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A combination of biocontrol agents improves the management of dry root rot *(Macrophomina phaseolina)* in greengram

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Summary. The biocontrol agents *Trichoderma viride* (strains Tv1 and Tv13), *Pseudomonas fluorescens* (Pf1 and Py15) and *Bacillus subtilis* (Bs16) were tested individually and in combination for their effectiveness against root rot of greengram caused by *Macrophomina phaseolina*. As regards the compatibility of the biocontrol agents with each other, *T. viride* strains were not compatible with *B. subtilis* (Bs16), but *P. fluorescens* strains were compatible with *B. subtilis* and *T. viride*. Of the biocontrol agents tested *in vitro* against *M. phaseolina*, combinations of *P. fluorescens*+*T. viride* (Pf1+Tv1, Pf1+Tv13 and Py15+Tv1) inhibited mycelial growth of the pathogen and they also promoted the growth of the greengram seedlings. A combination of Pf1+Tv1 was most effective in reducing root rot incidence under glass-house and field conditions as compared with other single or combined treatments or the untreated control. The activity of the defense-related enzymes peroxidase, polyphenol oxidase and phenyl alanine ammonia lyase was significantly greater in greengram plants treated with a talc based formulation containing Pf1+Tv1 followed by Pf1+Tv13 and Py15+Tv1, than in plants receiving other treatments or the untreated control. Moreover, a combination of Pf1+Tv1 followed by Pf1+Tv13 and Py15+Tv1 significantly increased yield under glass house and field conditions.

Key words: defense enzymes, *Pseudomonas fluorescens, Bacillus subtilis*, *Trichoderma viride*.

Introduction

Greengram (*Vigna radiata* [L.] Wilczek) is one of the most important and widely grown short-duration legumes worldwide. The crop is subject to diseases caused by fungi, bacteria and viruses. Of these diseases, root rot caused by *Macrophomina phaseolina* (Tassi.) Goid causes considerable losses (Raguchander *et al.,* 1993). Management of *M. phaseolina* using chemical fungicides has been the prevailing control method for over fifty years. Though fungicides have shown good results in controlling *M. phaseolina*, fungicide residue is a major problem and causes soil and environmental pollution, and human health hazards. In this context, the biocontrol of this disease may represent an ecofriendly strategy for managing *M. phaseolina* in crop plants. Mixtures of biocontrol agents will also have the advantage of exercising a broad spectrum activity, enhancing the efficacy and reliability of biological control generally and ensuring greater induction of defense enzymes over individual strains.

Single antagonistic strains often result in inconsistent disease control. One of the strategies for overcoming such inconsistent performance is to

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combine two or more beneficial microbes in a biocontrol preparation (Raupach and Kloepper, 1998). Combinations of biocontrol agents have the potential for a more extensive colonization of the rhizosphere, a more consistent expression of beneficial traits under a wider range of soil conditions, and of being antagonistic to a larger number of plant pests or pathogens than biocontrol strains applied individually. A combinatory approach has the potential to overcome problems that occur with individual biocontrol agents (Meyer and Roberts, 2002). Several researchers have tested different biocontrol strains in combination (Droby, 2001). The present study was undertaken to study the effectiveness of combinations of bacterial and fungal biocontrol agents against root rot of greengram and to develop ecofriendly management practices to control the disease.

Materials and Methods

Isolation of pathogen and maintenance of biocontrol agents

The root rot pathogen *M. phaseolina* was isolated from greengram plants showing typical root rot symptoms, and pure cultures of the pathogen were obtained by the single hyphal tip method (Rangaswami, 1972). The biocontrol agents *P. fluorescens* (Pf1 and Py15), *B. subtilis* (Bs16) and *T. viride* (Tv1 and Tv13) were obtained from the Culture collection section, Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, India.

Compatibility of biocontrol agents with each other

Plant growth promoting rhizobacteria (PGPR) strains were tested for their compatibility with each other following the method of Fukui *et al*. (1994). The compatibility of the fungal biocontrol agent with the PGPR strains was tested by their mycelial overgrowth on the PGPR strains without any inhibition zone, using the dual culture technique (Dennis and Webster, 1971).

In vitro testing of individual and combined biocontrol agents against *M. phaseolina*

The strains of the biocontrol agents were tested individually and in combination for their effectiveness against the mycelial growth of *M. phaseolina* by the dual culture technique (Dennis and Webster, 1971). Sterilized filter paper discs were spotted with 6 μl of culture filtrate of one of *P. fluorescens, B. subtilis* and *T. viride*. To test a combination of two biocontrol agents, 3 μl of each agent was spotted on the discs, and for a combination of three biocontrol agents, 2 μl was added. Each disc was placed on one side of a Petri dish (1 cm from the edge) containing a PDA medium (250 g potato, 20 g dextrose, 15 g agar and 1 l distilled water; pH 6.0 to 6.5). A mycelial disc (9 mm) of a seven-day-old culture of *M. phaseolina* was placed on the opposite side of the spot inoculation. Three replications were carried out for each treatment; the dishes were incubated at room temperature, and the inhibition zone was measured.

Effect of biocontrol agents on greengram growth *in vitro*

Preparation of inoculum

Pseudomonas fluorescens and *B. subtilis* strains were grown in conical flasks (250 ml) containing (for *P. fluorescens*) 100 ml of King's broth (KB) (peptone 20 g, di-potassium hydrogen phosphate 1.5 g, magnesium sulphate 1.5 g, glycerol 10 ml, distilled water 1 l; pH 7.0 to 7.2) and (for *B. subtilis*) 100 ml of nutrient broth (NB) (bactopeptone 5 g, beef extract 3 g, sodium chloride 5 g, distilled water 1 l; pH 6.8 to 7.2) for 48 h on a rotary shaker (150 rev min^{-1}) at 28±2°C. Cells were removed by centrifugation at 6000 *g* for 10 min at 4°C and washed in sterile water. The pellet was resuspended in a small quantity of sterile distilled water until to bacterial colonies of 3×10^8 cfu ml^{-1} were obtained. For dual strain inoculation, equal volumes of broth of the two strains were mixed and used for centrifugation (Fukui *et al*., 1994). Strains of the fungal antagonist *T. viride* were multiplied in *Trichoderma* special broth (TSB) by inoculating a disc of actively growing mycelium and incubating for 15 days. Then the mycelial mats along with the spores were harvested by filtering through Whatman No. 1 filter paper and the filtrate was mixed with sterile distilled water to produce colonies of 3×10^8 cfu ml^{-1}. To prepare a bacterial and fungal antagonistic mixture, equal amounts of the suspensions were mixed.

Seed bacterization

Seeds of greengram (cv. Co 6) were surface-sterilized with 2% sodium hypochlorite for 30 s, rinsed

in sterile distilled water and dried overnight. Ten ml of biocontrol inoculum containing 3×10^8 cfu ml^{-1} was placed in a Petri dish. One hundred mg of carboxy methyl cellulose (CMC) was added as an adhesive material. One gram of seeds was soaked in 10 ml of biocontrol suspension for 2 h and air-dried overnight in a sterile Petri dish.

Plant growth-promotion

The plant growth-promoting activity of the biocontrol agents was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Seed bacterization was done as above. Twenty-five seeds were placed on presoaked germination paper. The seeds were held in position with another presoaked germination paper strip and gently pressed. The polythene sheet along with the seeds was then rolled up and incubated in a growth chamber for 10 days. Three replications were carried out for each treatment. The root length and shoot length of individual seedlings were measured and the per cent germination of the seeds was calculated. The seedling vigour index was calculated using the formula, Vigour Index = (mean root length+mean shoot length) × % germination (Abdul Baki and Anderson, 1973).

Preparation of talc based bioformulation

A loopful of isolates of *P. fluorescens* and *B. subtilis* was inoculated into sterilized KB and NB respectively and incubated in a rotary shaker at 150 rpm for 72 h at room temperature (28±2°C). After 72 h, 400 ml of the bacterial broth suspension containing 9 ± 10^8 cfu ml^{-1}, 1 kg of carrier material (talc powder), 15 g calcium carbonate (to adjust the pH to neutral) and 5 g CMC (adhesive) were mixed under sterile conditions following the method described by Nandakumar *et al*. (2001). Isolates of *T. viride* were multiplied in molasses-yeast broth (30 ml molasses; 5 g yeast; plus water to a total volume of 1000 ml). The sterile broth was inoculated with an actively growing mycelial disc (9 mm) and incubated for 15 days. The biomass (3 ± 10^8 cfu ml^{-1}) along with the medium was incorporated into the sterile talc powder carrier material at the rate of 50 ml of suspension per 100 g of talc powder and thoroughly mixed with 500 mg CMC as described by Ramakrishnan *et al*. (1994). The mixture was shade-dried for 12 h and stored in polythene bags. The talc based formulation containing both fungal and bacterial antagonists was prepared by mixing equal amounts of the biocontrol agents at the time of application. In the case of bacterial mixtures, the bacterial strains were grown separately each in its respective broth and the strains were mixed to make up the formulation.

Pot culture study

Seeds were soaked in double the volume of sterile distilled water containing the talc based formulation (10 g kg^{-1} of seed). After 24 h, the suspension was drained off and the seeds were dried under shade for 30 min and used for sowing (Nandakumar *et al*., 2001). Carbendazim at the rate of 2 g kg^{-1} of seed was applied as a chemical fungicide. A pure culture of *M. phaseolina* was introduced into a sand-maize (19:1) medium and incubated for 15 days at room temperature for multiplication (Riker and Riker, 1936). The potting soil (red soil:sand:cow dung manure, 1:1:1 w:w:w) was incorporated with the fungus, and seeds of greengram were surface-sterilized with 0.1% mercuric chloride for 30 s, rinsed three times with sterile distilled water and sown at 20 seeds per pot. Five g of talc-based formulation per kg of soil was added 30 days after sowing. Carbendazim (0.1%) was applied as a soil-drench to the pots.

Assay of defense enzymes

Root samples of greengram were collected at 7-day intervals for 56 days after inoculation with *M. phaseolina* and pre-treatment with different bioformulations and bioformulation combinations. One g of root sample was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm and the supernatant was used to determine peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL).

Peroxidase activity was assayed as described by Hammerschmidt *et al*. (1982) and was expressed as changes in absorbance at 470 nm min^{-1} g^{-1} of fresh tissue. PPO activity was determined following the procedure given by Mayer *et al*. (1965) and was expressed as changes in absorbance at 470 nm min^{-1} g^{-1} of fresh tissue. PAL activity was assayed following the method of Ross and Sederoff (1992) and was expressed as nmoles of cinnamic acid min^{-1} g^{-1} of fresh tissue.

Field study

Seeds were soaked in double the volume of sterile distilled water containing the talc-based formulation (10 g kg^{-1} of seed). After 24 h, the suspension was drained off and the seeds were dried under shade for 30 min and sown (Vidhyasekaran *et al.*, 1997). Carbendazim at the rate of 2 g kg^{-1} of seed was applied as seed treatment. In the field, biocontrol agents were applied at 2.5 kg ha^{-1} 30 days after sowing. Carbendazim (0.1%) was applied as a soil-drench to the field.

Disease and yield assessment

The trial was laid out with eight treatments which were selected from the pot culture experiments replicated thrice in a randomized block design. Disease incidence was recorded 30, 45 and 60 days after sowing and % disease incidence was calculated using the formula: % disease incidence = (No. of infected plants/total No. of plants)×100. The yield attributing parameters number of pods/plant, number of seeds/pod, root length and shoot length were recorded. The grain yield after harvesting the crop was also recorded.

Statistical analysis

The glasshouse and field-trial data were analyzed using the IRRISTAT version 92-1 programme developed by the biometrics unit, International Rice Research Institute, The Philippines. Percent infection, growth and yield data were analyzed independently by trial. Data were subjected to analysis of variance (ANOVA). Disease incidence data were arcsine transformed before analysis. The treatment means were compared by Duncan's multiple range test (DMRT) (Gomez and Gomez, 1984).

Results

Compatibility among biocontrol agents

Strains of *P. fluorescens* and *B. subtilis* were tested *in vitro* for compatibility. Strains that overgrew each other were compatible with each other, whereas strains that were separated by an inhibition zone were incompatible. No inhibition zone formed between *T. viride* (Tv1 and Tv13) and *P. fluorescens* (Pf1 and Py15) indicating that these strains were compatible.

Effect of biocontrol strains on radial mycelial growth of *M. phaseolina*

All treatments significantly reduced mycelial growth of *M. phaseolina*. The combinations Pf1+Tv1, Pf1+Tv13 and Py-15+Tv1 achieved a higher percent inhibition, by 39.4, 38.9 and 38.3% respectively, than the single strains Pf1, Py15, Bs16, Tv1 and Tv13, whose inhibition was only 20.5, 16.1, 18.9, 20.6 and 20.6% respectively. Other combinations: Bs16+Tv1; Bs16+Tv13; Pf1+Py15+Bs16; Pf1+Bs16+Tv1; Pf1+Bs16+Tv13; Py15+Bs16+Tv1 and Py15+Bs16+Tv13 achieved a percent inhibition of *M. phaseolina* that was equal to, or lower than, the inhibition achieved by the strains applied singly. The combinations Pf1+Tv1; Pf1+Tv13 and Py15+Tv1 had inhibition rates comparable with those of the fungicide carbendazim (39.4%) (Table 1).

Effectiveness of PGPR and *T. viride* strains in promoting plant growth

The biocontrol combinations Pf1+Tv1; Pf1+Tv13 and Py15+Tv1 produced greengram seedlings with a significantly higher vigour index, 3943.3, 3825.6 and 3706.1, respectively, than did the individual biocontrol strains Pf1, Py15, Bs16, Tv1 and Tv13, whose vigour index was only 3030.6, 2807.0, 2921.6, 3040.4 and 2881.6 respectively. Interestingly, however, other combinations, Bs16+Tv1; Bs16+Tv13; Pf1+Py15+Bs16; Pf1+Bs16+Tv1; Pf1+Bs16+Tv13; Py15+Bs16+Tv1 and Py15+Bs16+Tv13 produced seedlings with a lower vigour index and a lower germination percentage, than seedlings treated with the single strains. The untreated control seedlings had the lowest vigour index, 2409.4 (Table 2).

Glasshouse study

Those treatments that had been most effective in inhibiting *M. phaseolina* mycelial growth and in promoting greengram seedling growth were selected for pot culture studies. Of these treatments, a combination of strains reduced the disease incidence of root rot more strongly than did the individual strains. Seed treatment and soil application of Pf1+Tv1; Pf1+Tv13 and Py15+Tv1 achieved the lowest root rot incidence, with reductions of 85.7, 82.3 and 78.6% respectively over the control 60 days after sowing, compared with reductions of 57.0, 63.0, 59.2, 57.0 and 63.0 percent with Pf1,

Table 1. Effectiveness of *Pseudomonas fluorescens*, *Bacillus subtilis* and *Trichoderma viride* in inhibiting radial mycelial growth of *Macrophomina phaseolina*.

Treatment strain(s)[a]	Mycelial growth of the pathogen (mm)[b]	Inhibition over control (%)
Pf1	71.5 de	20.5
Py15	75.5 efg	16.1
Bs16	73.0 ef	18.9
Tv1	71.5 de	20.6
Tv13	71.5 de	20.6
Pf1+Bs16	65.5 c	27.2
Pf1+Tv1	54.5 a	39.4
Pf1+Tv13	55.0 a	38.9
Py15+Bs16	68.5 cd	23.9
Py15+Tv1	55.5 a	38.3
Py15+Tv13	60.5 b	32.8
Bs16+Tv1	74.0 efg	17.8
Bs16+Tv13	73.0 ef	18.9
Pf1+Py15+Bs16	71.5 de	20.6
Pf1+Bs16+Tv1	75.5 efg	16.1
Pf1+Bs16+Tv13	76.0 fg	15.6
Py15+Bs16+Tv1	77.0 fg	14.4
Py15+Bs-16+Tv13	77.5 g	13.9
Carbendazim	54.5 a	39.4
Control	90.0 h	00.0

[a] Pf1, *P. fluorescens* strain Pf1; Py15, *P. fluorescens* strain Py15; Bs16, *B. subtilis* strain 16; Tv1, *T. viride* strain Tv1; Tv13, *T. viride* strain Tv13.

[b] Values are the means of three replications. Means followed by a common letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

Py15, Bs16, Tv1 and Tv13 applied individually (Table 3). The highest percent increase in grain yield was with the combination treatments Pf1+Tv1; Pf1+Tv13 and Py15+Tv1, which produced a significantly greater increase in grain yield than did the individual strains Pf1, Py15, Bs16, Tv1 and Tv13. The highest yield was achieved with Pf1+Tv1 (9.1 g plant^{-1}) which differed significantly from all other treatments, as well as from the untreated control which was only 3.2 g plant^{-1} (Table 4). The results suggest that disease inhibition by a combination of strains is related to an *in vitro* interaction between those strains.

Induction of defense related enzymes by biocontrol agents in greengram

A greater activity of PO and PPO was recorded in all the plants receiving the biocontrol treatment as compared with untreated plants. The increase lasted for 21 days with all biocontrol agents, after which there was a decline. Interestingly, when the biocontrol agents were incorporated into the soil 30 days after sowing, increased enzyme activity occurred for up to 19 days after application, after which there was a decline. A mixture of biocontrol agents caused a greater PO and PPO activity than did the untreated control or individual biocontrol agents. The greatest increase in PO activity was produced by combinations Pf1+Tv1 (80.6%); Pf1+Tv13 (77.6%) and Py15+Tv1 compared with lower increases by the individual strains Pf1 (68.7%); Py15 (67.2%); Bs16 (68.9%); Tv1 (69.1%) and Tv13 (68.3%). Similarly, the greatest activity of PPO occurred with Pf1+Tv1; Pf1+Tv13 and Py15+Tv1, compared with individual biocontrol strains which caused less activity (Fig. 1, 2). Levels of PAL increased significantly with all biocontrol treatments for up to 21 days, after which there was a decline. When the biocontrol agents were applied to the soil 30

Table 2. Effectiveness of seed treatment with *Pseudomonas fluorescens*, *Bacillus subtilis* and *Trichoderma viride* on plant growth promotion under *in vitro* conditions.

Treatment[a]	Germination (%)[bc]	Shoot length (cm)[b]	Root length (cm)[b]	Vigour index[b]
Pf1	89.3 (70.9) f	15.8 d-g	18.1 hi	3030.6 f
Py15	86.0 (68.0) i	15.1 f-i	17.5 ij	2807.0 gh
Bs16	88.3 (70.0) g	15.1 ghi	18.0 hi	2921.6 fg
Tv1	88.6 (70.3) fg	16.1 def	18.2 ghi	3040.4 f
Tv13	87.3 (69.1) h	15.5 e-i	17.5 ij	2881.6 fg
Pf1+Bs16	92.0 (73.6) d	17.6 c	19.6 def	3424.2 d
Pf1+Tv1	96.3 (79.0) a	19.2 a	21.7 a	3943.3 a
Pf1+Tv13	96.0 (78.5) ab	18.7 ab	21.2 ab	3825.6 ab
Py15+Bs16	90.6 (72.2) e	16.6 d	19.1 efg	3232.2 e
Py15+Tv1	95.6 (78.0) b	18.1 bc	20.7 bc	3706.1 bc
Py15+Tv13	94.6 (76.6) c	17.5 c	20.1 cd	3562.2 cd
Bs16+Tv1	82.0 (64.9) k	14.9 ghi	16.8 jk	2604.3 i
Bs-16+Tv13	82.6 (65.4) k	14.8 hi	16.8 jk	2612.3 i
Pf1+Py15+Bs16	85.6 (67.7) i	16.1 def	18.8 fgh	2987.1 f
Pf1+Bs16+Tv1	80.0 (63.4) l	14.7 i	17.0 jk	2533.1 ij
Pf1+Bs16+Tv13	84.3 (66.7) j	14.9 ghi	17.0 jk	2693.2 hi
Py15+Bs16+Tv1	80.3 (63.7) l	15.3 e-i	17.0 jk	2596.7 i
Py15+Bs16+Tv13	80.0 (63.4) l	15.7 d-h	16.8 jk	2604.0 i
Carbendazim	94.6 (76.6) c	16.3 de	19.8 de	3407.4 d
Control	78.0 (62.0) m	14.6 i	16.3 k	2409.4 j

[a] For strain abbreviations see Table 1.
[b] See Table 1.
[c] Data in parentheses are arcsine transformed values.

Table 3. Effectiveness of *Pseudomonas fluorescens*, *Bacillus subtilis* and *Trichoderma viride* against root rot incidence of greengram in pot culture.

Treatment strain(s)[a]	Germination[b]		Disease incidence[b]	
	%[c]	Increase over control (%)	%[c]	Decrease over control (%)
Pf1	85.3 (67.5) de	13.8 e	30.3 (33.4) i	57.0 i
Py15	84.3 (66.7) f	12.4 h	35.7 (36.7) g	63.0 g
Bs16	84.7 (67.0) f	12.9 g	32.3 (34.7) h	59.2 h
Tv1	85.0 (67.2) e	13.3 f	30.3 (33.4) i	57.0 i
Tv13	84.7 (66.9) e	12.9 g	32.3 (34.7) h	63.0 g
Pf1+Bs16	85.7 (67.7) d	14.2 d	27.3 (31.5) f	68.7 f
Pf1+Tv1	97.7 (81.2) a	30.2 a	12.5 (20.7) b	85.7 b
Pf1+Tv13	92.3 (73.9) b	23.1 b	15.5 (23.2) c	82.3 c
Py15+Bs16	85.3 (67.5) de	13.8 e	27.3 (31.5) f	68.7 f
Py15+Tv1	89.3 (70.9) c	19.1 c	18.7 (25.6) d	78.6 d
Py15+Tv13	85.7 (67.7) d	14.2 d	21.3 (27.5) e	75.6 e
Carbendazim	92.3 (73.9) b	23.1 b	8.5 (16.9) a	90.3 a
Control	75.0 (60.0) g	0.0i	87.3 (69.1) j	0.0 j

[a] For strain abbreviations see Table 1.
[b] See Table 1.
[c] Data in parentheses are arcsine transformed values.

Table 4. Effect of *Pseudomonas fluorescens*, *Bacillus subtilis* and *Trichoderma viride* on yield of greengram under pot culture conditions.

Treatment strain(s)[a]	No. of pods plant^{-1} [b]	No. of seeds pod^{-1} [b]	Grain yield g plant^{-1} [b]	Increase over control[b] (%)
Pf1	11.5 gh	10.0 g	4.5 g	43.0
Py15	11.0 hi	9.5 gh	4.1 hi	29.1
Bs16	10.5 i	9.0 h	3.7 j	16.8
Tv1	11.0 hi	10.0 g	4.3 gh	36.1
Tv13	10.5 i	9.5 gh	3.9 ij	23.4
Pf1+Bs16	13.0 de	12.0 de	6.1 e	93.0
Pf1+Tv1	15.0 a	15.5 a	9.1 a	187.7
Pf1+Tv13	14.5 ab	13.5 b	7.6 b	140.5
Py15+Bs16	12.0 fg	11.5 ef	5.4 f	70.0
Py15+Tv1	14.0 bc	13.0 bc	7.1 c	125.3
Py15+Tv13	13.5 cd	12.5 cd	6.6 d	108.9
Carbendazim	12.5 ef	11.0 f	5.4 f	70.3
Control	9.0 j	9.0 h	3.2 k	00.0

[a] For strain abbreviations see Table 1.
[b] See Table 1.

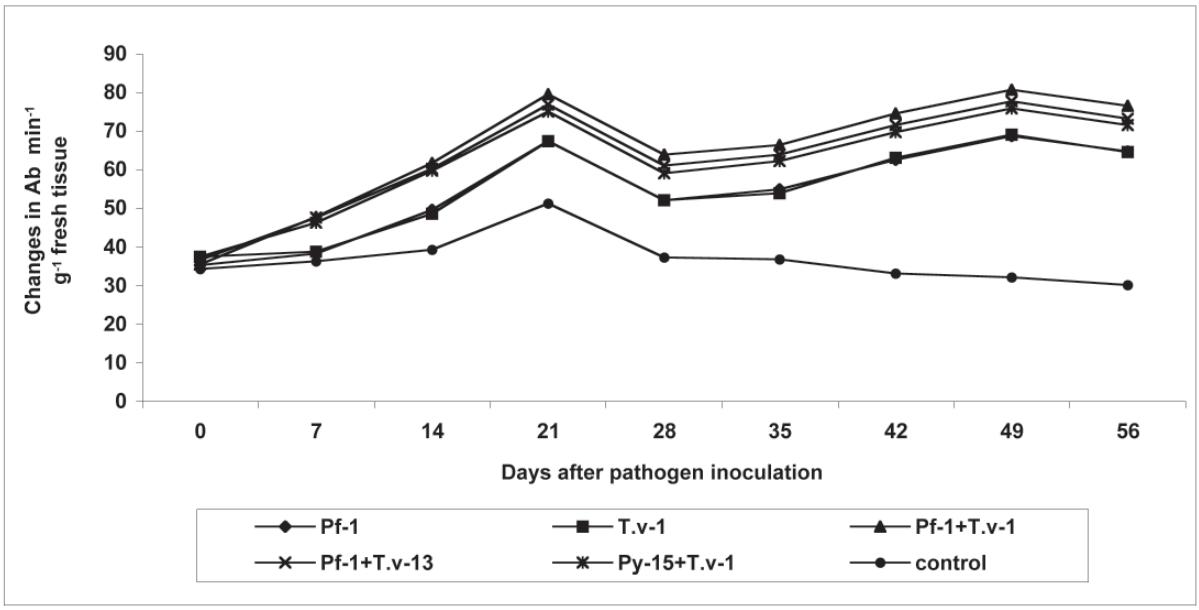

Fig 1. Peroxidase (PO) activity in greengram plants treated with *Pseudomonas fluorescens*, *Bacillus subtilis* and *Trichoderma viride* against root rot under greenhouse conditions. For strain code see Table 1.

days after sowing, PAL activity was again enhanced for up to 19 days after which there was a decline. In general, a combination of biocontrol agents produced a greater PAL activity than did single agents. PAL activity was greatest with the combinations Pf1+Tv1 (97.8%); Pf1+Tv13 (95.0%) and Py15+Tv1 (92.9%), followed by Py15+Tv13 (91.0%); Pf1+Bs16 (89.9%) and Py15+Bs16 (88.7%). A lower increase in PAL activity was recorded with the individual biocontrol agents Pf1 (79.23); Py15 (79.37); Bs16 (78.91); Tv1 (8036) and Tv13 (79.96) (Fig. 3).

Field study

The greatest reduction in root rot incidence was observed with the bioformulation mixtures Pf1+Tv1; Pf1+Tv13 and Py15+Tv1 (81.8, 78.7 and 77.5% respectively) followed by Py15+Tv13 (73.0%); Pf1+Bs16 (68.8%) and Py15+Bs16 (64.6%) as compared with the untreated control. The biocontrol agents not only reduced disease incidence

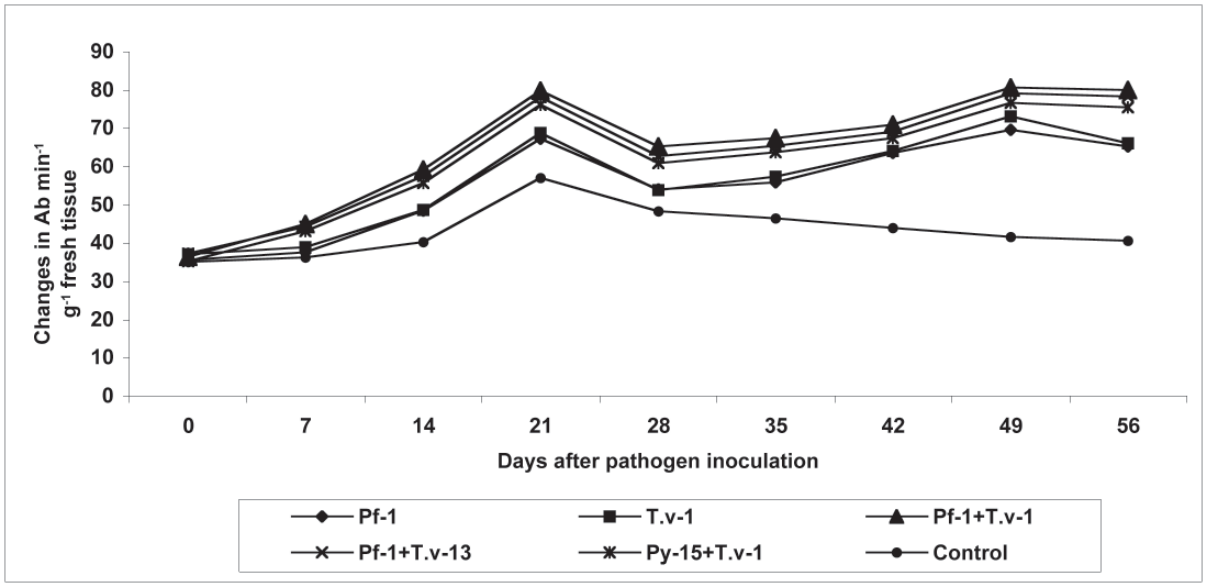

Fig. 2. Polyphenol oxidase (PPO) activity in greengram plants treated with *Pseudomonas fluorescens*, *Bacillus subtilis* and *Trichoderma viride* against root rot under greenhouse conditions.

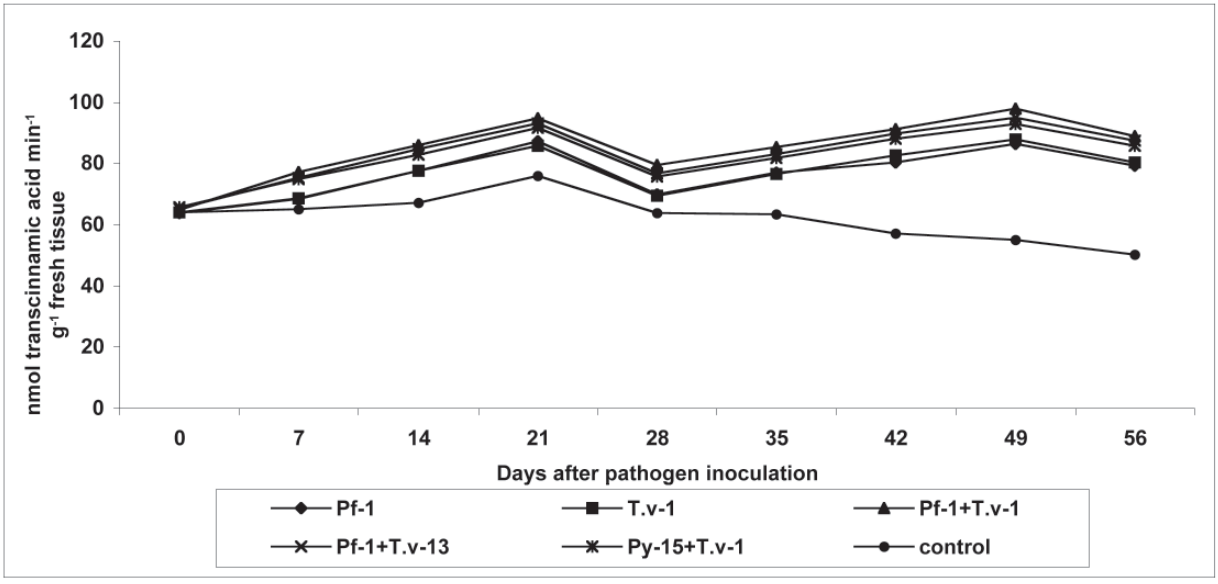

Fig. 3. Phenylalanine ammonia lyase (PAL) enzyme activity in greengram plants treated with *Pseudomonas fluorescens*, *Bacillus subtilis* and *Trichoderma viride* against root rot under greenhouse conditions.

Table 5. Effectiveness of *Pseudomonas fluorescens*, *Bacillus subtilis* and *Trichoderma viride* against root rot incidence of greengram under field conditions.

Treatment strain(s) [a]	Days after sowing [b,c]					Grain yield	
	30	45	60	Mean	Reduction over control (%)	Kg ha^{-1}	Increase over control (%)
Pf1+Bs16	4.0 (11.5) e	5.3 (13.3) f	11.1 (19.1) de	7.8 [f]	68.8	543.3 [d]	36.8
Pf1+Tv1	1.0 (5.7) b	2.6 (9.3) b	7.5 (15.4) b	4.5 [b]	81.8	833.3 [a]	58.7
Pf1+Tv13	1.6 (7.3) c	3.0 (9.3) c	8.5 (16.4) b	5.3 [c]	78.7	670.3 [b]	48.8
Py15+Bs16	4.3 (12.0) f	6.6 (14.9) g	12.6 (20.5)	8.8 [g]	64.6	416.7 [e]	17.3
Py15+Tv1	1.6 (7.3) c	3.3 (10.5) d	9.1 (17.0) bc	5.6 [d]	77.5	616.7 [c]	44.1
Py15+Tv13	2.6 (9.3) d	4.3 (12.0) e	10.3 (18.2) cd	6.7 [e]	73.0	543.3 [d]	36.7
Carbendazim	0.0 (1.0) a	1.3 (6.5) a	4.9 (12.1) a	2.7 [a]	89.3	572.0 [d]	39.8
Control	10.6 (19.00) g	21.3 (27.5) h	35.6 (36.4) f	24.9 [h]	0.0	343.3 [f]	00.0

[a] For strain abbreviations see Table 1.
[b] See Table 1.
[c] See Table 1.

but also increased yield: by 58.7% with Pf1+Tv1; by 48.8% with Pf1+Tv13 and by 44.1% with Py15+Tv13 as compared with the untreated control (Table 5).

Discussion

Multiple strain mixtures of microbial agents have been employed successfully against several plant pathogens in earlier studies. Mixtures include those of bacteria and fungi (Leibinger *et al.,* 1997), and those of bacteria and yeast (Janisiewicz and Bors, 1995). Against this background, talc-based formulations containing a mixture of bacterial biocontrol agents were tested against the greengram root rot agent *M. phaseolina in vitro* and in the field. Several authors have suggested that combinations of introduced biocontrol agents have to be compatible with each other for better and more consistent disease suppression (Raaijmakers *et al.,* 1995). In the present study, the *P. fluorescens* strains Pf1 and Py15 were compatible with *B. subtilis* and with *T. viride* strains Tv1 and Tv13. An important prerequisite for the effectiveness of strains appears to be the compatibility of the co-inoculated microorganisms (Raaijmakers *et al.,* 1995). Georgakopoulos *et al*. (2002) identified potentially useful and compatible antagonist combinations by cultivating antagonists in a liquid medium containing a substrate previously used by other antagonists of the prospective combination. Also, a single biocontrol strain may not grow equally well in a variety of environmental conditions (Fukui *et al*., 1994). In the present study, the combinations Pf1+Tv1; Pf1+Tv13 and Py15+Tv1 produced the greatest reduction in *M. phaseolina* mycelial growth, followed by Py15+Tv13; Pf1+Bs16 and Py15+Bs16 as compared with the agents applied singly. The lower mycelial pathogen growth may be due to antibiotics produced by the biocontrol agents, as has been reported by many workers (Ramamoorthy and Samiyappan, 2001, Viswanathan and Samiyappan, 2001). By contrast, some combinations, Bs16+Tv1; Bs16+Tv13; Pf1+Py15+Bs16; Pf1+Bs16+Tv1; Pf1+Bs16+Tv13; Py15+Bs16+Tv1 and Py15+Bs16+Tv13, inhibited mycelial growth no better or less than each strain individually. This may have been due to an incompatible reaction between strain Bs16 of *B. subtilis* and strains Tv1 and Tv13 of *T. viride*.

The combinations Pf1+Tv1; Pf1+Tv13 and Py15+Tv1, and to a less extent Py15+Tv13; Pf1+Bs16 and Py15+Bs16, increased greengram plant growth in pot culture and in the field more than did individual biocontrol strains. Similarly, a combined application of *T. viride+P. fluorescens* increased root and shoot length in chilli (Manoranjitham *et al*., 2000) and black gram (Babu and Seetharaman, 2002). Mixtures of PGPR strains achieved better disease suppression of sheath

blight in rice than when they were applied singly (Nandakumar *et al*., 2001). In contrast, the combinations Bs16+Tv1; Bs16+Tv13; Pf1+Py15+Bs16; Pf1+Bs16+Tv1; Pf1+Bs16+Tv13; Py15+Bs16+Tv1 and Py15+Bs16+Tv13 produced greengram plants with a lower vigour index and germination percentage than did each strain applied alone. This may have been due to an incompatibility between *B. subtilis* strain Bs16 and *T. viride* strains Tv1 and Tv13.

Biocontrol agents have a direct antagonistic activity not only by producing various metabolites, but also by inducing defense enzymes, which have recently been found to be a new way whereby plants defend themselves from pathogen attack (Bharathi *et al*., 2004). In the present study, greengram plants infected with *M. phaseolina* and treated with the bioformulation combinations Pf1+Tv1; Pf1+Tv13 and Py15+Tv1 and to a less extent by Py15+Tv13; Pf1+Bs16 and Py15+Bs16, had higher levels of PO and PPO activity than infected plants treated with single biocontrol agents.

Increased levels of PO and PPO have been shown to result from a number of resistant interactions involving plant pathogenic fungi, bacteria and viruses (Kandan *et al*., 2002). PAL was reported to be induced in cucumber by fluorescent pseudomonads against *P. aphanidermatum* (Chen *et al*., 2000), in tomato against *Fusarium oxysporum* f. sp. *lycopersici* (Ramamoorthy *et al*., 2002) and in bean against *Botrytis cinerea* (Zdor and Anderson, 1992). In the present study, plants infected with *M. phaseolina* and treated with the bioformulation combinations Pf1+Tv1; Pf1+Tv13 and Py15+Tv1 and to a less extent Py15+Tv13; Pf1+Bs16 and Py15+Bs16 had a greater PAL activity than infected plants treated with individual biocontrol agents. The results suggest that disease suppression by these combinations of biocontrol agents in both the pot culture and the field studies, was related to the *in vitro* interaction that occurred between the strains.

The biocontrol agents not only controlled dry root rot but also promoted plant growth, and this gives them an advantage over the use of chemical fungicides against root rot in disease management. The work has to be intensified to study the mechanisms involved in disease control by mixtures of biocontrol agents.

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