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Priyanka
Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad, Karnataka, India

Geeta Goudar
Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad, Karnataka, India

Corresponding Author:
Priyanka
Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad, Karnataka, India

In-vitro screening of fluorescent pseudomonads against *Colletotrichum truncatum* of soybean (*Glycine max* L. Merrill)

Priyanka and Geeta Goudar

Abstract
The present study was conducted on *in-vitro* screening of fluorescent pseudomonads against *Colletotrichum truncatum* of soybean. Out of 62 native fluorescent pseudomonads isolates from soybean rhizosphere, 38 isolates showed antagonistic activity against *Colletotrichum truncatum* under dual plate inoculation technique. The zone of inhibition ranges from 2.20 to 6.35 cm with per cent inhibition of 24.44% to 74.36%. Among 38 isolates, five isolates viz, BFP22, DFP62, DFP56, BFP42 and DFP54 were found potent with 45. 56 - 70.37 per cent inhibition of mycelial growth against *C. truncatum*. Five isolates were further tested for their growth promotional properties like, HCN production, siderophore production, IAA and GA production. The isolate DFP48 showed highest IAA production of (28.89 mg/25 ml), DFP54 recorded highest GA production of 18.52 mg/25ml, ten isolates exhibited strong HCN production and DFP54 showed highest siderophore production (36.37mm zone of clearance on CAS media).

Keywords: Fluorescent pseudomonads, Soybean, *C. truncatum*, biocontrol

Introduction
Anthracnose caused by *Colletotrichum truncatum* is one of the most important seed-borne fungal pathogen of soybean (Sinclair and Backman, 1989) [24]. As soybean is also called as "golden bean" and it is one of the fore most important oilseed crops in the world for its excellent protein (42-45%), oil (22%) and starch content (21%). It is a good source of vitamin-B complex, thiamine and riboflavin. In India, yield losses of 16-100 per cent have been reported due to this disease. Higher physiological seed quality ensures healthy seedlings establishment under wider range of environmental conditions (Copeland and Mc Donald, 2001). Disease free quality seeds production in Soybean is utmost important to sustain the productivity and maintain the quality of the crop. Severe seed infection by *C. truncatum* may be able to inflict considerable damage to the seeds after harvest, consequently posing a serious problem to the economy in the world trade. PGPR promote plant growth by various factors like ability to produce plant growth regulators, asymbiotic N2 fixation, antagonism against phytopathogenic microorganisms by production of siderophores, antibiotics and cyanide, solubilization of mineral phosphates and other nutrients (Sarvanakumar *et al*., 2007) [22]. Pseudomonas is diverse genus that occupies many different niches and exhibits versatile metabolic capacity (Clarke, 1982) [11].
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 a group of PGPR known to survive both in rhizosphere (Pierret *et al.*, 1991) [19] and phyllosphere. Strains of *Pseudomonas fluorescens* are known to show biological control activity against certain soil-borne and foliar phytopathogenic fungi and have the potential to produce known secondary metabolites such as siderophore, antifungal antibiotics, HCN and protease which showed antagonistic activity against *Macrophomina phaseolina, Rhizoctonia solani*, *Phytophthora nicotianae var. parasitica*, *Pythium* sp. and *Fusarium* sp. (Anand *et al*., 2010) [4]. The growing interest in non-chemical methods of pest and disease management are solely due to environmental and health hazards. The use of Fluorescent pseudomonads based bio control agents is not only safe for the farmers and consumers but it is also good for the environment.

Material and Methods
Sixty two fluorescent pseudomonads (FP's) were isolated from soybean rhizosphere collected from Belagavi and Dharwad districts of Northern Karnataka. These isolates were confirmed

based on fluorescence under UV light on King's B agar medium.

The seed-born or foliar pathogen (*C. truncatum*) used in this study was collected from Department of Plant Pathology, UAS Dharwad. *In vitro* antagonistic potential FP's isolates.

All the sixty two isolates were evaluated against the tested pathogen through dual culture technique of Sakthivel and Gnanamanickam (1987) [21]. The radial mycelial growth of test pathogen was recorded daily and compared with control plates. The radial mycelial growth of test pathogen and antagonist were measured periodically and the zone of inhibition of mycelial growth of test pathogen by antagonists was calculated as per formulae.

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) [26].

Production of volatile compounds

1. HCN production

Ability of the efficient FP's strains to produce HCN was assessed as per the method of Wei *et al*., (1996). 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

2. Siderophore production

Siderophore are iron chelating compounds also acts as antimicrobial compounds by increasing competition for available iron in the rhizosphere. Selected potent FP's isolates were evaluated for siderophore production qualitatively on chrome azurol-S agar (CAS) and zone of clearance of CAS media was recorded and calculated as described by Schwyn and Neilands (1987) [23].

Production of plant growth promoting substances

Isolates were also subjected to qualitative analysis for the production of indole acetic acid (IAA) (Bric *et al*., 1991) [9] and gibberlic acid (GA) (Brown and Lowbury, 1968) [10]. P-solubilization ability on Pikovaskayas medium. The diameter of the zone of TCP solubilization was measured.

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

Results and Discussion

The work focused on screening of potential FP's isolates for their antagonistic activity against *C. truncatum* of Northern Karnataka viz., Dharwad and Belagavi District, under *in vitro* condition and in future the potential isolates can be evaluated under pot culture experiments. The study also focused on functional properties of the FP isolates. Out of 62 FP's isolates, 38 isolates inhibited *C. truncatum* under *in vitro* condition truncatum the zone of inhibition varied from 2.20 to 6.35 cm with per cent inhibition of 24.44 to 74.36 (Table 1). The maximum percent inhibition of 74.36 was observed in BFP22, and the isolates DFP62 and DFP56 were on par with each other with per cent inhibition of 70.84 and 67.87 respectively, Three isolates showed least inhibition ranging from 24.44 to 28.33 per cent. 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; Ahmadzadeh *et al*., 2006; Rakh *et al*., 2011) [18, 2, 20]. The effectiveness of fluorescent pseudomonads against multiple pathogens is also known (Suneesh, 2004 [25]; Aly *et al*., (2015) [3]; Arif Fouzia *et al*., (2016) [6] and Megha *et al*., 2007b) [17].

Functional characterization of potential FP's isolates

Based on antagonistic activity against *C. truncatum* under in vitro condition five potential isolates were selected and studied for their functional properties viz., P- solubilization, HCN production, Siderophore production, IAA and GA production (Table 3). P-solubilization (TCP) on Pikovskaya's agar medium and displayed wide variations in the diameter of the zone of solubilization, which varied from 20.00-21.65mm. 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.

These isolates were shown strong HCN production (+++) (Table2). HCN is known to induce systemic resistance in plants (Wei *et al*., 1991) [28]. The 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. Voisard *et al*., (1989) [27] reported HCN production as a mechanism of bio-control of plant pathogens. Similarly, Ahmadzadeh and Sharifi-Tehrani (2009) [1] detected the production of HCN by six isolates of fluorescent pseudomonads and the strains exhibited good in vitro antifungal activity against Rhizoctonia solani.

Siderophore production by antagonistic isolates ranged from 21.97 to 36.37 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) [7]. These efficient fluorescent pseudomonads in the present study were screened for their ability to produce IAA and GA (Table 3) and these isolates exhibited significantly varying quantities of IAA (19.97µg to 28.03µg IAA/25 ml of broth) and GA of 12.19 to 18.52µg per 25 ml broth. The results obtained in this study are in line with the observation made by Khakipour *et al*., (2008) [14], 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) [16], who observed the production of GA3 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) [15]. Similarly Suneesh (2004) [26] 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.

Table 1: Antagonistic activity of fluorescent pseudomonad isolates against *Colletotrichum truncatum* (foliar and seed born pathogen) under *in vitro* condition

Sl. No.	Isolates	ZOI (cm)	Per cent inhibition
1	BFP1	-	-
2	BFP2	-	-
3	BFP3	-	-
4	BFP4	-	-
5	BFP5	-	-
6	BFP6	-	-
7	BFP7	2.90 (9.80) *	32.22 (34.57) *
8	BFP8	3.73 (11.14)	40.81 (39.69)
9	BFP9	3.60 (10.93)	39.66 (39.02)
10	BFP10	-	-
11	BFP11	-	-
12	BFP12	-	-
13	BFP13	-	-
14	BFP14	-	-
15	BFP15	-	-
16	BFP16	-	-
17	BFP17	-	-
18	BFP18	-	-
19	BFP19	-	-
20	BFP20	3.92 (11.41)	42.70 (40.79)
21	BFP21	-	-
22	BFP22	6.60 (14.88)	74.36 (59.56)
23	BFP23	-	-
24	BFP24	-	-
25	BFP25	-	-
26	BFP26	2.55 (9.18)	28.33 (32.15)
27	BFP27	2.52 (9.12)	27.96 (31.91)
28	BFP28	-	-
29	BFP29	-	-
30	BFP30	2.92 (9.83)	32.41 (34.68)
31	BFP31	-	-

Table 2: Qualitative analysis of five potent isolates for their antagonistic metabolites production (HCN and Siderophore)

Sl. No.	Isolate code	Per cent inhibition under *in vitro* condition	HCN production	Siderophore production (mm)
1.	BFP22	74.36	+ + +	21.97
2.	DFP62	70.84	+ + +	26.70
3.	DFP56	67.78	+ + +	24.00
4.	BFP42	63.22	+ + +	23.60
5.	DFP54	56.55	+ + +	36.37

Table 3: Plant growth promotional traits of potential isolates (P-solubilisation, IAA and GA production)

Sl. No.	Isolate code	P-solubilization (mm) (Qualitative)	IAA (µg/25 ml)	GA (µg/25 ml)
1.	BFP22	20.00	19.97	12.19
2.	DFP62	20.34	26.41	12.99
3.	DFP56	18.80	24.81	12.20
4.	BFP42	21.11	23.74	13.12
5.	DFP54	21.65	28.03	18.52

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