Plant Pathol. J. 20(1) : 75-80 (2004)

Combined Application of *Pseudomonas fluorescens* and *Trichoderma viride* has an Improved Biocontrol Activity Against Stem Rot in Groundnut

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(Received on November 24, 2003; Accepted on January 10, 2004)

In an attempt to develop effective biocontrol system for management of stem rot disease in groundnut, 57 bacterial isolates and 13 isolates of *Trichoderma* spp. were evaluated for their antagonistic activity against *Sclerotium rolfsii*. The antagonists were selected based on their ability to inhibit the external growth of *S. rolfsii* from infected groundnut seeds. Four isolates of *Pseudomonas fluorescens*, GB 4, GB 8, GB 10 and GB 27, and *T. viride* pq 1 were identified as potent antagonists of *S. rolfsii*. *T. viride* pq 1 produced extracellular chitinase and parasitized the mycelium of *S. rolfsii*. Under controlled environment conditions, *P. fluorescens* GB 10, GB 27, *T. viride* pq 1 and the systemic fungicide Thiram® reduced the mortality of *S. rolfsii* inoculated to groundnut seedlings by 58.0%, 55.9%, 70.0% and 25.9%, respectively compared to control. *In vitro* growth of *P. fluorescens* GB 10 and GB 27 was compatible with *T. viride* pq 1 and Thiram®. Integrated use of these two bacterial isolates with *T. viride* pq 1 or Thiram® improved their biocontrol efficacy. Combined application of either GB 10 or GB 27 with *T. viride* pq 1 was significantly effective than that with Thiram® in protecting groundnut seedlings from stem rot infection.

Keywords **:** *Arachis hypogaea*, fungicide tolerance, peanut, sclerotia, synergism

Stem rot incited by *Sclerotium rolfsii* is one of the major production constraints of groundnut (*Arachis hypogaea* L.) in majority of the tropical and subtropical countries. Stem rot causes pod yield losses of 10-25%, but under severe diseased conditions yield losses range up to 80% (Rodriguez-kabana et al., 1975). Absence of potential genetic resistance in cultivated genotypes and high costs of the existing chemical control methods necessitate alternate cost-effective strategies for management of stem rot. Among the available alternate disease management options, biological control appears promising.

Trichoderma spp. are effective in control of soil/seed-borne fungal diseases in several crop plants (Kubicek et al., 2001), including groundnut (Podile and Kishore, 2002). Major mechanisms involved in the biocontrol activity of *Trichoderma* spp. were competition for space and nutrients, production of diffusible and/or volatile antibiotics, and hydrolytic enzymes like chitinase and β-1,3-glucanase. These hydrolytic enzymes partially degrade the pathogen cell wall and leads to its parasitization (Kubicek et al., 2001). *Pseudomonas* spp. are effective root colonizers and biocontrol agents, by production of antibiotics and other antifungal metabolites including antibiotics, hydrogen cyanide and siderophores (O'Sullivan and O'Gara, 1992). A positive relationship was observed between the antifungal activity of chitinolytic *P. fluorescens* isolates and their level of chitinase production (Velazhahan et al., 1999). In recent years, more emphasis is laid on the combined use of biocontrol agents with different mechanisms of disease control, for improved disease control and also to overcome the inconsistent performance of the introduced biocontrol agents.

Majority of the existing biocontrol agents for management of soil-borne diseases, were isolated from the rhizosphere. There is a possibility to explore antagonists from other habitats as potent biocontrol agents. Endophytes, which colonize and reside in the internal plant habitats were proved effective in plant growth promotion and disease control in a wide range of crops (Manjula et al., 2002). Some biocontrol agents have also been isolated from the seed surfaces (Guanlin et al., 1997).

In the present study antagonistic bacteria and *Trichoderma* spp. isolated from the groundnut seed were evaluated for control of *S. rolfsii* infection in groundnut under controlled environment conditions. The fungicide tolerance and *in vitro* compatibility of the identified biocontrol agents were determined and exploited for effective management of stem rot disease.

Materials and Methods

Fungal and bacterial isolates. Groundnut pods were collected at

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harvest from different experimental fields of the International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India. The pods were sun dried and shelled under asceptic conditions. The seeds were plated on nutrient agar (NA) (Himedia, Mumbai, India) for isolation of bacteria and *Trichoderma* selective medium (Elad et al., 1981) for isolation of *Trichoderma* spp. The plates were incubated at 28°C for 72 h and observed for growth of bacteria and fungi. Single colony and single spore isolates of the observed bacteria and fungi were used in all the further experiments. *S. rolfsii* was isolated from infected groundnut seed and maintained on potato dextrose agar (PDA) (Himedia, Mumbai, India) at 4°C.

Identification of bacterial and fungal isolates. Selected anti-fungal bacterial isolates were identified using NFERM bacterial diagnostic kit (LACHEMA, Czech Republic). Identification of antagonistic *Trichoderma* spp. was done according to the identification key proposed by Rifai (1969).

Inhibition of seed carried *S. rolfsii*. Antagonistic bacteria and *Trichoderma* spp. were identified by their inhibition of external growth of seed-carried *S. rolfsii* and the antifungal activity of selected isolates was reconfirmed by dual culture test. Seeds of groundnut genotype ICGS 11, infected with *S. rolfsii* were collected from a seed lot, based on the visible symptoms of infection. The seeds were surface sterilized by treatment with 100 mg/ml chloramphenicol for 30 min. The seeds were washed thrice with sterile distilled water and resuspended in log phase bacterial cell suspension (10^9 CFU/ml) in 0.5% carboxy methyl cellulose (CMC) for 5 min. Bacterized seeds were dried under a sterile flow of air in a laminar flow. The cell number ranged from 10^6-10^7 CFU/seed. Similarly, seeds were also suspended in a spore suspension of *Trichoderma* spp. (10^8 conidia/ml) in 0.5% CMC for 30 min and the air-dried seeds had ~10^5 conidia/seed. Five treated seeds were plated on water agar in 9 cm diameter petri plates and incubated at 28°C. Seeds treated with 0.5% CMC alone were maintained as control. Seeds were observed under a photo microscope for external growth of *S. rolfsii*, after an incubation of 72 h. Seed germination was also recorded in each treatment. Ten seeds were maintained in each treatment and the experiment was repeated with three replications.

In dual culture test, the bacteria were streaked as a line on one edge of PDA (pH 6.1) in a 9 cm diameter petri plate. After 24 h of incubation at 30°C, a 7 mm disc of an actively growing culture of *S. rolfsii* was inoculated at the center. Plates inoculated with *S. rolfsii* alone were maintained as control. All the inoculated plates were further incubated for 72 h at 28°C and the colony diameter in each treatment was compared with that of control.

Mycelial discs of 7 mm diameter from actively growing cultures of *T. viride* pq 1 and *S. rolfsii* were inoculated at either end of PDA and incubated for 7 days at 28°C. The plates were observed at regular intervals of 24 h and the antifungal activity was recorded on a 1-5 rating scale (Bell et al., 1982). PDA plates inoculated with *S. rolfsii* alone were treated as control. The above experiments were repeated with three replications.

***In vitro* characterization of antifungal bacteria and *T. viride* pq 1.** The potent antifungal bacterial isolates were characterized for their *in vitro* production of hydrogen cyanide (HCN) and chitinase. *T. viride* pq 1 was tested for production of volatile antibiotics, and hydrolytic enzymes chitinase and β-1,3-glucanase. All the experiments were repeated with three replications.

Production of HCN. For qualitative determination of the production of HCN by antagonistic bacteria, the bacteria were inoculated on NA supplemented with 4.4 g/l glycine. Inoculated plates were inverted and a strip of sterilized filter paper saturated with 0.5% picric acid in 2% (w/v) sodium carbonate was placed in the upper lid. The plates were then sealed with parafilm and incubated for 72 h at 28°C. A change in color of the filter paper from yellow to light brown or reddish brown indicated the production of HCN (Bakker and Schippers, 1987).

Production of volatile antibiotics. The lower lids of two PDA petri plates containing PDA were inoculated separately with mycelial discs of *T. viride* pq 1 and *S. rolfsii*. The two lids were then inverted one over the other and sealed air-tight with parafilm. Plates inoculated with *S. rolfsii* and inverted over uninoculated plates were treated as control (Denis and Webster, 1971). The assembly was incubated at 28°C with 12 h photoperiod. After 96 h, colony diameter of *S. rolfsii* was measured in all the treatments.

Production of chitinase. Bacteria were spotted on minimal medium with 0.1% (w/v) colloidal chitin as a sole carbon source (Manjula, 1999). The inoculated plates were incubated for 120 h at 30°C and observed for clearing zones around the colonies.

Mycelial discs from actively growing culture of *T. viride* pq 1 were inoculated on Richards medium (potassium nitrate - 10 g, potassium dihydrogen orthophosphate - 5 g, magnesium sulphate - 2.5 g, ferric chloride - 0.02 g and distilled water - 1000 ml) with 1% colloidal chitin as sole carbon source, and incubated for 72 h at 28°C with a 12 h photoperiod. The plates were then observed for zone of chitin lysis around the mycelium.

Production of β-1,3-glucanase. *T. viride* pq 1 was inoculated on RM with 1% (w/v) lichenan and the plates were stained with 1% (w/v) congo red after an incubation for 72 h at 28°C. The stained plates were observed for clear zone around the mycelial growth.

Evaluation of antifungal strains for control of groundnut stem rot. Four antagonistic bacterial isolates *P. fluorescens* GB 4, GB 8, GB 10 and GB 27, and *T. viride* pq 1 were evaluated for control of groundnut stem rot in greenhouse. Six seeds of groundnut cv. TMV 2 treated with the antagonistic bacteria and *T. viride* pq 1 were planted in 15 cm diameter plastic pots filled with red alfisol, farm yard manure and sand (2:1:1). The population of the bacterial cells was ~10^7 CFU/seed and that of *T. viride* pq 1 was ~10^5 conidia/seed. Seeds coated with 0.5% CMC were treated as control. Seed treatment with the systemic fungicide Thiram® (Sudama Chemtech P. Ltd., Gujarat, India; 75% active ingredient), at a recommended dose of 2 g/kg seed, was also maintained as one of the treatment to compare the disease control efficacy of biocontrol agents. At 14 days after sowing (DAS), a 1 cm disc of an actively growing culture of *S. rolfsii* on PDA was placed at the stem base of each plant and covered with coarse sand up to a thickness of 1.5 cm (Ganesan and Gnanamanickam, 1987). The sand layer was kept moist and the greenhouse temperature was maintained at 27 ± 2°C. Inoculated plants were observed for mortality at 15 days after inoculation (DAI). Ten plants were maintained in each treatment and the experiment was repeated

with three replications.

***In vitro* compatibility of *P. fluorescens* or *T. viride* pq 1 and Thiram®.** *P. fluorescens* GB 10 and GB 27 effective in stem rot control were tested for their tolerance to *T. viride* pq 1. These three biocontrol agents were inturn tested for their tolerance to Thiram®, a commonly used seed dressing fungicide. The compatibility of *P. fluorescens* isolates and *T. viride* pq 1 was studied by co-culturing them on PDA. The two antagonist cultures were inoculated at a distance of 2 cm from each other and incubated at 28°C. Inoculated plates were observed for growth of both the cultures at regular intervals. To determine the fungicide tolerance, bacterial isolates were inoculated in 50 ml of LB broth in 250 ml conical flasks, added with Thiram® at final concentrations of 5, 10, 20, 30, 50, 100, 250, 500 and 1000 µg/ml. Inoculated flasks were incubated for 48 h on a rotary shaker at 30°C and 180 rpm. The cell growth in each treatment was monitored by measuring the optical density (OD) at 420 nm at regular intervals of 12 h, in comparison to control without any fungicide. Actively growing culture of *T. viride* pq 1 was transferred to PDA added with Thiram® at concentrations of 5-1000 µg/ml and incubated at 28°C. The fungal growth was observed at 96 h after inoculation and compared with control grown on PDA alone. The experiments were repeated with four replications.

Integrated use of biocontrol agents and fungicide for disease control. Groundnut seeds (cv. TMV 2) bacterized with *P. fluorescens* GB 10 and GB 27 were air-dried and coated with *T. viride* pq 1 or Thiram® (2 g/kg seed) before planting. Seeds treated with either the biocontrol agents or Thiram® alone were used for comparison and seeds treated with 0.5% CMC served as control. The pathogen inoculation and disease scoring were similar to as mentioned above. Ten plants were maintained in each treatment and the experiment was repeated with three replications.

Statistical analysis. Different treatments in all the experiments were arranged in a completely randomized block design. Data from all the experiments were analyzed by analysis of variance (ANOVA) using Genstat 5 statistical package. Least significant difference (LSD) at 1% level of significance (P=0.01) was used to compare the mean values of different treatments in an experiment.

Results

***In vitro* antifungal activity.** Single colony isolates of 57 bacteria (GB 1-GB 57) and thirteen isolates of *Trichoderma* spp. (pq 1-pq 13) were tested for inhibition of external growth of *S. rolfsii* from groundnut seeds. In different treatments, the external growth of seed carried *S. rolfsii* was in 3.3%-75.0% seeds compared to 76.7% in control. Eleven isolates significantly inhibited the external growth of *S. rolfsii* and none of them inhibited the seed germination. Four bacterial isolates, *P. fluorescens* GB 4, GB 8, GB 10 and GB 27 were highly effective in inhibition of seed-carried *S. rolfsii* ($P_{(0.01)}$=10.3). These isolates also reduced the radial growth of *S .rolfsii* in dual cultures by >35% (Fig. 1; $P_{(0.01)}$=7.6). The four bacterial isolates also increased the seed germination with *P. fluorescens* GB 27 being the most

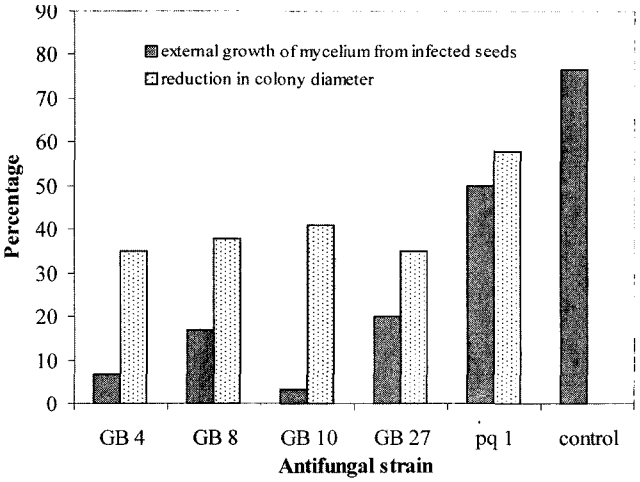

Fig. 1. Antagonistic activity of four isolates of *Pseudomonas fluorescens* - GB 4, GB 8, GB 10 and GB 27, and *Trichoderma viride* pq 1 against *Sclerotium rolfsii*. The antifungal activity of these isolates was determined by their inhibition of external growth of seed-carried *S. rolfsii* when applied as seed treatment and also by reduction in colony diameter in dual cultures.

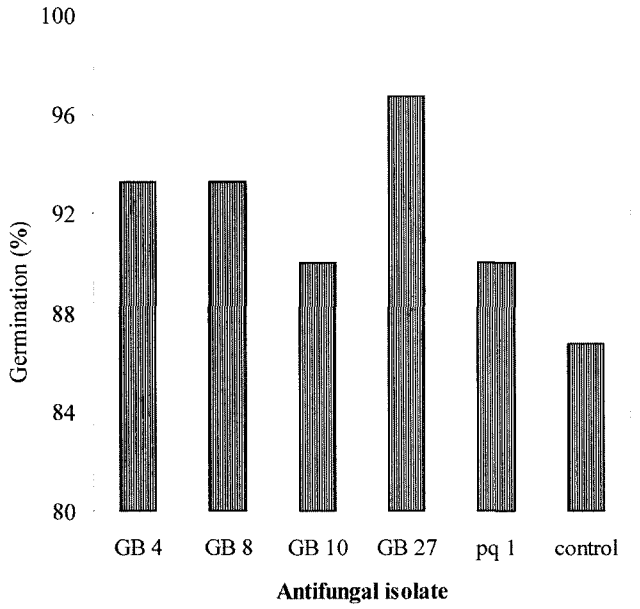

Fig. 2. Effect of the four antifungal isolates of *Pseudomonas fluorescens* - GB 4, GB 8, GB 10 and GB 27, and *Trichoderma viride* pq 1 applied as seed treatment on the *in vitro* germination of *Sclerotium rolfsii* infected groundnut seeds.

effective (Fig. 2; $P_{(0.01)}$=3.1).

T. viride pq 1 inhibited the external growth of seed-carried *S. rolfsii* and its radial growth in dual cultures by 50.0% and 58.0%, respectively (Fig. 1). In dual culture method *T. viride* pq 1 parasitized the mycelium of *S. rolfsii* and the antifungal activity was rated 4.0 on a 1-5 rating scale. Starting from 48 h after inoculation, hyphae of *T.*

Table 1. *In vitro* characterization of antifungal isolates of *Pseudomonas fluorescens* and *Trichoderma viride* pq 1

Isolate	Production of			
	HCN[a]	volatile antibiotics chitinase other than	cyanide	glucanase
P. fluorescens GB 4	+	ND[b]	–	ND
P. fluorescens GB 8	+	ND	–	ND
P. fluorescens GB 10	+	ND	–	ND
P. fluorescens GB 27	+	ND	–	ND
T. viride pq 1	ND	+	+	–

[a] HCN = Hydrogen cyanide.
[b] ND = not determined.

viride pq 1 were observed to coil around *S. rolfsii* hyphae and lead to their rupture. Parasitized *S. rolfsii* failed to produce scelrotia compared to abundant sclerotial production in control.

***In vitro* characterization of antifungal isolates.** The four antifungal bacterial isolates, *P. fluorescens* GB 4, GB 8, GB 10 and GB 27 produced HCN *in vitro* but failed to produce extracellular chitinase. In attempts to determine the production of volatile antibiotics by *T. viride* pq 1, colony diameter of *S. rolfsii* remained the same both in presence and absence of the antagonist. Thus, production of volatile antibiotics is not involved in the antagonistic activity of *T. viride* pq 1. *T. viride* pq 1 produced extracellular chitinase but not β-1,3-glucanase (Table 1).

Biocontrol of stem rot. In controlled environment conditions, *P. fluorescens* GB 8, GB 10 and GB 27 used as seed treatment, significantly (P=0.01) reduced the mortality of groundnut seedlings by 58.0-80.0% compared to control. Among the four bacterial isolates tested, GB 10 was most effective in stem rot biocontrol, closely followed by GB 27 ($P_{(0.01)}$=9.7) . *T. viride* pq 1 reduced the mortality of seedlings by >70.0% compared to control. All these biocontrol agents were significantly (P=0.01) effective than Thiram®, which reduced the incidence of stem rot by 25.9% (Fig. 3).

***In vitro* compatibility of *P. fluorescens* or *T. viride* with Thiram®.** In dual cultures, *P. fluorescens* GB 10 and GB 27 had no effect on the growth of *T. viride* pq 1 or vice versa. The two bacterial isolates were tolerant to Thiram® at all the test concentrations, as determined by OD of the growth medium. However, Thiram® even at the lowest concentration tested (5 µg/ml), completely inhibited the growth of *T. viride* pq 1.

Combined application of *P. fluorescens* and *T. viride* pq 1 or Thiram® for management of stem rot. Isolates of *P. fluorescens* GB 10 or GB 27 applied in combination with *T. viride* pq 1 were significantly effective (P=0.05 and 0.01 for GB 10 and GB 27, respectively, and based on the mortality value) than each bacterial isolate, *T. viride* pq 1 or Thiram®

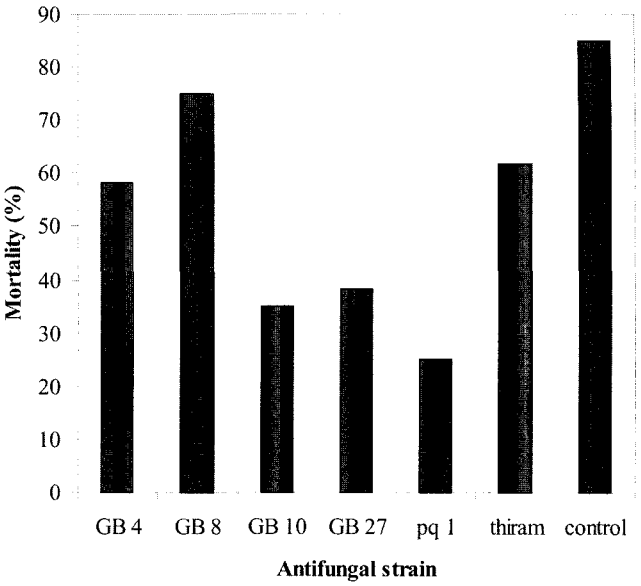

Fig. 3. Biocontrol potential of four antifungal isolates of *Pseudomonas fluorescens* - GB 4, GB 8, GB 10 and GB 27, and *Trichoderma viride* pq 1, applied as seed treatment, against stem rot infection in groundnut under controlled environment conditions. The antagonists were applied as seed treatment and at 14 days after sowing the seedlings were inoculated with *Sclerotium rolfsii*. Mortality of the inoculated plants was recorded at 15 days after inoculation.

in protecting the groundnut seedlings from stem rot infection. Furthermore, combined application of either of the bacterial isolate with *T. viride* pq 1 was effective than their combined application with Thiram®. Of all the

Table 2. Biological control of groundnut stem rot by the combined application of *Pseudomonas fluorescens* GB 10 or GB 27 and *Trichoderma viride* pq 1 or Thiram®

Treatment	Mortality (percentage control[a]) (%)
P. fluorescens GB 10	35.0 (58.0)
P. fluorescens GB 10 + *T. viride* pq 1	18.3 (78.0)
P. fluorescens GB 10 + Thiram®	28.3 (66.0)
P. fluorescens GB 27	36.7 (55.9)
P. fluorescens GB 27 + *T. viride* pq 1	16.7 (80.4)
P. fluorescens GB 27 + Thiram®	23.3 (72.0)
T. viride pq 1	25.0 (70.0)
Thiram®	61.7 (25.9)
Control	83.3 (0.0)
LSD (P = 0.01)	7.63

[a] Values in the parenthesis are the percentage disease control in each treatment over control. All the values are the mean of six replicates in two separate sets of experiments.

The two bacterial isolates had an *in vitro* compatibility with *T. viride* and Thiram®. All the treatments were applied as seed treatment and *Sclerotium rolfsii* was inoculated at 14 days after sowing. Mortality of the inoculated plants was observed at 15 days after inoculation.

different treatments, a combination of *P. fluorescens* GB 27 and *T. viride* pq 1 had the highest disease control ability (Table 2).

Discussion

An important finding of the present study is the identification of seed-associated microorganisms as biocontrol agents, which has not received much attention in the past. Though, we have not compared the efficacy of these microorganisms with those from other habitats, the results obtained indicate that seed-associated microorganisms can be a potential source of biocontrol agents. Microbial strains adapted to colonize the seed surface may also have an additional advantage in colonizing the plant root system by using the limited available nutrients and root exudates.

Inhibition of seed-carried *S. rolfsii* was used as a selection method to identify potent antifungal strains, as this system to some extent mimics the natural antagonism, though in majority of the instances stem rot infection in groundnut is due to soil-borne inoculum. The antifungal strains identified by this selection method also significantly inhibited *S. rolfsii* in the dual culture method. In dual culture assay, *T. viride* pq 1 inhibited the growth of *S. rolfsii*, parasitized and lysed the mycelium of *S. rolfsii*. *Trichoderma* spp. were known to penetrate and colonize both the sclerotia and mycelium of *S. rolfsii* (Henis et al., 1983). Degradation of chitin in the cell walls of *S. rolfsii* by chitinases of *Trichoderma* spp., facilitates their penetration of *S. rolfsii* mycelium (Haran et al., 1996). Since, sclerotia are the perennating bodies, which carry *S. rolfsii* from one season to the other, inhibition of sclerotial production can reduce the over all available inoculum of *S. rolfsii* in field.

Pseudomonas spp. are well known for production of broad spectrum antibiotics such as 2,4-diacetyl phloroglucinol and antibiosis was proved to be a major mechanism involved in their biocontrol activity (O'Sullivan and O'Gara, 1992). HCN and siderophores produced by *Pseudomonas* spp. were also involved in their antifungal activity. Voisard et al. (1989) observed that the suppression of black rot of tobacco was due to the production of HCN by *P. fluorescens*, and also HCN induced resistance in the host plant. A leaf colonizing *Pseudomonas* sp. constructed to overproduce HCN protected wheat from a leaf pathogen, *Septocia tritici* more effectively than the parent strain (Flaishman et al., 1996). In the present study, the four selected antifungal *P. fluorescens* isolates were observed to produce HCN *in vitro*, which might have contributed for their biocontrol ability in addition to antibiotics.

P. fluorescens GB 10, GB 27 and *T. viride* pq 1 were significantly effective than Thiram® in control of stem rot. Different isolates of *P. fluorescens* and *Trichoderma* spp. were identified as biocontrol agents of groundnut stem rot and other soil-borne diseases (Podile and Kishore, 2002; Ganesan and Gnanamanickam, 1987). In contrast to the mycelial inhibition in dual cultures, the four *P. fluorescens* isolates differed in their biocontrol ability possibly due to the differences in root colonization and production of antifungal metabolites in natural environments.

Synergism between different biocontrol agents and their compatibility with fungicides was often observed as an effective means for their integration with the existing disease management practices. Combined application of *P. fluorescens* GB 10 or GB 27 and *T. viride* pq 1 resulted in improved biocontrol than the combined application of GB 10 or GB 27 and Thiram®. Jetiyanon and Kloepper (2002) proposed a combinational use of different biocontrol agents for improved and stable biocontrol agents against a complex of diseases. Our results support the earlier observations that a combination of biocontrol agents with different mechanisms of disease control will have an additive effect and results in enhanced disease control compared to their individual application (Guetsky et al., 2002). The present study identified additional biocontrol agents for control of groundnut stem rot, which can be easily and stably integrated into the existing production practices.

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