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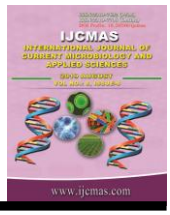

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Evaluations of Fluorescent Pseudomonads against Collor or Root Rot of Soybean Caused by *Sclerotium rolfsii*

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A B S T R A C T

Collor or root rot of soybean is an important soil-borne fungal disease caused by *Sclerotium rolfsii* causing up to 5-50 per cent of yield losses annually. The present investigation was undertaken on effect of fluorescent pseudomonads on collor or root rot management in soybean. Sixty two different pseudomonad isolates were evaluated for their antagonistic activity against *S. rolfsii* under *in vitro* condition. Per cent inhibition of mycelial growth of *S. rolfsii* by pseudomonads ranged from 22.59 to 70.37. Fifty one isolates showed antagonism against the pathogen. Five isolates BFP22, BFP38, DFP47, DFP48 and DFP62 were found potent with 45. 56 - 70.37 per cent inhibition of mycelial growth against *S. rolfsii*. They were further evaluated in greenhouse as seed treatment and soil application. Fluorescent pseudomonad isolate DFP48 was found potent and promising as it reduced the disease to the maximum extent of 21.96 per cent over pathogen alone control (56.01 %).

Introduction

Collor or Root rot is caused by *Sclerotium rolfsii* is one of the most widespread diseases of soybean and causes serious yield losses upto 5-50 per cent under favourable environmental conditions (Mahmood and Sinclair 1992). The pathogen has very wide host range and the resistance sources in soybean against this disease are rare. The pathogen survives as sclerotia in soil or in stubbles or on seeds and is disseminated by irrigation water (Premalatha and Dath, 1990). Fungicides for seed treatment (IRRI, 1980), soil application (Chen and Chu, 1973) and foliar spray (Dev and Mary, 1986) are being applied to control the disease. However, these treatments are expensive and add pollutants to the environment. Use of bio-control agents in plant disease management is an ecologically-friendly and cost effective strategy which can be used in integration with other management tactics for sustained crop yields. A successful bio-agent should not only be able to reduce the

disease but also contribute to crop growth promotion and yield. Among different bio-control agents, plant growth-promoting rhizobacteria (PGPR) are widely used in managing soil borne diseases of several field crops. PGPR group offers an effective means of antagonism against phytopathogens.

Besides, they also contribute to enhanced seedling growth and induced systemic resistance (ISR) against diseases and thereby increase in yield (Pathak *et al.,* 2004). In recent years, fluorescent pseudomonads have drawn attention worldwide because of production of secondary metabolites such as siderophore, antibiotics, volatile compounds, HCN, enzymes and phytohormones (Gupta *et al.,* 2001).

The ideal bio-control agent for the management of foliar infection and soil borne pathogen may be the one that can survive in both rhizosphere and phyllosphere. Among the various bio-control agents, fluorescent pseudomonads are known to survive both in rhizosphere (Park *et al.,* 1991) and phyllosphere (Wilson *et al.,* 1992). Considering such qualities of bio-control agent, the present study was aimed to screen the fluorescent pseudomonads for antagonism under *in vitro* and to evaluate their bio-control potentiality under glasshouse condition against *S. rolfsii* in soybean.

Materials and Methods

Sixty two fluorescent pseudomonads were obtained from 37 soybean rhizosphere samples collected from Dharwad and Belgavi districts, these isolates were confirmed based on fluorescence under UV light on King's B agar medium.

The collor or root rot fungal pathogen used in the study was collected from Department of Plant Pathology, UAS Dharwad.

In vitro antifungal activity

The dual inoculation technique of Sakthivel and Gnanamanickam (1987) was followed to study the antagonistic activity of the fluorescent pseudomonads. The fungal pathogens were grown on potato dextrose agar plates until they completely cover the agar surface. With the help of a sterile cork borer (10 mm diameter), discs of fungal growth from the plates was taken and placed at the center of the fresh PDA plates. Each test isolate was then streaked parallel on either sides of the fungal disc leaving 1.5 cm distance from the edge of the plate. The PDA plates inoculated with only fungal pathogens were considered respective controls. The plates were incubated at 30 °C for 96 h. The colony diameter of the fungus in control plate and the plates streaked with fluorescent pseudomonads were recorded. The zone of inhibition (ZOI) of each fungal pathogen by different isolates were calculated by using the following formula,

ZOI = Colony diameter (control plate) - Colony diameter (in dual inoculated plates)

The per cent inhibition of pathogen was assessed by using the formula given below (Vincent, 1927).

HCN production

Ability of the efficient fluorescent pseudomonad strains to produce HCN was assessed as per the method of Wei *et al.,* (1996). Whatman no.1 filter paper pads were placed inside the lids of the Petri plates and the plates were sterilized. Tryptic soya agar medium (TSA) amended with glycine (4.4 g/l) was sterilized and poured into the sterile plates. Twenty four hours old fluorescent pseudomonads strains were streaked on to the medium. The filter paper padding in each plate was soaked with two ml sterile picric acid

solution. Inoculated plates were sealed with parafilm in order to contain the gaseous metabolite produced by the antagonistic fluorescent pseudomonads and allowed for a chemical reaction with picric acid on the top. After incubation for a week at 28±1 ºC, the colour changes of the filter paper was noticed and the HCN production potential of the antagonistic fluorescent pseudomonads was assessed as per the following scoring.

No colour change: No HCN production

Brownish colouration: Weak HCN production

Brownish to orange: Moderate HCN production

Orange to reddish brown: Strong HCN production

Siderophore production

Siderophores act as antimicrobial compounds by increasing competition for available iron in the rhizosphere. Selected bacterial strains (BFP22, BFP38, DFP48, DFP47 and DFP62) were tested for production of siderophores, qualitatively on chrome azurol-S agar (CAS) as described by Schwyn and Neilands (1987)

PGP traits

These isolates were also subjected to qualitative analysis for the production of indole acetic acid (IAA) (Bric *et al.*, 1991) and gibberlic acid (GA) (Brown and Lowbury, 1968). P-solubilization ability on Pikovaskayas medium. The diameter of the zone of TCP solubilization was measured.

In vivo Evaluation of efficient isolates against *S. rolfsii* of soybean

Pot experiment was conducted with challenge inoculation of *S. rolfsii* along with appropriate control taking soybean as test crop. Earthen pots of 30 cm top diameter were filled with 10 kg of sterilized soil. Before sowing, the soil in each pot was mixed with 0.26 g urea, 1.5 g single superphosphate (SSP) and 0.12 g murate of potash (MOP) to supply 40: 80: 25 Kg N: P_2O_5: K_2O per ha on soil weight basis as per the package of practices. Half of the N was applied at the time of sowing and the remaining half was applied as top dressing after 30 days of sowing. The fungus, *S. rolfsii* causing collor or root rot disease in soybean was multiplied as a mixed inoculum in maize powder and sand (1:4) mixture. 10 mm disc (5-6 no.) of mycelial growth of the *S. rolfsii* was inoculated to sterilized flask containing maize powder and sand mixture and flask was incubated for 15-20 days at 30 ^{o}C. After 20 days flasks was completely filled with sclerotial bodies, which is commonly called as jaint culture. For pot application, top 200 gm of soil was inoculated with 5 per cent of pathogen inoculum (jaint culture) one day before sowing. The selected 5 fluorescent pseudomonad isolates were multiplied in King's B broth for 96 h at 30 ^{o}C under shaking conditions (175 rpm). These broth cultures were diluted to maintain the population of 10^8-10^9 CFU/ml and applied @ 10 ml per pot just one day after sowing. Soybean seeds were treated with respective isolates at the rate of 10 gm per kg of seed, 10-15 min prior to sowing and for the chemical control treatment, the seeds were treated with Carboxin 37.5 % + Thiram 37.5 % at the rate of 4 g/kg of seeds. Soybean seeds of JS-335 variety treated with respective FP isolate were used for the experiment by dibbling method. All the agronomic or cultural practices were followed as per the package of practices recommended for the soybean crop, except disease management. Observations on wilt incidence caused by *S. rolfsii* were recorded at 15, 30 and 60 DAS and per cent disease incidence was calculated by using the formula given by Mayee and Datar, 1986.

$$PDI = \frac{\text{No. of infected plants}}{\text{Total number of plants}} \times 100$$

Statistical analysis

The statistical analyzes of the data were carried out by employing completely randomized design (CRD). The critical differences were calculated at P = 0.01 for the *in-vitro* and pot culture experiments wherever F tests were significant and interpretation of the results was carried out in accordance with Pansey and Sukhatme (1985).

Results and Discussion

In vitro elevation of FP's isolates

The present study focused on evaluation FP isolates obtained from soybean rhizosphere of Dharwad and Belgavi districts against *S. rolfsii* (Collor or root rot fungal pathogen), the study also focused on functional properties of the FP isolates. Out of 62 isolates, 51 FP isolates inhibited *S. rolfsii* under *in vitro* condition (Table1). The zone of inhibition varied from 2.03 to 6.33 cm with percent inhibition of 22.59 to 70.37 per cent. Based on highest per cent inhibition five best isolates were selected for pot culture experiment.

The maximum percent inhibition of 70.37 was observed in BFP22, which was significantly superior over all other isolates. The isolates DFP62 and DFP48 were on par with each other with percent inhibition of 60.93 and 58.89 respectively. These observations are in line with the earlier reports on fluorescent pseudomonads against plant pathogenic fungi like *Fusarium, Rhizoctonia, Macrophomina, Pyricularia, Alternaria, Sclerotium, Colletotrichum, Pythium* and *Phytophthora* (Mercado-Blanco *et al.,* 2004; Bhatia *et al.,* 2005; Ahmadzadeh *et al.,* 2006; Rakh *et al.,* 2011; Vishwanath *et al.,* 2012; Manivannan *et al.,* 2012; Prasad *et al.,* 2013). The effectiveness of fluorescent pseudomonads against multiple pathogens is also known (Tripathi and Johri, 2002; Suneesh, 2004; Kandoliya and Vakharia, (2014); Aly *et al., (*2015); Arif Fouzia *et al.,* (2016) and Megha *et al.,* 2007b*).*

Functional Characterization of potent FP's isolates

Among These antagonistic isolates, five potential isolates were selected and studied for their functional properties *viz.,* P-solubilization, HCN production, Siderophore production, IAA and GA production (Table 2). P-solubilization (TCP) on Pikovskaya's agar medium and displayed wide variations in the diameter of the zone of solubilization, which varied from 20.00- 21.71mm. The extent of zone of solubilization may or may not correlate with the amount of P solubilized (Rashid *et al.,* 2004). Isolates of *Pseudomonas fluorescens* species differ in the ability to produce phosphatase enzyme and production of organic acids and hence showed different solubilization efficiency.

Important aspect of microbial antagonistic activity is best realized when it is applied for right cause. Therefore, understanding the mechanisms of antagonistic activity could be key to application of strains for specific purposes. These isolates were shown strong HCN production (+++). HCN is known to induce systemic resistance in plants (Wei *et al.,* 1991).

Voisard *et al.,* (1989) reported HCN production as a mechanism of bio-control of plant pathogens. Similarly, Ahmadzadeh and Sharifi-Tehrani (2009) detected the production of HCN by six isolates of fluorescent pseudomonads and the strains exhibited good *in vitro* antifungal activity against *Rhizoctonia solani*.

The siderophore production by antagonistic microorganisms is believed to be a mechanism of pathogen suppression. Siderophore production test using CAS agar plate has been used for rapid screening of potential beneficial bacterial isolates (Schwyn and Neiland, 1987). Siderophore production by antagonistic isolates ranged from 21.97 to 29.73 mm. Fluorescent pseudomonads offer an interesting biological system with their ability to promote plant growth directly through production of plant growth promoting substances (IAA and GA) and indirectly through control of plant pathogens and deleterious organisms or both (Bakthavatchalu *et al.*, 2012).

Seed bacterization with such organisms has emerged as a powerful technology to enhance plant growth and yield, besides providing protection against diseases. Earlier, Suneesh (2004) and Megha *et al.,* (2007a) made an attempt to characterize PGPR isolates of Western Ghats and studied their functional diversity. Their efforts helped in identifying several PGPR with novel traits useful in agriculture. The present study is complimented with the previous work done.

These efficient fluorescent pseudomonads in the present study were screened for their ability to produce IAA and GA and these isolates exhibited significantly varying quantities of IAA (19.97µg to 28.89µg IAA/25 ml of broth) and GA of 12.19 to 16.29 µg per 25 ml broth.

The results obtained in this study are in line with the observation made by Khakipour *et al.,* (2008), who reported that the IAA produced by *P. fluorescens* and *P. putida* strains varied from 0 to 31.6 mg/l and 0 to 24.08 mg/l, respectively. The variations in IAA production could be an inherent metabolic variability among the isolates (Leinhos and Vacek, 1994). Similarly Lenin and Jayanti (2012), who observed the production of GA_3 by isolates of *Pseudomonas* ranged from 6.21 to 6.80 µg per 25 ml broth. The variations in IAA production could be an inherent metabolic variability among the isolates (Leinhos and Vacek, 1994). Similarly Suneesh (2004) reported that all the 48 fluorescent Pseudomonads isolated from the moist deciduous forests produced GA in the range of 0.72 to 5.27 µg per 25 ml of broth.

In vivo evaluation of potential fluorescent pseudomonads against *S. rolfsii*

Among 62 fluorescent pseudomonad isolates screened against *S. rolfsii* under *in vitro* condition, five strains were found to be potent antagonists. Five strains of fluorescent pseudomonad viz., BFP22, BFP38, DFP48, DFP47 and DFP62 were selected for *in vivo* study.

All the selected efficient isolates were tested for their bio-control potential in soybean crop challenge inoculated with *S. rolfsii* under pot culture. Per cent disease control was calculated using PDI values at 15, 30 and 60 DAS. The data is represented in Table 3.

At 15 DAS, very less per cent disease incidence of 13.09 was observed in T_3 (DFP48 + *S. rolfsii*), the treatments T_4 (DFP47 + *S. rolfsii*), T_5 (DFP62+ *S. rolfsii*), T_2 (BFP38 + *S. rolfsii*) and T_1 (BFP22+ *S. rolfsii*) recorded the per cent disease incidence of 15.29, 16.21, 16.93 and 21.22 respectively. The treatment T_7 (*S. rolfsii*) recorded highest per cent disease incidence of 33.73 and no disease was observed in T_8 (absolute control). At 30 DAS, the percent disease incidence was increased (PDI) to 47.92 in case of pathogen alone inoculated treatment (T_7) and no disease in T_8 (absolute control). The least percent disease incidence of 19.89 was observed in T_3 (DFP48 + *S. rolfsii),* which was on par with the treatment T_4 (DFP47 + *S. rolfsii*) (19.96).

Table.1 Antagonistic activity of fluorescent pseudomonad isolates against *Sclerotium rolfsii* under *in vitro* condition

Sl. No.	Isolates	ZOI (cm)	Per cent inhibition (%)
1	BFP1	-	-
2	BFP2	3.73 (11.13) *	41.48 (39.28) *
3	BFP3	-	-
4	BFP4	3.12 (10.16)	34.63 (36.04)
5	BFP5	2.85 (9.72)	31.70 (34.53)
6	BFP6	2.98 (9.94)	33.15 (35.68)
7	BFP7	2.82 (9.66)	31.29 (34.27)
8	BFP8	3.78 (11.21)	42.04 (39.88)
9	BFP9	3.00 (9.97)	33.33 (35.41)
10	BFP10	-	-
11	BFP11	2.60 (9.27)	28.89 (32.86)
12	BFP12	3.50 (10.78)	38.89 (38.37)
13	BFP13	-	-
14	BFP14	-	-
15	BFP15	2.03 (8.19)	22.59 (29.24)
16	BFP16	2.52 (9.12)	27.96 (32.05)
17	BFP17	-	-
18	BFP18	-	-
19	BFP19	-	-
20	BFP20	-	-
21	BFP21	3.03 (10.02)	33.70 (35.50)
22	BFP22	6.33 (14.57)	70.37 (54.00)
23	BFP23	3.13 (10.18)	34.74 (36.30)
24	BFP24	3.25 (10.38)	36.11 (36.84)
25	BFP25	3.32 (10.49)	36.85 (37.06)
26	BFP26	2.42 (8.94)	26.85 (31.81)
27	BFP27	2.45 (9.00)	27.22 (32.05)
28	BFP28	-	-
29	BFP29	-	-
30	BFP30	3.37 (10.57)	37.41 (37.59)
31	BFP31	3.08 (10.11)	34.26 (35.90)
32	BFP32	3.32 (10.49)	36.85 (36.84)

33	**BFP33**	**3.37 (10.57)**	**37.41 (37.59)**
34	BFP34	3.20 (10.30)	35.56 (36.66)
35	BFP35	3.50 (10.78)	38.89 (38.37)
36	BFP36	3.35 (10.54)	37.22 (37.41)
37	BFP37	3.25 (10.38)	36.11 (36.84)
38	BFP38	4.10 (11.68)	45.56 (41.71)
39	BFP39	3.38 (10.59)	37.59 (37.76)
40	BFP40	3.12 (10.16)	34.63 (36.04)
41	BFP41	3.15 (10.22)	35.00 (36.62)
42	BFP42	4.47 (12.20)	49.63 (43.57)
43	BFP43	2.53 (9.15)	28.15 (32.63)
44	BFP44	3.32 (10.49)	36.89 (37.41)
45	DFP45	2.60 (9.27)	28.89 (32.87)
46	DFP46	3.20 (10.30)	35.56 (36.44)
47	DFP47	4.10 (11.68)	45.56 (41.50)
48	DFP48	5.30 (13.30)	58.89 (48.17)
49	DFP49	3.77 (11.19)	41.85 (39.84)
50	DFP50	3.82 (11.26)	42.41 (60.01)
51	DFP51	3.42 (10.65)	37.96 (56.97)
52	DFP52	3.32 (10.49)	36.85 (55.79)
53	DFP53	3.40 (10.62)	37.78 (56.38)
54	DFP54	3.37 (10.57)	37.41 (56.38)
55	DFP55	3.25 (10.38)	36.11 (55.26)
56	DFP56	3.32 (10.49)	36.85 (55.79)
57	DFP57	3.53 (10.83)	39.26 (57.88)
58	DFP58	3.58 (10.91)	39.81 (58.01)
59	DFP59	3.22 (10.33)	35.74 (54.93)
60	DFP60	3.42 (10.65)	37.96 (56.64)
61	DFP61	3.35 (10.54)	37.22 (56.31)
62	DFP62	5.48 (13.54)	60.93 (73.52)
63	Control	0.00 (1.00)	0.00 (1.00)
	S.m. ±	**0.094**	**0.752**
	C.D. @ 1 %	**0.350**	**2.791**

Table.2 Properties of FP isolates selected for pot culture studies against *sclerotium rolfsii*

Sl. No.	Isolate code	Per cent inhibition under *in vitro* condition	HCN production	P- solubilization (mm) (Qualitative)	Siderophore production (mm)	IAA (µg/25 ml)	GA (µg/25 ml)
1.	BFP22	70.37	+ + +	20.00	21.97	19.97	12.19
2.	BFP38	60.93	+ + +	21.02	24.00	25.00	16.29
3.	DFP48	58.89	+ + +	21.71	29.73	28.89	13.15
4.	DFP47	49.63	+ + +	20.96	23.47	22.55	12.85
5.	DFP62	45.56	+ + +	20.34	26.70	26.41	12.99

Table.3 Effect of fluorescent pseudomonad isolates on per cent disease incidence caused by *S. rolfsii*

Treatments	Per cent disease incidence		
	15 DAS	30 DAS	60 DAS
T_1: BFP22 + *S. rolfsii*	21.22 (4.71) *	23.78 (4.98) *	24.77 (5.08) *
T_2: BFP38 + *S. rolfsii*	16.93 (4.21)	24.81 (5.08)	24.96 (5.09)
T_3: DFP48 + *S. rolfsii*	13.09 (3.75)	19.89 (4.57)	21.96 (4.79)
T_4: DFP47 + *S. rolfsii*	15.29 (4.03)	19.96 (4.58)	22.71 (4.87)
T_5: DFP62 + *S. rolfsii*	16.21 (4.15)	21.16 (4.71)	24.39 (5.04)
T_6: (Carboxin 37.5 % + thiram 37.5 %) + *S. rolfsii*	13.59 (3.82)	27.55 (5.34)	20.98 (4.69)
T_7: *S. rolfsii* alone	33.73 (5.89)	47.92 (6.99)	56.01 (7.55)
T_8: Absolute control	00.00 (1.00)	00.00 (1.00)	00.00 (1.00)
S.Em. ±	**0.13**	**0.02**	**0.01**
C.D. @ 1 %	**0.57**	**0.07**	**0.05**

At 90 DAS, PDI increased to 56.01 in pathogen alone inoculated treatment (T_7) and no disease in T_8 (absolute control). Among the isolates tested, the lowest PDI of 21.96 was observed in treatment T_3 (DFP48 + *S. rolfsii*).

The FP isolates were used for both seed treatment and soil application (Susilowati *et al.*, 2011). The per cent disease incidence was calculated by observing the disease at 15, 30 and 90 DAS. As observed the percent disease was increased in all the treatments as the inoculation period was increased. The lowest PDI of 20.98 was observed in T_6 (carboxin 37.5 % + Thiram 37.5 % + *S. rolfsii*). Among FP inoculated treatments, the treatment T_3 (DFP48 + *S. rolfsii*) recorded lowest PDI of 21.96 followed by T_4 (DFP47 + *S. rolfsii*) with PDI of 22.71 (Fig. 5). Seed treatment followed by the soil application resulted in resistance towards the disease and the reduction of disease severity. The results are in line with the findings of Susilowati *et al.*, (2011), who reported the disease suppression by the *Pseudomonas* sp. CRB-17 (seed treatment and soil drenching) toward *F. oxysporum* was highest (100 %) in sterile soil but decreased into the lowest (15.7 %) in non-sterile soil. Fluorescent pseudomonads possess several properties that make them the bio-control agents of choice (Johri *et al.*, 1997). The siderophores are usually produced by various beneficial soil microbes. Among them fluorescent pseudomonads are also involved in inhibition of *S. rolfsii* which is positively correlated (r = +0.336) with production of siderophores by fluorescent pseudomonads (Indi, 2010). These fluorescent pseudomonad isolates showed good *in vitro* activity against antifungal activity against *R. solani* (Ahmadzadeh and Sharifi, 2009). The results are in line with the findings of Susilowati *et al.*, (2011), who reported the disease suppression by the *Pseudomonas* sp. CRB-17 (seed treatment and soil drenching) toward *F. oxysporum* was highest (100 %) in sterile soil but decreased into the lowest (15.7 %) in non-sterile soil.

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