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EVALUATION OF *PSEUDOMONAS FLUORESCENS* FROM RHIZOSPHERE SOIL AGAINST FRUIT ROT OF CHILLI CAUSED BY *COLLETOTRICHUM CAPSICI* (SYD) BUTLER AND BISBY

M. Charumathi and T. Suthin Raj

Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Chidambaram (Tamilnadu) India.

Abstract

The present study aimed to screen Plant Growth Promoting Rhizobacteria (PGPR) of *Pseudomonas fluorescens* for its effect on induction of resistance in chilli (*Capsicum annum* L.) against anthracnose disease under *in vitro* conditions. A total of 20 isolates were evaluated for their antagonistic activity against *Colletotrichum capsici* where Pf 1 native isolate was able to inhibit mycelial growth of the pathogen followed by Pf 11 and Pf 2. In dual culture assay, Pf 1 showed 93.41% inhibition whereas Pf 11 produced 76.81% inhibition of *C. capsici*. The mycelial dry weight of *C. capsici* decreased with an increasing concentration of culture filtrates of *P. fluorscens* and among them, Pf 1 showed a greatest inhibition compared to the other isolates. Seed treatment with *Pseudomonas fluorescens* resulted in a maximum enhancement of seed germination (97.60%) and seedling vigor (1287.34) with an increase in vegetative growth parameters.

Key words: *C. capsici*, *Pseudomonas fluorescens*, Chilli fruit rot.

Introduction

Chilli is the fourth most important vegetable crop in the world and first in Asia but its production poses significant risks. Anthracnose disease in chilli is one of the most important limiting factors of chilli production worldwide, especially in tropical and sub-tropical regions which causes both qualitative and quantitative yield loss (Than *et al*., 2008). The fungus *Colletotrichum capsici* affects both mature and immature chilli fruits and survives on seed as acervuli and micro sclerotia (Suthin Raj and Christopher 2009, Suthin Raj *et al*., 2014a). Anthracnose causes yield loss up to 50% in India (Sharma *et al*., 2005). Cultural methods, biological control, application of chemical fungicides and use of resistant cultivars are amongst the effective disease control measures that have been employed to control chilli anthracnose (Than *et al*., 2008). Although the management of anthracnose disease is still being extensively researched, commercial cultivars of *C. annuum* that are resistant to anthracnose have not yet been developed. Conversely, the dose and frequency of fungicides being applied to control the disease is costing too much for the small scale farmers often without significant benefit (Handisoa and Alemub 2017). Conventional synthetic fungicides need to be replaced by bio-fungicide as the former lost their effectiveness due to pesticide treadmill. Crop productivity is affected by environmental and genetic factors. Microbes that are beneficial to plants are used to enhance the crop yield and are alternatives to chemical fertilizers and pesticides. *Pseudomonas* and *Bacillus* species are the predominant plant growth-promoting bacteria. The antagonistic bacteria especially PGPR, *Fluorescent pseudomonads* are the most exploited bacteria for biological control of soil-borne and foliar plant pathogens. In past three decades, numerous strains of *Flurorescent pseudomonads* have been isolated from the rhizosphere soil and plant roots by several workers and their biocontrol activity against soil borne and foliar pathogens are reported (Vivekananthan, 2004). *Pseudomonas* spp. secrete several metabolites that trigger plant growth and prevent pathogen infection. Limited studies have been conducted to understand the physiological changes that occur in crops in response to *Pseudomonas* spp. to provide protection against adverse environmental conditions (Radhakrishnan *et al*., 2017). Environment friendly control tactics gained impetus due to growing socio-economic concerns. In pursuit of finding replacement of toxic pesticides, scientists working in these lines started trying biocontrol and such other substitutes.

In the past decades, therefore, quite a few scientific published information became available on this subject. This research was, therefore, initiated with the objective of managing chili anthracnose through antagonists under laboratory condition.

Materials and Methods

Isolation of pathogen

The pathogenic organism was isolated from the semi riped fruits of Chilli as a pure culture on potato dextrose agar (PDA) medium. Diseased fruit were cut into 3-mm pieces using a sterile scalpel. These were surface sterilized with 1% sodium hypochlorite solution for 30 sec., washed repeatedly in sterile distilled water and then aseptically transferred onto PDA plates. The plates were incubated at 28°C for 5-7 days. Aseptically, bits of mycelia were taken from the margin of colonies on PDA medium. These were subcultured on fresh medium. The purity of the culture was checked microscopically comparing the mycelia and conidial characters of the organism with those observed during a histopathological study. The culture was maintained in the same medium and stored at 4°C for further study.

Isolation of bacterial antagonist

20 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 done from 10^{-1} to 10^{-6}. One ml of suspension from 10^{-6} dilution was transferred and plated on K.B. agar medium and incubated at 30°C until colonies were observed (King *et al*., 1954). After 48 hours, a loopful of bacterial antagonist was purified by streak plateing and the purified culture was used for further studies.

Morphological characterization

Pure culture of the selected isolates were streaked on King's B. agar Petri plates separately for colony development. The individual colonies were examined for shape, size, structure of colonies and pigmentation.

Biochemical tests for *P. fluorescens*

For the identification of *P. fluorescens*, certain biochemical tests were conducted according to Bergey's Manual for Determinative Bacteriology.

Gram staining

A loopful 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 strain 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. and decolorized with 95 percent ethyl alcohol. The smear was washed in gentle stream of water and counter stained with saffranin for 30 seconds. The Gram negative cells appeared red in color and Gram positive cells appeared violet in color.

Table 1: Cultural characteristic of different isolates of *Pseudomonas fluorescens.*

Isolates	Cell shape	Colony type	Colour of the colony	Type of growth	Reaction to UV light fluorescence emission
Pf1	Rod	Round	Yellowish	Fast	Bright
Pf2	Rod	Round	Yellowish white	Fast	Bright
Pf3	Rod	Round	Dull yellowish	Fast	Dull
Pf4	Rod	Round	Yellowish	Fast	Bright
Pf5	Rod	Round	Yellowish	Fast	Bright
Pf6	Rod	Round	Yellowish white	Fast	Bright
Pf7	Rod	Round	Yellowish white	Fast	Bright
Pf8	Rod	Round	Yellowish	Fast	Bright
Pf9	Rod	Round	Yellowish	Fast	Bright
Pf10	Rod	Round	Yellowish white	Fast	Bright
Pf11	Rod	Round	Dull yellowish	Fast	Dull
Pf12	Rod	Round	Yellowish white	Fast	Bright
Pf13	Rod	Round	Yellowish	Fast	Bright
Pf14	Rod	Round	Yellowish	Fast	Bright
Pf15	Rod	Round	Yellowish	Fast	Bright
Pf16	Rod	Round	Dull yellowish	Fast	Dull
Pf17	Rod	Round	Yellowish white	Fast	Bright
Pf18	Rod	Round	Yellowish	Fast	Bright
Pf19	Rod	Round	Yellowish	Fast	Bright
Pf20	Rod	Round	Yellowish white	Fast	Bright

Starch hydrolysis

Filter paper was dipped in a dry 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 percent iodine solution. A colorless halo around the growth and blue color in the rest of the plates showed utilization of starch by the microorganism.

Fluorescent pigment

The test tubes containing sterilized Kings B. medium were inoculated with the isolate of *Pseudomonas* sp. incubated for five days and observed. Yellowish green fluorescent pigment observed under UV light (365 nm) indicated a positive result.

Efficacy of bacterial antagonists against *C. capsici* in *in vitro* (Dennis and Webster, 1971)

A 8 mm actively growing PDA culture disc of the pathogen was placed on a PDA Petri dish at one side, 1.5 cm away from the edge of the plate and incubated at room temperature 27±2°C. Forty-eight hours later, actively growing cultures of the respective test bacteria were separately streaked onto the medium at the opposite side of the plate, 1.5 cm away from the edge. This was done in three replications for each treatment and incubated. The medium with the pathogen alone served as a control. After 6 days, the radial growth of the pathogen was measured. The results were expressed as percent growth inhibition on the control.

Mycelial dry weight

Potato dextrose broth was prepared in 250 ml Erlenmeyer flasks and autoclaved. Aliquots of 10, 15 and 20 ml of culture filtrates of *P. fluorescens* grown for 48 h. were added to 40, 35 and 30 ml broth in flasks to give a final concentration of 20, 30 and 40%. 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.

Table 2: Biochemical characterization of different isolates of *Pseudomonas fluorescens.*

Isolates	Gram staining	Starch hydrolysis	Fluorescent pigmentation
Pf1	Negative	Negative	Positive
Pf2	Negative	Negative	Positive
Pf3	Negative	Negative	Positive
Pf4	Negative	Negative	Positive
Pf5	Negative	Negative	Positive
Pf6	Negative	Negative	Positive
Pf7	Negative	Negative	Positive
Pf8	Negative	Negative	Positive
Pf9	Negative	Negative	Positive
Pf10	Negative	Negative	Positive
Pf11	Negative	Negative	Positive
Pf12	Negative	Negative	Positive
Pf13	Negative	Negative	Positive
Pf14	Negative	Negative	Positive
Pf15	Negative	Negative	Positive
Pf16	Negative	Negative	Positive
Pf17	Negative	Negative	Positive
Pf18	Negative	Negative	Positive
Pf19	Negative	Negative	Positive
Pf20	Negative	Negative	Positive

Table 3: Evaluation of various isolates of *P. fluorescens* against *C. capsici* by dual culture technique.

S. No.	Isolates	Linear growth (mm)		%Growth inhibition*
		Antagonist	*C. capsici*	
1.	Pf1	69.13	20.87	76.81(61.21)[a]
2.	Pf2	62.02	27.98	68.91(56.11)[c]
3.	Pf3	54.10	35.90	60.11(50.83)[g]
4.	Pf4	43.81	46.19	48.68(44.24)[d]
5.	Pf5	55.23	34.77	61.37(51.57)[f]
6.	Pf6	57.13	32.87	63.48(52.82)[e]
7.	Pf7	45.13	44.87	50.14(45.08)[k]
8.	Pf8	44.97	45.03	49.97(44.98)[k]
9.	Pf9	59.70	30.30	66.33(54.53)[d]
10.	Pf10	50.12	39.88	55.69(48.27)[h]
11.	Pf11	67.27	22.73	74.74(59.83)[b]
12.	Pf12	46.03	43.97	51.14(45.65)[j]
13.	Pf13	40.21	49.79	44.68(41.95)[m]
14.	Pf14	53.64	36.36	59.60(50.53)[g]
15.	Pf15	57.81	32.19	64.23(53.27)[e]
16.	Pf16	43.14	46.86	47.93(43.81)[l]
17.	Pf17	45.57	44.43	50.63(45.36)[ik]
18.	Pf18	49.85	40.15	55.39(48.09)[h]
19.	Pf19	48.38	41.62	53.76(47.16)[i]
20.	Pf20	38.56	51.44	42.84(40.88)[n]
Control			90.00	

Mean of three replications, Figures with in parenthesis are arcsin transformation. Values in the column followed by common letters do not differ significantly by DMRT (P=0.05).

Effect of *P. fluorescens* on plant growth promotion (*in vitro*)

Preparation of inoculum of the antagonists: The isolates of *P. fluorescens* were grown in conical flasks (250 ml) containing 100 ml of King's B. broth for 48 hrs. on a rotary shaker (150 rev min.-1) at 28±20C. The cells were removed by centrifugation at 8000 rpm for 10 min. at 40^0C and washed in sterile water. The pellet was resuspended in a small quantity of sterile distilled water until bacterial colonies of 10^8 cfu/ml were obtained which were measured by the dilution plate technique.

Seed treatment with antagonist

Seeds of chilli were surface sterilized with two per cent sodium hypochlorite for 30 sec., rinsed in sterile dist. water and dried overnight. One gram of seed was soaked for 2 h. in ten ml of antagonist inoculum, taken in Petri dish and added with 100 mg of carboxyl methyl cellulose (CMC).

Plant growth promotion (*in vitro*) by Roll Towel Method

Plant growth-promoting activity of antagonists were

Table 4: Evaluation of various isolates of *P. fluorescens* against mycelial dry weight of *C. capsici.*

S. No.	Isolates	*Mycelial dry weight (mg/50 ml broth)			
		20%	30%	40%	Mean
1.	Pf1	218	99	20	112.33[a]
2.	Pf2	225	114	24	121.00[bc]
3.	Pf3	241	134	35	136.67[h]
4.	Pf4	230	120	24	124.67[d]
5.	Pf5	239	130	33	134.00[g]
6.	Pf6	232	124	27	127.67[e]
7.	Pf7	264	155	53	157.33[n]
8.	Pf8	262	153	40	151.67[m]
9.	Pf9	229	116	27	124.00[d]
10.	Pf10	250	143	42	145.00[j]
11.	Pf11	220	103	22	115.00[b]
12.	Pf12	256	148	47	150.33[l]
13.	Pf13	270	158	57	161.67[p]
14.	Pf14	247	137	37	140.33[i]
15.	Pf15	235	127	30	130.67[f]
16.	Pf16	267	157	55	159.67[o]
17.	Pf17	259	150	48	152.33[m]
18.	Pf18	248	140	30	139.33[i]
19.	Pf19	253	145	45	147.67[k]
20.	Pf20	310	150	50	170.00[q]
Control		540	540	540	540.00

Mean of three replications, Figures with in parenthesis are arcsin transformation. Values in the column followed by common letters do not differ significantly by DMRT (P=0.05).

assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Twenty five chilli seeds treated with antagonist were kept over presoaked germination paper. The seeds were held in position by placing an another presoaked germination paper strip over it and then it was gently pressed. The sheets along with seeds were then rolled and incubated in a growth chamber for 10 days. Three replications were maintained for each treatment. The root length and shoot length of individual seedlings were measured and the percent germination of seeds was also calculated. The seedling vigour index was calculated by the formula as described by Abdul Baki and Anderson (1973).

Vigor index (VI) = (Mean root length + Mean shoot length) × Germination (%)

Results and Discussion

The resident beneficial rhizosphere microbes form the first line of defense against the pathogen invading plants. These rhizosphere microbes (PGPR and PGPF) are known to inhibit pathogens by inducing resistance mechanism apart from increasing plant growth (Jetiyanon and Kloepper, 2002, Murali and Amruthesh, 2015). Due to their beneficial aspects, these microbes are commercially

Table 5: Plant growth promoting activity of *P. fluorescens* isolates on chilli cultivar (K 2) by the Roll Towel Method.

Isolates	Root length mean (in cm)*	Shoot length mean (in cm)*	Germination* %	Vigour index*
Pf1	3.94[a]	9.25[a]	97.60(80.02)[a]	1287.34[a]
Pf2	3.45[ab]	8.31[bc]	93.00(74.66)[c]	1093.68[c]
Pf3	2.45[cdef]	7.89[ed]	88.17(69.88)[j]	911.68[h]
Pf4	1.96[cef]	7.58[def]	77.22(61.49)[op]	736.68[r]
Pf5	3.15[abcd]	7.23[fghij]	90.56(72.11)[f]	940.01[g]
Pf6	3.19[abc]	7.15[ghij]	91.01(72.55)[ef]	941.04[f]
Pf7	2.02[ef]	6.96[j]	84.05(66.46)[j]	754.77[op]
Pf8	1.88[ef]	7.52[defg]	79.18(62.85)[n]	744.29[q]
Pf9	3.21[abc]	7.25[fghij]	92.23(73.81)[d]	964.73[d]
Pf10	3.05[abcd]	7.04[hij]	89.65(71.23)[gh]	904.57[j]
Pf11	3.49[ab]	8.24[bc]	94.32(76.21)[b]	1106.37[b]
Pf12	2.40[cdef]	7.76[de]	82.43(65.22)[l]	837.49[m]
Pf13	1.78[cdef]	7.42[efgh]	73.21(58.83)[r]	673.53[t]
Pf14	3.03[abcd]	7.09[hij]	89.76(71.34)[g]	908.37[i]
Pf15	3.12[abcd]	7.19[fghij]	91.32(72.87)[e]	941.50[e]
Pf16	1.72[ef]	7.39[efghi]	75.98(60.65)[q]	692.18[s]
Pf17	2.19[def]	6.98[ij]	89.00(70.63)[h]	816.13[n]
Pf18	3.29[abc]	7.27[fghij]	83.21(65.81)[k]	878.70[k]
Pf19	2.67[bcde]	7.97[b]	81.03(64.18)[m]	862.16[l]
Pf20	1.66[f]	7.36[efghij]	67.19(55.05)[s]	606.05[u]

Mean of three replications, Figures with in parenthesis are arcsin transformation. Values in the column followed by common letters do not differ significantly by DMRT (P=0.05).

exploited nowadays as bio-fertilizers and biopesticide for agricultural benefits. Compared to PGPF, PGPR are exploited most by due to their large availability, fast growing capability and compatible nature.

In the present study twenty isolates of *P. fluorescens* were isolated from the rhizhosphere soil of different localities. All the isolates were found to be effective against *C. capsici*. These isolates were further subjected to morphological and biochemical characterization and plant growth promotion activity. Based on the antagonistic potential and other characteristics, all the isolates of *P. fluorescens* were studied in detail for colony morphology, colour, growth type, fluorescence and cell shape. It is evident from the observations that all the isolates produced round shaped colonies and rod shaped cells (Table 1). The results of the biochemical tests performed for the identification of the effective native isolates *of P. fluorscens* showed that all of them produced similar results with regard to gram staining (negative), starch hydrolysis (negative) and fluorescent pigmentation (positive) (Table 2).

Antagonistic effect of the bacterial isolates were tested against the chilli anthracnose pathogen, *Colletotrichum capsici* by the standard dual culture method. The results indicated that all the isolates inhibited growth of the test fungus significantly (Table 3). A maximum inhibition of 76.81% was recorded by Pf 1 followed by Pf 11 and Pf 2. The present study is in according with that of Linu *et al*., (2017) who reported *Pseudomonas fluorescens* to show 90% radial growth inhibition of *Colletotrichum capsici*. George *et al*., (2009) reported the biological control of three *Colletotrichum lindemuthianum* races using *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas fluorescens* WCS365.

The mycelial dry weight of *C. capsici* decreased with and increasing concentration of culture filtrates of *P. fluorscens* in all isolates and among them Pf 1 and Pf 11 showed greatest inhibition compared to the other isolates on mycelial dry weight (Table 4). A similar result was revealed by Suthin Raj *et al*., (2014b) who evidenced the *Pseudompnas fluorescens* Pf 1 have the greatest inhibition of mycelial dry weight of *C. capsici*.

The culture filtrates of selected isolates also increased the germination of chilli seeds and induced plant growth promotion under *in vitro* conditions. Among the 20 isolates, Pf 1 recorded a maximum germination percent, increased the shoot length, root length and vigour index. It showed a highest germination percentage of 97.60, with shoot length 9.25 cm, root length 3.94 cm and vigour index 1287.34 followed by Pf 11 and Pf2 (Table 5). Similar observations were made in cucumber and tomato, wherein seed treatment with PGPR significantly enhanced seed germination and seedling vigor compared to untreated control apart from increasing vegetative plant growth parameters (Babu *et al*., 2015, Islam *et al*., 2016). Also, seed treatment with *Bacillu*s spp. have significantly enhanced plant height, shoot fresh and dry weights and some leaves in various crops (Kumar *et al*., 2011, Yuan *et al*., 2013).

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