplification was performed in a DNA thermal cycle (Yercaud Biotech, Salem).

Following amplification, 7 µl of product were analysed by electrophoresis at 100 V (1% agrose gel 0.2 µg of ethidium bromide ml^{-1}) in TAE buffer. Photograph was taken with the Nikon COOLOIXSI0 VR Camera and Gel Documentation was done in DGelDAS Software analysis tool (Yercaud Biotech, Salem).

Evaluation of *P. fluorescens* for the management of *C. capsici* under field conditions

The field trials were conducted at Shathankudi Village, Perambalur-District between December 2009 and March 2010 in a field with a history of chilli fruit rot incidence. Trials were laid out in plots (33 x 13 feet) arranged in a randomized block design. Thirty day-old seedlings were planted into the field plots in rows with row/plants spacing of 60 x 30 cm and a total population of 210 plants/plot. Three replicate plots were maintained for each treatment. Treatment application details and experimental observation were the same as in the green house experiment with below mentioned treatment schedule. Regular cultivation practices were followed as per the recommendation.

Treatment details

T_1: Application of *P. fluorescens* (seed treatment); T_2: application of *P. fluorescens* (prophylactic spray at 25 and 75 DAT); T_3: T_1 + T_2; T_4: seed treatment with mancozeb + spraying 50 and 75 DAT; T_5: control.

Disease incidence

The fruit rot incidence was assessed 100, 125 and 150 days after transplanting. The intensity of fruit rot was calculated using the

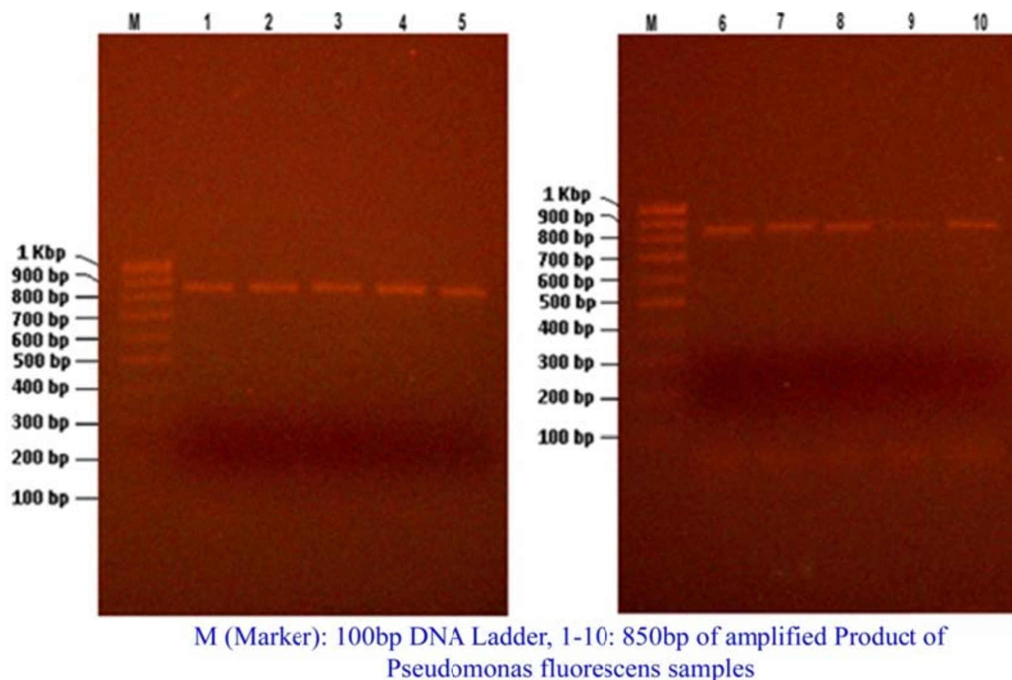

Figure 1. Agarose gel showing species-specific amplification for *P. fluorescens* using PCR.

percent disease index (PDI) grade chart proposed by Reddy et al. (2008); the percent disease index (PDI) was calculated using the Mc Kinney (1923) infection index:

$$PDI = \frac{\text{Sum of numerical ratings}}{\text{Total number of fruits observed}} \times \frac{100}{\text{Maximum category value}}$$

Plant growth parameters

Growth parameters *viz*., mean leaf area, mean plant height, mean no. of flowers/plant, mean no. of fruits/plant, mean fruit length and fruit yield were assessed for the plants at the time of harvest.

Experimental design and data analysis

The experiments were conducted using completely randomized design (CRD) with three replications. The significant difference, if any, among the means were compared by Duncan's multiple range test (DMRT). Whenever necessary, the data were transformed before statistical analysis following appropriate methods.

RESULTS

PCR assay to identify *P. fluorescens*

Ten isolates of *P. fluorescens* were collected from various parts of Tamilnadu and were confirmed by using PCR analysis. Primer sets 16SPSEfluF and 16SPSER (16SPSEfluF 5'-TG CATTCAAA-ACTGACTG-3'; 16SPSER 5'-AATCACACCGTG-GTAACCG-3') (obtained from Genei, Bangalore) were used to amplify the DNA 16S specific region for *P. fluorescens.* The size of the amplified single DNA fragment obtained was about 850 bp of 16SrRNA. All the tested isolates of *P. fluorescens* were amplified in a single DNA fragment of 850 bp of 16SrRNA (Figure 1). Hence all 10 isolates were confirmed as *P. fluorescens.*

In vitro evaluation of *P.fluorescens against C. capsici*

The results of the screening of 10 isolates of *P. fluorescens* against *C. capsici* on PDA plates are presented in Table 1. Among the isolates, *P. fluorescens* PI-1 appeared to be most effective against *C. capsici* showing 75.6% inhibition of colony growth and minimum mean mycelial dry weight in broth 146.5 mg/50 ml broth. It was followed by isolate PI-8 which showed 74.1% inhibition and minimum mean mycelial dry weight in broth of 153 mg/50 ml.

Isolates PI-9 and PI-10 showed minimum growth inhibition. All the isolates significantly had reduced mycelial growth of the pathogen over the control.

Mycelial dry weight

The mycelial dry weight of *C. capsici* decreased with increasing concentration of culture filtrates of *P. fluorescens* in all isolates and isolates P1 and P8 again showed the greatest inhibition (78 and 72%, respectively) on mycelia dry weight (Table 1).

Table 1. Evaluation of various isolates of *P. fluorescens* against *C. capsici* by dual culture technique.

Isolate	Linear growth (mm)		Growth inhibition (%)	Mycelial dry weight (mg/50 m/broth)				
	Antagonist	*C. capsici*		10%	20%	30%	40%	Mean
P. fluorescens (P. I_1) (TNAU-P.f_1)	68.00	22.00	75.56*[a] (4.33)**	260 (5.56)	200 (5.30)	104 (4.65)	22 (3.13)	4.66[a]
P. I_2 - Dharmapuri	60.60	29.40	67.32[b] (4.22)	305 (5.72)	228 (5.43)	114 (4.74)	30 (3.43)	4.83[bc]
P. I_3 - Marthandam	53.60	36.40	59.11[d] (4.05)	326 (5.79)	235 (5.46)	119 (4.78)	34 (3.55)	4.89[b]
P. I_4 - Aduthurai	51.20	38.80	56.89[e] (4.50)	338 (5.82)	249 (5.52)	123 (4.82)	37 (3.63)	4.94[b]
P. I_5 - Vallampadugai	54.50	35.50	60.55[d] (4.11)	330 (5.80)	239 (5.48)	121 (4.80)	36 (3.61)	4.92[b]
P. I_6 - Vadalore	62.00	28.00	68.88[b] (4.24)	282 (5.64)	224 (5.41)	111 (4.71)	34 (3.55)	4.83[bc]
P. I_7 - Kovilpatti	56.33	33.67	62.58[c] (4.15)	313 (5.74)	231 (5.44)	116 (4.76)	32 (3.49)	4.86 [b]
P. I_8 - Annamalainagar	66.70	23.30	74.11[a] (4.31)	266 (5.58)	208 (5.34)	107 (4.68)	27 (3.32)	4.72[ab]
P. I_9 - Cuddalore	48.72	41.28	54.13[f] (4.00)	354 (5.87)	256 (5.54)	136 (4.91)	40 (3.70)	5.01[cd]
P. I_{10} - Sivapuri	49.60	40.40	55.11[f] (4.02)	351 (5.86)	248 (5.51)	132 (4.89)	39 (3.68)	4.98[c]
Control		90.00		540 (6.29)	540 (6.29)	540 (6.29)	540 (6.29)	6.29[d]
Mean				5.79[d]	5.52[c]	4.91[b]	3.76[a]	

*Values in the column followed by common letters do not differ significantly by DMRT (P=0.05). **Figures in parenthesis are arcsin transformation.

Effect of *P. fluorescens* on incidence of fruit rot under field conditions

The results (Table 2) indicated that the application of *P. fluorescens* (seed treatment + prophylactic spray at 25 and 75 DAT) (T_3) significantly reduced the incidence of fruit rot at 100, 125 and 150 days after planting, respectively, as compared to the other treatments. This was followed by application of Mancozeb (seed treatment + prophylactic spraying 30 and 45 DAT) (T_4).

Effect of *P. fluorescens* and mancozeb treatments on growth and yield of *C. capsici* under field condition

Table 3 shows that all treatments have significantly enhanced the growth and fruit yield, as compared to the control. Among the combination, seed treatment + prophylactic spraying 25 and 75 DAT with *P. fluorescens* (T_3) significantly increased mean plant height, number of flowers/plant, mean number of fruits/plant, mean fruit length and fruit yield (375 g/plant) followed by spraying mancozeb (seed treatment + prophylactic spraying at 30 and 45 DAT) (T_4)

DISCUSSION

DNA sequence analysis has been used to characterize and analyze the taxonomic complexity of *P. fluorescens.* The present study revealed that the primer sets 16SPSEfluF and 16SPSER (16SPSEfluF 5'-TG CATTCAAA-ACTGACTG-3'; 16SPSER 5'-AATCACACCGTG-GTAACCG-3') (Genei, Bangalore) were used to amplify the DNA 16S specific region for *P. fluorescens*. The size of the amplified single DNA fragment obtained was 850 bp of 16S rRNA for *P. fluorescens*. All the tested isolates of *P. fluorescens* were amplified in single DNA fragments of 850 bp of 16SrRNA. A similar study was carried out by Scarpellini et al. (2004) who reported the amplification of single DNA fragment of 850 bp of 16S rRNA for *P. fluorescens*.

The results revealed that, all the ten isolates of *P. flourescens* (TNAU) and native isolates inhibited the growth of *C. capsici in vitro*. Similar results were observed by Vivekananthan et al. (2004, Bharathi et al. (2004), Srinivas et al. (2006), Muthukumar et al. (2010) and Anand et al. (2010). The above authors have reported that isolate *P. flourescens* as well as native isolates strongly inhibited the growth of *C. capsici* under laboratory condition.

Table 2. Effect of IDM formulation on fruit rot incidence under field condition.

Treatment	Fruit rot incidence on 100th day	Increase over control (%)	Fruit rot incidence on 125th day	Increase over control (%)	Fruit rot incidence on 150th day	Increase over control (%)
T_1 - Application of *P. fluorescens* (Seed treatment)	3.95[c]	49.68	7.44[cd]	49.04	9.70[c]	57.86
T_2 - Application of *P. fluorescens* (prophylactic spray at 25 and 75 DAT)	4.44[d]	43.43	8.05[d]	42.86	11.09[d]	38.82
T_3 - T_1 + T_2	2.50[a]	68.15	6.60[a]	54.79	8.33[a]	63.81
T_4-Seed treatment with mancozeb + spraying, 30 and 45 DAT	3.23 [b]	58.53	6.99 [b]	52.12	8.98 [b]	60.99
T_5 - Control	7.85 [e]		14.60[e]		23.02[e]	

*In a row under each treatment, value in the column followed by common letters do not differ significantly by DMRT (P = 0.05).

Table 3. Effect of IDM formulation on growth and yield attributes under field condition.

Treatment	Mean plant height (cm)	Mean no. of flowers/plant	Mean no. of fruits/plant	Mean fruit length (cm)	Fruit yield (g/plant)
T_1 - Application of *P. fluorescens* (seed treatment)	99.10[c]	162[c]	94[c]	7.10[c]	350[c]
T_2 - Application of *P. fluorescens* (Prophylactic spray at 25 and 75 DAT)	98.21[d]	160[d]	92[d]	6.76[d]	338[d]
T_3 - T_1 + T_2	109.06[a]	184[a]	110[a]	8.88[a]	375[a]
T_4 - Seed treatment with mancozeb + spraying, 30 and 45 DAT	101.29[b]	166[b]	96[b]	7.55[b]	352[b]
T_5 - Control	85.95[e]	150[e]	61[e]	4.20[e]	230[e]

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

In the present study, application of *P. fluorescens* (P.f1) (seed treatment + prophylactic spraying at 25 and 75 DAT) at 50 DAT significantly reduced the incidence of *C. capsici.* A significant reduction in *C. capsici* incidence from *P. fluorescens* (Pf-1) + chitin treatment was recorded in chilli plants by Bharathi et al. (2004). Similar conclusions on the management of plant diseases by different pseudomonad strains either as bacterial suspension or through different formulations have been reported by many workers (Vidhyasekaran et al., 1997; Viswanathan and Samiyappan, 1999). This may be due to fluorescent pseudomonas producing compounds like pseudobactin, HCN, salicylic acid, 2-hydroxy phenazine, oligomycin, pyoluteorin, pyrolnitrin, pyocyanin and 2,4-diacelyphloroglucinol which elicit induced systemic resistance in the host plant or interfere specifically with fungal pathogens (Ongena et al., 1999; Velazhahan et al., 1999; Dave and Dube, 2000; Gupta et al., 2001; Pandey et al., 2006; Hofte and Bakker, 2007; Reddy et al., 2008 and Muthukumar et al., 2010).

The results of the present experiment revealed the superiority of all the treatments in increasing the mean leaf area, mean plant height, mean number of flowers/plant, mean number of fruits/plant, mean fruit and fruit yield g/plant length over control. Application of P.f1 (seed treatment + prophylactic spraying at 25 and 75 DAT) to treated chilli plants was found to be more effective than the other treatments in improving growth

characters and yield parameters. Combination of bioagents *viz. P. fluorescens* (Pf-1) and *B. subtilis* along with soil application of organic amendments significantly increased growth characters and yield parameters (Reddy et al., 2008). Same phenomenon was observed by Almaghrabi et al. (2013). Combined application of *P. fluorescens* (Pf32, Pf93) + *B. subtilis* (B49) significantly increased the plant height, number of branches and number of bolls under field conditions in cotton (Salaheddin et al., 2010).

The present study indicated that application of *P. fluorescens* (seed treatment + prophylactic spray) will be effective in controlling the chilli fruit rot disease and increasing the plant growth and yield of chilli.

Conflict of Interest

The authors have not declared any conflict of interest.

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