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Short communication

Pseudomonas aeruginosa inhibits the plant cell wall degrading enzymes of *Sclerotium rolfsii* and reduces the severity of groundnut stem rot

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Abstract

Three hundred and ninety-three groundnut-associated bacterial strains, applied both as seed treatment and soil amendment, were evaluated for control of stem rot disease (caused by *Sclerotium rolfsii*) of groundnut in a controlled environment. Twelve strains significantly ($P=0.01$) reduced the incidence of stem, rot of which groundnut seed endophytes *Pseudomonas aeruginosa* GSE 18 and GSE 19 reduced the seedling mortality by 54% and 58%, compared to the control. In dual cultures, the 12 biocontrol strains reduced the mycelial growth of *S. rolfsii* by 32%–74% as compared to the control. Cell- free culture filtrates of *P. aeruginosa* GSE 18 and GSE 19 inhibited the activity *in vitro* of the cell wall-degrading enzymes (CWDE) polygalacturonase and cellulase by *S. rolfsii* up to a maximum of 55% and 50%, respectively, when measured 6 days after inoculation. *Pseudomonas aeruginosa* GSE 18 and GSE 19 with a known tolerance to thiram, a commonly used seed dressing fungicide, suppressed the growth of *S. rolfsii*, inhibited the activity of CWDE, and reduced the incidence of stem rot, suggesting the usefulness of these biocontrol strains as components in the integrated management of groundnut stem rot.

Stem rot or stem blight caused by *Sclerotium rolfsii*, a broad host range fungus, is the major soil-borne disease of groundnut (*Arachis hypogaea*). The disease has a global distribution and causes pod yield losses of 10–25% that can sometimes be up to 80% (Mehan et al., 1994). In addition, oxalic acid produced by *S. rolfsii* causes blue discolouration and affects the seed quality. Available sources of host plant resistance and chemical control need to be strengthened for effective management of groundnut stem rot (Podile and Kishore, 2002). Biological control using antagonistic *Pseudomonas fluorescens* appears to be a potential management tool for reducing the severity of groundnut stem rot (Ganesan and Gnanamanickam, 1987). Selection of broad-spectrum antifungal isolates with effective mechanisms of disease suppression for integrated disease management (IDM) may provide a better alternative to existing control strategies. Seed treatment or soil amendment with biocontrol agents contributes to the management of phytopathogenic fungi either by direct spatial and nutrient competition, antibiosis and/or indirect induction of host defense responses (Weller, 1988; Whipps, 2001). In the past, the selection of biocontrol agents was mostly based on their *in vitro* antifungal activity, which subsequently resulted in poor performance of several of the potent antifungal strains *in vivo*, since antibiotic production in the rhizosphere is dependent on a number of abiotic and biotic factors (Fravel, 1988; Raaijmakers et al., 2002). This selection criterion also eliminates the identification of biocontrol agents that

are exclusively involved in competition and induction of host defense responses. Hence, selection of biocontrol strains based on their *in vivo* performance is desirable for the identification of potential effective strains.

Necrotrophic fungal pathogens such as *S. rolfsii*, *Botrytis cinerea*, etc., produce toxins that inactivate the host cells, or cell wall degrading enzymes (CWDE) that degrade the plant cell wall polymers prior to fungal penetration. Pectinases and cellulases are important CWDE that break down pectin and cellulose, the two major constituents of plant cell walls, and facilitate fungal infection (Cooper, 1983). Production of oxalic acid and polygalacturonases are involved in the early stages of pathogenesis of *S. rolfsii* (Bateman and Beer, 1965). Specific inhibition of CWDE of *B. cinerea* by the secondary metabolites and protease of fungal biocontrol agents has been reported (Kapat et al., 1998; Elad and Kapat, 1999). The present study reports an attempt to select bacterial biocontrol strains for the control of stem rot disease from a large collection of groundnut-associated bacteria by screening *in vivo* under controlled environment conditions. Furthermore, the effect of cell-free culture filtrates of selected bacterial strains on the growth and activity *in vitro* of CWDE of *S. rolfsii* was determined to evaluate the suitability of these strains as components of IDM.

Three hundred and ninety-three groundnut-associated bacterial strains, collected from different habitats including the rhizosphere (63.6%), phylloplane (17%), geocarposphere (3.3%), and leaf (0.3%), root (1.3%) and seed endophytes (14.5%), were used in the present study. These strains were isolated earlier from groundnut plants in nine districts of Andhra Pradesh, India, and stored as glycerol stocks at −70 °C (Kishore et al., 2005a). *Sclerotium rolfsii* was isolated from stem rot-infected groundnut plants in the field and a single sclerotial isolate was maintained on potato dextrose agar (PDA) at 4 °C. The 393 groundnut-associated bacterial strains, applied both as seed treatment and soil amendment, were evaluated for control of stem rot disease in a controlled environment. A culture of each bacterium grown on Luria-Bertani (LB) agar in 90 mm diam Petri dishes for 48 h at 30 °C was transferred into 20 ml of 0.5% carboxy methyl cellulose (CMC). Groundnut seeds of cv. TMV 2, surface-sterilized by soaking in 0.02% (w/v) $HgCl_2$ for 5 min, were washed three times in sterile distilled water (SDW) and suspended in the above bacterial cell suspension for 30 min. Treated seeds were air-dried and the viable cell count as determined by dilution plating was 10^6–10^7 CFU seed^{-1}. Five bacterized seeds were planted in 15 cm diam plastic pots filled with a potting mixture consisting of alfisol, farm yard manure and sand (3:1:1), with CMC-treated seeds as the control. After germination, the seedlings were thinned to four in each pot. Bacterial cells grown in LB broth for 48 h at 30 °C and 180 rpm were suspended in 10 mM phosphate buffer (~10^8 CFU ml^{-1}), and 2 ml of the suspension was applied at the base of seedlings 5 days after sowing (DAS).

For artificial inoculation with *S. rolfsii*, dried sclerotia were germinated for 36–48 h on PDA at 28 °C, and a 1 cm disc of germinated sclerotia along with actively growing mycelium was used as the inoculum (Shokes et al., 1996). At 14 DAS, the inoculum disc was transferred to the stem base and covered with coarse sand up to a height of 1.5 cm that was maintained in a moist condition (Ganesan and Gnanamanickam, 1987). The temperature in the greenhouse was maintained 27 ± 2°C throughout the experiment. Observations were recorded in terms of seedling mortality 15 days after inoculation (DAI). The experiment was repeated with three replications in each treatment.

Twelve selected bacterial strains effective against stem rot in the greenhouse were tested for their effect on the growth of *S. rolfsii* in potato dextrose broth (PDB). About 100 μl of an individual bacterial culture grown overnight in LB broth and a 7 mm disc of actively growing mycelium of *S. rolfsii* were co-inoculated into 50 ml of PDB in 250 ml conical flasks. Dual inoculated flasks were incubated for 96 h at 28 °C and 125 rpm. The mycelium in each flask was separated by filtration, washed with SDW, dried in an oven at 60 °C until it attained a constant weight, and the dry biomass recorded. The experiment was conducted twice with three flasks in each treatment.

The effect of cell-free culture filtrates (CCF) of *Pseudomonas aeruginosa* GSE 18 and GSE 19 on the activity *in vitro* of CWDE, polygalacturonase and cellulase, by *S. rolfsii* was quantified. These two bacterial strains were isolated as endophytes from surface-sterilized groundnut seeds (Kishore et al., 2005a). *Sclerotium rolfsii* was grown in Richard's medium (RM) with a varied carbon source depending on the enzyme to be assayed – sodium

polypectate (Sigma Chemical Co., USA) for polygalacturonase and CMC for cellulase. CCF of *P. aeruginosa* GSE 18 and GSE 19 were obtained by filter sterilization of supernatants of cultures grown in LB broth for 48 h at 30 °C and 180 rpm. CCF were added to pre-cooled RM in 100 ml conical flasks at a final concentration of 25% (v/v) in 30 ml volume. The flasks were inoculated with a 7 mm disc of actively growing *S. rolfsii* and incubated at 28 °C and 125 rpm. The fungal mycelium was separated at 2, 4 and 6 DAI to measure the dry biomass and the culture filtrate was used as crude enzyme extract to determine the activities of CWDE. Three culture flasks were used as replicates of individual treatments and the experiments were repeated once.

The reaction mixture for polygalacturonase assays consisted of 400 μl of 0.25% sodium polypectate in 50 mM sodium acetate buffer pH 5.2 and 100 μl of enzyme extract (Collmer et al., 1988). The release of galacturonic acid in the reaction mixture was determined using dinitro salicylate (DNS) reagent (Miller, 1959) against the galacturonic acid standard. One unit of polygalacturonase activity was defined as the amount of enzyme that released 1 μM of galacturonic acid min^{-1} ml^{-1} of enzyme extract.

The reaction mixture for cellulase assays contained 300 μl of 1% CMC in 50 mM acetate buffer, pH 5.2, and 200 μl of enzyme extract. The amount of reducing sugar released was measured using DNS reagent. One unit of cellulase activity was defined as the amount of enzyme that catalyzed the release of 1 μM glucose min^{-1} ml^{-1} of enzyme extract under the assay conditions. Both the enzyme activities were expressed as units g^{-1} dry biomass of *S. rolfsii*.

Data from all the experiments were subjected to analysis of variance (ANOVA) using the Genstat 5 statistical package or regression analysis. Least significant difference (LSD) at 1% level of significance ($P = 0.01$) was used to compare the mean values of different treatments in *in vitro* antifungal and *in vivo* disease control tests.

Germinated sclerotia along with the actively growing mycelium, as inoculum, resulted in complete mortality of the groundnut plants in the control treatment. Of the 393 groundnut-associated bacterial strains, 12 strains isolated from the geocarposphere, phylloplane, rhizosphere and seed significantly ($P = 0.01$) reduced the mortality of *S. rolfsii*-inoculated groundnut plants as compared to the control. Two seed endophytes, *P. aeruginosa* GSE 18 and GSE 19, and a rhizobacterium, *Pseudomonas* sp. GRS 175, were more effective than other bacteria in reducing the plant mortality (33–58%) (Table 1). Of the bacterial strains from different groundnut habitats, the relative proportion of bacteria effective against stem rot, from

Table 1. Biological control of stem rot disease of groundnut by selected groundnut-associated bacterial strains and their effect on the growth of *Sclerotium rolfsii* in dual cultures

Bacterial strain[a]	Habitat	Plant mortality (%)	Biomass of *S. rolfsii* (mg)[b]
GGS 12	geocarposphere	81.3 ± 4.6	296.7 ± 12.5 (41)
GPS 21	phylloplane	75.0 ± 2.7	315.7 ± 18.9 (37)
GPS 38	phylloplane	81.3 ± 3.9	268.7 ± 21.8 (46)
Pseudomonas sp. GRS 175	rhizosphere	66.7 ± 4.0	142.6 ± 8.7 (71)
GRS 223	rhizosphere	72.9 ± 5.3	257.9 ± 15.9 (48)
GSE 3	seed	89.6 ± 5.3	267.9 ± 12.9 (46)
GSE 5	seed	79.2 ± 9.7	337.2 ± 23.7 (32)
GSE 6	seed	87.5 ± 3.0	276.8 ± 21.9 (45)
P. aeruginosa GSE 18	seed	45.8 ± 2.7	128.9 ± 9.7 (74)
P. aeruginosa GSE 19	seed	41.7 ± 2.8	140.4 ± 6.7 (72)
GSE 21	seed	87.5 ± 5.0	152.6 ± 7.0 (69)
P. aeruginosa GSE 30	seed	68.8 ± 5.7	161.5 ± 10.9 (68)
Control		100.0 ± 0.0	498.5 ± 35.9
LSD ($P = 0.01$)		9.7	28.7

[a]393 groundnut-associated bacterial strains were tested and those that significantly reduced the plant mortality 15 days after pathogen inoculation were listed.
[b]Actively growing bacteria and *S. rolfsii* were co-cultured for 96 h at 28 °C and 125 rpm, and the mycelial dry weight was recorded. Values in parenthesis indicate the percentage inhibition of biomass in each treatment with respect to control.
Data from six replications of a repeated experiment were analyzed by one-way ANOVA and least significant difference (LSD) at 1% level was used to compare different treatments.

each habitat was as follows: geocarposphere – 8%, phylloplane – 3%, rhizosphere – 1% and seed endophytes – 12%.

The 12 selected biocontrol strains inhibited the growth of *S. rolfsii* in liquid medium, to a maximum of 74% in the presence of *P. aeruginosa* GSE 18 closely followed by *P. aeruginosa* GSE 19 and *Pseudomonas* sp. GRS 175 (Table 1). There was a significant correlation between the inhibition of fungal biomass and biocontrol activity of the 12 strains ($r = 0.71$; $P < 0.01$).

The percentage inhibition of *S. rolfsii* enzyme activity by CCF of GSE 18 and GSE 19 varied with the incubation period and the inhibition ranged >50% compared to control, measured 6 DAI. Inhibition of polygalacturonase activity by CCF was maximum at 4 DAI, whereas inhibition of cellulase activity was maximum at 6 DAI (Figure 1). Inhibition of biomass was maximum at

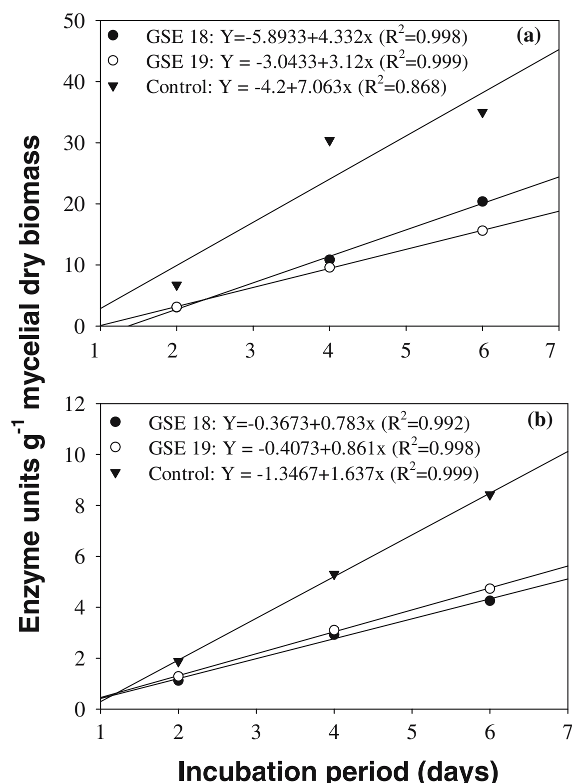

Figure 1. Effect of cell-free culture filtrates (CCF) of *Pseudomonas aeruginosa* GSE 18 and GSE 19 on the production of cell wall degrading enzymes of *Sclerotium rolfsii* – (A) Polygalacturonase and (B) Cellulase. Richard's medium with 25% (v/v) CCF and varied carbon source (sodium polypectate for polygalacturonase and carboxy methyl cellulose for cellulase) was used to determine the enzyme activity.

4 DAI, whereas the inhibition of enzyme activities continued up to 6 DAI.

The relationship between *in vitro* antibiosis and *in vivo* disease control by the biocontrol agents based on several screening programmes is not clear (Fravel, 1988). Failure of several potent antifungal strains as disease protectants in natural environments could be due to the variation in their establishment and production of antifungal metabolites in relation to several biotic and abiotic factors including soil type, nutrition and environment. In some instances the ability to induce systemic resistance in host plants determines the efficacy of biocontrol agents. Thus, to identify biocontrol agents with different modes of action, in the present study we selected bacterial strains for stem rot control based on their performance in a controlled environment. We observed a good correlation between the inhibition of *S. rolfsii* biomass in liquid culture and control of stem rot disease by the selected bacterial strains. However, variation in greenhouse performance among the 12 selected antifungal bacterial strains could be due to their difference in soil/rhizosphere establishment and activation of host defense responses.

Groundnut seed endophytes, *P. aeruginosa* GSE 18 and GSE 19 effectively reduced stem rot severity in the greenhouse. These two strains were antagonistic to eight fungal pathogens of groundnut and induced hyphal deformations in *S. rolfsii* (Kishore et al., 2005b). Biocontrol efficacy of GSE 18 and GSE 19 is substantiated by the earlier reports on beneficial effects of endophytic bacterial strains in the rhizosphere (Sessitsch et al., 2004). *Pseudomonas* spp., being broad-spectrum antifungal and rhizosphere competent, are most commonly identified as biocontrol agents of soil/seed-borne fungi (O'Sullivan and O'Gara, 1992). A combination of *P. fluorescens* and *Trichoderma viride* had an improved biocontrol activity against groundnut stem rot disease (Manjula et al., 2004). *Pseudomonas* spp. was also effective in the control of *S. rolfsii* infection in chickpea by induction of phenolic acids (Singh et al., 2003). Following their application as a seed treatment, *P. aeruginosa* GSE 18 and GSE 19 rapidly colonized groundnut rhizosphere to control collar rot disease (Kishore et al., 2005b). In addition, these two biocontrol agents were tolerant to the commonly used seed dressing fungicide thiram (Kishore et al., 2005b), and hence may be of practical significance for the

economic control of different soil-borne fungal diseases of groundnut.

A direct relationship has been observed between the pathogenicity and CWDE production ability of phytopathogenic fungi (Campion et al., 1997). Specific inhibition of CWDE retains the integrity of plant cell walls and reduces fungal virulence. Bacterial and fungal biocontrol agents are known to inhibit the activity *in vitro* of fungal CWDE that in turn reduced their pathogenicity *in vivo*. *Trichoderma harzianum* inhibited the production of cutin esterase, exo- and endo-polygalacturonases, pectin lyase and pectin methyl esterase by *Rhizoctonia solani* in nutrient medium (Bertagnolli et al., 1996) and *B. cinerea* on bean leaf surfaces (Kapat et al., 1998; Elad and Kapat, 1999). A calcium-dependent endoproteinase of *Bacillus megaterium* inactivated the cellulase, pectinase and pectin lyase of *R. solani* (Bertagnolli et al., 1996). We observed a similar inhibition of *S. rolfsii* CWDE by CCF of *P. aeruginosa* GSE 18 and GSE 19. Inhibition of the *in vitro* activity of CWDE of *S. rolfsii* by *P. aeruginosa* GSE 18 and GSE 19 may have an additional role in the control of stem rot disease.

The ability of *P. aeruginosa* GSE 18 and GSE 19 to significantly reduce the incidence of stem rot and inhibit the growth of *S. rolfsii* and its CWDE coupled with tolerance to thiram suggests their usefulness as components of IDM of groundnut stem rot, provided they are non-toxic to animals. Furthermore, the broad-spectrum antifungal activity of these strains may help in the suppression of other soilborne fungal diseases of groundnut.

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