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In vitro antagonistic activity of *Pseudomonas* spp. against *Rhizoctonia soloni*

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The present research work deals with the *in-vitro* study of antagonistic activity of *Pseudomonas* spp. against plantpathogenic fungi *Rhizoctonia soloni.* The bacterial strains were isolated from rhizospheric soil of Korea district of Chhattisgarh. A total of 28 bacterial cultures were isolated from 25 representative soil samples collected from five blocks of Korea district of Chhattisgarh, out of which four were identified as *Pseudomonas* spp. (PKS10- *Pseudomonas syringae*, PKM11- *Pseudomonas syringae*, PKJ25- *Pseudomonas alcaligenes* and PKB27- *Pseudomonas alcaligenes*) from Plant Pathology Division, IARI Delhi. These four *Pseudomonas* spp. were examined for their ability to antagonize *R. soloni* in *in vitro* plate assay by dual culture inoculation along with the standard check (Pmtcc- *Pseudomonas* isolate from IMTECH Chandigarh). The antagonistic activity was interpreted by restricted growth zone of the fungal pathogen in dual culture. The diameter of hyphal growth of the fungi in dual culture with isolates PKS10, PKM11, PKJ25, PKB27, Pmtcc and control were 3.82, 3.68, 2.73, 3.41, 3.25 and 7.3 cm respectively. All the four *Pseudomonas* isolates PKS10, PKM11, PKJ25, PKB27 and Pmtcc (standard check) inhibited the hyphal growth of *Rhizoctonia* spp. by 47.67, 49.58, 62.60, 53.28 and 55.47% respectively. One among four *Pseudomonas* isolates, (PKJ25) *P. alcaligenes* was found to suppress the growth of fungal pathogen significantly in dual culture by 62.60% and was more effective than other isolates. This study suggests that *P. alcaligenes* isolates might be used as potential biological control agents against plant pathogenic fungi *Rhizoctonia soloni*.

Key words: Antagonism, biological control, *Pseudomonas alcaligenes, Rhizoctonia soloni*.

INTRODUCTION

Rhizoctonia solani has been encountered as one of the potent soil-borne fungal pathogens, which develops in both cultured and non-cultured soils. *R. solani* are highly destructive phytopathogens (Curtis et al., 2010), known to cause symptoms of damping- off and root rot diseases to wide range of vegetable and crop plants including tomato (Abu-Taleb et al., 2011; Karima et al., 2012). Various methods have been reported for controlling of damping-off disease but biological control is an efficient and ecofriendly methods. Many microbial species are involved in the biocontrol of phytopathogens such as *Trichoderma viride* (Hafez et al., 2013), *Pseudomonas*

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Table 1. Occurrence, identities, sources and abbreviation of *Pseudomonas* in Rhizospheric soil of Korea district of Chhattisgarh.

S/N	Bacterial genus identified from Rhizospheric soil	District	Block	Field	Crops
1	*Pseudomonas syringae* (PKS10)	Korea	Sonhat Block	Kailashpur	*Oryza sativa*
2	*Pseudomonas syringae* (PKM11)	Korea	ManendragarhBlock	Barbaspur	*Abelmoschus eculentus*
3	*Pseudomonas alcaligenes* (PKB27)	Korea	Baikunthpur Block	Dumaria	*Lycopersicon esculentum*
4	*Pseudomonas alcaligenes* (PKJ25)	Korea	Janakpur Block	Umarwaah	*Oryza sativa*

fluorescens and *Bacillus subtilis* (Sivasakthi et al., 2014). Adhikari et al. (2013) reported the antagonistic nature of rhizospheric bacteria against *R. solani*.

Pseudomonas spp. is one of the most promising groups of rhizospheric bacterial inhabitants that are extensively investigated to be used as biocontrol of pathogens in agriculture (O'Sullivan and O'Gara, 1992; Ganeshan and Kumar, 2006; Maurya et al., 2014). They show antagonistic activity against diverse phytopathogens such as *Pythium* spp. (Leoper, 1988) and *Rhizoctonia* spp. (Howell and Stipanovic, 1979). Various species of *Pseudomonas* are thought to play an important role in plant growth promotion and disease suppression (Kloepper et al., 1980; Jayaswal et al., 1990). In particular *P. fluorescence* (Howell and Stipanovic, 1979; Weller and Cook, 1983) and *P.cepacia* (Hebbar et al., 1992; Jayaswal et al., 1990) have attracted a considerable attention on account of their potential for biological control. Shalini and Srivastava (2008) screened out antifungal activities of *P. fluorescence* against phytopathogenic fungi. Antifungal activity of fluorescent Pseudomonads against *R. solani* is correlated to the production of secondary metabolites (Mina et al., 2013; Sharma et al., 2014; Mezeal, 2014). *Pseudomonas aeruginosa* rhizobacterial isolates PTR-3 exhibited antagonism of over 68.9% by restricting *in vitro* mycelial growth of *R. solani* up to 1.9 cm (Kamei et al., 2014).

In the present investigation the *in vitro* biocontrol efficacy of *Pseudomonas* spp. isolated from Korea district of Chhattisgarh is reported against *R. soloni*; they are able to antagonize plant pathogenic fungi in *in-vitro* condition, hence can be used as potential biocontrol agent.

MATERIALS AND METHODS

Isolation and characterization of *Pseudomonas*

In the present investigation soil samples were collected from randomly selected locations in the field region from Korea district of Chhattisgarh by composite sampling method (Walworth, 2004).

Korea District is North-Eastern District of Chhattisgarh State of India. Geographically, state lies in Latitude between 23° 02' 42" to 23 deg. 44' 46" North and Longitude between 81° 46' 42" to 82 deg. 33' 43" East. Height from Sea Level is 700 Meters. The District is bound on the North by Shidhi District of Madhya Pradesh, on the South by Bilaspur Districts, on the East by its parent District Surguja and on the West by Shahdol District of Madhya Pradesh (Figure 1). Temparature (Average) is 32°C (Max) and 17°C (Min); land area covers 5978 Sq. Km and forest area is 59.03%. The Climate is ideal with a beautiful monsoon, a mild summer and a bearable winter. Average Rainfall is 1410.9 mm, Soil type red-yellow and major crop-paddy (http://korea.gov.in/glance.htm (National Informatics centre Korea *Chhattisgarh).* Korea district is divided into 5 blocks- Baikunthpur, Manendragarh, Khadgawan, Sonhat and Janakpur.

All total of 25 soil samples were collected from 5 blocks of agro based areas of Korea district of Chhattisgarh, five representative soil samples from each block (Baikunthpur, Manendragarh, Khadgawan, Sonhat, Janakpur), during May- June 2009, all these were drawn from post harvested fields (Venkateswarlu et al., 1984). Total of 28 bacterial cultures were isolated from 25 soil samples of 5 blocks of Korea district of Chhattisgarh.

Isolation of rhizospheric bacteria was carried out by serial 10-fold dilutions technique (Pandey et al., 2006) on Nutrient agar and Pseudomonas agar base (all from Hi Media). Four out of 28 bacterial cultures were identified as *Pseudomonas* spp. by Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, Pusa campus, New Delhi, India - 12, for there confirmation through molecular marker (16s rRNA and 16s rDNA sequencing) (Kanimozhi and Panneerselvam, 2010). Isolated and identified bacterial cultures were characterized by studying cultural characteristics of individual isolates on nutrient agar, nutrient broth and Pseudomonas agar base medium. Morphological characteristics of bacterial cultures were studied microscopically. The bacteria were also tested for their biochemical reaction, antibiotic sensitivity and tolerance of pH (from 3 to 11 at intervals of 2 pH units), temperature (from 4.0 to 40°C) and salt concentration (from 0.5-20% NaCl) by growing them on Nutrient Agar Medium and broth.

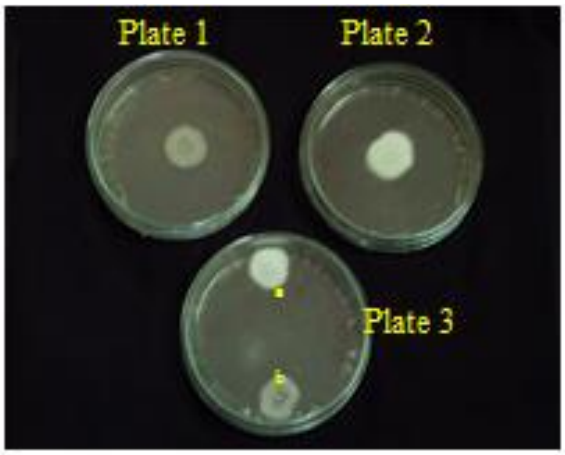
Isolation of fungal pathogen

Fungal pathogen *R. soloni* was isolated from stem of infected tomato plantlet with damping – off disease from local field. The fungal parthogen was characterized by microscopic examination (Ganesan and Gnanamanickam, 1987) and its pure cultures were maintained on PDA for further use (Devi et al., 1989).

Figure 1. Political map of Korea district of Chhattisgarh, India.

Assay for *in vitro* antagonism

A loopful of bacterial culture was placed (5mm in diameter) at one edge on the periphery of PDA plate and mycelial discs (5mm in diameter) were cut from actively growing fungal culture and placed opposite to the bacterial inoculation on PDA plate (Picture 1) (Ganesan and Gnanamanickam, 1987; Podile et al., 1988; Babu et al., 2000).



Picture 1. Pure culture plate of *Pseudomonas spp.* (plate 1) and *Rhizoctonia soloni* (plate 2). Dual culture plate of antagonism by *Pseudomonas* against *Rhizoctonia soloni* on PDA (plate 3). (a) *Rhizoctonia soloni* (b) *Pseudomonas* isolate PKJ25.

Zone of inhibition was recorded after 1 week of incubation, by measuring the restricted growth zone between the edges of fungal and bacterial colonies (Picture 2). Plate with pure *Pseudomonas* inoculum corresponding to pure fungal inoculum was taken as control. Inoculated Petri plates were incubated at 25±1°C for 07 days (Picture 3). The assays of dual culture interaction were conducted in triplicates in Completely Randomized Design and repeated twice. The per cent inhibition of mycelial growth of the pathogens was calculated using the following formula (Perveen and Bokhari, 2012):

$$I = (C - T/C) \times 100$$

Where, I = Inhibition (%) or antagonistic effect, C = colony diameter of test fungus in control plate and T = colony diameter of the same test fungus in dual culture against *Pseudomonas* as antagonist.

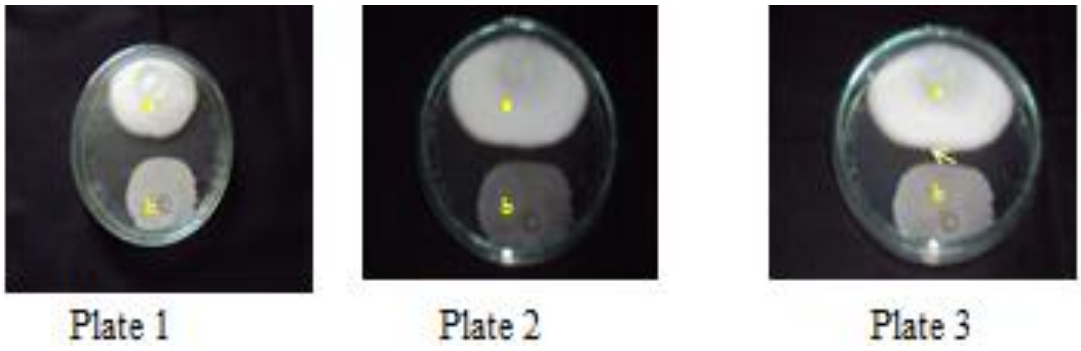
All four isolates of *Pseudomonas* (PKS10, PKM11, PKJ25 and PKB27) were tested *in vitro* for their antagonistic activity against test plant pathogens, *Rhizoctonia* spp. (causal agent of damping - off of tomato) along with standard check Pmtcc (*Pseudomonas* isolate from MTCC Chandigarh used as standard) in comparison to control (pure cultures of fungal pathogen taken as control for in vitro assay).

RESULTS AND DISCUSSION

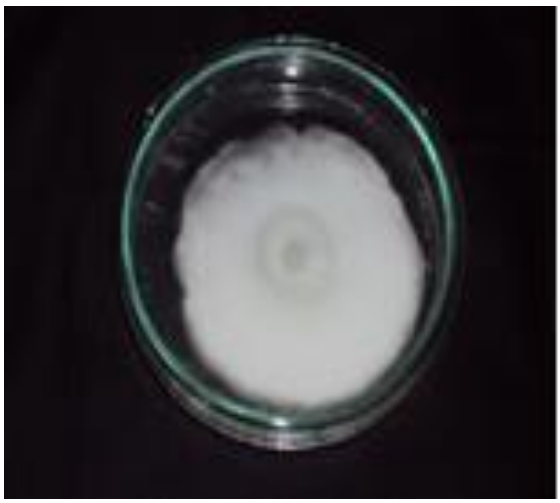
Two among four *Pseudomonas* isolates belong to *spp. P. syringae* (PKS10 and PKM11) and *P. alcaligenes* (PKJ25 and PKB27). These four isolates of *Pseudomonas* were from rhizosphere of *Oryza sativa* (Kailashpur, Sonhat Block), *Abelmoschus esculentus* (Barbaspur, Manendragarh Block), *Oryza sativa* (Umarwah, Janakpur Block) and *Lycopersicum esculentum* (Dumaria, Baikunthpur Block) respectively. Table 1 explains the *Pseudomonas* isolates, identities, sources and abbreviation.

Characterization of different *Pseudomonas* isolates

Results of morphological, biochemical and physical



Picture 2. Dual culture plate of antagonism by *Pseudomonas* spp. against *Rhizoctonia soloni*. (a) *Rhizoctonia soloni* (b) *Pseudomonas* isolate PKJ25: Plate 1(after 3 days of incubation), Plate 2 (after 5 days) and Plate 3 (after 7 days of incubation).



Picture 3. Pure culture plate of *Rhizoctonia soloni* on PDA.

characterization of *Pseudomonas* spp. are given in Table 2. *Pseudomonas* isolates PKS10 and PKM11 formed creamy colonies on Nutrient agar Medium and yellow colonies on Pseudomonas agar medium whereas isolates PKJ25 and PKB27 formed white colonies on NAM and PAB, PKS10 and PKM11 showed surface growth while PKJ25 and PKB27 showed turbidity in liquid medium. These isolates had the morphological features like singly arranged, flagellated, non-endospore forming, non –capsulated, motile, Gram negative rods. The isolates PKS10 and PKM11 showed growth from 25-37□C (optimum temp. 35□C), could tolerate pH range 5–11 (optimum pH for PKS10- 7 and PKM11-9) and salt concentration up to 5%. Isolates PKJ25 and PKB27 were able to withstand temperature as high as 45□C, high concentration of NaCl upto 5% and a wide range of initial pH from 5 to 11 (optimum pH for PKJ25-5 and PKM11-5). Similar characteristics were described for bacteria belonging to genus *Pseudomonas* and species *fluorescens* (Sharma et al., 2007; Malviya and Singh, 2012) and *putida* (Pandey et al., 2006; Malboobi et al., 2009b) respectively. The result interpreted by Malboobi et al. (2009a) in their investigation on PSB tolerance to extreme climates supported our findings. They intensively examined the isolates for tolerance toward high temperature, high concentration of NaCl and wide range of pH and found that all PSB strains survived at high temperature and could tolerate concentration of 2.5% NaCl and alkaline pH. All four isolates were sensitive to gentamicin (<10 µg ml^{-1}), polymyxin B (<300 µg ml^{-1}), chloremphenicol (<5 µg ml^{-1}) but isolate PKS10 was resistance to Ampicillin (<10 µg ml^{-1}). All the isolates tested positive for amylase, catalase, cellulase and chitinase activity but isolate PKJ25 and PKB27 showed negative test for protease and urease activity while all isolates were negative for gelatinase. Production of chitinolytic enzyme is an important means for identification of fungal inhibitors (Malviya and Singh, 2012; Kamei et al., 2014).

Result of *in vitro* antagonistic effect of different *Pseudomonas* isolates against plant pathogenic fungi under dual culture technique is depicted in Table 3. Among 4 of the *Pseudomonas* isolates, isolate PKJ25 inhibited the mycelial growth and was inhibitory to the *Rhizoctonia soloni* as compared to the other three and Pmtcc. The diameter of hyphal growth of the fungi with isolates PKS10, PKM11, PKJ25, PKB27, Pmtcc and control were 3.82, 3.68, 2.73, 3.41, 3.25 and 7.3 cm respectively. All the four *Pseudomonas* isolates PKS10, PKM11, PKJ25, PKB27 and Pmtcc inhibited the hyphal growth of *Rhizoctonia*by 47.67, 49.58, 62.60, 53.28 and 55.47% respectively.

in-vitro antagonism *Pseudomonas* isolate against *R. soloni*

Our result suggests *Pseudomonas* isolate PKJ25 *(P. alcaligenes)* was efficient and significantly suppressed the vegetative growth of the test fungi *Rhizoctonia* by

Table 2. Characterization of different *Pseudomonas* isolates.

Characteristics	Bacterial Isolates			
	PKS10	**PKM11**	**PKJ25**	**PKB27**
Colony characteristics in NAM				
Colony colour	Cream	Cream	White	White
Colony size (after 24 h of incubation)	6 mm	5 mm	5 cm	5.5 cm
Form	R	R	I	I
Margins	Undulate	Lobate	Undulate	Undulate
Elevation	Flat	Flat	Flat	Flat
Optical feature	Opaque	Opaque	Opaque	Opaque
Colony characteristics in PAB				
Colony colour	Yellow	Yellow	White	White
Colony growth (after 24 h of incubation)	+++	+	++	+
Form	I	C	I	I
Margins	Undulate	Undulate	Lobate	Lobate
Elevation	Flat	Flat	Flat	Flat
Optical feature	Opaque	Opaque	Opaque	Opaque
Growth in liquid media	Surface	Surface	Turbid	Turbid
Microscopic Examination				
Gram staining	Negative	Negative	Negative	Negative
Shape	Rod	Rod	Rod	Rod
Arrangement	Single	Single	Single	Single
Flagella	Polar flagella	Polar flagella	Polar flagella	Polar flagella
Motility	+	+	+++	+
Endospore	Negative	Negative	Negative	Negative
Capsule	Negative	Negative	Negative	Negative
Biochemical Reactions				
Catalase	Positive	Positive	Positive	Positive
Amylase	Positive	Positive	Positive	Positive
Protease	Positive	Positive	Negative	Negative
Gelatinase	Negative	Negative	Negative	Negative
Urease	Positive	Positive	Negative	Negative
Cellulase	Positive	Positive	Positive	Positive
Chitinase	Positive	Positive	Positive	Positive
Lactose fermentation (acid fermentation)	Positive	Positive	Positive	Positive
Antibiotic sensitivity test				
Ampicillin	Negative	Negative	Positive	Positive
Ciprofloxacin	Positive	Positive	Positive	Positive
Polymyxin B	Positive	Positive	Positive	Positive
Gentamycin	Positive	Positive	Positive	Positive
Physical factors affecting bacterial growth				
Effect of molecular O_2 (Aerobic/ Anaerobic)	Aerobic	Aerobic	Aerobic	Aerobic
Effect of Osmotic pressure/salt tolerance Concentration of NaCl (0.5%-20%)	5%	5%	5%	5%
pH tolerance (3pH-11pH)	5pH-11pH	5pH-11pH	5pH-11pH	5pH-11pH
Optimum pH	7pH	9pH	5pH	5pH
Temperature tolerance (4 - 50°C)				
Temperature tolerance (4 - 50°C)	25- 37°C	25- 37°C	25-45°C	25- 45°C
Optimum temperature	37°C±2	37°C±2	37°C±2	37°C±2

R, Regular; I,Irregular; C,circular; +, slight; ++, medium; +++, abundant .

Table 3. *In vitro* antagonistic effect of different *Pseudomonas* isolates against plant pathogenic Fungi under dual culture technique.

S. No.	*Pseudomonas* isolates	Fungi pathogen	
		Rhizoctonia soloni	
		Colony diameter (cm)	Inhibition%
1	PKS10	3.82	47.67
2	PKM11	3.68	49.58
3	PKJ25	2.73	62.60
4	PKB27	3.41	53.28
5	Pmtcc	3.25	55.47
6	Control	7.30	00.00

Result represents the mean of three replications per strain. Pure culture of fungal pathogen was taken as control.

restricting the hyphal growth, in *in vitro* condition with 62.60% inhibition followed by Pmtcc and PKB27 with 55.47 and 53.28% inhibition respectively. In similar study, Podile et al. (1988) reported inhibitory effect of *P. aeruginosa* and *P. fluorecens* on plant pathogenic fungi *R. solani, Sclerotium rolfsii.* Devi et al. (1989) suggested that antagonistic bacteria *Pseudomonas fluorescent* isolates (Pfr1-14) obtained from rice rhizosphere suppressed the rice ShB pathogen, *R. solonii in vitro* by inhibiting mycelial growth and sclerotial germination. Similar report of antagonistic effect of Fluorescent *Pseudomonas* was reported by Khan and Zaidi (2002) for *R. soloni* and *Fusarium oxysporium.* Akhtar and Siddiqui (2009) suggested the use of plant growth promoting rhizobacteria for the biocontrol of root-rot disease complex of chickpea and their studies showed that the three *Pseudomonas* spp. had inhibitory effect on *Macrophomina phaseolina*; *P. alcaligenes was* one of the biocontrol agent. Jayaraj et al., (2007) tested 08 fluorescent Pseudomonads isolated from tomato rhizosphere and observed highest growth inhibition (15.5 mm) of *Pythium aphanidermatum* and controlled damping off of tomato by 68.5%.

The antagonistic nature of *P. aeruginosa* rhizobacterial isolates PTR-3 and PCF-3 against *R. solani* was also reported by Kamei et al. (2014). Their finding suggest that rhizobacterial isolates PTR-3 restricted mycelial growth of *R. solani* up to 1.9 (cm) and were found to exhibit antagonism of over 68.9%. Sharma et al. (2014) reported that *Pseudomonas* spp. isolates showed antifungal activity against *Rhizoctonia* spp. in the range of 7.27-53.84% inhibition. Also, *P. fluorescens* isolate restricted the linear growth of *R. solani* by 81.3% as reported by Mezeal (2014).

Thus, present study receives strong support from the above observations and the information generated through this study will help for future studies on the antagonistic affect of native microorganisms on soil in Chhattisgarh (India) and consequently for the maintenance of native microorganisms as microbial antagonists for enhancement of crop production.

Conflict of interests

The authors did not declare any conflict of interest.

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