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ISOLATION AND EVALUATION OF NATIVE STRAINS OF *PSEUDOMONAS FLUORESCENS* FOR BIOLOGICAL CONTROL OF CHICKPEA WILT CAUSED BY *FUSARIUM OXYSPORUM*F. SP. *CICERI*

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ABSTRACT
In this study, antagonistic effect of twenty isolates of *Pseudomonas fluorescens* isolated from rhizosphere of Chickpea was evaluated against *Fusariumoxysporum* f.sp. *ciceri* as potential bio-control agent*in-vitro* and *in-vivo*.Six isolates, Pf18 (80.1%), Pf4 (79.8%),Pf20(76.4%),Pf19(73%), Pf13 (72.6%) and Pf14 (70.3%) were selected according to their high antagonistic efficiency in *in-vitro* which was shown as maximuminhibition % in dual culture assay. Green house experiment the same six isolates effectively reduced the percentage of wilted plants, percentage ranges from 4-18% at 30 DAS, 14-30% at 60DAS and 30.6-39.4 % at 90 DAS and the best disease control was achieved by isolate Pf18 (41.75%) over control. In field trial isolate Pf14 was found significantly superior than other isolates in increasing the growth parameter and germination percentage (96.7-98%) over control of chickpea plant. These isolates significantly reduced the percentage of wilted plants (30.5-36.6 %) compared with untreated control (81.6%) at 90 DAS. Thus, our results indicate that native isolates of *Pseudomonas fluorescens* improve growth parameter in plants and can help in the bio control of pathogen.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important pulse crop and India India is the largest producer and consumer of chickpeas in the world and ranks first in the world as per the latest report of Food and Agriculture Organization (FAO, 2013). It was grown on about 11.9 million hectares in 2010. While in 2012-13 (second advance estimates) record production of chickpea is 8567.8 thousand tone. The diseases are one of the main constrains for the low production of this crop (Godhani *et al.,* 2010). *Fusarium* wilt caused by *Fusariumoxysporum* is one of the major soil and seed borne disease, this facultative saprophyte pathogen can survive in soil up to six years in the absence of susceptible host (Haware *et al.,* 1986) and then it may acquire the ability to overcome different environmental stress and biological competition which indicates to the existence of physiological races. Fungicidal application as seed or soil treatment, however, has been found to be ineffective against these pathogens as the propagules are capriciously distributed in the soil and often beyond the reach of chemicals (Campbell, 1989). However, the effectiveness of host resistance is curtailed by the occurrence of pathogenic races in Foc (Haware and Nene, 1982; Jiménez-Diaz *et al*., 1989; Jiménez-Gasco *et al*., 2004).

Biological control, therefore, holds promise as a strategy for disease management and it is environment friendly too. Antagonistic bacteria, fluorescent pseudomonads have been widely used against a number of phytopathogens (Bell *et al.,* 1982; Rini and Sulochana, 2006). In recent years, attempts were also made to use a consortium of biocontrol agents to get persistent control of plant pathogens (Chaube and Sharma, 2002). Keeping this in view and the growing importance of biological control agents, the present study was carried out to evaluate the biocontrol efficiency of native isolates of fluorescent pseudomonas against *F. oxysporum* and to study their nature of action.

MATERIALS AND METHODS

Isolation of *Fusariumoxysporum*f.sp.*ciceri*

Diseased chickpea plants showing typical symptoms wilt were collected from experimental field of Department of Plant Pathology, SHIATS Allahabad. The pathogen *Fusariumoxysporum*f. sp. *ciceri* was isolated from the freshly infected root on PDA medium. Freshly infected chickpea stem and roots were washed thoroughly with distilled water. A small portion of diseased tissues along with a portion of adjacent healthy tissueswere cut into small pieces (3 mm in length) and then surface sterilized with 0.1% $HgCl_2$ for 30 seconds. The pieces then were rinsed thrice with sterilized distilled water. The surface sterilized and rinsed pieces were inoculated aseptically on sterilized patriplates containing PD Amedium. The inoculated patriplates were incubated at 20 to 25ºC for five to six days. When the fungal colony developed, a small cut of single mycelium is transferred on another patriplatescontaining PDA medium to obtain pure culture

(Patel *et al.,* 2011). The pure culture were maintained throughout the period of investigation by periodic sub culturing on fresh media and stored in refrigerator at 4ºC (Plate 2).

Isolation of *Pseudomonas fluorescens*

Isolation of *P. fluorescens*was made from rhizhosphere of chickpea field from different regions of Allahabad district. The 10 cm rhizhosphere soil particles loosely adhering to the roots were gently teasted out and the roots were cut into small pieces and mixed well. The soil thus obtained was crushed in a sterile mortar and pestle and shaken with 100mL of sterile distilled water for 10-20 min. to obtain standard soil suspension. Isolation of *P. fluorescens*was made by following the serial dilutions and pour plate method using the specific King's B medium (King *et al*., 1954)

Pour plate method

King's B medium, a selective one (Kings *et al.,* 1954) was used for the isolation of *P. fluorescens*. One ml of soil suspension from aliquot dilutions (10^5 to 10^8) was aseptically added to sterile petriplates containing twenty ml of sterile medium and incubated at 28 ± 2ºC for 48 hrs. 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 4ºC for further use (Meera and Balabas kar, 2012) (Plate 1). For the identification of *P. fluorescens*, certain biochemical tests were conducted according to Bergey's Manual for Determinative Bacteriology (Breed *et al.,* 1989).

Dual culture assay

The strains of *Pseudomonas fluorescens* were evaluated against*Fusariumoxysporum*f.sp. *ciceri* in laboratory by dual culture techniques on PDA(Kaur *et al.,* 2003) to screen out the most efficacious one. Petri dishes (90 mm) containing nutrient agar medium were inoculated with *Pseudomonas* and*Fusariumoxysporum*f.sp. *ciceri*at equal distance from the periphery of the plate. Inoculated plates were incubated at 25ºC in BOD incubator and the radial growth of pathogen (*foc)* was measured at interval of 24 hours upto7 days after incubation. Controls without *pseudomonas* were maintained and each treatment was replicated thrice. Observations were recorded after 7 days of inoculation on area covered by the *Pseudomonas* strains and inhibition of mycelial growth of pathogenic fungi by each strain was recorded). The bacterial isolates showing maximum zone of inhibition was selected for further studies.

Percentage growth inhibition was calculated as per formulaby Arora and Upadhyay (1978) given below

$$\% \text{ of growthinbition} = \frac{\text{colony growth in control plate} - \text{colonygrowthintreatdplate}}{\text{colony growth in control plate}} \times 100$$

Evaluation of antagonistic microorganism in greenhouse

To test the efficacy of *P. Fluorescens* strains in the control of chickpea wilt disease under greenhouse conditions, chickpea seeds were sown in pots containing field soil into which was incorporated *Fusarium oxysporum* f. sp. *ciceri* cultures grown on sand-maize medium. Four seeds (variety "Uday") were sown per pot and 3 pots per strain were maintained. The fungal culture was incorporated in the ratio of 1:19(sand- maize inoculums/soil).The seeds were treated with cell suspension in water (10^9cfu/ml) of the selected *P. fluorescens* strains. The wilt incidence was assessed upto 90 days after sowing (Vidhyasekaran and Muthamilan, 1995).

Evaluation of antagonistic microorganism in field

The experiment was carried out in Randomized Complete Block design replicated thrice. Sick field was fully prepared and properly manure. The seeds of highly susceptible chickpea variety,' Uday' were treated with different strains of *Pseudomonas* and left for 30 minutesin shade for natural drying (Patel *et al.,* 2011). Then the seeds were sown in randomized block design with three replication of each treatment. Sowing of chickpea was done on 28th October 2013 in plot size of 2×2 m^2 with spacing of 30×10 cm row to row and plant to plant, respectively. A control plot was maintained by treating the seeds of chickpea alone. Data was recorded on the wilt incidence (%), shoot and root length (cm).

Data analysis

The observation were recorded analyzed statistically in completely randomized design (factorial) for *in vitro* experiment and in randomized black design for field experiment Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Isolation of Pseudomonas fluorescens isolates

Antagonistic effect of twenty strains of *Pseudomonas fluorescence viz*., Pf1,Pf2, Pf3, Pf4,Pf4.....Pf20 were studied *in vitro* against *Fusariumoxysporum*f.sp. *ciceri*in PDA medium by dual culture methods. Theresult showed that all the antagonistic strains of *Pseudomonas* used in the present study, restricted the mycelia growth of *Fusariumoxysporum*f.sp. *ciceri* significantly. Out of all these antagonistics strain six found significantly highest mycelial growth inhibition against pathogen. They were selected for further studies.

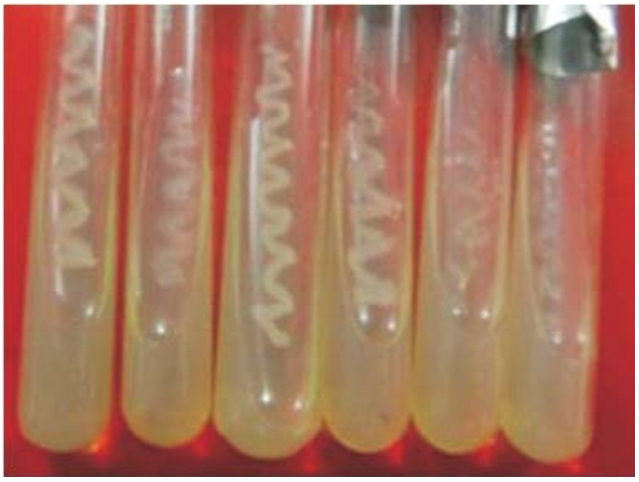

Plate 1: Pure culture of *P. fluorescens ciceri*

Table 1: *In vitro* screening of *Pseudomonas fluorescens* isolates against *Fusariumoxysporum*f.sp. *ciceri* (dual culture).

Pseudomonas fluorescens Isolates	Mean mycelial growth of three replications (mm) (*Foc*)	% of growth Inhibitions (7th day of inoculation)
Pf1	25.7	61.8
Pf2	25.2	62.3
Pf3	28.2	59.0
Pf4	9.4	79.8
Pf5	25.3	62.2
Pf6	18.8	69.4
Pf7	29.8	57.2
Pf8	28.7	58.5
Pf9	26.2	61.2
Pf10	27.3	60.2
Pf11	26.8	58.5
Pf12	28.3	58.9
Pf13	15.9	72.6
Pf14	18.0	70.3
Pf15	21.7	66.2
Pf16	27.8	61.7
Pf17	25.4	62.1
Pf18	9.2	80.1
Pf19	15.6	73.0
Pf20	12.5	76.4
Control	90.3	-
CV±	2.09	
CD@5%	0.88	

Table 2: Effectof selected*Pseudomonasfluorescens* isolates on Fusarium wilt of chickpea in green house condition

Treatment	% of wilt incidence(DAS) 30	60	90	% of disease control
Control	24	48	80	-
Foc+Pf4	4	21.4	34.6	36.75
Foc+Pf13	16	26.0	33.4	38.25
Foc+Pf14	10	14.6	34	37.50
Foc+Pf18	6	28.6	30.6	41.75
Foc+19	18	30.6	39.4	30.75
Foc+20	8	18.6	34	37.50
Sem±	0.20	0.99	0.12	-
CD@5%	0.60	0.28	0.35	-

Dual culture assay

The result of dual culture indicates that all the isolates of antagonist inhibits the growth of test fungus significantly (Table1). The maximum mycelia growth inhibition were recorded in Pf18 (80.1%) and the minimum inPf7(57.2%).In isolates Pf4 (79.8%), Pf13 (72.6%), Pf14 (70.3%), Pf19 (73%) and Pf20 (76.4%) shows above 70% mycelia growth inhibition, respectively (Plate 3). This is on conformity of the finding made by (Krishnamurthy and Gnananamanikam, 1998) they reported that antagonists of *Pseudomonas* spp. against several fungus both *invivo* and *in vitro* condition. Koche *et al*. (2013) *Pseudomonas fluorescens* obtained from citrus rhizosphere inhibiting the mycelial growth *Phytophthora*spp. Up to 38.88%. Fluorescent Pseudomonads produce secondary metabolites with antibiotic activities and suppressed many soil borne diseases (Thomashow and Weller, 1996). Kumar *et al*. (2007) suggested the extracellular secretion of antifungal by *Pseudomonas fluorescens*and also suggested a significant role of secondary metabolites such as antibiotics siderophore in suppression of fungal pathogens. Similar finding reported by Kaur *et al*. (2007) reported that 14 out of 96 *Pseudomonas*isolates from chickpea rhizosphere were highly antagonistic to *F. oxysporum sp*.

Effect of antagonists under green house experiment

The soilapplication of native bacterial isolates in green house experiment was found effective in controlling wilt incidence. The bacterial antagonist shows (Table 2) least wilt incidence by (4%) in 30 DAS in Pf4 comparing to control (24%).But, at 60 DAS isolate Pf14 shows minimum wilt incidence (14.6%) compare to control (48%).At 90 DAS 80% wilted plant in control where as isolatePf18 shows least wilt incidence (30.6%) followed by Pf13 (33.4%), Pf14-20 (34%), Pf4 (34.6) and Pf19 (39.4%), respectively. Percentage of disease control is maximum in strain Pf18 (41.75%) over control (Plate: 4). Inhibition of chickpea root pathogen by *Pseudomonas* was also reported by (Selvarajan and Jeyarajan, 1996, Kaur *et al*., 2007; Mane and Mahendra Pal, 2008). Goel *et al*. (2002) also reported that *Pseudomonas* strains as potential biocontrol agents against *Rhizoctoniasolani*, *Pythium*spp. and

Plate: 2.*Pure culture of F. oxysporum*f.sp.

Plate 3: Dual culture of native isolates of *P. fluorescence*aginst*F. oxysporum*(7th DAI)

Table 3: Effect of selected *Pseudomonas fluorescenc*e isolates on Fusarium wilt of chickpea in field trial.

Treatments	% of seed germination	% of wilt incidence (DAS) 30	60	90	Shoot length(cm)	Shoot weight(cm)	Root length(cm)	Root weight(cm)
Control + Foc	89.9	21.1	59.4	81.6	31.03	8.70	12.53	3.57
Foc + Pf4	95.4	13.3	28.3	36.6	44.3	14.2	14.4	4.67
Foc + Pf13	98.0	8.8	22.7	31.1	45.3	15.2	15.3	5.37
Foc + Pf14	96.7	8.3	22.7	30.5	48.0	18.5	19.1	8.03
Foc + Pf18	97.1	5.5	23.3	31.6	45.0	15.5	14.8	6.50
Foc + Pf19	96.4	10.0	24.4	32.7	46.5	16.5	16.1	6.70
Foc + Pf20	97.4	6.6	27.7	36.1	44.2	14.27	13.3	5.20
Sem ±	2.69	0.28	1.57	1.53	0.21	0.18	0.19	0.39
CD@5%	8.07	0.85	4.72	4.59	0.64	0.53	0.53	1.77

Plate 4: Antagonists effect on the growth of Chickpea under green house experiment

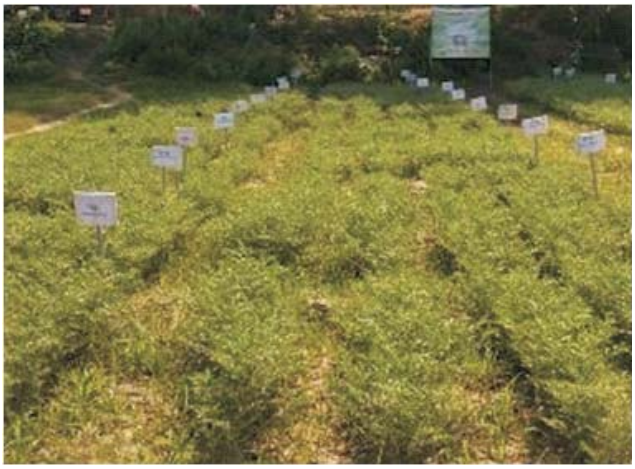

Plate 5: Antagonists effect on the growth of chickpea under field experiment

*Fusariumoxysporum*f. sp*ciceri*under culture condition as well as field experiment.

Effect of antagonists in field experiment

Among the 6 isolates (table3) maximum shoot-root length (48, 19.1cm) and weight (18.5, 8.03cm) were recorded in isolate Pf14 respectively. *Pseudomonas*spp. responsible for increasing root elongation was also reported (O' Sullivan and O' Gara, 1992). The bacterial isolates also increase the seed germination percent (98-95%) comparing to control (89.9), isolate Pf13 shows maximum seed germination. (Rudresh *et al*., 2005) shows positive effect of *Pseudomonas fluorescens* isolates on growth parameters. Minimum wilt incidence were recorded in Pf18 (5.5%; 30 DAS), Pf13- 14(22.7%; 60 DAS) and Pf14 (30.5%; 90DAS) comparing to control (21.1, 59.4and81.6%;30, 60 and 90 DAS, respectively) (Plate: 5).Fluorescens Pseudomonas isolates were Effective to reduce the incidence of several soil borne pathogen including *Fusariumoxysporum* (Kaur *et al.,* 2003). Kumar *et al.* (2013) observed in field trial that seed treatment with *fluorescent pseudomonas* gave significant reduction in disease incidence.

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