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Fluorescent Pseudomonads As Prospective Bioinoculants For Sunflower (*Helianthus annus* L.)

A. Suresh, Srinivas Munjam, V. Praveen Kumar, V. Krishna Reddy and S. Ram Reddy

Department of Microbiology, Kakatiya University, Warangal-506009, India

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Email: suresh.addepalli2007@gmail.com

ABSTRACT

Fluorescent pseudomonads (FLPs) play a significant role in the plant growth promotion and health. Hence, the presence of FLPs and their ability to colonize the root surface determine the extent of plant growth. The aim of the present investigation was to isolate, screen and characterize the FLPs from the rhizosphere of sunflower. FLPs were isolated from the rhizospher of sunflower grown in different agroedaphic location of Telangana (India). Rhizospher soils supported more FLPs than the non-rhizosphere soil. Out of 150, 20 FLP isolates were found to be efficient isolates since these exhibited the ability to produce plant growth promoting substances like IAA, GA, siderophores, protease, cellulase and chitinase apart from phosphate solubilization. Based on these characteristics five isolates viz, MBPS-1, MBPS-30, MBPS-14, MBPS-66 and MBPS-79 were selected and further evaluated for their possibility of use as bioinoculants. Through the selected five isolates varied in individual attributes, the present studies indicate that they can be projected as potential bioinoculants for sunflower crop.

INTRODUCTION

Fluoresent pseudomonads is a very large and important family of Gram negative bacteria. They belong to a group of chemoheterotrophic bacteria with versatile functions and are predominantly present in the soil. They have the ability to colonize rhizosphere of a wide variety of crops including cereals, pulses, oilseeds and vegetables (Johri et al., 1997). The genus Pseudomonas includes both fluorescent and non fluorescent species. The fluorescent species produce water soluble yellow green pigments and fluoresce under low wavelength UV radiation.

They are equipped with multiple mechanisms for biocontrol of phytopathogens and plant growth promotion and hence are being used widely as bioinoculants (Pierson and Weller 1994; Baagnasco et al., 1998; Deelip et al., 1998; Yogesh et al., 2005).They produce a wide variety of antibiotics (Kraus & Loper, 1995; Deepti and Johri, 2003), chitinolytic enzymes, siderophores (Seong & Shin, 1996; Suryakala et al., 2004), HCN (Mondal et al., 2000) and growth promoting hormones like IAA (Glick, 1995), gibberellic acid (Safak and Nilufer, 2004). Solubilization of inorganic phosphates by fluorescent pseudomonads was also reported (Rodriguez & Fraga, 1999; Arti and Patel, 2003; Srivastava et al., 2004). Fluorescent pseudomonads are not host specific but because of their possible adaptation to the root exudates of a particular host, they may be partially host specific (Kishore et al., 2005). Recently Ali et al.(2009) reported that fluorescent pseudomonad also confer thermo tolerance to the crop plants. Thus, in view of their multiple beneficial effects on plant growth, a large number of investigatiors have been investing their studies on fluorescent pseudomonads associated with different crop plants in order to develop them as bioinoculants individually or in combination with other organisms. Fluorescent pseudomonads isolated from a particular host may prove to be ideal candidates for that host as bioinoculants. The aim of the present investigation was to isolate, screen and characterize the FLPs from the rhizosphere of sunflower and an attempt was made to evaluate the five strains of FLPs for antifungal activity against selected four phytopathogenic fungi viz *Macrophomina phaseolina, Colletotrichum falcatum, Fusarium oxysporum, Curvularia lunata*.

MATERIALS AND METHODS

Sample collection and isolation of fluorescent pseudomonads

Isolation of fluorescent and non fluorescent pseudomonads were made from both rhizosphere and non rhizosphere soils of sunflower (*Helianthus annus*) grown in five different agroedaphic regions (Khammam, Hasanparthy, Kothagudem, Kodamuru and Paluvalpula (T.S)) of plants in the age group of 30, 60 and 90 days. Samples collected from the field were carried to laboratory in an ice-box and stored in a refrigerator and isolation and enumeration of FLPs and non-FLPs were made within 12 hours of sample collection.

Soil serial dilution followed by spread plate technique was adopted. Dilutions ranging from 10-2 to 10-6 were standardized and used for isolations. Number of viable colonies were enumerated and recorded as number per gram of sample taken. King's B medium was employed for fluorescein detection of FLPs. The colonies without fluorescein were treated as non-FLPs.

Studies on PGPR traits

Indole acetic acid (IAA) production

IAA production was tested by Salkowski colorimetric technique (Glickmann & Dessux, 1995).

Gibberellic acid (GA) production

GA production was determined by the method suggested by Cho et al., (1979).

Siderophore production

Deferrated glassware and iron deficient MM9 medium was used in these experiments. Production of siderophores was tested and confirmed by FeCl3 test (Jalal and Dick, 1991) and the assay was performed by spectrophotometry (Meyer & Abdullah, 1978). Quantitative assay for siderophore production was performed by the method suggested by Reeves et al., (1983).

Hydrogen cyanide (HCN) production

Production of HCN by an isolate was tested by the method of Lorck (1948) and Castric (1977).

Phosphate solubilization

Twenty four hours old cultures of fluorescent pseudomonads were spot inoculated on to Pikovskayas agar medium (Pikovskaya, 1948). The diameter of the colony and halozone developed due to phosphate solubilization were measured after 72 hours. Phosphate solubilization on solid medium was expressed in terms of solubilization efficiency (SE).

$$SE\ (\%) = \frac{Z - C}{C} \times 100$$

Where, Z is solubilization zone, C is colony diameter

Quantitative assay of phosphate solubilization was made by molybdophosphoric acid blue method (Koering & Johnson, 1942).

Cellulase production

Cellulase production by the fluorescent pseudomonads was tested by the method suggested by Cattelan et al., (1999). Quantitative assay was made by the method suggested by Miller (1959).

Chitinase production

Chitinase production by the FLPs was tested by the method

bsuggested by Lim et al. (1991). Quantitative assay for chitinase was performed by the method suggested by Boller and Mauch (1988).
Protease production Production of protease was tested by spot inoculation method as suggested by Maurhofer et al.(1995). Quantitative assay was performed by the method suggested by Morihara et al. (1964).

Antagonistic activity

Antagonism of test organisms was tested by the method of Fokkema (1973).

RESULTS AND DISCUSSION

Distribution of fluorescent pseudomonads

The results presented in table -1 reveal that in general, the rhizosphere of sunflower cultivated under different agro-edaphic conditions harboured more FLPs than the non-rhizosphere soil. The ratios of FLPs and non FLPs in rhizosphere soils ranged from 0.09 to 0.258 that varied with both the type of soil and age of the plant. In case of non-rhizosphere soil, the ratio ranged between 0.04 to 0.202. Thus a wide variation is evident in the distribution of pseudomonads in rhizosphere and non-rhizosphere soils. It is also evident from the table that the population of both types of pseudomonads varied with the type of soil. This differential distribution of FLPs can be explained in terms of different edaphic factors and variation in the agronomic practices of sunflower. The maximum population of both types of pseudomonads was recorded at the age of 30 days of plant growth. It may be due to the vigorous growth and metabolism of the plant at this age, during which many metabolites are likely to be released which serve as the nutrients for rhizospheric organisms. Subsequently, the populations decreased slowly and steadily. Interestingly, such type of tendency was not observed in case of non-rhizosphere soil. Gloria and Leda (2006) reported the fluorescent pseudomonads associated with the rhizosphere of several crops. Suresh et al. (2014) reported the distribution and plant growth promoting abilities of fluorescent pseudomonads associated with pearl millet. Considerably lower counts of fluorescent pseudomonads in rhizosphere of various monocot crops were reported by Miller et al.(1959). Among the factors affecting the bacterial colonization of roots, host plants play a major role. Thus the size and composition of rhizobacteria communities have been described as plant dependent.

Plant growth promoting attributes

A total of 150 FLP isolates obtained with different colony morphology were screened for traits that are expected to be involved in plant growth promotion (PGP) and plant health promoting activities. Twenty isolates (8.0%) were proved to be positive for at least two traits examined (Table-2). Out of 20 isolates, eleven isolates were able to produce detectable amounts of indole-3-acetic acid. Significant amounts of gibberellic acid was also produced by eight isolates. Siderophore production in iron free medium was observed in eleven isolates. Production of HCN and ammonia were observed in four and twelve isolates respectively. Similarly, production of protease in six isolates, cellulase in eight isolates and chitinases in six isolates was observed. Solubilization of inorganic phosphates (tri and di calcium) by FLPs was observed in eight isolates. Among all, five isolates viz, MBPS-1, MBPS-14, MBPS-30, MBPS-66 and MBPS-79 were found positive for more number of traits tested and are treated as promising isolates. They have shown the relative efficacy ratios of 0.66 to 0.77. These strains were further evaluated for other attributes. Screening of rhizobacteria for their plant growth promoting activities was made by Chaiharn et al.(2008). The ability of pseudomonads to produce auxin can significantly affect the plant growth (Khakipour et al.,2008). As an arsenal of beneficial effects exerted, FLPs are known to produce IAA (Ahmad et al., 2005), GA (Basiacik and Nilufer, 2004) and other plant growth regulators. Some species of pseudomonads can also produce different levels of HCN that are toxic to certain pathogenic fungi (David and Gara,1994). Under conditions of low iron regime, the pseudomonad isolates studied, produced yellow-green iron binding peptides, the siderophores (Rached and Ahmed, 2005). Similarly plant growth promoting activites of fluorescent pseudomonads isolated from the Iranian soil was reported by Abbas et al.(2009).

Phosphate solubilization

A critical perusal of the table-3 reveals the ability of phosphate solubilization by selected five fluorescent pseudomonad strains. The present strains are capable of solubilizing Ca3 (PO4)2 in the liquid medium. The maximum amount of soluble phosphates was released by MBPS-66 and the least by MBPS – 1. The initial pH of the medium was 5.9. However due to the microbial production of organic acids the decrease of pH in the culture medium from 5.9 to 4.9 was observed. Sridevi et al. (2007) reported the phosphate solubilization by *Rhizobium* isolates from Crotalaria spp. Adhyan et al. (2008) reported the efficient phosphorus solubilization by mutant strain of *Xanthomonas campestris* with different carbon and nitrogen sources. Kundu et al. (2009) reported the biodiversity of phosphate solubilizing bacteria in rhizosphere of

Table 1: Distribution of fluorescent pseudomonads in rhizosphere and non-rhizosphere soils of sunflower grown

Place	Age of the crop (DAS)	Rhizzosphere			Non-rhizosphere		
		Fluorescent	Non-fluourscent	Ratio	Fluorescent	Non-fluorescent	Ratio
Khammam	30	6.5*	38.75	0.171	2.75	36.0	0.076
	60	3.5	42.35	0.082	1.4	26.25	0.053
	90	2.35	29.5	0.079	1.2	48.75	0.024
Hasanparthy	30	5.25	35.55	0.147	3.4	32.5	0.104
	60	3.05	20.5	0.148	2.5	12.35	0.202
	90	2.3	13.75	0.167	1.3	32.5	0.04
kothagudem	30	6.16	39.5	0.155	1.64	40.12	0.04
	60	4.5	30.25	0.148	1.0	39.82	0.025
	90	2.26	26.17	0.086	0.0	46.12	0.0
Kodamuru	30	8.8	34.25	0.258	2.7	42.5	0.063
	60	3.66	30.5	0.09	2.25	25.55	0.088
	90	2.7	31.66	0.085	1.1	42.35	0.025
Paluvalpula	30	11.5	62.5	0.184	2.75	35.0	0.078
	60	12.6	69.61	0.181	1.64	32.61	0.05
	90	9.64	56.1	0.171	0.0	377.9	0.0

Values are expressed in colony forming units (10^4 CFU/g)

Table 2 : Production of growth promoting substances by fluorescent pseudomonads isolated from rhizosphere of sunflower

S.No	Isolate	IAA like compounds	Gibberellic acid	Siderophores	HCN	Ammonia	Cellulase	Protease	Chitinase	P solubilization	Relative efficacy
1	MBPS-1	+	+	+	--	+	--	+	+	+	**0.77**
2	MBPS-2	--	+	+	--	+	--	--	--	--	0.33
3	MBPS-3	+	--	--	--	--	--	--	--	--	0.11
4	MBPS-4	--	--	--	+	--	--	--	--	--	0.11
5	MBPS-5	+	--	--	--	+	--	--	--	--	0.22
6	MBPS-6	+	--	--	--	--	--	+	+	+	0.44
7	MBPS-7	+	+	--	--	+	--	+	--	+	0.55
8	MBPS-8	--	--	+	--	+	--	--	--	--	0.22
9	MBPS-9	--	--	+	--	--	+	--	+	--	0.22
10	MBPS-10	--	+	--	--	+	+	--	--	--	0.33
11	MBPS-11	--	--	+	--	+	--	--	--	--	0.22
12	MBPS-12	--	+	--	--	--	+	--	--	--	0.22
13	MBPS-13	+	--	+	--	+	+	--	+	--	0.55
14	MBPS-14	+	--	+	+	+	+	+	--	+	**0.77**
15	MBPS-30	+	+	+	--	+	+	--	--	+	**0.66**
16	MBPS-16	+	--	+	--	--	--	+	--	+	0.44
17	MBPS-66	+	--	+	+	+	+	--	+	+	**0.77**
18	MBPS-18	--	+	--	+	--	--	--	+	--	0.33
19	MBPS-19	--	--	--	--	+	--	--	--	--	0.11
20	MBPS-79	+	+	+	--	--	+	+	--	+	**0.77**
% of isolate		55	40	55	25	60	40	30	30	40	

+ = Positive, -- = Negative

Table 3: Phosphate solubilization by the five selected fluorescent pseudomonad strains

		Number of days									
		1		3		5		7		10	
S.No	Isolate	pH	P released µg/ml	pH	P released µg/ml	pH	P released µg/ml	pH	P released µg/ml	pH	P released µg/ml
1	MBPS-1	5.5	2.2	4.25	40.6	4.68	29.1	3.96	22.2	3.82	39.9
2	MBPS30	5.9	4.7	4.69	19.6	4.56	27.6	3.26	32.6	3.34	46.3
3	MBPS-14	5.1	5.4	5.25	26.12	4.37	26.1	3.98	29.11	3.56	61.5
4	MBPS-66	5.9	8.7	4.25	31.1	4.3	36.3	4.77	40.5	3.34	**65.1**
5	MBPS-79	5.5	7.8	5.26	29.13	4.45	26.6	4.06	26.61	3.49	46.6
6	Control	4.9	1.7	4.13	19.6	4.63	25.5	3.66	21.3	2.99	26.1
	LSD 0.05		0.26		0.04		0.01		0.03		0.67

Values are mean of three replicates and significant at $p < 0.05$

Table 4 : Antifungal activity of selected fluorescent pseudomonad strains against four pathogenic fungi

S.No	Isolate	*Macrophomina phaseolina*	*Colletotrichum falcatum*	*Fusarium oxysporum*	*Curvularia lunata*
1	MBPS-1	0.5	1.4	1.7	1.5
2	MBPS-30	0.1	1.5	1.7	1
3	MBPS-14	0.6	1.2	1.9	1.1
4	MBPS-66	1.7	1.7	2.7	1.3
5	MBPS-79	1.1	0.7	2.5	1.2
6	Control	0.5	0.5	1.5	0.6

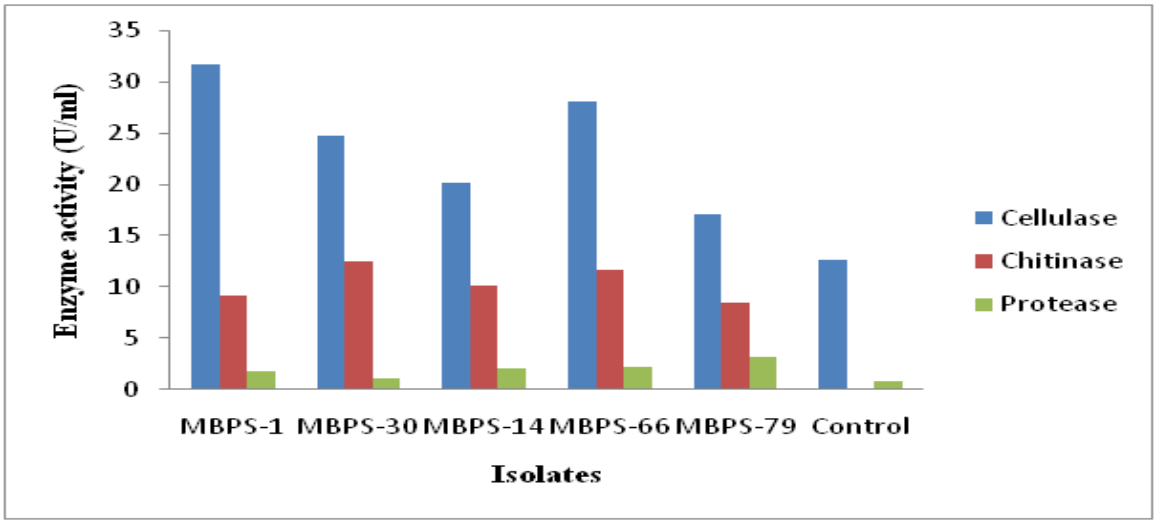

Figure 1. Plant growth promoting properties of selected fluorescent pseudomonad strains

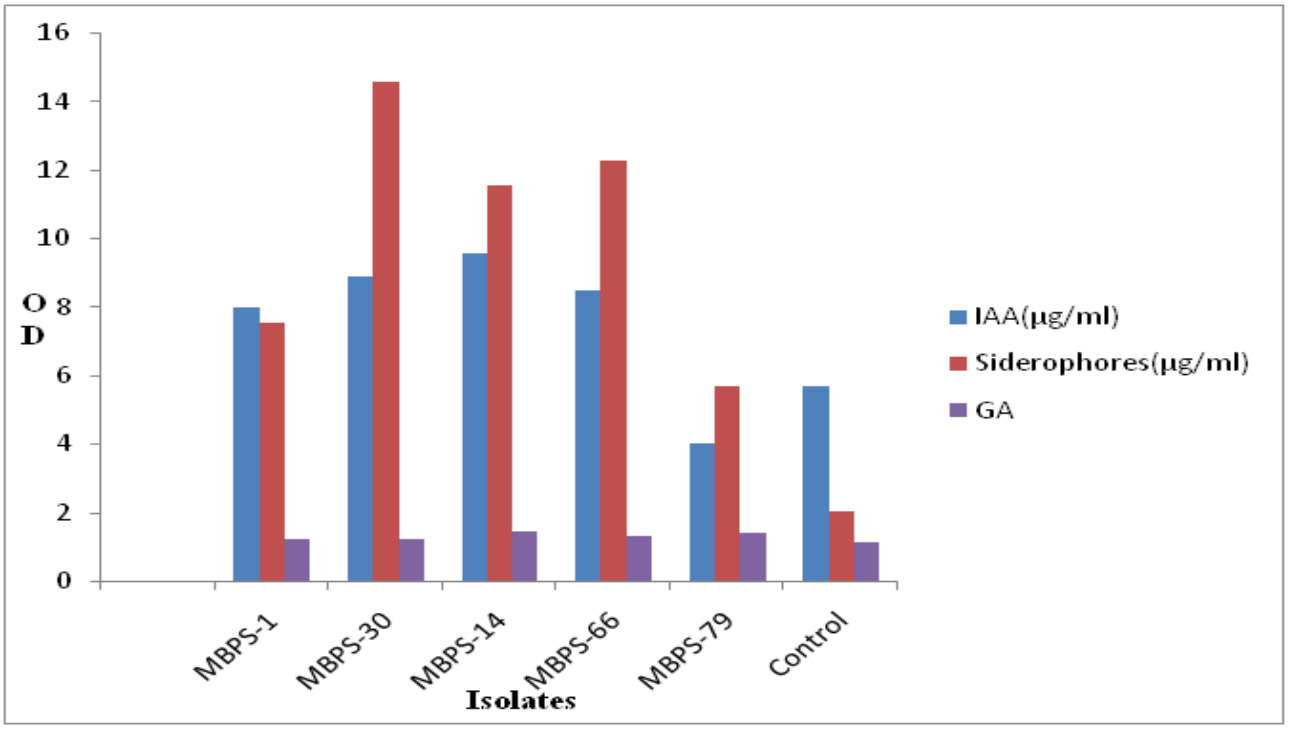

Figure 2. Assay of cell wall degrading enzymes produced by selected fluorescent pseudomonad strains.

chickpea, mustard and wheat grown in different regions of Haryana. Suresh et al. (2014) attributed the enhanced growth and yield of rice cultivated under system of rice intensification (SRI) is due to fluorescent pseudomonads.

Production of IAA, GA and siderophores

A critical perusal of the text fig-1 reveals that all five isolates were able to produce IAA, however, in negligible amounts. MBPS-14 produced highest amount of IAA while, MBPS-79 least. Enhanced production of IAA by Pseudomonas strains grown in PVK broth without tryptophan was recorded by Glickmann and Dessux (1995). Suresh and Reddy (2010) reported the production of hydrogen cyanide and IAA by fluorescent pseudomonads of rhizosphere soils of some crop plants. Increase in IAA production in medium supplemented with tryptophan was reported by Ahmad and Khan (2005). Out of five isolates, MBPS-14 produced highest amount of GA while, MBPS-79 least. Gopal (2004) reported that Pseudomonas treatment significantly increased plant growth, dry matter production and yield of Ashwagandha. Cheruth et al. (2008) reported that plant growth regulators and fungicides alter growth characteristics in *Catharanthus roseus*. Urszula et al. (2008) reported that the gibberellic acid signaling repressor RGL2 inhibits Arabidopsis seed germination by stimulating abscisic acid synthesis and AB15 activity. Text fig-1 also reveals that all the five strains were able to produce siderophores. However, the ability varied with the strain. MBPS-30 and MBPS-66 were found to be highest producers, whereas MBPS-79 was the least producer. Fluorescent pseudomonads are known to produce several kinds of siderophores such as pyoverdine, pyochelin (Dave and Dube, 2000). Siderophores efficiently chelate the iron in the root environment and deprive the pathogenic microorganisms. This iron deficiency leads to an impaired growth of the deleterious micro-organisms. Rached and Ahmed (2005) reported the effect of iron and growth inhibitors on siderophores production by *Pseudomonas fluorescens*.

Production of enzymes

Results pertaining to production of cellulases, chitinases and proteases by the present five strains of fluorescent pseudomonads are presented in text fig - 2 MBPS-79 isolate produced highest amount of protease followed by MBPS-66 and MBPS-14. The least quantity of protease production was released by MBPS-1. Ruchi et al.(2014) while evaluating the growth promoting attributes reported the enzyme production by fluorescent pseudomonads which were associated with apple and pear. With regard to production of cellulases, MBPS- 1 has shown highest production followed by MBPS-30 and MBPS -66. The least quantity of cellulase production was observed in MBPS-79. Mukesh Kumar et al. (2012) reported the optimization of *Bacillus cereus* MRK1 for cellulase production and its biostoning activity. Tabao and Monsalud (2010) made that the characterization and identification of high cellulase producing bacterial strains from Philippine mangroves.

Highest chitinase production was observed in MBPS-30 followed by MBPS-66 and MBPS-14. The least quantity of chitinase was recorded in MBPS-79. Similarly, chitinase production by fluorescent pseudomonads isolated from sugarcane rhizosphere on different

substrates was studied by Viswanathan and Samiyappan (2001). They observed that chitinase production was significantly higher when chitin was amended to Kings B medium. Shanmugaiah et al. (2008) reported that the optimization of cultural conditions for production of chitinase by *Bacillus laterosporous* MML2270 isolated from rice rhizosphere soil.

Antifungal activity

FLPs have been widely tested for biocontrol activity against fungal pathogens because of their rapid growth rate and their ability to colonize rhizosphere rapidly and their ability to suppress the soil borne pathogens. They also produce highly potent broad-spectrum antifungal molecules against a variety of phytopathogens, thus acting as effective biocontrol agents (Srivastava and Shalini, 2004). FLPs colonizing roots of a wide range of crop plants are reported to be antagonistic to soil borne plant pathogens (Siddiqui et al., 2006). In the present study, an attempt was made to evaluate the five strains of FLPs for antifungal activity against selected four phytopathogenic fungi viz *Macrophomina phaseolina, Colletotrichum falcatum, Fusarium oxysporum, Curvularia lunata* and the results are presented in table-4. It is evident from the results that these strains caused inhibition zones ranging from 0.1 to 2.7 cm (diameter) for different phytopathogenic fungi. However, the antifungal activity varied both with the FLP strain as well as test fungi. MBPS-66 showed more inhibitory activity against all the four pathogenic fungi. In case of fungi, the inhibitory effect of all the five isolates was more pronounced on *C. falcatum*. Supraja et al.(2011) studied the plant growth promotion and biocontrol properties of local isolates of fluorescent pseudomonads.

CONCLUSION

The results of the present investigations clearly indicate that fluorescent pseudomonads out number the non-fluorescent pseudomonads in the rhizosphere of sunflower. Through the fluorescent pseudomonads occur in large numbers, only a limited number of them are able to exhibit plant growth promoting attributes. In the selected five isolates, the ability of phosphate solubilization, production of IAA and siderophores varied with the strain. Similarly, enzyme producing capacities also varied. MBPS-66 isolate showed antibiosis against all the four pathogenic fungi investigated. In general, the present studies indicate that the selected fluorescent pseudomonads are able to show plant growth promoting and plant pathogenic inhibitory ability and they can be proposed as bioinoculants for sunflower.

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Disclosure statement

No potential conflict of interest was reported by the author.

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