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Full Length Research Paper

Effects of single and combined inoculations of selected *Trichoderma* and *Bacillus* isolates on growth of dry bean and biological control of *Rhizoctonia solani* damping-off

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Six *Trichoderma* isolates, *Trichoderma atroviride* strain 3A, *T. atroviride* strain 6, *Trichoderma harzianum*, strain SY, *Trichoderma pseudokoningii*, an unidentified strain *Trichoderma* sp. strain 2F and *T. harzianum* strain kmd and three *Bacillus subtilis* isolates, *B. subtilis* B69, *B. subtilis* B77 and *B. subtilis* B81, were tested *in vivo*, singly and each in combination for growth promotion of dry beans and biological control of damping-off caused by *Rhizoctonia solani* in cucumber. All fungal and bacterial isolates were applied as seed treatments in greenhouse and rhizotron studies. Greenhouse trials showed that combined inoculations of *T. atroviride* strain 6 and *B. subtilis* B69 gave the highest growth promotion of bean in terms of seedling dry biomass (43.0% over uninoculated control). Rhizotron studies supported these findings, where it was shown that root biomass and root area were increased. However, results obtained for bean yield trials were inconsistent and had no correlation with the seedling trials (P = 0.87 and P = 0.35). No increase was obtained in protein or fat content of bean seed for any of the selected isolates and/or their combinations tested in two separate greenhouse yield trials. In the biological control trials, single inoculations of *T. harzianum* strain kmd, *T. atroviride* strain 3A and *T. harzianum* strain SY gave the highest percentage survival of cucumber plants in the greenhouse. None of the *Trichoderma* plus *Bacillus* combinations were better than the single inoculations of *T. harzianum* strain kmd, *T. atroviride* strain 3A and *T. harzianum* strain SY. The performances, particularly of *B. subtilis* B69 and B81 were enhanced when combined with *T. atroviride* strain 3A, *T. atroviride* strain 6, *T. harzianum* strain SY or *T. harzianum* strain kmd. The performance of each of the *Trichoderma* and *Bacillus* combinations was better than the *Bacillus* isolates used alone. This study showed that there was potential in using mixtures of *Trichoderma* and *Bacillus* for improving plant growth and disease control.

Key words: *Bacillus*, dry bean, plant growth promotion, *Trichoderma.*

INTRODUCTION

Increases in crop yield and plant disease control have been observed following seed and/or seedling treatments Prakash, 1996; Kim et al., 1997; Utkhede et al., 1999; Harman, 2000; Rabeendran et al., 2000; Egamberdiyeva, 2007; Kohler et al., 2007; Felici et al., 2008). When inoculated, these organisms act via a series of mechanisms to control plant pathogens leading to a decrease in disease levels with a corresponding increase in crop yield (Podile and Prakash, 1996; Elad, 2000).

The genus *Trichoderma* belongs to the Deuteromycetes (Samuels, 1996) class of fungi and has

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been exploited as biological control agents against a range of plant pathogenic fungi due to their antagonistic properties towards plant pathogens (Papavizas, 1985; Chet, 1987). Some strains of *Trichoderma* have been widely used as biological control agents as well as plant growth promoters (Ousley et al., 1994; Harman, 2000; Rabeendran et al., 2000). *Bacillus* spp. is Gram-positive bacteria and numerous strains have shown biological control activity on a wide range of crops (Cook and Baker, 1983; Leifert et al., 1995; Podile and Prakash, 1996; Utkhede et al., 1999). Several isolates have also been found to promote plant growth (Probanza et al., 1996) and in some cases, increase nodulation in legumes (Podile, 1995). These *Bacillus* spp. strains are appealing candidates as inoculants for biological control against plant pathogens due to their ability to form endospores that are tolerant to heat and desiccation, giving them extended shelf lives compared with other biological control agents such as *Pseudomonas* spp. (Petras and Casida, 1985; Young et al., 1995).

Several reports in the literature indicate that combinations of biological control agents and plant growth promoting rhizobacteria (PGPR) can increase disease suppression (Guetsky et al., 2002), improve crop yields and enhance nutrient uptake by plants (Alagawadi and Gaur, 1988; Alagawadi and Gaur, 1992) over single organism inoculations. For example, Alagawadi and Gaur (1992) reported that combined inoculations of *Azospirillum brasilense* and *Pseudomonas striata* or *Bacillus polymyxa*, improved nitrogen and phosphorus uptake and consequently, increased sorghum grain yield compared with organisms inoculated individually. In a separate study, Jisha and Alagawadi (1996) reported an increase in sorghum (*Sorghum bicolor* L. Moench) yield when inoculated with combined formulations of *B. polymyxa* or *P. striata* and *Trichoderma harzianum* Rifai. However, information pertaining to combined inoculations of *Trichoderma* and *Bacillus* species on plant growth and especially on disease control appears to be very sparse, even though both *Bacillus* and *Trichoderma* species are well known for their biological control and plant growth promoting properties.

This study tested the hypothesis that combinations of selected *Trichoderma* and *Bacillus* isolates could enhance disease control and/or improve seedling growth, establishment and overall yield. Using six *Trichoderma* and three *Bacillus* isolates as an example, the hypothesis was tested under greenhouse conditions to evaluate the effect of single and combined inoculations of these two groups of organisms on growth promotion of dry beans and biological control of *Rhizoctonia solani* Kühn damping-off in cucumber.

MATERIALS AND METHODS

Sources of fungal and bacterial isolates

Six *Trichoderma* and three *Bacillus* isolates were used in this study. The *Trichoderma* isolates were *Trichoderma atroviride* P. Karsten (strain 3A); *T. harzianum* Rifai (strain SY); *Trichoderma pseudokoningii* Rifai, *T. atroviride* P. Karsten (strain 6), *Trichoderma* sp. strain 2F (unidentified strain) and *T. harzianum* Rifai strain kmd. Three *Bacillus subtilis* isolates were also used. These were Isolates B69, B77 and B81. The *Trichoderma* isolates were obtained from the Discipline of Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, South Africa. *T. atroviride* (strain 3A and 6) were previously found to exhibit biological control properties against *R. solani* (Yobo et al., 2004). The three *B. subtilis* strains were isolated and screened for biological control and growth promotion properties by Kubheka (2003). *B. subtilis* Isolates B69 and B81 were reported to have biological control properties against *R. solani,* while Isolate B77 was reported to exhibit growth promotion properties (Kubheka, 2003).

Preparation of *Trichoderma* and *Bacillus* inocula

The *Trichoderma* isolates were all grown and formulated by Plant Health Products, Nottingham Road, South Africa, according to a protocol used for commercial production of *Trichoderma* biological control agents. Kaolin was used as a carrier and each of the formulated isolates contained approximately 10^8 spore/g (M. Morris, Plant Health Products, (Pty) Ltd., Nottingham Road, Republic of South Africa; personal communication).

The three *Bacillus* isolates, B69, B77 and B81 were cultured separately in 250 ml conical flasks containing 100 ml of sterilized tryptone soy broth (Merck) medium. Each flask was inoculated with a loopful of *Bacillus* isolate cultured on tryptone soy agar (Merck) (30°C, 48 h). Three replicates were made for each isolate and incubated at 30°C for 72 h in a water bath shaker at 150 rpm (GFL® 1083, Labortechnik). Cell suspensions were centrifuged at 9000 *g* for 20 min (Beckman J2 HS centrifuge). Cell pellets were then, resuspended and washed twice with sterile distilled water. Final cell pellets were diluted with sterile distilled water to approximately 500 ml. Cell numbers were determined by dilution plate technique and adjusted to approximately 10^9 cfu/ml for each of the *Bacillus* isolate.

Sources of seeds

Dry bean (*Phaseolus vulgaris* L.) cv PAN 148 seeds were used for the growth promotion study. The untreated seeds were obtained from Pannar Seeds (Pty) Ltd., Greytown, Republic of South Africa. Seeds of cucumber (*Cucumis sativus* L.) cv. Ashley were obtained from Starke Ayres Seed Company Ltd., Republic of South Africa. An appropriate number of cucumber seeds (which had previously been treated with the fungicide thiram) were washed with distilled water seven times to considerably reduce fungicide residues on seeds. The washed seeds were air-dried under laminar flow bench overnight and tested with the pathogen in the greenhouse before use.

Seed treatment procedure: *Trichoderma*, *Bacillus* and their combinations

Seed treatments were performed as previously described by Yobo et al. (2010). Briefly, Kaolin formulations of each *Trichoderma* isolate (4 g) were separately mixed with 2% (w/v) sterile carboxymethyl cellulose (CMC) sticker suspensions (20 ml) to form slurry. Approximately 120 dry bean seeds were mixed into each slurry suspension, allowing for a 30 min contact period before being removed and air-dried on a laminar flow bench for 12 to 18 h prior to use. Inoculant densities were determined using a dilution plating technique with an average of 10^6 cfu/seed being achieved.

Seed treatments with the *Bacillus* isolates were prepared as

described earlier for the *Trichoderma* isolates. An average inoculant density of 10^6 and 10^7 cfu/seed for each *Bacillus* isolate was achieved. For each of the *Trichoderma*-*Bacillus* combination treatment, *Trichoderma* formulations in kaolin powder were separately mixed with the respective *Bacillus*-CMC suspension prior to seed treatment.

Growth promotion trials with dry bean

Treated dry bean seeds were planted into Speedling® 24 trays (24 cells per tray; 37 x 37 mm wide and 61 mm deep) filled with composted pine bark growth medium (Seedling mix, Gromed, South Africa). A total of 28 treatments, made up of six *Trichoderma* and three *Bacillus* isolates and their combinations were planted. Control seeds were treated with kaolin only. Three replicate trays were made for each treatment. The trays were watered with tap water, placed in a germination room (20 to 24°C) for 2 days and subsequently, moved to a polycarbonate greenhouse tunnel (22 to 26°C) and arranged in a randomised block design where they were irrigated three times daily by micro jet overhead irrigation (Inverted mini wobbler, Sennenger, U.S.A). The irrigation water was maintained at 20°C by means of a temperature controlled heating system (Pro Heat 2000 Plus, Republic of South Africa) and was supplemented with NPK soluble fertilizer (3:1:3(38) complete) (Ocean Agriculture, Mulders Drift, Republic of South Africa) at a rate of 1 g/l. Growth of seedlings was monitored for 5 weeks. Thereafter, seedlings were harvested at their base at soil level, placed in a paper bag and dried at 70°C for 48 h to determine the total dry biomass of seedlings per plot (tray). Only above-ground stems and leaves were weighed. The experiment was repeated and results pooled for statistical analysis.

Rhizotron studies

To confirm whether the result obtained in seedling trials could be reproduced, one *Trichoderma* isolate (*T. atroviride* strain 6), the three *Bacillus* isolates (B69, B77 and B81) and a combination of *T. atroviride* strain 6 and *Bacillus* isolate B69 were used in rhizotron studies to assess their effect on root and shoot growth of dry bean seedlings. These treatments were chosen based on the seedling trials results. Briefly, the rhizotrons were made out of two plexiglass (100 x 150 mm) plates held together with butterfly screws and separated by a silicone tube spacer (15 mm diameter). The nature and design of the rhizotrons was similar to that described by James et al. (1985). Using a small scoop, the rhizotrons were filled with Umgeni sand that had been previously sifted (2 mm pore size sieve) and steam pasteurised (100°C at 40 pounds pressure) for 60 min. Dried bean seeds were treated as previously described and for each treatment, four rhizotrons were planted with one seed per rhizotron. Each rhizotron was covered with aluminum foil to prevent daylight from reaching the roots, watered with tap water and left in a germination room for 2 days. They were then moved into a growth chamber maintained at 18 to 25°C and 60% relative humidity (Controlled Environment Research Unit, University of KwaZulu-Natal, Republic of South Africa). A 12 h daylight period was maintained with a light intensity of 302.03 PAR/$\mu mol.m^2.s^1$ being achieved. On germination, each seedling was watered daily (25 ml) with NPK soluble fertilizer [3:1:3(38) complete] at a rate of 0.5 g/l. The volume of water was increased to 50 ml per rhizotron after 2 weeks and subsequently, to two watering a day (mornings and evenings) from the third week till the end of the experiment. Seedlings growth was monitored for 5 weeks.

Root area measurements (image analysis)

Replicate seedlings from each treatment and rhizotron were harvested at the base of the plant after 5 weeks of growth. The roots were carefully washed five times in basins containing tap water, placed in plastic bags and refrigerated until root area measurements could be performed. Root samples from replicate treatments were finely spread on a scanner and covered with a graph paper to allow for calibration of the system. Images were then captured, calibrated and root area measurements taken using Soft Imaging System (SIS®) 3.0 image analysis software. Four measurements were made per replicate root sample and the mean area measurement determined.

Shoot and root dry biomass measurements

Roots (after image analysis) and shoots of seedlings from each rhizotron were both dried at 70°C for 48 h in an oven and their respective dry biomass were determined using a laboratory weighing machine (OHAUS Precision Plus, model 34BL99, Dynamics Corporation of America, New Hartford, Connecticut, USA). The experiment was repeated twice and results pooled for statistical analysis.

Dry bean yield trials

Two successive greenhouse trials were established to determine the effect of *Trichoderma* and *Bacillus* isolates as well as their combinations on dry bean yields. Plastic growing bags (5 l volume) were filled with approximately 4 l of composted pine bark growing medium. Dried bean seeds were treated as previously described. A total of 28 treatments, including an untreated control were planted. The treatments comprised of six *Trichoderma* isolates and three *Bacillus* isolates assessed individually and in combinations. For each treatment, two seeds were planted into each of the four plastic bags (four replicates per treatment) giving a total of 112 plastic bags. The bags were arranged in a randomised block design in a polycarbonate greenhouse tunnel maintained between 22 to 26°C and drip irrigated twice a day. The irrigation water was supplemented with NPK soluble fertilizer (3:1:3(38) complete) at a rate of 1 g/l and plant growth was monitored until harvest. To avoid possible competition between plants, seedlings were thinned to one plant per plastic bag one week after germination. Bean pods were allowed to mature and dry completely before being harvested. Once dried, bean pods from each plant were harvested separately. The pods were shelled and seeds from each plant were weighed using a laboratory weighing machine (OHAUS Precision Plus, model 34BL99, Dynamics Corporation of America, New Hartford, Connecticut, USA). The experiment was repeated once.

Determination of percentage protein and fat contents of bean seeds

Percentage protein and fat contents of the dry beans were determined for the following nine randomly selected treatments: *T. atroviride* strain 3A, *T. pseudokoningii*, *T. atroviride* strain 3A + *Bacillus* B77, *T. atroviride* strain 3A + *Bacillus* B81, *T. pseudokoningii* + *Bacillus* B81, *T. atroviride* strain 6 + *Bacillus* B69, *T. atroviride* strain 6 + *Bacillus* B77 and the untreated control. Percentage protein was analysed in a LECO FP2000 nitrogen analyzer (LECO Corporation, Michigan, USA) using the AOAC International (2002) methods of analyses. A Buchi 810 Soxhlett fat extractor (Buchi Laboratoriums-Technik AG, Posfach, Germany) was used for fat extraction and the percentage fat was calculated on the gravimetric analysis using the AOAC International (2002) methods of analyses.

Biological control of *R. solani* damping-off

Growth and preparation of pathogen inoculum

R. solani (PPRI accession number 03212) previously isolated from diseased cabbage (*Brassica oleracea* L. var. *capitata*) seedlings was sub-cultured onto V8 agar medium incubated at 26 ± 1°C until agar plates were fully colonized.

Greenhouse seedling trial

Cucumber seeds were treated as previously described for dry bean growth experiments. Speedling® 24 trays were half filled with composted pine bark (Potting Mix, Gromed). Pathogen inoculation was achieved by placing a 4 mm square V8 agar plug of *R. solani* in the centre of each cell directly on top of the growth medium. The cells were then, filled with growth medium and the treated seeds planted. Controls using seeds coated solely with kaolin were also established. Non-infested control trays received 4 mm agar plugs with no *R. solani*, whereas *R. solani* infested control trays received plugs with *R. solani*. Three replicate trays were established for each treatment. The trays were watered and treated as previously described for dry bean growth trials. Treatments were arranged in a randomised block design with 29 treatments and three replicates. Seedling survival was rated after 4 weeks. Seedlings that survived after 4 weeks were harvested at their base at soil level and subsequently, dried at 70°C for 48 h to determine the total dry weight of seedlings per plot (tray). Only above-ground stems and leaves were weighed. The experiment was repeated once.

Statistical analysis

A general linear model (GLM) was used to run an ANOVA on all data collected. If the ANOVA was significant, ($P \leq 0.05$) the means were separated using the Students Newman Keul's test using SAS (1987).

RESULTS

Seedling trials

Table 1 shows that a combined inoculation of *T. atroviride* strain 6 and *Bacillus* B69 gave the highest averaged seedling dry biomass (33.1 g/plot) which was significantly greater ($P < 0.05$) than 19 of the 28 treatments including the uninoculated control. This was closely followed by single inoculations of *Bacillus* B77, B69 and a combined inoculation of *T. harzianum* strain kmd + *Bacillus* B69 with mean dry seedling biomasses of 32.2, 31.2 and 28.9 g/plot, respectively. *Bacillus* B77 when used alone gave significantly higher dry seedling biomass (32.2 g/plot) than the uninoculated control (23.1 g/plot).

Only four treatments, *T. atroviride* strain 6 + *Bacillus* B69, *T. harzianum* strain kmd + *Bacillus* B69, single inoculations of *Bacillus* B77 and B69 gave over 20% increase in seedling dry biomass (43.4, 25.3, 39.3 and 34.9%, respectively) compared with the uninoculated control (Table 1).

Growth promotion studies in rhizotrons

The *Bacillus* and *Trichoderma* isolates and combinations selected for the rhizotron studies were based on the results obtained from the *in vivo* greenhouse seedling studies. The data in Table 2 reveals an increase in the shoot and root dry biomass and root area of bean seedlings as a result of inoculations with *Bacillus* B69, *Bacillus* B77, *T. atroviride* strain 6 and a combination of *T. atroviride* strain 6 and *Bacillus* B69. Maximum shoot dry biomass was obtained when *T. atroviride* strain 6 and *Bacillus* B69 were co-inoculated. This treatment showed significant ($P < 0.05$) increase in the shoot dry biomass over the uninoculated control and *Bacillus* B81 alone, but did not differ significantly from *Bacillus* B69, *Bacillus* B77 and *T. atroviride* strain 6 when used alone. The combined inoculation of *T. atroviride* strain 6 and *Bacillus* B69 gave the highest dry root biomass of all the treatments. This was the only treatment that showed significant ($P < 0.05$) increase in root dry biomass over *Bacillus* B81 when used alone.

The root area measurements was maximal for *Bacillus* B69 followed by combined inoculation of *T. atroviride* strain 6 + *Bacillus* B69 and single inoculations of *T. atroviride* strain 6 and *Bacillus* B81 (Table 2). However, none of these treatments were significantly different ($P > 0.05$) from the uninoculated control (Table 2). In all cases, except the dry root biomass, the combined inoculation of *T. atroviride* strain 6 + *Bacillus* B69 was better than any of the bacterial or fungal inoculants used in isolation.

Dry bean yield trial in tunnels

Increases or decreases in yield between treatments in the two separate trials were very disparate (data not shown). For example, compared with the uninoculated control, *Trichoderma* spp. strain 2F + *Bacillus* B81, *Trichoderma* spp. strain 2F + *Bacillus* B69 and *T. harzianum* kmd + *Bacillus* B69 reduced yield in Trial 1, but increased yield in Trial 2 by 19.7, 16.0 and 2.0%, respectively, in contrast to decreases in yield of -2.6, -0.3 and -17.6% in Trial 1. Combined inoculations of *T. atroviride* strain 3A + *Bacillus* B77 and *T. atroviride* strain 3A + *Bacillus* B81 were consistently better in the two trials than inoculations of *Bacillus* B77 and B81 alone. However, this increase was not better than the single inoculation of *T. atroviride* strain 3A that consistently increased yield in the two trials (data not shown). Most of the combined inoculations, however, gave a lower yield than the uninoculated control.

Single inoculations of *T. harzianum* kmd, *T. atroviride* strain 3A and *Bacillus* B69 consistently increased yield in Trial 1 and 2, respectively compared with the uninoculated control. Increases of 0.7, 23.7 and 6.9 and 37.3, 8.4 and 6.5%, respectively were observed in Trials 1 and 2 for the three isolates compared with the uninoculated control (data not shown).

Nonparametric analysis using the cluster groupings showed that there was no correlation between the

Table 1. Dry biomass of bean seedling as influenced by single and co-inoculations of *Trichoderma* and *Bacillus* isolates in Speedling®24 trays grown under greenhouse conditions after 4 weeks.

Isolate/combination/treatment	Mean dry seedling biomass plot^{-1} (g) after 4 week [a]	% Dry seedling biomass compared to uninoculated control (4 weeks)
Uninoculated Control	23.12^{cd}	0
Trichoderma spp. strain 2F	23.14 ^{cd}	0.95
T. atroviride strain 3A	23.33 ^{cd}	0.91
T. pseudokoningii	25.46 ^{abc}	10.12
T. atroviride strain 6	23.66 ^{d}	2.34
T. harzianum strain SY	22.97 ^{cd}	- 0.65
T. harzianum kmd	26.82 ^{abcd}	16.65
Bacillus B69	31.21 ^{abc}	34.99
Bacillus B77	32.21 ^{ab}	39.32
Bacillus B81	24.51 ^{cd}	6.01
Trichoderma spp. strain 2F + *Bacillus* B69	25.34 ^{bcd}	9.60
Trichoderma spp. strain 2F + *Bacillus* B77	23.59 ^{cd}	2.03
Trichoderma spp. strain 2F + *Bacillus* B81	24.34 ^{cd}	5.28
T. atroviride strain 3A+ *Bacillus* B69	24.44 ^{cd}	5.70
T. atroviride strain 3A+ *Bacillus* B77	27.05 ^{abcd}	17.00
T. atroviride strain 3A+ *Bacillus* B81	23.09 ^{cd}	- 0.13
T. pseudokoningii + *Bacillus* B69	26.87 ^{abcd}	16.22
T. pseudokoningii + *Bacillus* B77	25.98 ^{bcd}	12.37
T. pseudokoningii + *Bacillus* B81	23.51 ^{cd}	1.69
T. atroviride strain 6 + *Bacillus* B69	33.16 ^{a}	43.45
T. atroviride strain 6 + *Bacillus* B77	23.67 ^{cd}	2.38
T. atroviride strain 6 + *Bacillus* B81	25.38 ^{bcd}	9.78
T. harzianum strain SY + *Bacillus* B69	25.35 ^{bcd}	9.65
T. harzianum strain SY + *Bacillus* B77	25.38 ^{bcd}	9.78
T. harzianum strain SY + *Bacillus* B81	23.71 ^{cd}	2.55
T. harzianum kmd + *Bacillus* B69	28.99 ^{abcd}	25.39
T. harzianum kmd + *Bacillus* B77	25.16 ^{bcd}	8.82
T. harzianum kmd + *Bacillus* B81	22.99 ^{cd}	- 0.56
F-ratio	3.50	
P-level	0.0001	
CV (%)	10.31	
Significance	* * *	

[a]Values followed by different letters within a column are significantly different (Students Newmans Keul's test, P = 0.05)

Table 2. Dry shoot and root biomass and root area of bean seedlings as influenced by single and dual inoculations of *Trichoderma* and *Bacillus* isolates in rhizotrons grown under growth chamber conditions after 5 weeks.

Isolate/combination/treatment	Mean dry shoot biomass (g) after 5 week	% Dry shoot biomass compared to uninoculated control (5 weeks)	Mean dry root biomass (g) after 5 weeks	% Dry root biomass compared to uninoculated control (5 weeks)	Mean root area (mm^2) after 5 weeks	% Root area compared to uninoculated control (5 weeks)
Uninoculated control	2.47[b]	0	1.40[ab]	0	17476.20[a]	0
Bacillus B69	3.48[ab]	40.89	1.59[ab]	13.57	23639.52[a]	35.27
Bacillus B77	3.29 [ab]	33.20	1.63 [ab]	16.47	20931.60[a]	19.77
Bacillus B81	2.53 [b]	2.43	1.20[b]	- 14.71	23240.08[a]	32.98
T. atroviride strain 6	3.14[ab]	27.13	1.60 [ab]	14.23	23308.74 [a]	33.37
T. atroviride strain 6 + *Bacillus* B69	3.96[a]	60.32	1.91[a]	36.43	23352.40 [a]	33.63
F-ratio	3.41		2.75		2.43	
P-value	0.02		0.05		0.07	
% CV	19.73		18.58		14.13	
Significance	* *		*		ns	

[a] Values followed by different letters are significantly different (Students Newmans Keul's test, P = 0.05).

performance of the isolates/combinations and/or treatments in the seedling trial and the two yield trials (Table 3). Similarly, no correlation was found between the two yield trials as shown in the Chi square (χ^2) table (Table 3).

Determination of percentage protein and fat contents of bean seeds

No significant increase in percentage protein and fat content of bean seeds was observed between the selected isolates/combinations and/or treatments for the two yield trials (Table 4).

Biological control of *R. solani* damping-off

Percentage seedling survival for the controls ranged from 36.1% for the *R. solani* infested control to 98.6% for the non-infested control plants (P = 0.0001) (Table 5). The mean dry seedling biomass yield for the *R. solani* infested control was 40.0% of the yield obtained for the non-infested control (P = 0.0001). *T. harzianum* kmd, substantially reduced pre- and post-emergence damping- off caused by *R. solani*. Compared with the *R. solani* infested control, *T. harzianum* kmd significantly increased seedling survival from 36.1 to 78.4% and dry shoot biomass from 40.0 to 89.3% (Table 5). None of the three *Bacillus* plus *T. harzianum* kmd combinations were better than *T. harzianum* kmd alone in terms of seedling survival and dry shoot biomass. Compared with the *R. solani* infested control, applications of *T. atroviride* strain 3A and *T. harzianum* strain SY significantly increased percentage seedling survival from 36.1 to 63.8 and 61.1% and dry shoot biomass from 40.0 to 71.5 and 70.7%, respectively. Combinations of these two *Trichoderma* isolates with the *Bacillus* isolates did not give better percentage seedling survival and dry shoot biomass compared with *T. atroviride* strain 3A and *T. harzianum* strain SY used alone.

The best combinations were *T. atroviride* strain 3A + *Bacillus* B81, *T. atroviride* strain 6 + *Bacillus* B69 and *T. atroviride* strain 6 + *Bacillus* B81 with percentage seedling survival of 61.1, 59.0 and 59.0%, respectively. Percentage seedling survival achieved by *T. atroviride* strain 6 + *Bacillus* B69 and *T. atroviride* strain 6 + *Bacillus* B81 was higher than *T. atroviride* strain 6 and *Bacillus* B69 and B81 alone (Table 5).

In all cases, either used alone or in combination with the three *Bacillus* isolates, *Trichoderma* spp.

Table 3. Chi square (χ^2) test of association between: (1) seedling performance and yield performance and (2) yield trials 1 and 2 performances of isolates/combinations and/or treatments in the greenhouse.

Parameter	Chi square (χ^2)	P- value	Significance
Seedling trial versus yield trial 1	4.05	0.87	ns
Seedling trial versus yield trial 2	9.99	0.35	ns
Yield trial 1 versus yield trial 2	13.66	0.13	ns

ns, Not significant (P > 0.05).

Table 4. Percentage protein and fat content of bean seed for selected isolates/combinations and/or treatments from yield trials 1 and 2.

Isolate/combination/treatment	Yield trial 1		Yield trial 2	
	Protein (%)	Fat (%)	Protein (%)	Fat (%)
Uninoculated control	22.25 [a]	0.83 [a]	23.08 [a]	0.74 [a]
T. atroviride kmd	23.25 [a]	0.79 [a]	23.35 [a]	0.76 [a]
T. atroviride strain 3A	23.38 [a]	0.83 [a]	24.10 [a]	0.80 [a]
T. atroviride strain 3A + *Bacillus* B77	22.75 [a]	0.81 [a]	23.53 [a]	0.81 [a]
T. atroviride strain 3A + *Bacillus* B81	24.57 [a]	0.75 [a]	22.79 [a]	0.86 [a]
T. pseudokoningii	22.10 [a]	0.75 [a]	22.22 [a]	0.87 [a]
T. pseudokoningii + *Bacillus* B81	23.83 [a]	0.75 [a]	23.56 [a]	0.81 [a]
T. atroviride strain 6 + *Bacillus* B69	23.49 [a]	0.78 [a]	23.39 [a]	0.83 [a]
T. atroviride strain 6 + *Bacillus* B77	22.04 [a]	0.73 [a]	21.38 [a]	0.86 [a]
F-ratio	0.98	1.28	0.75	1.13
P-value	0.48	0.31	0.65	0.39
CV (%)	6.53	7.19	9.12	8.99
Significance	ns	ns	ns	ns

[a] Values followed by the same letter within a column are significantly different (Students Newmans Keul's test, P > 0.05); ns, Not significant (P > 0.05).

strain 2F and *T. pseudokoningii* did not enhance disease control.

DISCUSSION

The major objective of this work was to investigate whether plant growth promotion and biological control could be enhanced through combined applications of selected *Trichoderma* and *Bacillus* isolates. From the growth promotion trials, it was demonstrated that a combined application of *T. atroviride* strain 6 and *B. subtilis* B69 achieved the highest bean seedling dry biomass (+43.4%) compared with the uninoculated control. This result was later confirmed in rhizotron studies in pasteurised sand with *T. atroviride* strain 6 + *B. subtilis* B69 giving the highest shoot and root dry biomass. However, these growth improvements were not reflected in the two successive dry bean yield trials conducted in this study and neither were they increased in neither protein nor fat content in bean seeds observed. Biological control trials in the greenhouse demonstrated that single inoculations of *T. harzianum* kmd, *T. atroviride* strain 3A and *T. harzianum* strain SY significantly reduced *R. solani* damping-off. A general trend indicates that combined inoculations of *Trichoderma* and *Bacillus* isolates tend towards an increased suppression of damping-off better than single inoculations of the *Bacillus* isolates. This increase was consistent with combinations of *T. atroviride* strain 3A, *T. harzianum* kmd, *T. atroviride* strain 6 or *T. harzianum* strain SY with *B. subtilis* B69 or *B. subtilis* B81. Different mechanisms of action for the *Trichoderma* and *Bacillus* isolates may explain why some combinations of the two organisms increased plant growth and disease control than some single inoculations. The growth promotion results are in agreement with studies by Jisha and Alagawadi (1996) who demonstrated that mixtures of a strain of *B. polymyxa* and *T. harzianum* increased the growth of sorghum better than each organism used alone. The *Trichoderma* and *Bacillus* isolates used in this study were primarily selected for biological control purposes, except *B. subtilis* B77 that was previously shown to enhance plant growth. One would therefore, not expect the

Table 5. Seedling survival and dry biomass of cucumber as influenced by single and dual inoculations of *Trichoderma* and *Bacillus* isolates in the greenhouse after 4 weeks of growth.

Isolate/combination/treatment	Mean number of surviving seedling after 4 week [a]	Seedling survival after 4 week (%)	Mean dry biomass after 4 week [a]	% Dry biomass compared to uninoculated control (4 weeks)
Non-infested control	23.67 [a]	98.68	18.59[a]	100
R. solani infested control	8.67 [h]	36.13	7.44 [ij]	40.02
Trichoderma spp. strain 2F	10.67 [fgh]	44.46	8.70[hij]	46.80
T. atroviride strain 3A	15.33[c]	63.88	13.30 [c]	71.54
T. pseudokoningii	10.50[fgh]	43.75	8.05 [hij]	43.30
T. atroviride strain 6	12.00 [defgh]	50.00	9.99[efghi]	53.74
T. harzianum strain SY	14.67[cd]	61.13	13.15 [c]	70.74
T. harzianum kmd	18.83[b]	78.46	16.61 [b]	89.35
Bacillus B69	10.50 [fgh]	43.75	8.82[ghij]	47.44
Bacillus B77	8.83[h]	36.79	8.42[hij]	45.29
Bacillus B81	11.83 [defgh]	49.29	9.78 [efghi]	52.61
Trichoderma spp. strain 2F + *Bacillus* B69	10.17[fgh]	42.36	7.34 [ij]	39.48
Trichoderma spp. strain 2F + *Bacillus* B77	11.33[efgh]	47.21	9.01[fghij]	48.47
Trichoderma spp. strain 2F + *Bacillus* B81	11.00[efgh]	45.83	7.42 [ij]	39.91
T. atroviride strain 3A+ *Bacillus* B69	13.00[cdefg]	54.17	11.40[cdefg]	61.32
T. atroviride strain 3A+ *Bacillus* B77	10.77 [fgh]	44.88	8.20[hij]	44.11
T. atroviride strain 3A+ *Bacillus* B81	14.67 [cd]	61.13	12.57[cd]	67.62
T. pseudokoningii + *Bacillus* B69	9.83 [gh]	40.96	7.72 [hij]	41.53
T. pseudokoningii + *Bacillus* B77	11.67 [defgh]	48.63	9.42[fghij]	50.67
T. pseudokoningii + *Bacillus* B81	10.00[fgh]	41.67	7.77 [hij]	41.80
T. atroviride strain 6 + *Bacillus* B69	14.16[cde]	59.04	12.65[cd]	68.05
T. atroviride strain 6 + *Bacillus* B77	8.67[h]	36.13	6.65[j]	35.77
T. atroviride strain 6 + *Bacillus* B81	14.17 [cde]	59.04	13.28 [c]	71.44
T. harzianum strain SY + *Bacillus* B69	12.83 [cdefg]	53.46	10.33[defgh]	55.57
T. harzianum strain SY + *Bacillus* B77	10.33 [fgh]	43.04	8.88[ghij]	47.77
T. harzianum strain SY + *Bacillus* B81	13.33 [cdef]	55.54	10.58[defgh]	56.91
T. harzianum kmd + *Bacillus* B69	12.83 [cdefg]	53.46	11.55[cdef]	62.13
T. harzianum kmd + *Bacillus* B77	10.83 [fgh]	45.13	8.66 [hij]	46.58
T. harzianum kmd + *Bacillus* B81	13.17[cdefg]	54.88	11.97[cde]	64.39
F-ratio	22.12		24.81	
P-level	0.0001		0.0001	
CV (%)	9.42		9.64	
Significance	* * *		* * *	

[a] Values followed by different letters within a column are significantly different (Students Newmans Keul's test, $P < 0.05$); ***, significant at $P \leq 0.001$.

selected *Trichoderma* and *Bacillus* isolates to exhibit marked plant growth promotion. The increases in bean seedling growth by selected *Trichoderma* and *Bacillus* isolates and their combinations were attributed to possible factors such as increased mineral uptake, siderophore production and/or possible production of plant growth promoters (Kumar and Dube, 1992). All the *Trichoderma* and *Bacillus* isolates used in this study produced siderophores *in vitro* as one of the biological control traits (Yobo, 2005) and might have contributed to an increase in seedling growth.

Reports on *Trichoderma* and *Bacillus* as plant growth promoters have mainly been focused on seedling growth and development where their performances have been found to vary in consistencies (Kleifeld and Chet, 1992; Ousley et al., 1993, 1994; Shishido et al., 1995; Rabeendran et al., 2000). In this study, increase in growth in the bean seedling trial was found to be consistent with increase in growth in the rhizotron study.

Plant growth promoting rhizobacteria (PGPR) have been reported to increase crop yields (Kloepper et al., 1989; Jisha and Alagawadi, 1996; Harman, 2000; Mathre et al., 2000). However, results obtained during the yield trials were erratic and by no means conclusive, which raises the question whether increase in seedling performance by PGPR translates into actual increase in crop yield. The erratic performance by the isolates and/or combinations in the yield trials could partly be accounted for by some factors, which were thought to have a direct effect on yield. A possible inhibitory effect of the *Trichoderma* and *Bacillus* isolates to one another was ruled out, as this was not observed during the seedling trials in the speedling trays and rhizotron. Moreover, greenhouse conditions provide a favourable environment for plant growth. An optimal environment is more likely to mask the effect of the *Trichoderma* and *Bacillus* isolates. It is more likely that the fungal and bacterial effect on plant growth could be seen under periods of stress as found in the field (Rabeendran et al., 2000). Erratic and inconsistent performances of bacterial PGPR have been reported under field conditions (Schroth and Becker, 1990). Although, Kloepper et al. (1989) reported increase in yield as a result on inoculation of bacterial PGPR to a range of crops, decreases in yield were also common in trials.

A significant observation in this study was that plant growth promotion/seedling vigour appeared to have little or no corresponding effect on yield. Schroth and Becker (1990) noted that “early growth promotion often is not accompanied by higher yield”. It is therefore necessary that growth measurements be taken throughout the duration of a yield/growth promotion trial, as “increased yield is desirable but not essential to demonstrate efficacy” (Schroth and Becker, 1990). The authors therefore, maintain that increase in seedling vigour could be attributed to an increase in mineralization as a result of *Trichoderma* and *Bacillus* inoculations. The *Trichoderma* and *Bacillus* isolates used in this study were found to substantially increase nitrogen content of dry bean plants compared with the unfertilized control plants (Yobo et al., 2009).

Various *Trichoderma* and *Bacillus* spp. have been reported as being able to successfully control several plant pathogens (Rytter et al., 1989; Kim et al., 1997; Koch, 1999; Utkhede et al., 1999; Zhang et al., 1999; Lewis and Lumsden, 2001). The results obtained for the biological control trials support the finding of Koch (1999) and Lewis and Lumsden (2001), both of which demonstrated that *Trichoderma* sp. formulations were able to reduce damping-off caused by *R. solani*. Our results indicate that none of the *Trichoderma* and *Bacillus* isolates competitively controlled *R. solani* damping-off better than *T. harzianum* kmd. Only two of the *Trichoderma* isolates, *T. atroviride* strain 3A and *T. harzianum* strain SY gave percentage plant stands of 64 and 61% compared with 78% plant stand by *T. harzianum* kmd. These three *Trichoderma* isolates were shown to be hyperparasitic against *R. solani in vitro* and also exhibited chitinase activity and siderophore production (Yobo, 2005). These mechanisms are all thought to contribute to biological control (Hadar et al., 1979; Kumar and Dube, 1992; Menendez and Godeas, 1998). Enhanced biological control by *T. harzianum* kmd compared with the other *Trichoderma* isolates may have resulted from the additive action of antibiosis, as it was the only *Trichoderma* isolate that inhibited *R. solani in vitro*, suggesting the production of an anti-inhibitory compound (Yobo, 2005). None of the *Bacillus* isolates were able to achieve a 50% plant stand. The highest plant stand (49%) was recorded by *Bacillus* B81. Both *B. subtilis* B69 and B81 inhibited *R. solani in vitro* and also produced siderophores (Yobo, 2005).

Using bacterial/bacterial and fungal combinations to improve biological control has been suggested and studied (Duffy et al., 1996; Raupach and Kloepper, 1998; Guetsky et al., 2002). To the best of our knowledge, reports on the feasibility of combining *Trichoderma* and *Bacillus* spp. to improve biological control is sparse. Although, limited studies have been carried out on the combined effect of *Trichoderma* and *Bacillus* spp. on plant growth that has been cited in this study, there is still a lack of information on the combined effect of these two organisms on biological control. Our results indicate that none of the combinations were better than *T. harzianum* kmd, *T. atroviride* strain 3A and *T. harzianum* strain SY. The best combination was *T. atroviride* strain 3A + *Bacillus* B81 with a plant stand of 61.1% which was equal in performance to *T. harzianum* strain SY. The performances, particularly of *B. subtilis* B69 and B81, were enhanced by combinations with *T. atroviride* strain 3A, *T. atroviride* strain 6, *T. harzianum* strain SY or *T. harzianum* kmd. This suggests that the *Trichoderma* isolates were largely responsible for the control of *R. solani* damping-off in this study. This also suggests a

possible synergism between these two organisms leading to a better biological control than the two *Bacillus* isolates used alone.

In vitro compatibility test between the *Trichoderma* and *Bacillus* isolates (Yobo, 2005) showed that the *Trichoderma* and *Bacillus* isolates did not inhibit each other. Although, *in vitro* compatibility tests may predict the feasibility of using two organisms together, this may not apply to all combinations as factors such as competition is difficult to test *in vitro*.

Applications of *Trichoderma* and *Bacillus* isolates increased dry bean yield in some treatments but were not consistent. Mechanisms for this were not established and this requires further investigation to understand the inconsistencies observed in bean yield Trials 1 and 2.

The results presented here suggest that there are possibilities of enhancing biological control of plant diseases and/or increase seedling growth and establishment through mixtures of *Trichoderma* and *Bacillus* spp. Several reports have shown that individual *Trichoderma* and *Bacillus* spp. could suppress plant pathogen activities as well as promote plant growth. A combination of these two organisms, as shown in this study, could lead to an increase in disease suppression and plant growth. The likelihood of this combination performing maximally will depend on the modes of action and compatibility of the intended isolates to be combined. Complementary modes of action between these two organisms, if exploited, could lead to increased synergism and activity especially under variable environmental conditions (Raupach and Kloepper, 1998) and in situations where more than one plant pathogen exists. Mixtures of these two organisms could be be used in conjunction with a reduced rate of fungicide applications (Yobo et al., 2010). The *Bacillus* beneficial to organic farming (Raupach and Kloepper, 1998) or could isolates were the main contributors to growth promotion in this study as with the *Trichoderma* isolates during the biological control trials. Essentially, combinations of *Trichoderma* and *Bacillus* spp. isolates did not antagonise each other *in vitro* (Yobo, 2005). Hence, the different niche occupancy by these two organisms when used as a mixture could aid in sourcing of nutrients and controlling possible minor and major plant fungal plant pathogens leading to improved plant growth and biological control.

In practice, *Trichoderma* and *Bacillus* spp. are easier to formulate than other organisms such as fluorescent pseudomonads due to production of spores by the *Trichoderma* and *Bacillus* spp. The feasibility of producing the two organisms as a mixture and as a commercial product may not be feasible due to a high production and registration cost that may be involved compared with the cost incurred by producing a single strain (Schisler et al., 1997).

This work in part suggests that the combinations of *Trichoderma* and *Bacillus* isolates could play a major role in integrated plant disease management as well as a role in biofertilisation. The two organisms could thus, result in a possible additive effect, leading to enhanced plant growth and biological control.

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