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Antagonistic potential of fluorescent pseudomonads and control of charcoal rot of Chickpea caused by *Macrophomina phaseolina*

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Abstract: *The effectiveness of plant growth promoting rhizobacteria especially Pseudomonas fluorescens isolates were tested against charcoal rot of chickpea both in green house as well as in field conditions. Most of the isolates reduced charcoal rot disease and promoted plant growth in green house. A marked increase in shoot and root length was observed in P. fluorescens treated plants. Among all the P. fluorescens isolates Pf4-99, was found most effective in the improvement of chickpea crop in green house as well as in field. Pf4-99 effectively promoted plant growth and produced indole acetic acid in culture medium. This isolate also inhibited the mycelial growth of the M. phaseolina under in vitro conditions and reduced the disease severity. Potential isolate (Pf4-99) also significantly increased the biomass of the chickpea plants, shoot length, root length and protein content of the chickpea seeds. A part from these, the total number of seeds per plant and their weight were also enhanced. The colonization of Pf4-99 reduced the incidence of seed mycoflora by which indirectly enhanced the seed germination and vigour index of seedlings. The observations revealed that isolate Pf4-99 is quite effective to reduce the charcoal rot disease both in field and greenhouse, and also increases seed yields significantly. Therefore, this isolate appears to be an efficient biocontrol agent against charcoal rot disease as well as yield increasing rhizobacterium.*

Key words: *Pseudomonads, Biocontrol, Chickpea, Charcoal rot, Macrophomina phaseolina*

Introduction

The increasing demand for a steady food supply to the growing world population will require controlling of plant diseases that reduced crop yield subsequently. In order to control the plant diseases, biological control is gaining greater attention due to low cost and ecofriendly application. Chickpea (*Cicer arietinum* Linn.) is the world's third most important pulse crop and India accounting about 75% of the world chickpea production (FAO, 1993). Charcoal rot, caused by *Macrophomina phaseolina*, is an economically important disease of chickpea. The soil borne fungus *M. phaseolina* is endemic to temperate and tropical regions of the world and can infect over 500 different hosts (Wyllie, 1998). The incidence of charcoal rot of chickpea is most common in India and it severely limits crop yield (ICRISAT, 1988; Pandey and Singh, 1990 ; Srivastava, *et al.,* 2001).

Disease management strategies primarily depend on sanitary practices and well timed fungicide applications. However development of fungicide resistance within population of *M. phaseolina* became a problem, and alternative approaches that can be incorporated into integrated pest management of chickpea charcoal rot disease are needed. Biological control is coming up as an alternative strategy for disease management, which is also ecology-conscious and environment friendly. Several fungal (*Trichoderma* sp.) and bacterial (*Pseudomonas* sp. and *Bacillus* sp.) antagonists, have been successfully used as biocontrol agents in the control of seed and soil borne pathogens like *Sclerotium rolfsii, Rhizoctonia solani, Fusarium oxysporum* and *Sclerotinia sclerotiorum* in the various crops. (Sharma *et al.,* 1999; Mukhopadhyay *et al.,* 1992; Raguchander *et al*., 1997; Sankar and Jeyarajan, 1996; Abrahm Mathew and Gupta, 1998; Kehri and Chandra, 1991). Application of *Trichoderma viride, Pseudomonas* and *Bacillus* sp. have been found to substantially control seedling, root rots and stalk rots of maize caused by *Fusarium graminearum*, when used as seed inoculant (Chen *et al*., 1999; Chang and Kommedahl, 1986; Kommedahl and Chang, 1975). *Pseudomonas cepacea* was also found to inhibit a range of soil borne fungal pathogens including *Fusarium graminearum, Fusarium moniliforme* and *M. phaseolina* (Hebbar *et al*., 1992). Incidence of charcoal rot was substantially reduced after seed treatment of mungbean and sunflower by *Trichoderma harzianum, Gliocladium virens* and *Streptomyces* sp. (Hussain *et al*., 1990). Plant growth promoting *Pseudomonas* and *Bacillus* species generally employ an array of mechanisms like antibiosis, site competition, HCN production, siderophore production, fluorescent pigments and/or antifungal volatiles (Weller, 1988; Voisard *et al*., 1989; Cartwright *et al.,* 1995; Gardener *et al.,* 2000; Pal *et al.,* 2000 ; Validov *et al*., 2005 ; Singh *et al*., 2006) to antagonize pathogens.

The purpose of this study was to assess the biocontrol efficacy of the *Pseudomonas fluorescens* (Pf4-99) to control the charcoal rot of chickpea caused by *M. phaseolina* under natural field condition. Chemical agent carbendazim, a fungicide, was also used in this study to compare the efficacy of both control agents.

Materials and Methods

Plant material, bacterial isolates and pathogen: Seeds of *Cicer arietinum* cultivar Radhey highly susceptible to charcoal rot were

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used for the study. Seeds were surface sterilized for 2 min with 70% ethanol followed by 2% sodium hypochlorite (10 min) and rinsed in sterile distilled water (SDW). Fluorescent pseudomonads were isolated from rhizosphere soils of chickpea from different locations of Varanasi (India) and grown on King's B (KB) medium on a rotary shaker (50 rpm) at 30± 2°C. Isolation and identification were done according to the method described by Yeole and Dube (1997). The charcoal rot pathogen *M. phaseolina* was isolated from infected chickpea and designated as M.ph. The fungus was grown on the synthetic medium.

Plant growth promotion: Surface sterilized chickpea seeds were sown in plastic pots (20 cm dia., 2 seeds pot^{-1}) filled with soil containing M.ph and *P. fluorescens*. The inoculum of M.ph was prepared in sterile sand : maize meal medium (50g sand +1.5g maize meal + 10 ml water) and was incubated for 15 days at 28±1°C. The inoculum (5 % w/w) was mixed thoroughly in double autoclaved sandy loam chickpea field soil. The isolates of *P. fluorescens* were added separately (30 ml initial level 10^8 cells ml^{-1}) to each pot, the following treatments were conducted in chickpea roots; i. control pots with only sterilized soil, ii. M.ph control pots containing soil and the pathogen, iii. *P. fluorescens* treated seeds sown in pots containing one of 5 isolates of *P. fluorescens*, and iv. soil containing isolates of *P. fluorescens* + M.ph. All experiments were repeated three times with five replicates of each treatment. Experiments were carried out in green house in a complete randomized design (CRD).

Determination of plant vigour and disease severity index: Plant growth promoting activity of fluorescent pseudomonads was assessed based on the seedling vigour index by standard roll towel method (ISTA, 1993). The vigour index was calculated by using the formula of Abdul Baki and Anderson (1973):

Vigour Index = (Mean shoot length + Mean root length) x Germination (%)

Determination of disease severity index : The disease severity index was calculated as described by Bhattacharya *et al*., (1985). The extent of infection by *M. phaseolina* was indicated by the presence of dark brown lesion and also by the presence of microsclerotia of the fungus on root systems. Healthy and infected plants were divided into four groups as follows:

Healthy plants	1 = No root rot symptoms,
Slightly infected plants	2 = Dark brown to black spots on collar as well as on primary roots
Heavily infected plant	3 =Weak and stunted plants with rotting of roots,
Plants dead	4 = Dead and fallen plants

Lesions on the entire root system and the disease severity index (D.I.) were calculated as follows:

$$D.I. = \frac{0\,(H^n) + 1\,(S^n) + 2\,(H^{n*}) + 3\,(D^n)}{\text{Total number of plants examined}}$$

Where -

(H^n) = Number of healthy plants
(S^n) = Number of slightly infected plants
(H^{n*}) = Number of heavily infected plants
(D^n) = Number of dead plants

Test for antagonism: Fungal culture was grown on PDA at 28±2°C for dual culture bioassay. For this, 1 mm fungal disk taken from master plate was placed on PDA:KB (1:1, v/v) plates and incubated overnight at 28±2°C. *P. fluorescence* culture was spot inoculated on the edges of the plate after 1 d and incubated at 30± 2°C for 72 hr. Inhibition of fungal mycelium around the bacterial colonies were scored positive. The percentage inhibition of the growth of pathogen was calculated with the help of the formula given by Whipps (1997).

Production of indole acetic acid (IAA) : Production of indole acetic acid (IAA) by different fluorescent pseudomonads in the medium was assessed by the method of Gorden and Paleg (1957). The fluorescent pseudomonads were grown in tripticase soya broth for 30 h. One ml of cell free culture medium was allowed to react with 2 ml of Salkowsky reagent (1 ml of 0.5M $FeCl_3$ in 50 ml of 35% $HClO_4$) and incubated at 28 ± 2°C for 30 min. the absorbance was read at 530 nm. A standard curve was prepared using IAA and the presence of IAA in the culture filtrate was quantified as mg/ml.

Field experiments :

The experimental site: The seeds of *Cicer arietinum* cv. Radhey susceptible to charcoal rot were used. Seeds were surface sterilized as described earlier. Two field experiments were laid out at the research field, Banaras Hindu University, Varanasi. The experiments were repeated for the two consecutive Rabi seasons of 2002-2003 and 2003-2004 in order to obtain high degree of reliability and precision in the results. Antagonistic and growth promontory efficiency of *Pseudomonas* isolate Pf4-99 and carbendazim was checked under natural field conditions. The field survey was made in adjacent area of Banaras Hindu University, Varanasi, to have an idea of the occurrence of the charcoal rot disease of chickpea caused by *Macrophomina phaseolina*.

Experimental design : Field trial was performed in randomized block design (RBD) with the following details:

Plant to plant distance	20 cm
Row to row distance	20 cm
Number of plants / row	30
Total number of plants / replicates	30
Number of replicates	3

***P. fluorescens* treatments:** Treatments were employed in pot experiment as replicated in field trial. In the field trial *P. fluorescens* were grown in King'S B Medium at 30± 2°C for 48 hr. Bacterial cells were washed by centrifuging at 5000xg for 10 min. Pellets

were resuspended in 0.1 M $MgSO_4$ at a concentration of 10^8 cfu ml^{-1}.

In addition, carbendazim 0.2% (w/v) was included as a standard chemical treatment alone or in combination with *P. fluorescens*. Treatments given in the field trial with chickpea were as follows:

1. Absolute control : no *P. fluorescens*, no Pathogen
2. Positive control : only *P. fluorescens* (10^8 cfu g^{-1}) at the time of sowing.
3. Negative control : only pathogen (10^4 cfu g^{-1}).
4. Seed treated by both *P. fluorescens* as well as *M. phaseolina*
5. Only carbendazim at the time of sowing.

Inoculation of pathogen: *Macrophomina phaseolina* inoculum (MPI) was prepared as described earlier. *M. phaseolina* was inoculated in chickpea seedlings 14 d after sowing @10^4 cfu g^{-1}; the humidity level was 80±2 % at temperature, 24±2°C.

Determination of crude protein content in chickpea seeds: Crude protein content in seeds was calculated by the following formula.

Crude protein content in seeds (%) = Nitrogen content in seed (%) x 6.25

The nitrogen content in mature seeds was estimated according to the method described by Snell and Snell (1949), by using Nessler's reagent.

Results and Discussion

Among the twenty *P. fluorescens* isolates tested, five isolates Pf2-99, Pf4-99, Pf11-99, Pf14-99 and Pf21-99 were found to increase plant vigour and produced higher amount of IAA under laboratory conditions. Among these five isolates, *P. fluorescens* isolate, Pf4-99 effectively increased plant vigour and produced the maximum amount of IAA in culture medium (data not shown). In this study, *P. fluorescens* isolate Pf4-99 also displayed antagonistic activity against (M. ph), which was tested on the basis of extra-cellular capacity of fungal inhibition in dual plate assay. Pf4-99 exhibited antifungal activity against (M.ph) on King's B medium:synthetic medium (1:1). *In vitro* assessment in dual plate assay resulted in 8-10.8 mm inhibition zone after 3 d of inoculation of *P. fluorescens* isolate Pf4-99 against *M. phaseolina* (Table 1), which suggested the extracellular secretion of antifungals by this pseudomonad. Thomashow *et al*. (1990), Meziane *et al*. (2005) and Maurhofer *et al*. (1998) have suggested a significant role for secondary metabolites such as antibiotics, siderophores of *Pseudomonas* in suppression of fungal pathogens. Stability of inhibition zone in the dual plate assay suggested that besides extracellular secretion of active molecules, cell density could have also governed the mycelial inhibition.

Table - 1: Efficacy of *P. fluorescens* isolates on inhibition of mycelial growth of *M. phaseolina*

P. fluorescens isolates	Inhibition zone (mm)
Pf2-99	8.7[b]
Pf4-99	10.8[d]
Pf11-99	8.3[b]
Pf14-99	9.6[c]
Pf21-99	7.2[a]
Control	-

Value shown is a mean of five replicates. Means followed by the same letter indicates no significant difference (p=0.05) by Duncan's Multiple Range Test.

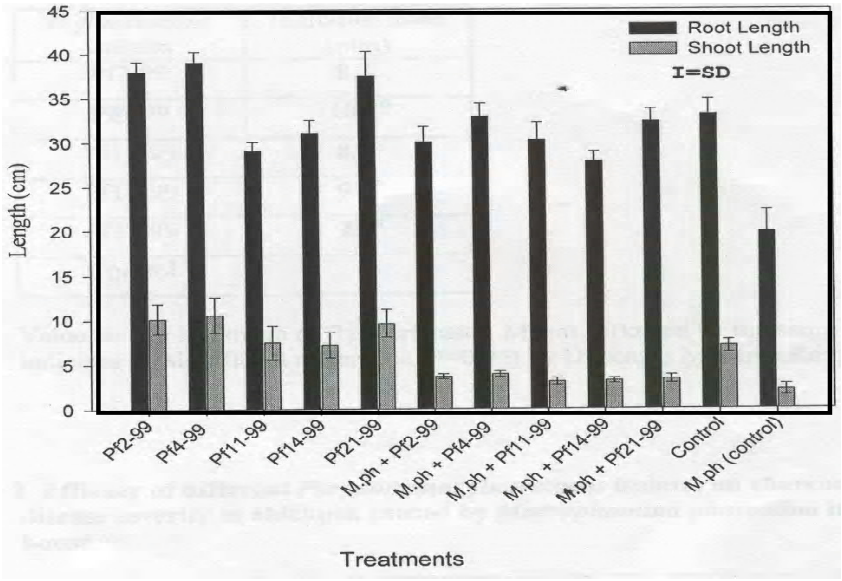

Fig. 1: Growth promotion of chickpea by different *P. fluorescens* isolates

Plant growth promoting bacteria including *Pseudomonas* spp. have been reported to stimulate the development of healthy root system (Germinda and Walley, 1996) and rapid root colonization by beneficial bacteria (Bolton *et al*., 1990). The results showed that all the five isolates reduced the charcoal rot disease by 27-45% in green house against control (Table 2). Maximum 45 % reduction in disease severity was observed with Pf4-99 followed by Pf21-99 with 39 % (Table 2). A marked increase in shoot and root length was observed in *P. fluorescens*-treated plants (Fig. 1). The effect was more pronounced in roots as compared to shoots, with record of 34.6 % increase in root length in Pf4-99 treated plants, whereas increase in shoot length was only 16 % as compared to M.ph. control (Fig. 1). In general, M.ph. inhibited root development in combination with *P. fluorescens* isolates or alone as 70 % and 44 % reductions in root length were recorded for plants grown in M.ph-infested and M.ph + Pf4-99 inoculated pots, respectively (Fig.1).

Table - 2: Efficacy of different *Pseudomonas fluorescens* isolates on charcoal rot disease severity in chickpea caused by *Macrophomina phaseolina* in green house

Treatment	Disease severity[1]	Plant height* (cm)
Pf2-99	29.6	30.0
Pf4-99	24.2	35.5
Pf11-99	29.3	31.4
Pf14-99	31.6	28.7
Pf21-99	26.5	29.0
Control	43.5	24.0
$LSD_{0.05}$	2.62	2.22

[1]Disease index was calculated according to Bhattacharya, *et al*., (1985).
*Value shown is a mean of five replicates. Means followed by the same letter indicates no significant difference (p=0.05) by Duncan's Multiple Range Test.

It is evident from Table 4 that when plants treated with Pf4-99 and M.ph. in field condition, it gave an effective control of charcoal rot of chickpea during two successive years of trial and it was found statistically at par in the action. Control of charcoal rot disease by the treatment of Pf4-99 was assessed and compared with that of carbendazim. 26% disease reduction was observed in plants treated with Pf4-99, which nearly equaled with that of the application of carbendazim. Inoculation with Pf4-99 and M.ph. combinely was also found to be quite effective in reducing the disease (19% & 42%) occurrence in comparison to absolute and M.ph control respectively (Table 3).

Table - 3: Effect of Pf4-99 and carbendazim on the incidence of charcoal rot of chickpea under field condition, during 2002-2003 and 2003-2004

Treatments	Disease severity %		Disease* severity % (Mean)
	2002-2003	2003-2004	
Absolute control	42	44	43^{b}
M.ph Control	58	62	60^{a}
Pf4-99	30	33	31.5^{d}
Pf4-99 + M.ph	37	37.3	37.15^{c}
Carbendazim	29.6	30.1	29.85^{de}
$LSD_{P=0.05}$			3.8

*Disease severity index was calculated according to Bhattacharya *et al*., 1985)
Data are means of 3 replicates

Plant height was recorded after 60 days of growth in all sets of field experiment. Highest plant height was observed with Pf4-99, which exhibited 29.4% increase against the control. Plant height was also found to be increased (16.9%) in bacterized plant challenged with M.ph. while no prominent increase in plant

Table - 4: Effect of Pf4-99 and carbendazim on plant growth (cm) of chickpea under field

Treatments	Plant height (cm)		Plant height (cm) (Mean)
	2002-2003	2003-2004	
Absolute control	31.4	31.8	31.60^{c}
M.ph control	23.9	23.1	23.50^{d}
Pf4-99	40.2	41.6	40.90^{a}
Pf4-99 + M.ph	37.1	36.4	36.75^{b}
Carbendazim	32.7	32.67	32.68^{c}
$LSD_{P=0.05}$			2.1

Data are means of 3 replicates

Table - 5: Total protein content (%) of seed of chickpea after Pf4-99 and carbendazim treatment

Treatments	Nitrogen content (%)		Nitrogen content (%) (Mean)	Protein* content (%) (Mean)
	2002-2003	2003-2004		
Absolute control	3.12	3.00	3.06	18.82^{b}
M.ph control	2.74	2.72	2.73	17.08^{c}
Pf4-99	3.29	3.29	3.29	20.61^{a}
Pf4-99 + M.ph	3.29	3.25	3.24	20.28^{a}
Carbendazim	3.09	3.08	3.08	19.34^{ab}
$LSD_{P=0.05}$			.027	0.99

Data are means of 3 replicates
*Protein content (%) = Nitrogen content (%) X 6.25

Table - 6: Efficacy of Pf4-99 and carbendazim on seed yield of chickpea under field condition

Treatments	Seed weight (g plant^{-1})	100 seed weight (g)	Seed yield (t ha^{-1})
Control	72.40^{c}	18.89^{d}	2.02^{b}
M.ph control	57.60^{d}	16.66^{e}	1.38^{c}
Pf4-99	86.26^{a}	26.30^{a}	2.45^{a}
Pf4-99 + M.ph	78.50^{b}	24.50^{b}	2.23^{a}
Carbendazim	72.65^{c}	22.22^{c}	2.12^{b}
$LSD_{P=0.05}$	3.72	0.89	0.62

Data are means of 3 replicates

height could be observed in plants treated with carbendazim (Table 4). The above observation proved that Pf4-99 could enhanced chickpea growth promotion (Fig. 1) and suppressed the diseases caused by *M. phaseolina* both in pot experiment (Table 2) as well as in field trial (Table 3). Different workers have also been reported that *P. fluorescens* was effective biocontrol agent for different fungal pathogens (Meyer *et al*.,1992; Buysens *et al*., 1996; Reimmann *et al*., 1988 ; Singh *et al.*, 2006). *P. fluorescens* could act as strong elicitors of plant defense reactions (M' Piga *et al*., 1997). Recent studies imply that prior application of fluorescent pseudomonads strengthens host cell wall structure that results in restriction of pathogen invasion in plant tissue (Benhamou *et el*., 2000; Chen *et al*., 2000 ; Conrath *et al.,* 2002 ; Dwivedi and Johri, 2003).

Yield of plant treated with isolates Pf4-99 was found to be enhanced (86.26 g plant^{-1}) against absolute control plants (72.4 g plant^{-1}) while plant treated with Pf4-99 and M.ph. showed plant yield 78.5 g plant^{-1} (Table 6). Yield of carbendazim treated plant was equal to that of absolute control plant. The hundred seeds weight was also high in Pf4-99 treated plant as compared to other treatments (Table 6). In Pf4-99 treated plants, the total seed yield of 2.45 t ha^{-1} was recorded against 2.02 t ha^{-1} in control plants. Plants treated with carbendazim showed the seed yields 2.11 t ha^{-1} (Table 6). The maximum protein content in mature chickpea seed (20.36 %) was observed in bacterized plants challenged with M.ph. The plant treated with carbendazim and Pf4-99 individually showed 19.34 and 20.61% protein content respectively. The protein content in mature chickpea seed in absolute control plant was 18.82% (Table 5). This results of increasing protein content and higher yield with the use of bioagent is in the agreement with the observation made in higher seedling's establishment and higher yield by Raguchander *et al.* (1997), Das *et al.* (1998) and Sharma *et al.* (1999).

In conclusion, the observations revealed that *P. fluorescens* isolate Pf4-99 was found to protect the chickpea plant from charcoal rot incidence with the additional benefit of increased yield, under field conditions. Therefore, this isolate appears to be an efficient biocontrol agent against charcoal rot disease as well as yield increasing rhizobacterium.

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