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Antibiotic potential of plant growth promoting rhizobacteria (PGPR) against *Sclerotium rolfsii*

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High performance liquid chromatographic (HPLC) analysis of culture filtrates of plant growth promoting rhizobacteria (PGPR) and medium of inhibitory zone of interaction of *Sclerotium rolfsii* with PGPR, viz. *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* 4, *Pseudomonas fluorescens* 4 (new) and *Pseudomonas* sp. varied from sample to sample. In all the culture filtrates of PGPRs, *P. aeruginosa* had nine phenolic acids in which ferulic acid (14.52 μg/ml) was maximum followed by other phenolic acids. However, the culture filtrates of *P. fluorescens* 4 had six phenolic acids with maximum ferulic acid (20.54 μg/ml) followed by indole acetic acid (IAA), caffeic, salicylic, *o*-coumeric acid and cinnamic acids. However, *P. fluorescens* 4 culture filtrate had seven phenolic acids in which salicylic acid was maximum (18.03 μg) followed by IAA, caffeic, vanillic, ferulic, *o*-coumeric and cinnamic acids. *Pseudomonas* sp. also showed eight phenolic acids where caffeic acid (2.75 μg) was maximum followed by trace amounts of ferulic, salicylic, IAA, vanillic, cinnamic, *o*-coumeric and tannic acids. The analysis of antibiosis zone of PGPRs showed fairly rich phenolic acids. A total of nine phenolic acids were detected in which caffeic acid was maximum (29.14 μg/g) followed by gallic (17.64 μg/g) and vanillic (3.52 μg/g) acids but others were in traces. In *P. aeruginosa*, antibiosis zone had seven phenolic acids where IAA was maximum (3.48 μg/g) followed by *o*-coumeric acid (2.08 μg/g), others were in traces. The medium of antibiosis zone of *P. fluorescens* 4 and *P. fluorescens* 4 new had eight phenolic acids in which IAA was maximum with other phenolic acids in traces.

Keywords: PGPRs; culture filtrate; zone of antibiosis; phenolic acids

Introduction

Secondary metabolites, such as alkaloids, terpenoids, sterols and phenolic compounds, are the constituents which play a major role in plant defence mechanisms (Nicholson and Hammerschmidt 1992; Kuc 1995). Among them, phenolic compounds and their derivatives are reported to inhibit wide range of soil-borne phytopathogens (Stoessl 1983; Sarma et al. 2002; Maurya et al. 2005, 2007). They are also formed in response to host–pathogen interaction as part of an active defence response (Nicholson and Hammerschmidt 1992; Maurya et al. 2007). Van Peer et al. (1991) reported the accumulation of phenolic compounds in carnation by a *Pseudomonas* sp. that minimised the intensity of *Fusarium* wilt. Similarly, successful

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protection of pea from *Erysiphe pisi* through foliar application of plant growth promoting rhizobacteria (PGPR) has been demonstrated by Singh et al. (2000).

Sclerotium rolfsii is one of the most important soil-borne fungal phytopathogens which cause collar rot as well as leaf spot diseases in mono and dicotyledonous crop plants (Maurya et al. 2007). Singh et al. (2003a) reported that the application of PGPRs effectively manages the collar rot of chickpea (*Cicer arietinum*) caused by *S. rolfsii* under field conditions.

Looking at the antifungal activity of phenolic acids excreted by PGPRs, the present study was conducted to investigate the secretion of phenolic compounds in culture filtrate and in the zone of antibiosis of PGPRs and *S. rolfsii*.

Materials and methods

Preparation of culture filtrates of PGPRs

Czapeck's Dox broth medium was used for culturing PGPRs. Each bacterial isolate was inoculated separately in a conical flask in triplicate. The culture filtrates from a 10–12 day-old broth medium were used for the analysis of phenolic acids by high performance liquid chromatography (HPLC).

Screening of antibiosis potentials of PGPRs

Dual cultures

One 5 mm disk of a pure culture of *S. rolfsii* was placed at the centre of a Petri dish containing Czapeck's Dox agar medium. A rectangular line was made in Petri dishes with the suspension of bioantagonistic PGPRs (Pag, Pf4, Pf4 new and *P.* sp. (5×10^{-8} cfu/ml)), placed surrounding the fungal inoculums of *S. rolfsii* (Figure 1). Each isolate of PGPRs was plated in triplicate. Plates were incubated for 72 h at 25 ± 2°C.

Extraction of phenolic acids from culture filtrates and medium of antibiosis zone between PGPRs and S. rolfsii

Culture filtrates of PGPRs and medium of antibiosis were taken for isolation of phenolic acids. One millilitre of culture filtrate was mixed with ethyl acetate and shaken well which formed a biphasic layer. Upper layer of biphasic was collected and evaporated in a Petri dish and then re-suspended in 95% ethanol. The culture medium (1 g) of 6–8-day-old antibiosis zone was initially collected with the help of scalpel and macerated in a pestle–mortar. Finely crushed samples were suspended in 5–10 ml of ethanol–water (80:20; v/v) and collected in screw-capped specimen tubes. The suspension was subjected to ultra-sonication (Branson Sonifier, USA) for 15 min at 4°C followed by centrifugation at 7500 *g* for 15 min. The residue was re-extracted twice and the supernatant was pooled prior to evaporation under vacuum (Buchi Rotavapor Re Type). Dried samples were re-suspended in 1.0 ml HPLC grade methanol by vortexing and filtered through a membrane filter (pore size 0.45 μm, Millipore) before HPLC analysis.

High performance liquid chromatographic (HPLC) analysis

High performance liquid chromatography of isolated materials was performed on HPLC system (Shimadzu Corporation, Japan) equipped with two Shimadzu LC-10

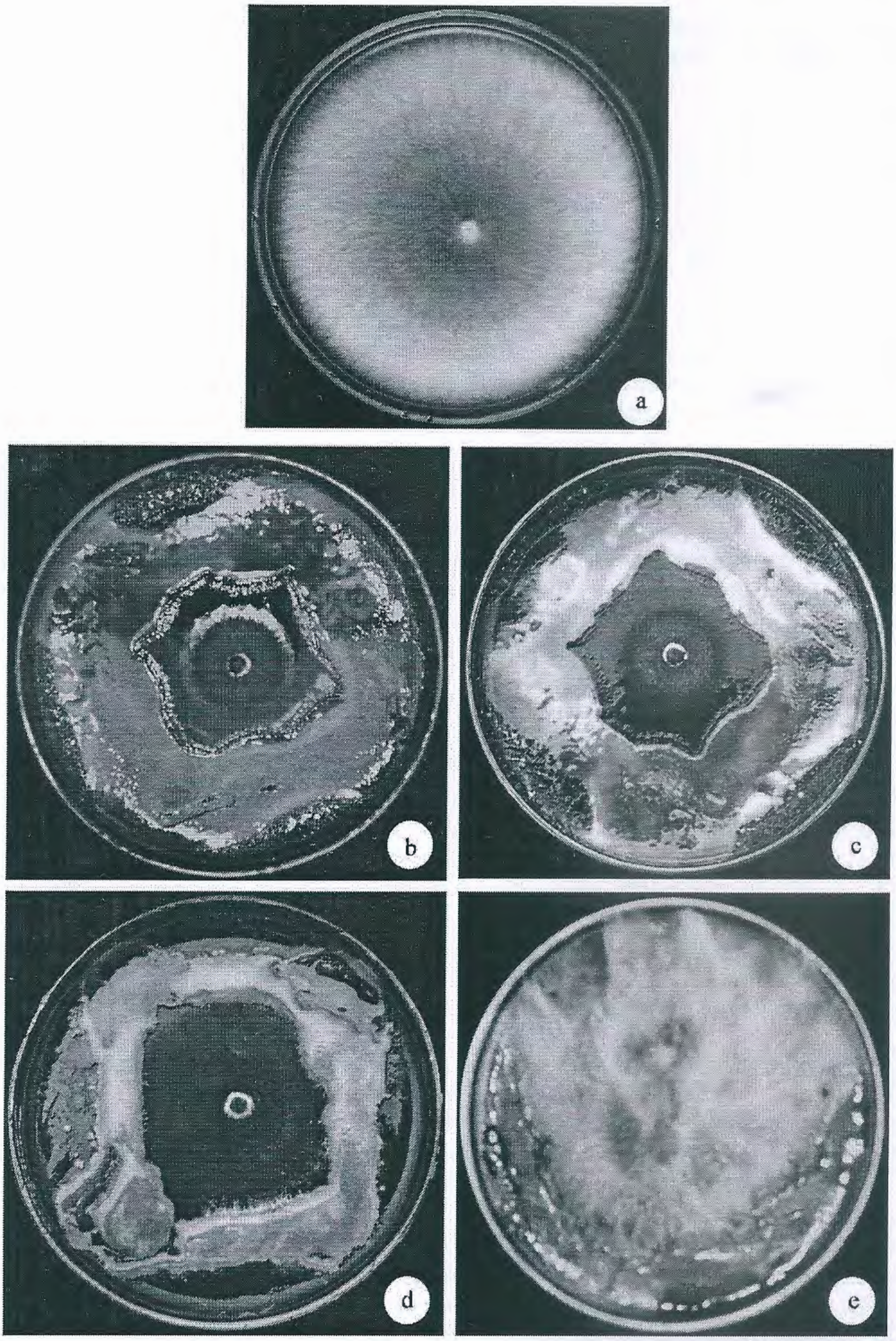

Figure 1. Antibiosis potential of plant growth promoting rhizobacteria against *Sclerotium rolfsii*, (a) Control, (b) *Pseudomonas fluorescens* strain 4, (c) *P. fluorescens* strain 4 (new), (d) *Pseudomonas* sp., and (e) *Pseudomonas auriginosa*.

AT*VP* reciprocating pumps, a variable UV-VIS detector (Shimadzu SPD-10 A*VP*), an integrator and CLASS-VP software for data recording and processing (Shimadzu). Reversed phase chromatographic analysis was carried out in isocratic conditions using C-18 reversed phase HPLC column (250 × 4.6 mm i.d., particle size 5 μm) Luna 5μ C-18 (2), Phenomenex, USA at 25°C. Running conditions included mobile phase methanol 0.4% acetic acid (80:20, v/v), flow rate 1.0 ml/min, injection volume 5 μl and detection at 290 nm (Singh et al. 2003a; Maurya et al. 2007). Samples were injected thrice in the sample loop and the means of the peak areas of individual compounds were taken for quantification. Phenolic acids were used as internal and external standards and those present in the samples were identified by comparing retention time (Rt) of the standards. Further confirmation of phenolic acids was done by co-injection of the standards. Amounts of individual compounds were calculated by comparing peak areas of reference compounds with those in the samples run under the similar elution conditions.

Results and discussion

High performance liquid chromatographic analysis of culture filtrates of PGPRs and the medium of antibiosis zone of PGPRs and *S. rolfsii* showed several phenolic acids, viz. tannic (TA, Rt. 2.94 min), gallic (GA, Rt., 3.10 min), caffeic (Caf-A, Rt. 3.48 min), vanillic (VA, 3.76 min), ferulic (FA, Rt. 4.02), *o*-coumaric (O-Cou-A, Rt. 4.64 min), indole acetic acid (IAA, 5.05 min), cinnamic (CA, Rt. 6.67 min) and salicylic acids (SA, Rt. 7.86 min), which were identified qualitatively and quantitatively. The culture filtrate of *P. aeruginosa* had nine phenolic acids where ferulic acid (14.52 μg/ml) was maximum followed by vanillic (8.74 μg/ml), caffeic (3.75), IAA (3.30), gallic (3.19) and salicylic (1.61) acids. Other phenolic acids were in traces. The culture filtrate of *P. fluorescens* 4 had six phenolic acids with maximum ferulic acid (20.54 μg/ml) followed by IAA (12.95 μg), caffeic (11.35 μg), salicylic (7.19 μg), *o*-coumeric acid (4.45 μg) and cinnamic (1.05 μg) acids. But the culture filtrate of *P. fluorescens* 4 new isolate had seven phenolic acids where salicylic acid (18.03 μg) was maximum followed by IAA (6.74 μg), caffeic (5.70 μg), vanillic (4.69 μg), ferulic (4.25 μg), *o*-coumeric (2.74 μg) and cinnamic (0.85 μg) acids, and *Pseudomonas* sp. had eight phenolic acids with a maximum of caffeic acid (2.75 μg) followed by ferulic (2.64 μg), salicylic (2.59 μg) and IAA (2.43 μg). However, vanillic, cinnamic, *o*-coumeric and tannic acids were in traces (Table 1).

High performance liquid chromatographic analysis of the medium of antibiosis zone between PGPRs and *S. rolfsii* showed that a good number of phenolic acids of *P. aeruginosa*, *P. fluorescens* 4, *P. fluorescens* 4 new and *Pseudomonas* sp. *Pseudomonas* sp. had fairly rich phenolic acids where caffeic acid (29.14 μg/g) was maximum followed by gallic (17.64 μg), vanillic (3.52 μg), *o*-coumeric (2.76 μg), IAA (1.77 μg) and ferulic (1.25 μg) acids. However, salicylic, cinnamic and tannic acids were in traces. The medium of inhibitory zone of *S. rolfsii* and *P. aeruginosa* had seven phenolic acids, where IAA (3.47 μg/g) was maximum followed by caffeic (2.52 μg/g), ferulic acid (1.88 μg), vanillic (1.61 μg/g) and *o*-coumeric (0.98 μg/g) acids, but cinnamic and tannic acids were in traces. Similarly, the medium of antibiosis zone of *P. fluorescens* 4 and *S. rolfsii* had eight phenolic acids where IAA (2.60 μg/g) was maximum followed by caffeic (1.75 μg), ferulic (1.24 μg/g), vanillic (1.06 μg/g) and *o*-coumeric acids, but cinnamic, salicylic and tannic acids were in traces. The medium of antibiosis zone of *P. fluorescens* 4 new and *S. rolfsii* also had

Table 1. Phenolic acids in culture filtrates of some plant growth promoting rhizobacteria (PGPRs).

Name of PGPRs	Phenolic acids (μg/g fresh wt.)								
	TA	GA	Caf-A	VA	FA	O-Cou-A	IAA	CA	SA
Control	0.77±0.01	1.64±0.05	1.14±0.16	1.02±0.24	1.05±0.02	1.76±0.05	1.07±0.03	0.11±0.01	0.12±0.02
P. aruginosa	0.19±0.01	3.19±0.16	3.75±0.07	8.74±0.56	14.52±1.12	1.63±0.12	3.30±0.02	0.18±0.01	1.61±0.12
P. fluorescens 4	UDL	UDL	11.35±1.20	UDL	20.54±2.14	4.45±0.24	12.95±0.52	1.05±0.04	7.19±0.42
P. fluorescens (new)	0.29±0.02	UDL	5.73±0.08	4.51±0.12	3.84±0.02	1.52±0.06	6.23±0.07	1.37±0.04	17.41±1.02
Pseudomonas sp.	0.06±0.01	UDL	2.75±0.05	0.83±0.01	2.64±0.08	0.70±0.01	2.43±0.02	0.13±0.01	2.59±0.06

Table 2. Phenolic acid secretion in inhibitory zone of the culture medium of *S. rolfsii* interacted with plant growth promoting rhizobacteria (PGPRs).

	Phenolic acids (μg/g fresh wt.)								
Treatments	TA	GA	Caf-A	VA	FA	O-Cou-A	IAA	CA	SA
Control	0.77±0.01	1.64±0.05	1.14±0.16	1.02±0.24	1.05±0.02	1.76±0.05	1.07±0.03	0.11±0.01	0.12±0.02
Pag × S. rolfsii	0.08±0.002	2.11±0.08	0.79± 0.03	1.33±0.12	1.77±0.07	2.03±0.05	3.48±0.50	0.14±0.02	0.13±0.01
Pf4 × S. rolfsii	0.11± 0.01	UDL	1.75±0.09	1.06±0.13	1.24±0.14	0.61±0.01	2.60±0.12	0.10±0.01	0.05±0.01
Pf4 new × S. rolfsii	0.09±0.007	UDL	2.52±0.06	1.61±0.23	1.88±0.08	0.98±0.02	3.47±0.76	0.11±0.01	0.03±0.01
Pseudomonas sp.	0.04±0.001	17.64	29.14±0.12	3.52±0.14	1.25±0.12	2.76±0.01	1.77±0.01	0.16±0.02	0.82±0.06

Note: TA, tannic; GA, gallic; Caf-A, caffeic; VA, vanillic; FA, ferulic; O-Cou-A, coumeric; IAA, indole acetic acid; CA, cinnamic, and SA, salicylic acid. ±Standard error.

eight phenolic acids where caffeic acid (2.23 μg/g) was maximum followed by cinnamic (1.60 μg), ferulic (1.17 μg/g), vanillic (1.11 μg/g) and *o*-coumeric (0.74 μg/g) acids and IAA (0.58 μg/g) but salicylic and tannic acids were in traces. (Table 2 and Figure 1).

According to Rakh et al. (2011), rhizobacteria *Pseudomonas* cf. *monteilii 9* had shown strong antagonistic activity against *S. rolfsii* and also produced diffusible antibiotic, volatile metabolites, hydrogen cyanide and siderophore which affect *S. rolfsii* growth *in vitro*. Singh et al. (2003b) reported that *P. fluorescens* NBRI-N6 and *P. fluorescens* successfully controlled the collar rot disease of betelvine (*Piper betle* L.) caused by *S. rolfsii*. Rovera et al. (2008) reported that *P. aurantiaca* has ability to produce wide range of secondary metabolites. Among phenolic acids, ferulic acid is highly antifungal against *S. rolfsii in vitro* (Sarma and Singh 2003). It also plays an important role in the formation of lignin in non-host plant resistance against *S. rolfsii* invasion (Maurya et al. 2007). Sarma and Singh (2003) also reported that ferulic acid plays a role in inhibition of collar rot of chickpea caused by *S. rolfsii*. The presence of vanillic acid which is considered as precursor of ferulic acid in most of the test samples indicates the probability of further production of ferulic acid. The synthesis of ferulic acid does not always take place through the usual phenyl propanoid pathway as it is also reported to be synthesised in the host after pathogen ingress through some alternative pathway (Nicholson and Hammerschmidt 1992). It is also well known that salicylic acid is a signalling molecule which plays a role in inducing systemic resistance in plants. The presence of indole acetic acid, ferulic and salicylic acids indicates that PGPRs are not only the growth promoters, they can also inhibit the ingress of pathogens and have ability to induce resistance in the plants.

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