Biological control of collar rot disease with broad-spectrum antifungal bacteria associated with groundnut

G. Krishna Kishore, S. Pande, and A.R. Podile

Abstract: Bacteria associated with 6 habitats of groundnut were evaluated for their broad-spectrum antifungal activity and suppression of collar rot (*Aspergillus niger*) of groundnut. Three hundred and ninety-three strains were tested against 8 fungal pathogens of groundnut including 5 necrotrophic fungi, *Aspergillus flavus*, *A. niger*, *Rhizoctonia bataticola*, *Rhizoctonia solani*, and *Sclerotium rolfsii*, and 3 biotrophic fungi, *Cercospora arachidicola*, *Phaeoisariopsis personata*, and *Puccinia arachidis*. *Pseudomonas* sp. GRS 175, *Pseudomonas aeruginosa* GPS 21, GSE 18, GSE 19, and GSE 30, and their cell-free culture filtrates were highly antagonistic to all the test fungi. The cell-free culture filtrates of these bacteria were fungicidal and induced mycelial deformations including hyphal bulging and vacuolization in necrotrophic fungi. The cell-free culture filtrates at 10% (*v*/*v*) concentration significantly inhibited the spore germination of biotrophic fungi. In the greenhouse, *P. aeruginosa* GSE 18 emerged as an effective biocontrol agent of collar rot closely followed by *P. aeruginosa* GSE 19. The bacterium applied as a seed treatment reduced the pre-emergence rotting and postemergence wilting by >60%. *Pseudomonas aeruginosa* GSE 18 effectively colonized the groundnut rhizosphere, both in native and in *A. niger* infested potting mixtures. Ninety-day-old peat formulation of *P. aeruginosa* GSE 18 had biocontrol ability comparable with the midlog-phase cells. *Pseudomonas aeruginosa* GSE 18, tolerant to thiram, in combination with the fungicide had an improved collar rot control. The present study was a successful attempt in selection of broad-spectrum and fungicide tolerant biocontrol agents that can be a useful component of integrated management of collar rot.

Key words: Arachis, biocontrol, crown rot, peanut.

Résumé : L'activité anti-fongique à large spectre de bactéries associées à 6 habitats de l'arachide, ainsi que leur potentiel de suppression de la rouille de l'arachide causée par *Aspergillus niger* ont été évalués. Trois cent quatre-vingt-trois souches ont été testées sur 8 pathogènes fongiques de l'arachide, incluant 5 champignons nécrotrophes : *Aspergillus flavus*, *A. niger*, *Rhizoctonia bataticola*, *Rhizoctonia solani* et *Sclerotium rolfsii*; et 3 champignons biotrophes : *Cercospora arachidicola*, *Phaeoisariopsis personata* et *Puccinia arachidis.* Les souches de *Pseudomonas* sp. GRS 175 et *P. aeruginosa* GPS 21, GSE 18, GSE 19 et GSE 30 ainsi que les filtrats acellulaires de culture (CAF) qui en sont issus ont été fortement antagonistes envers tous les champignons testés. Les CAF de ces bactéries ont été fongicides et ont causé des déformations du mycélium, incluant le renflement des hyphes et la vacuolisation chez les champignons nécrotrophes. Une concentration de 10 % (*v*/*v*) de CAF a inhibé significativement la germination des spores des champignons biotrophes. En serre, *P. aeruginosa* GSE 18 s'est révélé être un agent de lutte biologique efficace contre la rouille, suivi de près par *P. aeruginosa* GSE 19. Une application bactérienne pour traiter les graines a réduit l'émergence de la rouille et le flétrissement subséquent de plus de 60 %. *Pseudomonas aeruginosa* GSE 18 a colonisé efficacement la rhizosphère de l'arachide, tant en condition de base qu'en condition d'infestation par *A. niger* des mélanges d'empotage. Une formulation de tourbe âgée de 90 jours contenant *P. aeruginosa* possédait une activité de contrôle biologique comparable à celle de cellules en phase mi-logarithmique de croissance. *Pseudomonas aeruginosa* GSE 18 tolérante au thiram, combinée au fongicide, améliore le contrôle de la rouille. La présente étude a été un fructueux essai de sélection d'agents de lutte biologique à large spectre tolérants aux fongicides qui peuvent constituer des composantes utiles d'une lutte intégrée contre la rouille.

Mots clés : Arachis, lutte biologique, rouille, arachide.

[Traduit par la Rédaction]

Received 16 June 2004. Revision received 27 October 2004. Accepted 2 November 2004. Published on the NRC Research Press Web site at http://cjm.nrc.ca on 14 April 2005.

G. Krishna Kishore[1] **and A.R. Podile.**[2] Department of Plant Sciences, University of Hyderabad, Hyderabad 500 046, India.
S. Pande. Pathology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, 502 324, India.

[1]Present address: Pathology, ICRISAT, Patancheru, 502 324, India.
[2]Corresponding author (e-mail: arpsl@uohyd.ernet.in or podilerao@yahoo.com).

 doi: 10.1139/W04-119

Introduction

Biological suppression was proved to be a reliable component of integrated management of phytopathogenic fungi following greenhouse (Paulitz and Belanger 2001) and also field demonstration of several biocontrol agents. However, variation in the performance of biocontrol agents resulting from interactions with the native microflora, environmental conditions, and nutrient availability that affect the root colonization (Weller 1988) remain as a major bottleneck for large-scale use of the available biocontrol agents. Combined use of different biocontrol agents or integration of biocontrol agents with other disease management options, with identifiable differences in their mechanisms of action, has improved disease protection and the activity spectrum of biocontrol agents (Jetiyanon and Kloepper 2002). The combined use of reduced doses of fungicides and biocontrol agents offered an effective control of soil-borne diseases where chemical control alone was unaffordable (Duffy 2000; Kondoh et al. 2001).

Broad-spectrum antifungal biocontrol organisms are required for use in different cropping systems and also for control of multiple diseases in a single crop. A few of the available biocontrol agents mostly belonging to *Pseudomonas* spp. show a broad-spectrum antifungal activity by virtue of volatile and diffusible antibiotics (Haas and Keel 2003; Viji et al. 2003). Several studies failed to establish a definite relationship between in vitro antibiosis and in vivo disease control (Fravel 1988), indicating that biocontrol is a complex process in which different mechanisms act in synergy.

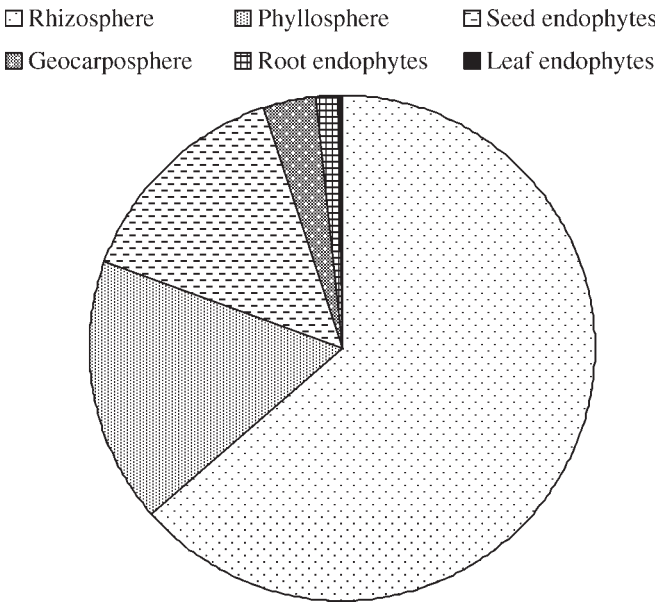
Collar rot caused by *Aspergillus niger* van Tieghem is of considerable importance in warm and temperate groundnut growing areas. The fungus causes pre-emergence rotting of groundnut seed and the infected seed fails to germinate. In emerged young seedlings, *A. niger* infection results in sudden wilting (Middleton et al. 1994). In earlier studies, we identified *Bacillus subtilis* AF 1 (Podile and Prakash 1996) and *Trichoderma viride* Pers. ex S.F. Gray A 14 (Kishore et al. 2001) as useful biocontrol agents for control of collar rot disease. Biocontrol action of *B. subtilis* AF 1 involved the induction of lipoxygenase activity in treated groundnut seedlings (Sailaja et al. 1998) and alteration of the phytoalexin metabolism for the host's benefit (Sailaja and Podile 1998). Use of these biocontrol agents with other management practices was limited by their fungicide sensitivity (authors' unpublished data). In the present study, attempts were made for selection of broad-spectrum antifungal bacteria that are effective against collar rot disease in vivo and compatible with fungicides.

Materials and methods

Bacterial strains

Three hundred and ninety-three groundnut-associated bacteria from 9 districts of Andhra Pradesh, India, were used. The bacteria represented 6 habitats of groundnut (Fig. 1): rhizosphere (63.6%), phyllosphere (17.0%), geocarposphere (3.3%), leaf endophytes (0.3%), root endophytes (1.3%), and seed endophytes (14.5%). The bacterial strains were designated based on their habitat of isolation: GRS, rhizosphere; GPS, phyllosphere; GGS, geocarposphere; GSE, seed endophytes; GRE, root endophytes; GLE, leaf endophytes, where G stands for groundnut.

Fig. 1. Percentage of bacterial strains from different habitats of groundnut tested in the present study.



Fungal strains

Eight fungal pathogens were isolated from infected groundnut plants from the farmers' fields. Five of them were necrotrophic soil/seed-borne fungi (*A. niger*, *Aspergillus flavus* Link ex Fries, *Rhizoctonia bataticola* (Tassi) Goid., *Rhizoctonia solani* Kuhn, and *Sclerotium rolfsii* Sacc.) that were maintained on potato dextrose agar (PDA) medium at 4 °C. Three of them were biotrophic foliar pathogens (*Cercospora arachidicola* Hori, *Phaeoisariopsis personata* (Berk. & Curt.) v. Arx, and *Puccinia arachidis* Speg.) that were maintained by the detached leaf technique (Subrahmanyam et al. 1983).

In vitro antifungal activity

Antifungal activity of 393 bacterial strains against soil/seed-borne fungi was determined by dual-culture assay and against foliar pathogens by in vitro spore germination assay. Antifungal activity of cell-free culture filtrates (CCF) of stationary-phase cultures of selected broad-spectrum strains against soil/seed-borne and foliar fungi was tested by inhibition of radial growth and biomass and in vitro spore germination assay, respectively.

Dual culture assay

Bacteria were inoculated as a line on one edge of a 90-mm-diameter petri plate containing PDA medium, pH 6.1 and incubated at 30 °C. After 24 h, a 5-mm-diameter actively growing fungus was inoculated at the center. Dual inoculated plates, with fungus alone as control, were incubated at 28 °C with a 12-h photoperiod. The inhibition zone between the 2 cultures was measured 3 d after inoculation for *R. bataticola*, *R. solani*, and *S. rolfsii* and 8 d after inoculation for *A. flavus* and *A. niger*.

Table 1. Antifungal activity of selected bacterial strains against 8 fungal pathogens of groundnut.

	Inhibition zone (mm)[b]					Inhibition of spore germination (%)[c]		
Bacterial strain[a]	AF	AN	RB	RS	SR	CA	PP	PA
Pseudomonas aeruginosa GPS 21	10.0	14.0	15.5	15.5	14.5	99.0	99.1	99.3
Pseudomonas sp. GRS 175	10.0	14.0	15.5	15.5	14.5	99.0	99.1	99.3
GRS 223	0	6.5	8.5	7.5	10.0	38.2	32.9	52.1
GRS 224	2.0	19.0	14.0	13.0	13.0	58.2	49.3	92.2
GRS 225	0	18.0	7.0	15.0	0	81.3	97.7	98.3
GSE 5	0	10.0	21.0	21.0	8.5	52.2	45.0	85.4
GSE 6	0	11.0	11.0	14.5	7.5	62.2	39.9	89.0
P. aeruginosa GSE 18	11.5	9.5	23.0	16.0	16.0	98.2	97.7	98.9
P. aeruginosa GSE 19	11.0	7.5	23.5	23.0	12.5	98.3	97.7	97.8
GSE 23	6.5	17.0	13.5	11.0	5.0	72.9	68.2	20.5
P. aeruginosa GSE 30	13.5	11.0	20.5	21.5	14.5	100.0	100.0	100.0
Control	0	0	0	0	0	0	0	0
LSD (0.01)	3.8	5.2	5.7	7.2	7.4	34.3	36.5	30.0

Note: AF, *Aspergillus flavus*; AN, *Aspergillus niger*; RB, *Rhizoctonia bataticola*; RS, *Rhizoctonia solani*; SR, *Sclerotium rolfsii*; CA, *Cercospora arachidicola*; PP, *Phaeoisariopsis personata*; PA, *Puccinia arachidis*.

[a]Bacterial strains antagonistic to 6 or more test fungi are shown. Unidentified bacterial strains were given the strain designations described in the Materials and methods.

[b]The values are the mean of 9 replications from 3 experiments. The mean values for AF, AN, RB, RS, and SR represent the inhibition zone measured in dual-culture assay. For AF and AN, the inhibition zone was measured 8 d after inoculation, and for RB, RS, and SR, the inhibition zone was measured 3 d after inoculation.

[c]The mean values for CA, PP, and PA represent the percent inhibition of spore germination with respect to the control in the in vitro spore germination assay. Conidia were observed for germination 24 h after inoculation for CA and PP, whereas urediniospores of PA were observed 8 h after inoculation.

Inhibition of fungal radial growth by CCF

Fifty millilitres of Luria–Bertani (LB) broth in 250-mL flasks was inoculated with 500 µL of overnight-grown bacterial culture in LB broth. After incubation for 48 h at 30 °C and 180 r·min^{-1}, the culture was centrifuged for 10 min at 14 500*g* and 4 °C. The supernatant was filter sterilized to obtain CCF. The CCF were added to autoclaved PDA medium, just before pouring the plates, at final concentrations of 10%, 25%, and 50% (*v*/*v*), and a 5-mm disc of an actively growing fungus was inoculated at the center of 90-mm-diameter petri plates. The plates were incubated at 28 °C and observed for radial growth at 48-h intervals up to 8 d after inoculation.

Inhibition of fungal biomass by CCF

The CCF were added to autoclaved and precooled potato dextrose broth (PDB) in 100-mL flasks at concentrations of 10%, 25%, and 50% (*v*/*v*) to a final volume of 30 mL. Each flask was inoculated separately with a 5-mm disc of actively growing fungus and incubated at 28 °C in a shaker at 125 r·min^{-1}. Mycelium was harvested after 96 h, oven-dried, and the biomass recorded.

In vitro spore germination assay

Fresh spores of *C. arachidicola*, *P. personata*, and *P. arachidis* were harvested with a cyclone spore collector and suspended in sterile distilled water with 0.01% Tween 20. The concentration was adjusted to 10^5 spores·mL^{-1}. Midlog-phase cells of the bacterial strains were resuspended in 0.5% (*w*/*v*) dextrose and adjusted to 2×10^8 CFU·mL^{-1}. Fifty microlitres each of the fungal spore and bacterial cell suspensions with dextrose solution as control was mixed well on a cavity slide. Similarly, the spore suspension was mixed with an equal volume of CCF at a final concentration of 10%, 25%, and 50% (*v*/*v*). The slides were placed in humid chambers and incubated in the dark at 24 ± 1 °C. Conidia of *C. arachidicola* and *P. personata* were incubated for 24 h, and urediniospores of *P. arachidis* were incubated for 8 h. Immediately after incubation, a drop of lactophenol – cotton blue (40 mL of glycerol, 20 mL of lactic acid, 20 g of phenol, and 5 mL of 1% aqueous cotton blue) was added to each slide to prevent further growth and the spore germination was observed under a microscope. In each replication, 100 spores were observed and the percent inhibition with respect to the control was calculated.

Effect of CCF on hyphal morphology

The effect of CCF of 5 broad-spectrum antagonistic bacteria, *Pseudomonas* sp. GRS 175 and *Pseudomonas aeruginosa* GPS 21, GSE 18, GSE 19, and GSE 30, selected as above, on the hyphal structure of 5 soil/seed-borne fungi was studied. A 4-mm disc from the edge of the fungal colony grown on PDA medium in the presence of CCF (10% or 25% (*v*/*v*) depending on the fungal growth) was fixed on a glass slide. The mycelium was stained with lactophenol – cotton blue solution and observed under a photomicroscope.

Characterization of bacterial strains

All of the bacterial strains were evaluated in vitro for qualitative production of extracellular enzymes, viz. chitinase, glucanase and protease, and metabolites, viz. siderophores (Schwyn and Neilands 1987) and HCN (Bakker and Schippers 1987). Production of chitinase, glucanase, (Cantwell and Mc Connell 1983) and protease (Hankin and Anagnostakis 1975) was characterized as lysis of colloidal chitin, lichenan, and gelatin, respectively, in minimal media. The bacteria were rated as +, ++, and +++ for production of each enzyme or metabolite based on the visual observations of the lytic zone or color development. The tests were conducted in triplicate and repeated once.

Table 2. Effect of cell-free culture filtrates (CCF) of selected antifungal bacterial strains on the radial growth of 5 necrotrophic soil/seed-borne fungal pathogens of groundnut.

		Colony diameter (mm)[b]					Mycelial dry mass (mg)[c]				
Bacterial strain	CCF (%)[a]	AF	AN	RB	RS	SR	AF	AN	RB	RS	SR
GRS 175	50	25.0	10.0	0	40.0	0	168.3	37.8	81.8	72.5	90.8
GRS 175	25	50.5	54.3	0	41.8	0	236.3	80.3	148.8	92.0	101.5
GRS 175	10	85.8	83.8	33.5	56.8	42.8	277.3	274.0	240.3	235.3	114.3
GPS 21	50	25.5	12.8	0	0	0	189.8	66.0	95.3	95.8	104.0
GPS 21	25	58.3	57.0	0	35.0	0	232.5	109.3	140.5	141.8	127.0
GPS 21	10	87.0	85.5	35.0	54.8	39.3	280.0	281.3	258.8	252.8	158.0
GSE 18	50	31.8	0	0	0	0	198.8	49.3	130.8	38.8	97.5
GSE 18	25	62.3	27.3	42.3	0	0	255.8	69.8	170.0	72.3	129.5
GSE 18	10	87.3	58.5	50.3	32.0	30.8	292.5	166.5	284.8	115.5	140.5
GSE 19	50	32.5	0	0	0	0	186.8	78.3	103.8	43.3	71.5
GSE 19	25	61.0	36.5	0	31.0	0	209.3	81.5	118.5	83.8	79.0
GSE 19	10	87.8	60.8	40.8	53.5	42.3	278.0	198.5	210.8	146.3	131.3
GSE 30	50	56.3	0	0	0	0	160.8	95.8	129.5	83.0	90.3
GSE 30	25	82.5	44.3	36.5	17.5	24.5	165.8	151.8	146.0	129.3	152.5
GSE 30	10	88.0	77.0	37.5	49.3	59.3	292.0	283.3	247.8	226.0	261.0
Control	50	88.0	89.8	90.0	90.0	90.0	289.8	277.0	274.3	291.0	446.5
Control	25	87.5	90.3	90.0	90.0	90.0	289.3	288.5	273.5	279.5	425.3
Control	10	87.8	89.8	90.0	90.0	90.0	279.8	289.8	285.8	276.8	442.8
LSD (0.01)		4.87	3.74	4.53	2.97	5.9	23.81	21.12	18.3	14.1	13.6

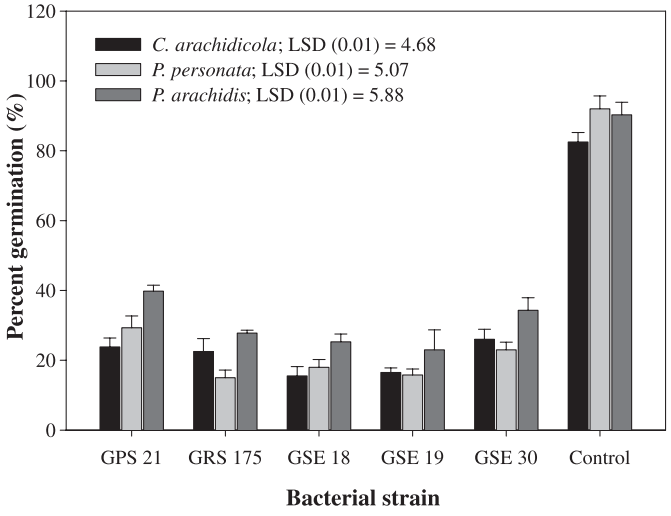
Note: AF, *Aspergillus flavus*; AN, *Aspergillus niger*; RB, *Rhizoctonia bataticola*; RS, *Rhizoctonia solani*; SR, *Sclerotium rolfsii*.

[a]CCF of stationary-phase cultures were added to precooled PDA or PDB.

[b]A 5-mm disc of an actively growing mycelium was used as inoculum. Inoculated plates were incubated at 28 °C and the colony diameter was measured 8 d after inoculation for AF and AN and 4 d after inoculation for RB, RS, and SR.

[c]The inoculated flasks were incubated at 28 °C and 125 r·min^{-1} and the dry biomass was determined 96 h after inoculation. The values are the mean of 9 replications from 3 experiments.

Fig. 2. Effect of cell-free culture filtrates (10% *v*/*v*) of 5 selected antifungal strains, *Pseudomonas* sp. GRS 175 and *Pseudomonas aeruginosa* GPS 21, GSE 18, GSE 19, and GSE 30, on the in vitro spore germination of 3 biotrophic foliar fungal pathogens of groundnut. In the presence of cell-free culture filtrates at 25% (*v*/*v*) concentration, no spore germination was observed among the 3 fungi. Each data point is the mean of 9 replications from 3 experiments, and error bars indicate the standard error among the 9 replications.



Selected bacterial strains were identified at the Microbial Type Culture Collection and GenBank, Institute of Microbial Technology (IMTECH), Chandigarh, India.

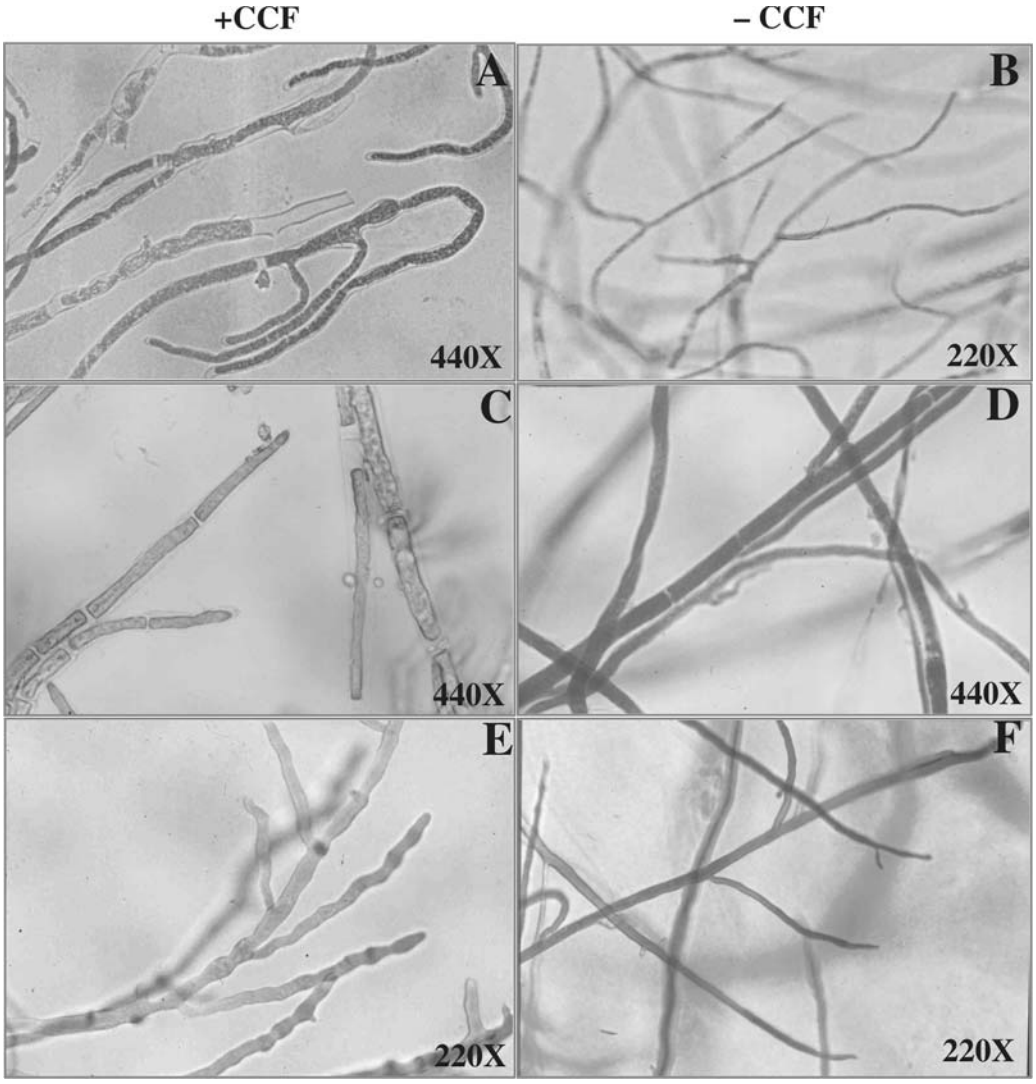
Selection of biocontrol strains

Aspergillus niger was multiplied on autoclaved sorghum seed for 8 d at 30 °C. The profusely sporulating culture was mixed with potting mixture consisting of red alfisol, farmyard manure, and sand (2:1:2) at 40 g·kg^{-1}. *Aspergillus niger* infested potting mixture was placed in the top 6 cm of the 15-cm-diameter pots. The pots were watered and left in the greenhouse for 48 h. Bacteria grown in 90-mm-diameter petriplates with LB agar medium for 48 h were scraped into 20 mL of 0.5% carboxymethylcellulose. Groundnut seeds (cv. TMV 2) surface sterilized with 0.02% $HgCl_2$ were suspended in the above cell suspension for 30 min and dried under a flow of sterile air in a laminar flow. Eight bacterized seeds (10^6–10^7 CFU·seed^{-1}) were sown in each pot with carboxymethylcellulose-treated seeds as control. A temperature of 30 ± 2 °C and minimal soil moisture were maintained all through. Incidence of collar rot was measured as pre-emergence rotting at 7 d after sowing and postemergence wilting at 20 d after sowing.

Survival of the biocontrol agents in groundnut rhizosphere

Survival and multiplication of *P. aeruginosa* GSE 18 and GSE 19 in groundnut rhizosphere, both in native and in *A. niger* infested potting mixture, were determined by using rifampicin resistance as a marker. *Pseudomonas aeruginosa*

Fig. 3. Effect of cell-free culture filtrates (CCF) of *Pseudomonas* sp. GRS 175 on the hyphal morphology of soil-borne necrotrophic fungal pathogens of groundnut. (A) Backward growth of *Sclerotium rolfsii* hyphae in the presence of CCF compared with the progressive growth in (B) the control; (C) cytoplasmic granulation of *Rhizoctonia solani* compared with (D) the control; (E) hyphal bulging of *Rhizoctonia bataticola* compared with (F) the control.



GSE 18-R_1 and GSE 19-R_1, spontaneous mutants of GSE 18 and GSE 19, with resistance to rifampicin were obtained by plating the cell suspension (~10^9 CFU·mL^{-1}) on LB agar medium with 100 µg rifampicin·mL^{-1}. Inoculated plates were incubated at 30 °C for 96 h. Mutant colonies were characterized and evaluated for stability of their antibiotic resistance by subculturing 20 times on LB agar medium with 100 µg rifampicin·mL^{-1}. Stable mutants were used for seed bacterization and their survival in the groundnut rhizosphere was determined at regular intervals of 5 d up to 30 d after sowing.

At each sampling, apparantly healthy seedlings were uprooted and a 2- to 3-cm portion from the middle of the root alone with the tightly adhering potting mixture was suspended in 50 mL of 10 mmol·L^{-1} phosphate buffer, pH 7.0. The suspension was incubated for 1 h at 30 °C and 180 r·min^{-1}. Serial dilutions were plated in triplicate on LB agar medium with 100 µg rifampicin·mL^{-1}. Inoculated plates were incubated at 30 °C for 48 h and the observed colony numbers were expressed as log CFU·g^{-1}.

Control of collar rot with *P. aeruginosa* GSE 18-R_1 in combination with seed dressing fungicide (thiram)

Pseudomonas aeruginosa GSE 18-R_1 was able to grow in LB agar medium and broth with 2 mg thiram (bis-(dimethyl thiocarbamoyl)disulfide)·mL^{-1}, a seed dressing fungicide. The bacterium in combination with thriam was evaluated for collar rot control. GSE 18-R_1 was formulated in sterile neutralized peat (Biocare Technology Pvt. Ltd., Australia) packed in high molecular mass and high-density polyethylene bags. Midlog-phase culture of GSE 18-R_1 grown in LB broth was resuspended in 10 mmol·L^{-1} phosphate buffer, pH 7.0, at a 100-fold dilution and added to peat at 50% (*v*/*w*). The bags were sealed and thoroughly kneaded to ensure uniform adsorption of the bacterial cells into peat and incubated at 30 °C. A ninety-day-old peat-based formulation

Fig. 4. In vitro evaluation of bacterial strains for production of metabolites or enzymes that contribute to antifungal activity. The percentage of strains positive for individual characteristics among the total 393 bacterial strains and 77 antifungal strains was compared.

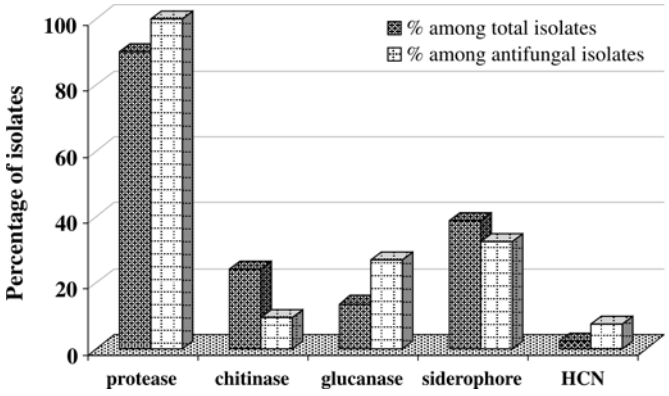
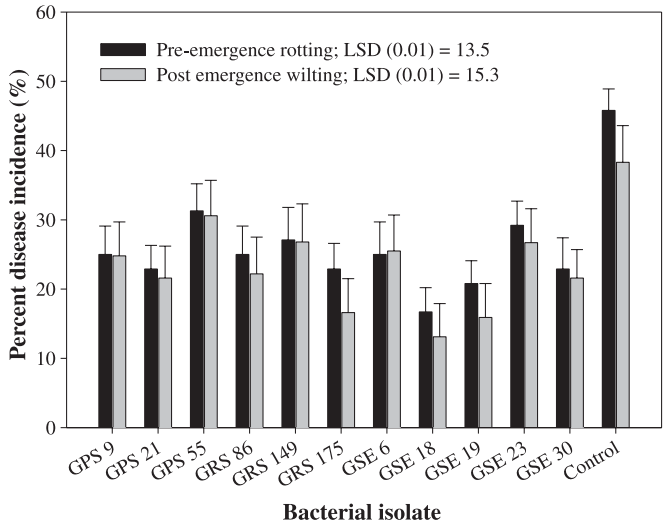


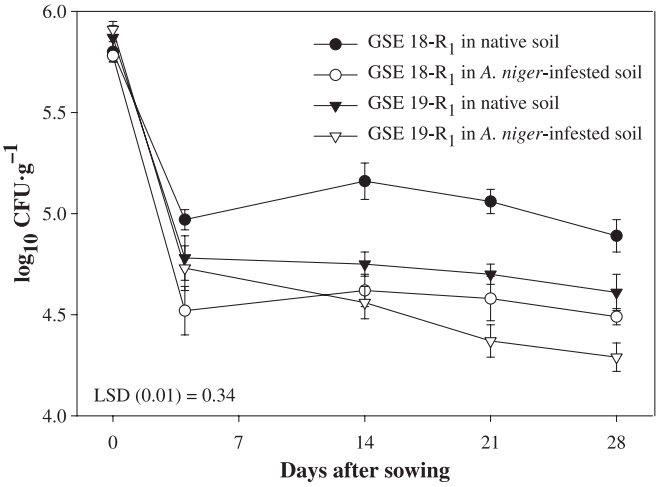
Fig. 5. Greenhouse selection of biocontrol agents effective against collar rot disease of groundnut. Seeds of groundnut cv. TMV 2, bacterized separately with 393 bacterial strains, were planted in *Aspergillus niger* infested potting mixture. Pre-emergence rotting was observed 7 d after sowing and postemergence wilting observed 20 d after sowing. Data points are the mean of 72 seeds in 9 replications from 3 experiments.



of *P. aeruginosa* GSE 18-R_1 with log 6.5 CFU·g^{-1} was used for seed bacterization. One hundred grams of surface-sterilized groundnut seeds (cv. TMV 2) was mixed thoroughly with 2 g of the formulation using 0.5% (*w*/*v*) carboxymethylcellulose as a binder and air-dried. Seeds treated with sterile peat served as control. Eight bacterized seeds were planted in 15-cm-diameter pots filled with *A. niger* infested potting mixture. In addition to collar rot severity, growth of seedlings in each treatment was measured as root length, shoot length, and dry mass at 20 d after sowing.

In different treatments, bacterized seeds were air-dried and treated with thiram at concentrations of 0.1, 0.25, 0.5, 1.0, and 2.0 g·(kg seed)$^{-1}$ and planted in *A. niger* infested potting mixture. Seeds treated with 0.5% carboxymethylcellulose were used as control.

Fig. 6. Populations of *Pseudomonas aeruginosa* GSE 18-R_1 and GSE 19-R_1, rifampicin-resistant mutants of GSE 18 and GSE 19, in the rhizosphere of groundnut, both in native and in *A. niger* infested potting mixtures. Bacteria were applied as seed treatment and their rhizosphere populations were monitored by dilution plating on LB agar with 100 µg rifampicin·mL^{-1}. Data points are the mean of 9 replications from 3 experiments.



Data analysis

All of the experiments were conducted as 3 replications and repeated twice, unless mentioned otherwise, in a completely randomized block design. The data were subjected to analysis of variance using the Genstat 5.0 statistical package. Mean values of different treatments in each experiment were compared at the 1% ($P = 0.01$) or 5% ($P = 0.05$) level of significance.

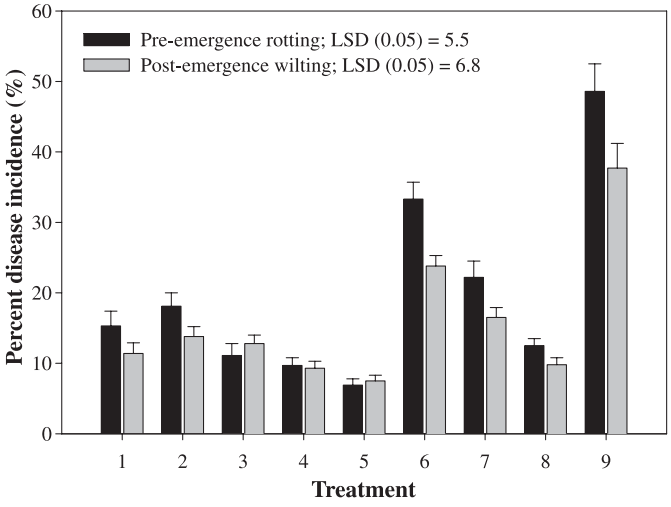
Results

In vitro antifungal activity

Of the 393 bacterial strains evaluated, 77 had a significant ($P = 0.01$) antifungal activity and 11 were inhibitory to 6 or more fungi (Table 1). Five bacterial strains, *Pseudomonas* sp. GRS 175 and *P. aeruginosa* GPS 21, GSE 18, GSE 19, and GSE 30, were highly effective against all 8 test fungi. Of the 77 antifungal strains, the number of strains effective against each fungus was as follows: *A. flavus*, 10 (13.0%); *A. niger*, 27 (35.1%); *C. arachidicola*, 58 (75.3%); *P. personata*, 53 (68.8%); *P. arachidis*, 65 (84.4%); *R. bataticola*, 47 (61.0%); *R. solani*, 53 (68.8%); *S. rolfsii*, 34 (44.2%).

At the highest concentration tested, i.e., 50% (*v*/*v*) concentration, CCF of GPS 21, GRS 175, GSE 18, GSE 19, and GSE 30 reduced the radial growth of *A. flavus* from 36.0% to 71.0% and were not inhibitory to *A. flavus* at 10% (*v*/*v*) concentration (Table 2). CCF of *P. aeruginosa* GSE 18, GSE 19, and GSE 30 at 50% (*v*/*v*) concentration completely inhibited the radial growth of *A. niger*, *R. bataticola*, *R. solani*, and *S. rolfsii*. Mycelial discs from all the treatments with complete inhibition of radial growth failed to grow when transferred to PDA medium 12 h after inoculation.

Fig. 7. Effect of *Pseudomonas aeruginosa* GSE 18-R_1 and thiram, individually and in combination, on the severity of collar rot of groundnut. Treatments: 1, seed treatment with midlog-phase cells of GSE 18-R_1; 2, seed treatment with peat formulation of GSE 18-R_1; 3, seed treatment with midlog-phase cells of GSE 18-R_1 and thiram at 0.5 g·(kg seed)$^{-1}$; 4, seed treatment with midlog-phase cells of GSE 18-R_1 and thiram at 1.0 g·(kg seed)$^{-1}$; 5, seed treatment with midlog-phase cells of GSE 18-R_1 and thiram at 2.0 g·(kg seed)$^{-1}$; 6, seed treatment with thiram at 0.5 g·(kg seed)$^{-1}$; 7, seed treatment with thiram at 1.0 g·(kg seed)$^{-1}$; 8, seed treatment with thiram at 2.0 g·(kg seed)$^{-1}$; 9, control. Data points are the mean of 9 replications from 3 experiments.



The reduction in fungal biomass in the presence of 25% (*v*/*v*) CCF varied from 86.7% (*P. aeruginosa* GSE 18 versus *R. solani*) to 31.4% (*P. aeruginosa* GSE 18 versus *A. flavus*) (Table 2). At a concentration of 10% (*v*/*v*), culture filtrate of *P. aeruginosa* GSE 18 was highly effective against *A. niger* and *R. solani* (62.4% and 66.1% reduction in biomass, respectively), and culture filtrate of *Pseudomonas* sp. GRS 175 was effective against *S. rolfsii* and *R. bataticola* (77.4% and 49.1% reduction in biomass, respectively).

Culture filtrates of the 5 selected bacteria at ≥25% (*v*/*v*) completely inhibited the spore germination of *C. arachidicola*, *P. personata*, and *P. arachidis*. The CCF remained highly effective against the 3 fungi up to 10% (*v*/*v*) concentration (Fig. 2).

In CCF-amended PDA medium, the hyphal growth direction of the test fungi was observed to be perpendicular or backward rather than progressive (Figs. 3A and 3B). An increase in hyphal diameter, granulation of the cytoplasm, and vacuolization of the cytoplasm (Figs. 3C and 3D) were commonly observed in all of the fungi grown in the presence of CCF of GRS 175 and GPS 21. In the presence of *P. aeruginosa* GSE 18, GSE 19, and GSE 30, hyphae showed only an increase in diameter; the tips remained unbranched and coiled. CCF of *Pseudomonas* sp. GRS 175 and *P. aeruginosa* GPS 21 induced more frequent hyphal bulging in *R. bataticola* (Figs. 3E and 3F) than in the other 4 fungi.

Characterization of bacterial strains

Of the 393 bacterial strains tested, 95 (24.2%) produced extracellular chitinase, 54 (13.7%) produced β-1,3-glucanase, 354 (90.1%) produced protease, 153 (38.9%) produced siderophores, and 10 (2.5%) produced HCN (Fig. 4). Compared with total strains, the percentage of chitinase and siderophore producing bacteria was less among the antifungal strains, whereas the percentage of glucanase-, protease-, and HCN-producing strains was high among the antifungal strains.

Selection of biocontrol strains

Eleven bacterial strains, 3 each from rhizosphere and phylloplane and 5 seed endophytes, significantly ($P = 0.01$) inhibited the pre-emergence rotting (Fig. 5). *Pseudomonas aeruginosa* GSE 18 and GSE 19 reduced pre-emergence rotting by 63.5% and 54.4%, respectively, and postemergence wilting by 65.6% and 59.9%, respectively, compared with the control. Two bacterial strains, *Achromobacter xylosidans* GRS 149 and *Bacillus megaterium* GPS 55, although not antagonistic to *A. niger* in dual-culture assay, reduced pre-emergence seed rotting by 40.8% and 31.6%, respectively, compared to control.

Survival of the biocontrol agents in groundnut rhizosphere

Stationary-phase cells of *P. aeruginosa* GSE 18-R_1 and GSE 19-R_1, applied as seed bacterization, differed in their survival in the groundnut rhizosphere compared with native and *A. niger* infested potting mixtures (Fig. 6). Starting from 7 d after sowing, the rhizosphere population of the bacterial strains was significantly ($P = 0.05$) higher in native soil than *A. niger* infested soil. At 28 d after sowing, *P. aeruginosa* GSE 18-R_1 and GSE 19-R_1 had a mean survival of log 4.5 and 4.6 CFU·g^{-1} in native soil compared with log 4.5 and 4.3 CFU·g^{-1} in *A. niger* infested soil.

Collar rot control by *P. aeruginosa* GSE 18-R_1 in combination with thiram

The ninety-day-old peat formulation of *P. aeruginosa* GSE 18-R_1 had suppressive effects on the incidence of collar rot, observed as both pre-emergence rotting and post-emergence wilting (Fig. 7). The peat formulation of *P. aeruginosa* GSE 18-R_1 had a biocontrol activity comparable ($P = 0.05$) with the midlog-phase cells. In addition, the formulation was closely comparable with the midlog-phase cells in groundnut growth promotion, measured as root length, shoot length, and dry mass, in the native potting mixture (Table 3).

Combined application of fungicide tolerant *P. aeruginosa* GSE 18-R_1 and thiram signifcantly ($P = 0.05$) improved the control of pre-emergence rotting of groundnut seeds in the *A. niger* infested potting mixture (Fig. 7). Integrated use of the bacterium with <0.5 g thiram·(kg seed)$^{-1}$ had an insignificant effect on both pre-emergence rotting and postemergence wilting compared with the individual application of the biocontrol agent. GSE 18-R_1 in combination with thiram at ≥1 g·(kg seed)$^{-1}$ was significantly ($P = 0.05$) more effective than either component applied alone.

Discussion

In the present study, bacterial strains from diverse habitats of groundnut with broad-spectrum antifungal activity were

Table 3. Effect of seed bacterization with *Pseudomonas aeruginosa* GSE 18 on the growth of groundnut in the presence of collar rot pathogen.

Treatment	*Aspergillus niger* infestation	Root length (cm)	Shoot length (cm)	Dry mass (g)
Midlog-phase cells	–	15.3±1.2	11.1±1.0	2.74±0.21
Midlog-phase cells	+	12.2±1.1	10.0±0.9	2.43±0.16
Peat formulation	–	15.2±1.1	11.1±1.2	2.72±0.18
Peat formulation	+	11.6±1.3	9.3±0.7	2.31±0.24
Control	–	12.7±1.5	9.9±0.7	2.39±0.16
Control	+	10.6±0.9	8.4±0.8	2.13±0.18
LSD (0.01)		1.5	1.3	0.18

Note: A 90-day-old peat formulation of *P. aeruginosa* GSE 18 at 2 g·(kg seed)$^{-1}$ was used for seed bacterization of groundnut (cv. TMV 2). Treated seed was planted in potting mixtures infested with or without *A. niger* for evaluation of collar rot biocontrol. Data points are the mean ± SE of 9 replications from 3 experiments.

isolated, selected, and applied as seed treatment for control of collar rot in groundnut with or without thiram combination. The frequency of antifungal strains was high among the seed endophytes compared with the rhizobacteria and phylloplane bacteria. The selected antifungal strains differed in their activity spectrum and 11 (2.8%) strains were effective against 6 or more fungi. Variation in the spectrum of antifungal activity of microorganisms or their metabolites is not uncommon (Leifert et al. 1995).

Pseudomonas sp. GRS 175 and *P. aeruginosa* GPS 21, GSE 18, GSE 19, and GSE 30 were highly inhibitory against 8 fungal pathogens of groundnut. *Pseudomonas* spp. quite often emerged as potent antagonists in several screening programs. Among a collection of 849 bacterial strains, 6 broad-spectrum antifungal strains were identified as *P. aeruginosa* (Viji et al. 2003). Pseudomonads are widely distributed in diverse agricultural ecosystems (Garbeva et al. 2004) and are good colonizers of plant root systems owing to their competitive advantage.

Broad-spectrum activity of *Pseudomonas* spp. contributes to their in vitro antifungal activity and in vivo disease control (Haas and Keel 2003). Lee et al. (2003) isolated a new antibiotic, aerugine, with protective activity against *Phytophthora* disease of pepper and anthracnose of cucumber from the culture filtrates of *Pseudomonas fluorescens* MM-B16. Mycelial discs incubated in the presence of CCF of the 5 selected pseudomonads in the present study lost their viability. Such fungicidal activity of the extracellular metabolites of antagonists is an additional advantage in the reduction of pathogen populations in natural environments. Secondary metabolites of *P. aeruginosa* are often reported to possess antifungal, plant growth promoting, and biocontrol activities (Bano and Musarrat 2003).

Macroscopic observation of the soil/seed-borne fungi in the presence of CCF of *Pseudomonas* spp. showed reduced and compact colonies, in contrast with normal spread in the control. The microscopic observation of backward growth of the terminal hyphae correlated with the reduced colony diameter and fluffy growth of the fungi in the presence of CCF. Additionally, hyphal distortions including degradation of cytoplasm, vacuolization, and bulging were also observed. These observed hyphal deformations were commonly observed in several other antagonist–pathogen interactions. In the presence of CCF of *Bacillus* spp., hyphae of *Colletotrichum capsici* and *Colletotrichum gloeosporioides* were thickened, vacuolated, and swollen at their tips (Meon 1994). CCF of *Bacillus brevis* induced a characteristic swelling of the hyphal tips of *Fusarium udum*, and the hyphae appeared bulbous with shrunken and granulated cytoplasm (Bapat and Shah 2000). Formation of bulbous structures by *F. udum* was also observed in the presence of CCF of *B. subtilis* AF 1 (Podile and Laxmi 1998).

The lytic action of chitinase, β-1,3-glucanase, and protease could be the major antifungal mechanism or supplement the antagonistic action of other antifungal metabolites produced by the same bacterial strain (Podile and Prakash 1996; Fridlender et al. 1993). However, there existed contrasting reports on the relationship between the production of lytic enzymes and antifungal activity of different antagonists. A strong correlation was observed between the chitinolytic potential of different bacterial strains and in vitro lysis of fungal mycelium (Renwick et al. 1991), whereas such a correlation was absent in the chitinoytic bacteria isolated from cereal grain (Frandberg and Schnwer 1998). In this report, correlation between in vitro chitinase production and antifungal activity was not seen.

Pseudomonas aeruginosa GSE 18 and GSE 19 emerged as effective biocontrol agents of collar rot disease of groundnut. Different mechanisms including effective rhizosphere colonization, production of extracellular antibiotics, lytic enzymes, and siderophores, and activation of host defense responses together might contribute to the observed disease control. Biocontrol efficacy of *P. aeruginosa* in control of fusarium wilts of chickpea and pigeonpea and charcoal rot of groundnut in pathogen-infested soils by *P. aeruginosa* (Anjaiah et al. 2003) and *Pseudomonas* sp. GRC_2 (Gupta et al. 2002), respectively, has been shown.

We observed that 2 bacterial strains, *A. xylosidans* GRS 149 and *B. megaterium* GSE 6 (siderophore producers), were not antagonistic to *A. niger* but reduced pre-emergence rotting by iron starvation in the rhizosphere. These 2 strains were potent siderophore producers and might have inhibited *A. niger* in the rhizosphere by iron starvation. Suppression of fusarium wilt from siderophore-mediated competition by *P. putida* WCS 358 has been shown (Duijff et al. 1994). There was no definite correlation between the in vitro antifungal activity of groundnut-associated bacterial strains and their ability to control collar rot disease. These observations support the role of mechanisms other than antibiosis in suppression of collar rot.

Peat formulation of *P. aeruginosa* GSE 18 was equally effective as fresh cells in plant growth promotion and control of collar rot disease. The effectiveness of a dry peat-based formulation of *P. fluorescens* has been reported in the control of fusarium wilt of chickpea (Vidhyasekaran and Muthamilan 1995) and sheath blight of rice and growth stimulation of rice (Rabindran and Vidhyasekaran 1996). Induction of fungicide tolerance in biocontrol agents has been explored to the advantage of biocontrol systems (Yamaguchi et al. 1998). Combined application of fungicide-tolerant biocontrol agents and fungicide improved the control of groundnut stem rot (Manjula et al. 2004), damping off of tomato (Kondoh et al. 2001), and postharvest rotting of pear fruits (Frances et al. 2002). *Pseudomonas aeruginosa* GSE 18-R_1 was tolerant to thiram and significantly improved the disease control in combination with half of the recommended dose of thiram. Combined application of thiram and *P. aeruginosa* GSE 18-R_1 creates a selective advantage for the biocontrol agent by reducing the populations of the pathogen and other competitive microorganisms. Broad-spectrum antifungal bacterial strains associated with groundnut have a great potential to control collar rot in combination with thiram. With a further combination of available levels of host plant resistance, it would be possible to control the collar rot more effectively.

Acknowledgements

The Andhra Pradesh – Nethelrands Biotechnology Programme, Institute of Public Enterprise, Hyderabad, India, supported part of this work as a research grant to A.R. Podile and a Senior Research Fellowship to G. Krishna-Kishore; we also thank the UGC–SAP and DST–FIST programs of the Department of Plant Sciences, University of Hyderabad.

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