Original research papers

Biocontrol of *Sclerotium rolfsii* using antagonistic activities of pseudomonads

Abstract

Thirty well characterized pseudomonad isolates for plant growth-promoting traits were screened for their antagonistic activities against 20 isolates of *Sclerotium rolfsii*. Out of the 30 pseudomonad isolates, PUR46 was found to be best against all 20 isolates of *Sclerotium rolfsii,* because of its unique ability to suppress the growth of mycelia as well as the sclerotia formation of most of the *S. rolfsii* isolates *in vitro* conditions. In our previous study, PUR46 was also found to be positive for growth promoting traits like phosphorus solubilization and ammonification. The results suggested that expression of one or more of the traits like antagonistic activity against *S. rolfsii* and solubilization of tri-calcium phosphate may help in controlling the pathogen besides enhancement of plant growth. In this study, our investigations clearly indicate that PGPR isolate PUR 46 may be exploited to be used as potential biocontrol agents against *S. rolfsii* in agriculture system.

Keywords: Pseudomonad, *Sclerotium rolfsii,* Plant growth-promoting traits, Antagonistic activities

Introduction

Sclerotium rolfsii Sacc. is a polyphagous fungal plant pathogens around the world in the equatorial zone between the 45 ºN and S latitudes where conditions are warm, humid and rainy. *S. rolfsii* is a devastating soil-borne fungus with a wide host range of crop plants and weeds in which the pathogen causes a great economic loss (Punja 1985; Sarma et al. 2002; Kator et al., 2015). Though the fungus is seed and soil borne, soil borne inoculums are more important in causing infection and disease development. Management of *S. rolfsii*, a major soil borne plant pathogen, through application of fungicides has been proved to be an enigma, as its broad host range and almost worldwide distribution precludes such strategy. In recent years, biological control of plant diseases involving indigenous microorganisms like plant growth-promoting rhizobacteria (PGPR) has proved to be a promising and ecofriendly strategy, especially, against soil-borne plant pathogens, because rhizosphere bacteria are ideal for use as biocontrol agents as they can provide first hand defense for plant roots against the attack by various soil borne plant pathogens (Weller 1988; Thomashow and Weller 1990; Dowling and O'Gara 1994; Selvakumar et al. 2013). Among the rhizobacteria, *Pseudomonas* spp. are emerged as the largest and most promising group of biocontrol agents owing to their potential of rapid and aggressive colonization, rhizosphere abundance, catabolic versatility, and their capacity to produce a diverse array of antifungal compounds (Anurutha and Gnanamanickam1990; Yeole and Dube 2000; Sivaprasad 2002; Saharan et al. 2011). Pseudomonads provide different mechanisms for suppressing plant pathogens (Salman et al. 2013; Kumar 2013; Beneduzi 2012). They include competition for

nutrients and space (Elad and Baker 1985; Elad and Chet 1987), antibiosis by producing antibiotics viz., pyrrolnitrin, pyocyanine, pyoluteorin, phenazines and 2, 4-diacetyl phoroglucinol (Pierson and Thomashow 1992) and production of siderophores (fluorescent yellow green pigment), viz., pseudobactin which confines the accessibility of iron required for the growth of pathogens (Lemanceau et al. 1992; Gull and Hafeez 2012). The production of lytic enzymes such as chitinases and β-1, 3 glucanases which degrade chitin and glucan present in the cell wall of fungi (Frindlender et al. 1993; Lim et al. 1991; Potgieter and Alexander 1996; Velazhahan et al. 1999), HCN production (Defago et al. 1990) and degradation of toxin produced by pathogen are some key mechanisms exist in PGPR (Borowitz et al. 1992; Duffy and Defago 1997). Several species of *Pseudomonas* are known to protect plant through eliciting induced systemic resistance (ISR) in plants (Garcia, 2012; Sarma et al. 2002; Singh et al. 2003, Mari et al. 1997; Wei et al. 1991). Therefore, biocontrol agents have emerged to grasp promise in disease management. Since biological control is an important component of integrated disease management, it is important to look for broad spectrum antifungal isolates of PGPR which are active against specific pathogens and further evaluate the antagonists for wider application. Hence the present investigation was taken up to screen and identify potent pseudomonad isolates among thirty isolates for traits associated with biocontrol of *S. rolfsii*. The proposed study would provide the information on exploiting the *Pseudomonad* sp, as an ecofriendly and sustainable alternative to the existing chemicals for growth promotion and management of diseases caused by *S. rolfsii*.

Materials and methods

Test organisms (*Sclerotium rolfsii)*

Twenty isolates of *S. rolfsii* were used in present investigation were obtained from the Department of Mycology and Plant Pathology, BHU, Varanasi. All the isolates were sub-cultured into fresh medium at 30 days intervals and stored at 4 ºC.

Rhizobacteria

Soil isolates of *Pseudomonas* spp. as reported earlier (Sahni and Prasad 2018) was used in present study.

In vitro screening of bacterial antagonists against *S. rolfsii* isolates

The 20 isolates of *S. rolfsii* were used in the present study. Initial *in vitro* screening of *Pseudomonads* spp. against the *S. rolfsii* isolates was performed in KMB medium. All pseudomonads isolates were screened for their antagonism by dual culture assays. The actively growing mycelial disc (8 mm diameter) of the respective isolate of *S. rolfsii* was placed at the centre of the Petri plate containing KMB medium and the respective bacterial isolate was streaked 4 cm away from the pathogen in a rectangular fashion and incubated at 28°C for 4 days. The petriplate inoculated with pathogen alone in the absence of antagonist served as control and the experiment was done in triplicates. The radial growth of fungal mycelium on each plate was measured and the percent inhibition of growth over control (absence of antagonists) was determined using the formula:

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

Where, I = inhibition of mycelial growth, C = growth of pathogen in control plate and T = growth of pathogen in dual cultures.

Sclerotia quantification :The actively growing mycelial disc (8 mm diameter) of the respective isolate of *S. rolfsii* was placed at the centre of the Petri plate containing KMB medium and the respective bacterial isolate was streaked 4 cm away from the pathogen in a rectangular fashion and incubated at 28°C for 10 days. The petriplate inoculated with pathogen alone in the absence of antagonist served as control and the experiment was done in triplicates. The number of sclerotia formation on each plate was counted and the percent inhibition of sclerotia formation over control (absence of antagonists) was determined using the formula:

$$S = 100 (C - T) / C$$

Where, S = percentage of sclerotia reduction, C = Number of sclerotia formation in control plate and T =Number of sclerotia formation in dual cultures.

Results

Screening of pseudomonad isolates for antagonistic activity against different isolates of *S. rolfsii*

All the 30 pseudomonad (Table 1) isolates were evaluated for their potential as biocontrol agent against *S. rolfsii*. They were screened for their antagonistic efficiency over a spectrum of *S. rolfsii* isolates collected from wide range of hosts, following dual culture technique (Johnson and Curl 1972) (Table 2). Results showed that pseudomonad isolates varied in their ability to inhibit *S. rolfsii in vitro*. Among 30 pseudomonad isolates studied, 7 isolates (R1, R2, C1, C3, C5, CRM1 and PUR46) showed differences in inhibition pattern and exhibited various interactions with different isolates of *S. rolfsii.* This comprising inhibition of *S. rolfsii* at a distance and slight inhibition, e.g. PUR46 against *Cicer arietinum* (DL2), whereas some isolates (R1, R2, C1, C3, C5, CRM1 and PUR46) restricted the growth of some of *S. rolfsii* isolates at the point of interface, e.g. R1, C1, C3 and C5 against *Artrica* sp. isolates of *S. rolfsii*. Similar types of interactions were also observed by R1, R2, C1, C3, C5 and CRM1 against *Cladium* sp. isolate of *S. rolfsii*. However, other 23 isolates were found to overgrow by all tested isolates of *S. rolfsii*.

However, among the various pseudomonad isolates , PUR46 was found to be the best in antagonistic activity over a large number of *S. rolfsii* isolates showing maximum inhibition with clear inhibition zone for six *S. rolfsii* isolates, namely, *Artrica* sp., *Bombax malabaricum*, *Cicer arietinum* (DL2), *Cladium* sp., *Coccinia indica* and BGT soil (Figure 1) whereas, it restricted the growth of four *S. rolfsii* isolates viz., *Amorphophallus companulatus*, *Ficus religiosa, Rauvolfia serpentine* and LPG, at the point of interface. The pseudomonad isolate R2 was next best in antagonistic activity against *S. rolfsii* isolates *in vitro*, which showed clear inhibition zone for three *S. rolfsii* isolates, viz., *Artrica* sp., *Cicer arietinum* (DL2), and *Coccinia indica* while it restricted the growth of three isolates, namely from *Bombax malabaricum*, *Cladium* sp. and BGT soil at the point of interface.

Comparative studies of inhibition pattern of different isolates of *S. rolfsii* by the pseudomonad isolate PUR46 by dual culture technique

Present investigation indicated differential sensitivity of different isolates of *S. rolfsii* towards PUR46 (Table 3), showing differences in percent inhibition of mycelial growth, and lysis pattern as well as percent reduction in sclerotia formation over control. It restricted the growth of four *S. rolfsii* isolates at the point of interface, in which three isolates from *Amorphophallus companulatus*, *Rauvolfia serpentine* mycelia, whereas *Ficus religiosa* isolate was forced to incomplete lysis leading to 95.20 % inhibition in sclerotia number over control (Figure 2). However, it was overgrown by ten isolates of *S. rolfsii*, where total lysis of mycelia was observed in

137 *Cladium sp*. (L) isolate in the advanced stage of antagonism (Table 3). Incomplete
138 lysis was observed in six isolates of *S. rolfsii,* causing poor development and reduction
139 in sclerotial number (72.31 to 100 % inhibition of sclerotia over control) (Table 3),
140 whereas three isolates showed deformation of mycelia with reduced number of
141 sclerotia (15.62 to 46.50 % inhibition over control). Interestingly, PUR46 showed
142 clear inhibition zone against six isolates of *S. rolfsii*. It reduced maximum 82.56 %
143 linear growth of mycelia in *Cicer arietinum* isolate (DL2), 75.90 % in *Bombax*
144 *malabaricum* isolate, while approximately 50 % in *Artrica* sp. and BGT soil isolates,
145 whereas less than 50 % in *Coccinia indica* and *Cladium* sp. isolates of *S. rolfsii* (Table
146 3).

147 Thus, our results clearly indicated that *Pseudomonas fluorescence* isolate
148 PUR46 was best in antagonistic activity over a large number of *S. rolfsii* isolates
149 (Figure 2), and identified as highly potential bioagent against *S. rolfsii*.

150 **Discussion**

151 **Plant growth-promoting attributes**

152 Fluorescent *Pseudomonas* spp. are important for biological control (Ganeshan
153 and Kumar 2005) as they can suppress diseases caused by phytopathogenic fungi
154 (Salman et al. 2013; Weller 1988; Thomshow and Weller 1988) and are candidates as
155 hosts for the delivery of genes. *Pseudomonas* spp. secretes biocontrol toxin to the
156 plant rhizosphere (Obukowiez et al. 1986; Van Elsas et al. 1991; Araujo et al.1994). In
157 present investigations, 30 pseudomonad isolates, 12 isolates produced fluorescent
158 pigment on KBM, and most of them caused total lysis of mycelia of *S. rolfsii* (DL2).

However, PSB2, R2 and A3 were negative in fluorescent pigment production but showed strong antibiosis against *S. rolfsii* and caused total lysis. So, antagonistic activity of the pseudomonads against *S. rolfsii* is not linked strictly with fluorescent pigmentation.

In vitro evaluation of antagonists for antimicrobial activity

The initial analysis of the pseudomonad isolates for their antagonistic activity against a large number of *S. rolfsii* isolates *in vitro*. It was observed that some isolates inhibited the growth of *S. rolfsii*. This suggested that some pseudomonad isolates can produce inhibitory metabolites against *S. rolfsii* that checked the growth of *S. rolfsii* isolates. The inhibitory property of the isolates reflects the inherent potential of the pseudomonads to produce inhibitory metabolites against *S. rolfsii*. A plethora of reports says that many bacteria produce antibiotics or antifungal proteins for their survival (Kudryashova et al., 2005; Antoun and Prévost, 2005). These antimicrobial factors play an important role in controlling several plant diseases (Kumar et al. 2013; Beneduzi et al. 2012; Okamoto et al. 1998; O'sullivan and O'Gara 1992; Thomashow and Weller 1988).

Our results clearly indicate that different isolates of *S. rolfsii* showed differential sensitivity towards a pseudomonad isolate resulted in differences in inhibition pattern. Different pseudomonad isolates also showed differences in inhibition pattern against a same *S. rolfsii* isolate and it might be attributed due to variable antifungal activity possessed by different pseudomonad spp. It is known that

the extent of inhibition zone formation is related to the ability of the organism to produce inhibitory metabolites against the test organism (Sivaprasad 2002).

Our findings indicated that the period of incubation played a highly significant role with inhibition in the beginning followed by maximum differential lysis of *S. rolfsii* in the advanced stage of antagonism. As a result, the natural fluffy growth of the fungal pathogen was suppressed and lead to total lysis of mycelia or partial lysis resulting in poor development of sclerotia, with reduced number and size. PUR46 produced differential lysis in different isolates of *S. rolfsii* indicating its strong antagonistic potential.

Conclusion: Our investigations clearly indicate that out of 30 PGPR isolates, PUR 46 was found to be best as potential biocontrol agents against S. rolfsii which may be exploited to be used as potential biocontrol agent against S. rolfsii in agriculture system.Thus screening and identification of novel bioagent PUR46 reflects its potential to suppress *S. rolfsii* and suggest usefulness of this super bioinoculant as component of IDM of *S. rolfsii.* Although the occurrence of growth promoting traits *in vitro* does not assurance that an isolate will promote plant growth in nature, it is therefore considered essential to assess the performance of this isolate under natural environment conditions. If the potential of this isolate is confirmed, it could in future be used as component of IDM, which will help in developing cost effective integrated biological control methods in agriculture to combat the pathogen *S. rolfsii*.

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321 Table 1. Habitat of Pseudomonas isolates.

322

S.No.	Pseudomo-nad isolates	Habitat (Host rhizosphere)
1	A1	Arhar
2	A2	Arhar
3	A3	Arhar
4	R1	Rajma
5	R2	Rajma
6	R3	Rajma
7	P1	Pea
8	P2	Pea
9	P3	Pea
10	P4	Pea
11	M1	Mungbean
12	L1	Lentil
13	L2	Lentil
14	L3	Lentil
15	L4	Lentil
16	C1	Chickpea
17	C2	Chickpea
18	C3	Chickpea
19	C4	Chickpea
20	C5	Chickpea
21	C6	Chickpea
22	C7	Chickpea
23	CRM1	Soil
24	CRM2	Soil
25	CRM3	Soil
26	KB133	Soil
27	PUR46	Soil
28	PUR171	Soil
29	PSB1	Soil
30	PSB2	Soil

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Table 2. Screening of pseudomonad isolates against different isolates of *Sclerotium rolfsii* on the basis of inhibition pattern of pathogen by dual culture technique

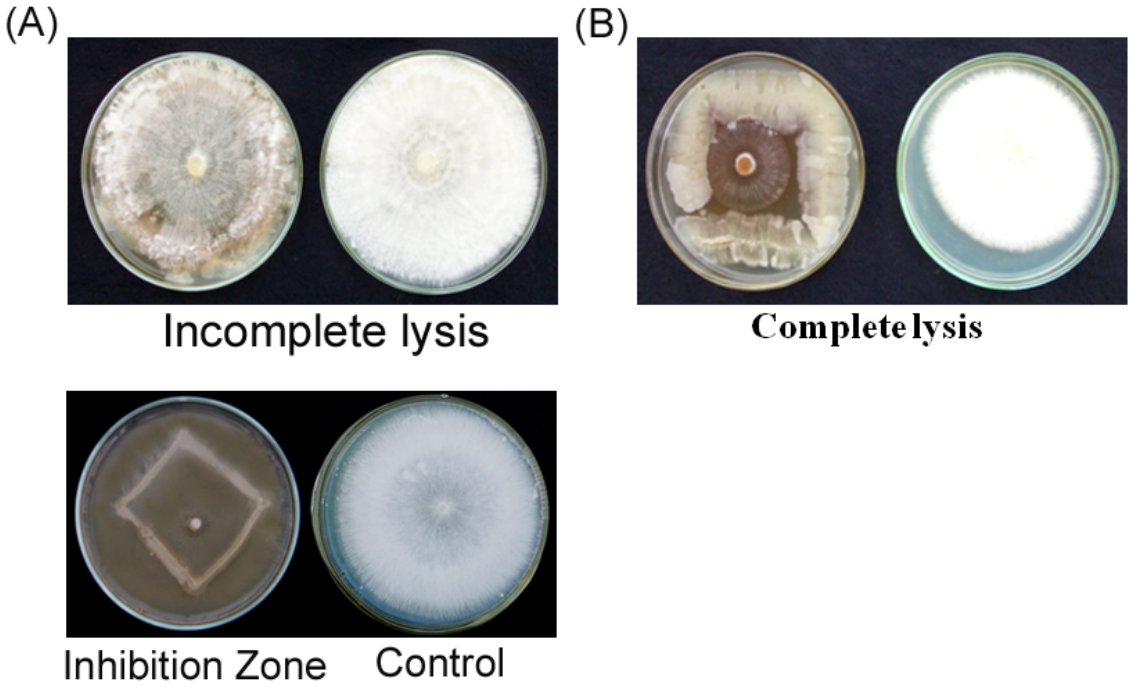
Isolates of *Sclerotium rolfsii*	Pseudomonad isolates																												
	A1	A2	A3	R1	R2	R3	P1	P2	P3	P4	M1	L1	L2	L3	L4	C1	C2	C3	C4	C5	C6	C7	CRM1	CRM2	CRM3	KB133	PUR46	PUR171	PSB1	PSB2
Artrica sp.	I_0	I_0	I_0	C_g	P_i	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	C_g	I_0	C_g	I_0	I_0	I_0	I_0	I_0	I_0	P_i	I_0	I_0	I_0
Amorphophallus companulatus	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	I_0	I_0
Blepharis boerhaviaefolia	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0
Bombax malabaricum	I_0	I_0	I_0	C_g	C_g	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	C_g	I_0	C_g	I_0	I_0	C_g	I_0	I_0	I_0	P_i	I_0	I_0	I_0
Cicer arietinum	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0
Cicer arietinum (DL2)	I_0	I_0	I_0	C_g	P_i	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	C_g	I_0	C_g	I_0	I_0	C_g	I_0	I_0	I_0	P_i	I_0	I_0	I_0
Cladium sp.	I_0	I_0	I_0	C_g	C_g	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	C_g	I_0	C_g	I_0	I_0	C_g	I_0	I_0	I_0	P_i	I_0	I_0	I_0
Cladium sp. (L)	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0
Coccinia indica	I_0	I_0	I_0	I_0	P_i	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	I_0	I_0	C_g	I_0	I_0	C_g	I_0	I_0	I_0	P_i	I_0	I_0	I_0	I_0
Cynodon dactylon	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0
Ficus religiosa	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	I_0	I_0
Glycine max	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0
Hemidesmus indicus	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0
Lycopersicon esculentum	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0
Morus nigra	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0
Phaseolus vulgaris	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	C_g	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0
Rauvolfia serpentina	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	I_0	I_0
Vigna radiata	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0
BGT soil	I_0	I_0	I_0	I_0	C_g	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	C_g	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	P_i	I_0	I_0	I_0
LPG	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	I_0	C_g	I_0	I_0	I_0

P_i = Pathogen inhibited by pseudomonad isolate; C_g = Cessation of growth of pathogen at line of contact; I_0 = Pseudomonad isolate overgrow by pathogen.

Table 3. Comparative studies of inhibition pattern of different isolates of *Sclerotium rolfsii* produced by pseudomonad isolate PUR46 by dual culture technique

Isolates of *Sclerotium rolfsii*	Interaction with pathogen	Inhibition zone (mm) ‡	Percent inhibition of mycelial growth over control	Lysis pattern	(No. of sclerotia/plate after interaction) ‡	Percent reduction of sclerotial no. over control
Artrica sp.	**P_i**	7.30	51.33 (45.41)	**TL**	-	-
Amorphophallus companulatus	**C_g**	-	-	**TL**	-	-
Blepharis boerhaviaefolia	I_0	-	-	IL	0.00	**100.00** (89.43)
Bombax malabaricum	**P_i**	15.67	75.90 (61.17)	**TL**	-	-
Cicer arietinum	I_0	-	-	DM	183.67	15.62 (29.48)
***Cicer arietinum* (DL2)**	**P_i**	15.70	**82.56** (65.58)	**TL**	-	-
Cladium sp.	**P_i**	28.67	21.80 (27.91)	**TL**	-	-
Cladium sp. (L)	I_0			**TL**	-	-
Coccinia indica	**P_i**	26.33	31.32 (34.22)	**TL**		
Cynodon dactylon	I_0	-	-	IL	34.67	82.87 (65.61)
Ficus religiosa	**C_g**	-	-	IL	6.00	95.20 (77.44)
Glycine max	I_0	-	-	IL	17.70	90.05 (71.67)
Hemidesmus indicus	I_0	-	-	DM	136.00	47.00 (43.33)
Lycopersicon esculentum	I_0	-	-	IL	52.33	72.31 (58.38)
Morus nigra	I_0	-	-	DM	132.67	46.50 (42.97)
Phaseolus vulgaris	I_0	-	-	IL	12.33	92.00 (73.61)
Rauvolfia serpentina	**C_g**	-	-	**TL**	-	-
Vigna radiata	I_0	-	-	IL	0.00	**100.00** (89.43)
BGT soil	**P_i**	29.30	53.74 (47.05)	**TL**	-	-
LPG	**C_g**	-	-	**TL**	-	-

P_i = Pathogen inhibited by pseudomonad isolate; C_g = Cessation of growth of pathogen at line of contact; I_0 = Pseudomonad isolate overgrow by pathogen; TL = Total lysis; IL = Incomplete lysis; DM = Deformed mycelia; ‡ = Mean of three replication; Values in the parentheses are arc sin transformed values.



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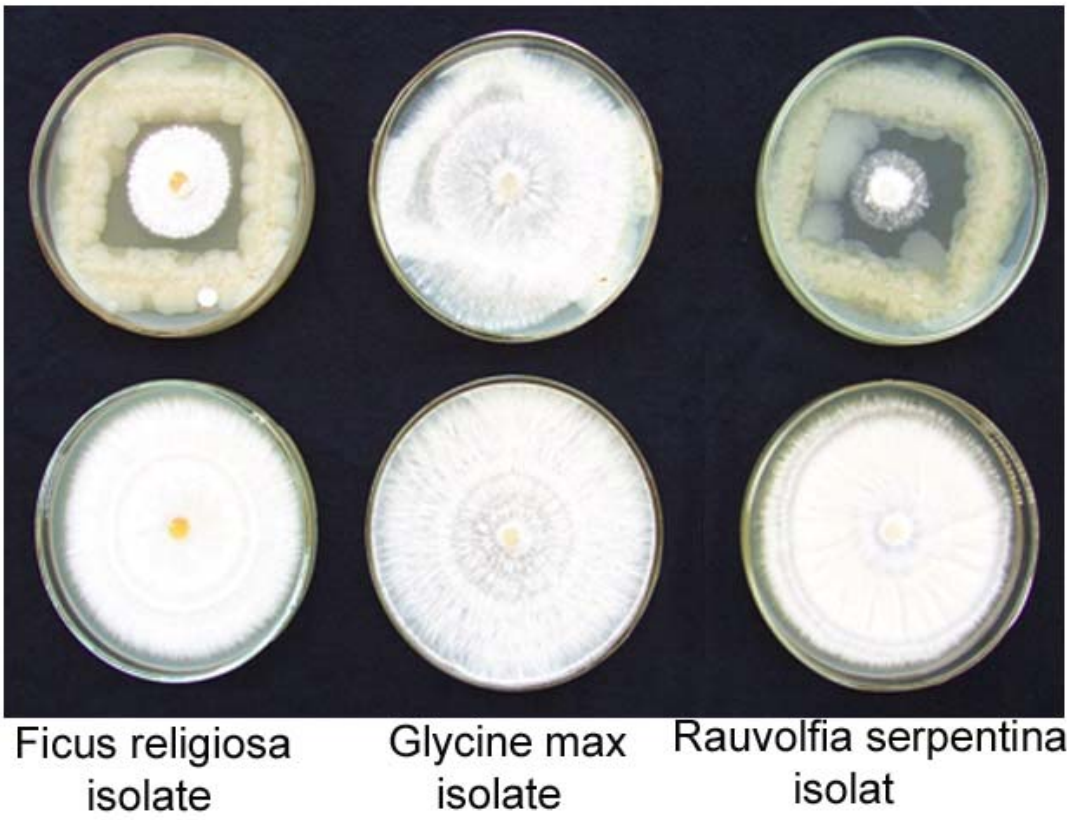
339 Figure 1. Lysis pattern of different isolates of S. rolfsii by Pseudomonas isolates. (A)
340 Incomplete lysis; (B) Complete lysis; (C) Inhibition zone *vs* control.



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343 Figure 2. Inhibition patter of different isolates of *S. rolfsii* by Pseudomonand isolates

344 PUR46.