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Metabolites of rhizobacteria antagonistic towards fungal plant pathogens

Garima JHA, Vanamala ANJAIAH*

Department of Botany, University of Delhi, Delhi - 110 007, India

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Abstract - Biological control of insect, plant pathogens and weeds is the only major alternative to the use of pesticides in agriculture and forestry. A double-layer technique was used for isolation of antagonistic bacteria from rhizosphere against plant pathogenic fungi. Four potential rhizobacteria was selected in dual culture plate method based on their antifungal activity against several soil-borne fungal plant pathogens. The selected rhizobacteria, identified based on their morphological, biochemical and molecular traits, belong to the species of fluorescent *Pseudomonas* (SAB8, GM4) and *Bacillus* (A555, GF23). The active antifungal metabolites produced by these strains in culture filtrates were tested for the growth inhibition of *Fusarium semitectum* used as test fungus. The active fraction of antifungal metabolite/(s) from fluorescent *Pseudomonas* (SAB8, GM4) and their effects on hyphal growth were observed under microscope. Two kinds of alterations were detected: inhibition of hyphal tip elongation and an extensive branching of hyphae with closer septa.

Key words: antagonism, antifungal compounds, *Bacillus*, fluorescent *Pseudomonas*, phytopathogenic fungi.

INTRODUCTION

Soil-borne plant diseases, caused by fungal pathogens, are very destructive resulting into great economic loss for crop production. Biological control of soil-borne diseases offers an attractive alternative or supplement to use of fungicides for the management of plant diseases. Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since they provide the front line defence for roots against soil-borne pathogens. Fluorescent *Pseudomonas* strains as biocontrol agents have been investigated intensively for their production of antifungal metabolites in the recent years (Maurhofer *et al.*, 1992; Thomashow and Weller, 1996). *Bacillus* spp. were also shown to be potential candidates for biocontrol agents as they are abundant in the soil and produce heat resistant spores apart from their active metabolites (Asaka and Shoda, 1996; Milner *et al.,* 1996). Several mechanisms have been proposed to explain pathogen suppression (Thomashow and Weller, 1996), which include competition for nutrients, production of metabolites such as antibiotics, siderophores and HCN (O'Sullivan and O'Gara, 1992; Thomashow and Weller, 1996). Biocontrol of soil-borne diseases can be accomplished by introducing the antagonistic microorganisms either through seed (seed bacterisation) or through incorporation into the soil (soil drench) with various techniques (Whipps, 1992).

Considering the potential environmental and health hazards and drawbacks in the chemical measures of plant disease control, the present investigation is aimed at isolating potential bacterial antagonists against fungal pathogens from the rhizosphere of plants. In this paper, we describe the isolation and characterisation of rhizobacteria in relation to their antifungal metabolites against phytopathogens. Production of antifungal compounds in culture filtrates and their *in vitro* antagonistic effects against various soil-borne fungal pathogens were investigated.

MATERIALS AND METHODS

Microorganisms and media. Phytopathogenic fungi such as *Fusarium semitectum, Fusarium udum, Fusarium oxysporum* f. sp. *ciceri, Fusarium oxysporum* f. sp. *lycopersici, Aspergillus flavus* (toxigenic and non-toxigenic), *Aspergillus niger, Rhizoctonia solani* and *Sclerotium rolfsii* were obtained from NBAIM (National Bureau of Agricultural Important Microorganisms), IARI, New Delhi, and maintained on Potato Dextrose Agar (PDA). All bacterial isolated strains were maintained on Nutrient Agar (NA). All the growth media, including Tryptone Glucose Yeast Extract Agar (TGYEA), Pseudomonas Agar Fluorescein (PAF), Pseudomonas Agar Base (PA), Nutrient broth used in this study were obtained from Hi-media Ltd., Mumbai.

Isolation of antagonistic rhizobacteria*.* Isolation of antagonistic strains were done from the rhizosphere soil, which was collected from healthy plant growing beside disease plant, serially diluted in sterile distilled water and tested using double layer technique (DLT). In DLT, spore suspension (10^8 colony forming units ml^{-1}) of *Fusarium semi-*

* Corresponding author. Address: Genetic Transformation Laboratory, ICRISAT, Patancheru (P.O.), A.P. - 502 324; India; E-mail: a_vanamala@yahoo.com

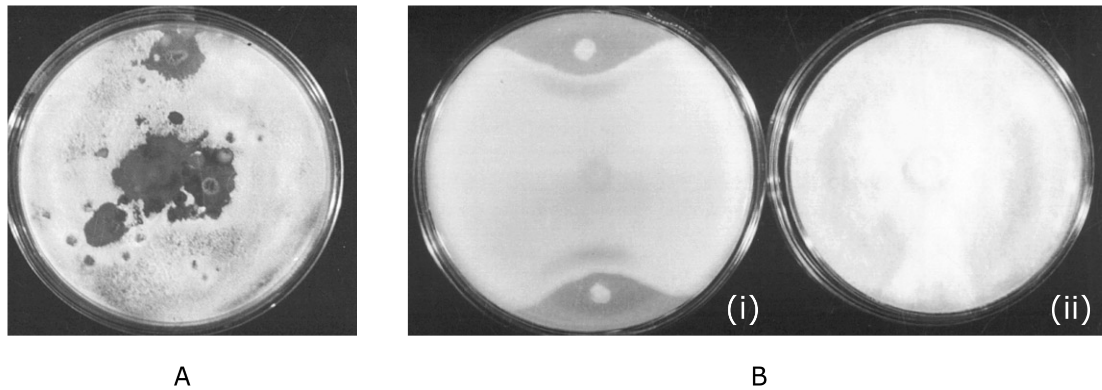

FIG. 1 - *In vitro* fungal growth inhibition by rhizobacteria. A: Double layer technique for isolation of antagonistic rhizobacteria. B: Dual culture plate technique for selection of promising bacterial antagonists; (i) antagonist rhizobacteria with test fungus, (ii) control plate with test fungus alone.

tectum was spread uniformly on the first layer of TGYEA plates (15 ml medium); after adsorption of the spores on the medium, a second layer of TGYEA (10 ml) was added and then 0.1 ml of rhizosphere soil dilutions were spread uniformly on the second layer. Plates were incubated at 28 ºC for 3-4 days till the fungal growth was visible on the second layer. Antagonistic rhizobacterial colonies were chosen on the bases of the presence of a fungal growth inhibition zone around the colony (Fig. 1A). Around 125 antagonistic bacteria were isolated and purified three times on nutrient agar plates. The potential antagonistic bacteria were further selected in dual culture plate method (Anjaiah *et al*., 1998) based on their *in vitro* antagonism against various plant pathogenic fungi on TGYEA plates incubated at 28 ºC for 5-6 days. The radial growth inhibition of fungi was calculated after incubation period. TGYEA plates inoculated with fungal plug alone served as controls (Fig. 1B).

Characterisation of rhizobacteria. The selected four rhizobacteria, SAB8, GM4, A555, and GF23, were characterised for morphological, physiological, biochemical and molecular traits to identify the strains at the genus level. The morphological traits were based on their colony morphology, Gram staining and spore staining; physiological traits were based on their growth, pigmentation and fluorescence under UV on iron limiting media (PA, SA, and PAF); and sporulation test was carried out on NA by heat killing the vegetative cells. Biochemical tests such as amylase, protease, gelatinase, lipase, cellulase and catalase activity were performed as described by Stukus (1997). Further the strains were characterised for molecular traits using multiplex PCR amplification for the presence of the outer membrane lipoprotein genes *opr*I and *opr*L as described earlier (De Vos *et al*., 1993).

Detection of antifungal compounds. For isolation of non-volatile antifungal compounds, bacteria were inoculated in nutrient broth, grown in an incubator shaker at 28 ºC with 200 rpm for two days and the cultures were centrifuged at 10000 rpm for 20 min. The supernatants were collected and filter sterilised through 0.2 µm Nalgen filters. Various concentrations (10, 20, 40%) of culture supernatants were incorporated in TGYEA plates. A plug from an actively growing colony of *F. semitectum* was placed in the centre of each plate and incubated at 28 ºC for 4 days. Three replicates were maintained for each strain. The same volume of sterile distilled water added in TGYEA plate served as control.

For testing heat stability of antifungal compounds present in spent medium, the autoclaved 30% of cell-free spent medium was added to the fresh sterile TGYE liquid broth, inoculated with a fungal plug and incubated on a shaker for 4 days. The weights of fungal mycelium were recorded and compared to the control obtained with the same volume of sterile water added.

To test the chitinase activity, the selected bacteria were inoculated on synthetic colloidal chitin nutrient agar medium (Basha and Ulaganathn, 2002) and incubated at 28 ∞C for 48 h. A whitish area around the colony was recorded indicating the extra cellular chitinase activity.

The production of volatile antifungal compounds was tested by pair-plate method as described by Laha *et al.* (1996). The test fungus, *F. semitectum*, was inoculated in one TGYEA plate and the bacterial strain in another one. Both the plates were joined end to end and sealed with Para-film and incubated at 28 ºC for 3 to 6 days. Plates inoculated with the fungus only, adjoined with uninoculated TGYEA plate served as control. Fungal growth was determined recording the colony diameter.

The antifungal compounds produced by *Pseudomonas* strains, SAB8 and GM4, were extracted into ethyl acetate from the culture supernatants of bacteria, grown for 48 h in Glucose Casamino Acid Yeast Extract (GCY) broth (Anjaiah *et al*., 1998). The concentrated fractions of extracted compounds were dissolved in 1 ml of 50% methanol and tested for the growth inhibition of *Fusarium semitectum* in agar wells made in GCY agar medium. The same volume of 50% methanol was added to the control wells. Further, microscopic observations were made in the antagonism plates of both the control and the extracted antifungal compounds added wells for the elongation of hyphal tips of mycelium.

RESULTS AND DISCUSSION

The selected rhizobacteria, SAB8, GM4, A555, and GF23, showed *in vitro* a broad spectrum antagonism against various pathogenic fungi, such as *Aspergillus niger, A. flavus* (toxigenic and non-toxigenic), *F. semitectum, F. oxysporum*

TABLE 1 - *In vitro* antagonism spectrum of rhizobacteria against various fungal plant pathogens

Fungal pathogens	Fungal growth inhibition (%) by antagonistic rhizobacteria			
	Strain SAB8	Strain GM4	Strain A555	Strain GF23
Aspergillus niger	66	66	57	69
A. flavus (toxigenic)	71	56	31	66
A. flavus (non-toxigenic)	70	70	70	70
Fusarium semitectum	63	75	45	50
F. oxysporum udum	40	37	42	62
F. oxysporum f. sp. *ciceri*	46	35	48	65
F. oxysporum f. sp. *lycopersici*	44	38	44	75
Rhizoctonia solani	43	31	69	57
Sclerotium rolfsii	29	54	29	36

f. sp. *ciceri, F. udum, F. oxysporum* f. sp. *lycopersici, Rhizoctonia solani,* and *Sclerotium rolfsii* (Table 1). Several publications also reported the broad-spectrum antagonistic activity of bacteria (*Bacillus* and *Pseudomonas*) against wide range of fungal pathogens isolated from the rhizosphere (Podile and Prakash, 1996; Anjaiah *et al*., 1998).

Morphological characterisation of selected four rhizobacteria revealed that two strains, SAB8 and GM4, were non-sporulating and Gram-negative rods. Growth on different media, production of greenish yellow pigment (siderophore) and fluorescence under UV on PA and PAF media indicated that these two strains were fluorescent pseudomonads. Fluorescent *Pseudomonas* strains, SAB8 and GM4, also showed protease, gelatinase, and catalase activity (data not shown). The other two strains, A555 and GF23, were found to be aerobic sporulating Gram-positive bacilli. They showed amylase, protease, catalase, cellulase and lipase activity (data not shown). Further, molecular characterisation of these strains showed the amplification of outer membrane lipoprotein gene, *opr*I, for strains SAB8 and GM4, and no amplification for strains A555 and GF23 in multiplex PCR (data not shown). This confirmed that the strains SAB8 and GM4 were rRNA group I fluorescent pseudomonads (De Vos *et al*., 1993).

The percentage of radial growth inhibition of *F. semitectum* rose increasing the concentration of culture filtrate in the medium (Table 2). The inhibitory effect of the culture filtrates was probably due to the production of antifungal compounds by rhizobacteria. Strain GF23 induced maximum growth inhibition compared to other strains at all concentrations of culture filtrates tested (Table 2).

TABLE 2 - Percentage of growth inhibition of *Fusarium semitectum* on TGYE agar medium in the presence of cell-free spent medium of selected rhizobacteria

Rhizobacteria	Fungal growth inhibition (%) by cell-free spent medium		
	10%	20%	40%
SAB8	4	15	62
GM4	6	9	77
A555	3	6	13
GF23	13	32	79
Control	0	0	0

In another experiment, the autoclaved cell-free spent medium tested for growth inhibition of *F. semitectum* in TGYE liquid broth, showed *in vitro* antagonism indicating that the compounds responsible for antagonism were thermostable. The autoclaved cell-free spent medium of strain GM4 showed maximum growth inhibition (Fig. 2).

Of all the strains tested for the chitinase activity on nutrient chitin agar medium, strain GF23 only produced extracellular chitinase enzyme apart from the antifungal compounds, which could have additive effect for maximum fungal growth inhibition.

The concentrated fraction of antifungal compound dissolved in 50% methanol showed *in vitro* antagonism against *F. semitectum*. Further, microscopic analysis revealed that the active fraction of extracted antifungal compound/(s) stunted the growth of hyphal tips of mycelium that lead to an increase of branching along the hyphae in comparison to control (Fig. 3).

Strains of rhizobacteria belonging to the group of *Bacillus* and *Pseudomonas* spp. were known to exhibit antagonism against phytopathogens by the production of antifungal compounds (Thomashow *et al*., 1990; O'Sullivan and O'Gara, 1992; Podile and Prakash, 1996). Mondal *et al.* (2000) reported extraction of 4 major phenolic compounds I, II, III and IV from the cell-free culture filtrate of *Pseudomonas fluorescens* (CRb-26), which were responsible for inhibition of growth of *Xanthomonas axonopodis* pv. *malvacearum.* Earlier reports indicated that extra cellular compounds from *Bacillus subtilis* AF (Podile and Prakash, 1996) and *Bacillus* strain BC121 (Basha and Ulaganathn, 2002) culture filtrates had significant growth retarded effect on *A. niger* and *Curvularia lunata* respectively. Milner *et al*. (1996) studied the role of two antibiotics in disease suppression from culture filtrate of *Bacillus cereus* UW85.

The production of HCN is one of the bacterial traits shown to be involved in the suppression of plant pathogens in the rhizosphere (Voisard *et al.,* 1989). However, none of the rhizobacteria in this study was able to produce HCN *in vitro.* All the rhizobacteria showed a small degree of fungal growth inhibition in pair plate method indicating the production of some volatile antifungal compounds. Among, strain GF23 showed maximum fungal growth inhibition by volatile compounds at six days after incubation (data not shown).

The results of present study indicated that the antagonism exhibited by these rhizobacteria against soil-borne

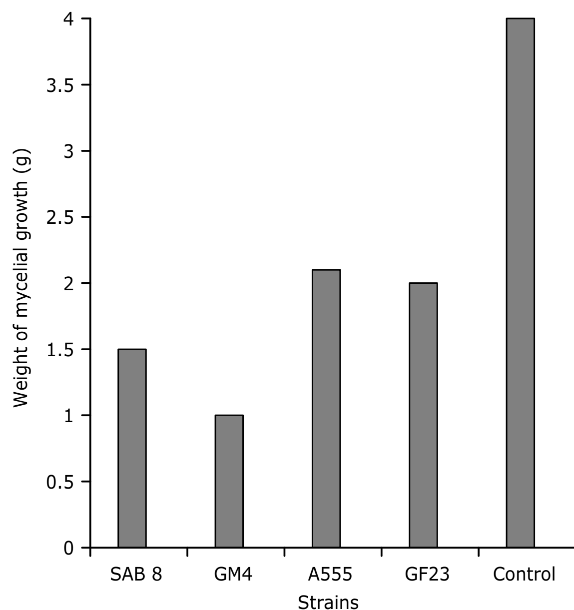

FIG. 2 - Mycelial growth of *Fusarium semitectum* measured in TGYE broth in the presence of autoclaved cell-free spent medium of selected rhizobacteria.

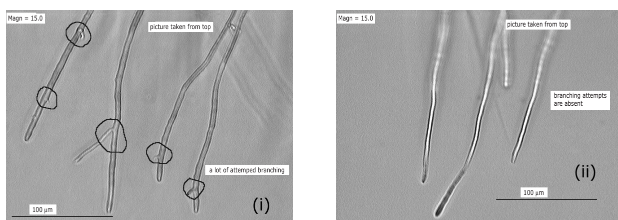

FIG. 3 - Microscopic examination of mycelium hyphal growth of *Fusarium semitectum* in presence of extracted antifungal compound/s (i) and control (ii).

fungi is possibly through the production of antifungal antibiotics. The utilisation of beneficial rhizobacteria to increase crop yield and to reduce the use of chemicals is indeed an attractive perspective.

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