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Effect of Combined Use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 on Biological Control of *Rhizoctonia solani* on *Solanum melongena* and *Capsicum annuum*

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Abstract: A combination of two compatible biological control agents, *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01, both antagonistic to the pathogen *Rhizoctonia solani*, was used to control damping-off in *Solanum melongena* and *Capsicum annuum*. Radial growth of the mycelium of *R. solani* was inhibited by *T. harzianum* RU01 in dual Petri plate assay. *T. harzianum* RU01 was capable to invading the whole surface of the pathogen colony, sporulating on it and suppress the production of sclerotia of *R. solani*. Microscopic studies showed the hyphae of *R. solani* surrounded by the *T. harzianum* RU01 and subsequent disintegration. *B. subtilis* CA32 produced a zone of inhibition only with the pathogen and no sings of antagonism between the bacteria and *T. harzianum* RU01 on dual Petri plate assay. Significant plant protection was achieved when either *B. subtilis* added to the seeds or *T. harzianum* added to soil. However, when combine application of biocontrol agents, seed bacterization and *T. harzianum* application to soil, significantly enhanced the plant protection from *R. solani*. Soil application of *B. subtilis* and seed application of *T. harzianum* either singly or in combination did not protect from *R. solani* infection indicating that the importance of mode of application of biocontrol agents.

Key words: Antagonism, aubergine, chili, mycoparasitism, synergism

INTRODUCTION

Solanum melongena L. (brinjal, aubergine or egg plant) and *Capsicum annuum* L. (chili or hot pepper) are important vegetable and spice crops especially in South East Asia. In general, these kinds of small seed crops are grown first in seed beds as nurseries and 3-4 weeks old seedlings are used to transplant in fields. Several fungal, bacterial and viral plant pathogens cause different diseases on both crops during the seedling stage therefore some times farmers confront with problems of getting healthy planting materials to establish on fields at suitable time. Among the fungal diseases, damping-off caused by *Rhizoctonia solani* is very common in nurseries and one of the most important soil-borne pathogens in Sri Lanka (Kudagamage *et al*., 2003).

Currently, Rhizoctonia damping-off is managed by cultural practices and application of fungicides. However, these methods are not completely effective and Rhizoctonia disease remains a persistent problem in Sri Lanka (Kudagamage *et al*., 2003). Although, some fungicides offer significant protection against *R. solani*, their negative impacts on beneficial soil microorganisms and on environment can not be ignored. It is therefore necessary to develop alternative methods to control such soil-borne pathogens. One such alternative is biological control, in which microorganisms are selected for their ability to antagonize pathogens (Handelsman and Stabb, 1996). Biological control can be an effective means of control in many instance where chemical is not available or practical.

Biological control of Rhizoctonia diseases has been demonstrated and represents as an additional strategy that may provide effective and sustainable management (Brewer and Larkin, 2005). Although biological control of different soil-borne pathogens on chili cultivars have been reported by Mao *et al*. (1998), Sid Ahamed *et al*. (1999), Ramamoorthy *et al*. (2002) and Nakkeeran *et al*. (2006) biological control of damping-off caused by *R. solani* on *S. melongena* has not been reported.

It has been reported that application of a single strain of a biological control agent is less effective than chemical protection and its effect may not consistence (Weller, 1988). This low and unstable efficacy or variability of plant protection of biological control agents may be due to the complexity of the ecosystem (Guetsky *et al*., 2002). Possible approaches of enhancement of the efficacy of biological control agent against soil-borne pathogens has been reviewed and it has been identified that one way to improve biological control ability may be the use of mixtures or combinations of biocontrol agents (Spadaro and Gullino, 2005). Pierson and Weller (1994) reported that

combining different *Pseudomonas fluorescens* strains increased the control of wheat take-all compared to the strains used individually. Raupach and Kloepper (1998) showed that use of combined application of biocontrol bacteria significantly enhanced the plant protection against different cucumber pathogens. Apart from the bacterial combinations, some reports indicated that combine of bacterial with biological control fungi especially *Trichoderma* sp. showed increased plant protection than they showed when used individually. For example, Brewer and Larkin (2005) reported that combination of *Bacillus subtilis* and *Trichoderma virens* enhanced the resistance against *R. solani* on potato than these biocontrol agents used individually. A positive, probably synergistic, interaction between *Trichoderma* sp. strains and bacterial species has been reported for combined applications in the control of plant pathogens (Fogliano *et al*., 2002). Ezziyyani *et al*. (2007) reported that the combined application of *T. harzianum* and *Streptomyces rochei* was more effective in controlling Phytophthora root rot in bell pepper.

Therefore, the main objective of this study was to evaluate the efficacy of *B. subtilis* CA32 and *T. harzianum* RU01 individually and in combinations with respect to the mode of application of biocontrol agents on protection of brinjal and chili seedlings against damping-off caused by *R. solani* during the seedbed stage under greenhouse conditions.

MATERIALS AND METHODS

Experiment sites and place: The experiments reported in this study were conducted at the Department of Botany, University of Ruhuna, Sri Lanka during the period of 2001-2005. Pot experiments were conducted in the greenhouse located at the department.

Pathogen isolation and identification: Chili plants showing necrotic lesions at the crown or collar region were collected from commercial chili fields in Southern Province in Sri Lanka. The samples were washed under tap water, placed in 0.5% NaOCl for 2 min, rinsed twice in Sterile Distilled Water (SDW) and blotted on sterile paper towels. About 1 cm pieces having lesions were then placed on PDA plates amended with chloramphenicol (100 μg mL^{-1}) to prevent growing of bacteria. After incubation over night at room temperature 25±2°C in the dark, agar plug containing actively growing mycelium was transferred to a fresh PDA plate and repeated this procedure till get a pure colony. *R. solani* was identified by using the colony characters and structure of sclerotia described by Sneh *et al*. (1991). The pathogenicity of the fungal isolates on brinjal and chili was confirmed by following Koch's postulates.

Isolation of rhizospheric bacteria and *Trichoderma*: Rhizospheric bacteria were isolated healthy plants obtained from commercial chili fields in the southern Province of Sri Lanka. Roots from young (about 3-4 weeks old) healthy plants were selected and brought to the laboratory. Plants were gently shaken in order to remove large soil clumps and roots cut into about 2 cm pieces. Then the root pieces were macerated in 10 mL sterile saline (NaCl 0.85%, w/v). Serially diluted aliquots from the suspension were separately plated on Casamino acids medium (Casamina Acid Agar (CAA), 5.0 g Casamino acid, 1.20 g K_2HPO_4 and 0.25 g $MgSO_4.7H_2O$ in 1 L H_2O), King's B medium (KB, 20.0 g protease peptone, 2.0 g $K_2HPO_4.3H_2O$, 1.5 g $MgSO_4.7H_2O$ and 15 mL glycerol). Plates were incubated at room temperature for 2 days. One colony per colony type was purified on CAA plates. Strains were stored at -80°C in glycerol (20%, v/v).

Trichoderma sp. were isolated from soil samples obtained from the same field by using dilution Petri plate technique on Trichoderma Selective Medium (TSM) (Askew and Laing, 1993) and maintained on Potato Dextrose Agar (PDA). Identification was performed by using colony characteristics described by Bissett (1991).

Screening of antagonistic bacteria against *R. solani* in Petri plate assay: About 83 bacterial isolates were initially screened for the ability to inhibit *R. solani* on CAA and KB plates. In the preliminary screening single colonies were selected and patched along the perimeters of plates and incubated overnight at 28°C. The following day each plate was inoculated with a *R. solani* plug (5 mm diameter) at the centre. The plates were incubated at 28°C for 2-3 days and observed for inhibition of fungal growth. The bacterial isolates positive for antagonism were selected based on the inhibition zone and the strains were ranked. The Gram test was performed to separate bacterial isolates according to their morphology and staining response. Antagonistic bacterial isolates were identified based on their physiological profiling according to Bergey's Manual of Systematic Bacteriology (Palleroni, 1984).

Interaction between *Trichoderma* sp. and *R. solani* in Petri plate assay: Five millimeter diameter discs of *Trichoderma* sp. grown on PDA were removed and placed on one side of a Petri dishes containing PDA medium. Similar discs of *R. solani* isolate grown in the same

manner were placed on the opposite side of Petri plates. Cultures were observed daily while incubating at 28°C and recorded for antagonism or parasitism of *Trichoderma* isolates against *R. solani*. Three replicates were included.

Seed bacterization and soil application: Among the bacterial isolates the most antagonistic bacterial isolate, according to the *in vitro* Petri plate assay, spontaneous rifampicin mutant of *B. subtilis* CA32 was selected for greenhouse pot experiments. Fresh cells of the bacteria were obtained from stock cultures stored at -80°C and grown in CAA broth for overnight at room temperature (25±2°C) on a shaker at 80 rpm. From this 100 mL CAA broth in a 250 mL flask was inoculated and incubated for 48 h at room temperature (25±2°C) on an orbital shaker (80 rpm). The bacterial culture was centrifuged (6000 g for 10 min at 4°C) and the supernatant was discarded. The cell pellet was resuspended in sterile 0.85% NaCl and centrifuged again at the same conditions. The supernatant was discarded and washed bacterial cells were resuspended in Sterile Distilled Water (SDW). The concentration of cells in the suspension was adjusted to 10^8 cells mL^{-1} by using a spectrophotometer at OD_{600} by using a standardized graph and used for seed bacterization or soil application. For seed bacterization, a batch of 50 seeds at a time was immersed in 100 mL of the bacterial suspension for 30 min, placed on a blotting paper and dried for 2 h in a laminar flow. Soil inoculation was performed by adding 100 mL of the bacterial suspension (10^8 cell mL^{-1}) as a soil drench to cover the whole soil surface of each pot.

Inoculum production of *Trichoderma harzianum* RU01: Among the *Trichoderma* isolates, *T. harzianum* RU01 profusely growth on PDA plates, produced substantial amount of conidia and showed strong *in vitro* antagonism against *R. solani*. Therefore, *T. harzianum* RU01 was selected for further studies. Four discs (5 mm diameter) of actively growing *T. harzianum* RU01 were cut out from the PDA plate and transferred to a glass Petri dish (90 mm diameter) containing a mixture of coir dust and rice bran (3:1, v/v) that had been moistened with 25 mL distilled water and autoclaved for 20 min at 121°C for two consecutive days. The dishes were incubated at room temperature (25±2°C) in darkness for 15 days. Viability of the inoculum was confirmed by plating on PDA. This inoculum was used for soil inoculation (approximately 10^6 cfu g^{-1} soil). For seed inoculation, *T. harzianum* RU01 was grown on PDA in Petri plates for 7 days at room temperature (25±2°C) under dark to allow profuse sporulation. Sterile distilled water was added to each plate and a conidial suspension was obtained by scraping the colony surface with a sterile spatula and filter through cheese cloth. Conidial suspension was adjusted to 10^6 spores mL^{-1} and mixed (0.01%) Tween-20 as a wetting agent. Inoculation of seeds was performed as mentioned for bacterization.

Preparation of *R. solani* inoculum used to infest the potting mix: To prepare the pathogen inoculum, Potato Dextrose Broth (PDB) was used. One hundred milliliter of PDB in 250 mL flasks were inoculated with four mycelial discs (5 mm diameter) of *R. solani* on PDA. The flasks were incubated in the dark at room temperature (25±2°C) for 7 days. The mycelium mat was separated and homogenated in SDW. The inoculum of the pathogen was introduced to the upper 5 cm of soil (approximately 25 propagules of *R. solani* g^{-1} soil). The infested soil was covered with paddy straw and incubated for 3 days at room temperature (25±2°C) to give appropriate conditions to the pathogen.

Pot experiments under greenhouse conditions: The experiment was conducted in plastic pots (15 cm diameter) where the pots were filled with approximately 1200 g of soil (clay:sand:coir dust at 1:1:1, v/v). Fifty seeds per pot were sown in the pots 3 cm below the soil surface. Eight treatments were tested:

T_1 = Healthy control (uninoculated)
T_2 = Diseased control (inoculated with *R. solani*)
T_3 = Bacterized seeds
T_4 = *T. harzianum* added seeds
T_5 = *B. subtilis* added to soil
T_6 = *T. harzianum* added to soil
T_7 = *B. subtilis* to seed and *T. harzianum* to soil
T_8 = *T. harzianum* to seeds and *B. subtilis* to soil

The pots were arranged on greenhouse benches in a completely randomized block design. The experiment was repeated three times and three replicates were included per treatment. Plants were grown in a greenhouse at the Department of Botany, University of Ruhuna for 30 days (The pots were watered every day (50 mL/pot), 28-30°C day and 24°C night temperature, 80±5% relative humidity and 12 h light/dark photoperiod). The collar region (stem base) and the root system of each plant was evaluated to determine disease severity according to a 1-5 scale:

1 = No visible symptoms
2 = Light discoloration with one or two small lesions

3 = <25% stem base and root tissues covered with lesions; 25-50% stem base and root tissues covered with lesions
4 = 50-75% stem base and root tissues covered with lesions
5 = >75% stem base and root tissues covered with lesions combined with a severe reduction in the root system or dead plants

During this period, damping-off seedlings were randomly removed and plated on acidified PDA to determine the cause of damping-off.

Number of *R. solani* in soil and rhizosphere: Number of *R. solani* propagules in soil and on rhizosphere was assessed at the end of each experiment using Rhizoctonia selective medium according to Szczech and Shoda (2004). Five grams of soil were collected in flask from each pot representing different treatments closed to the root system and mixed thoroughly considered as the bulk soil. *R. solani* populations in the rhizosphere of both crops were assessed by macerating 1 g of fresh root in SDW and ten times serially diluted aliquots were plated on the above medium. Each sample was replicated three times.

Statistical analysis: In all the experiments, treatments were arranged in a randomized complete block design. Data were analyzed using SPSS 10.0 for Windows. Mean separation was accomplished using Duncan's Multiple Range Test. The statistical significance was determined at (p = 0.05).

RESULTS

Pathogen identification and characteristics: *Rhizoctonia* isolate grown on PDA or CAA turned brown after about 7 days of incubation and the sclerotia were light brown and turned dark brown after few days. However, AG-group determination was not done. The pathogenicity of the *Rhizoctonia* isolate was performed with different local chili and brinjal cultivars. The *Rhizoctonia* isolate was pathogenic in all tested local cultivars of both plant species. Therefore, the isolate was identified as a virulent strain of *R. solani*.

Identification and characterization of *Trichoderma* sp.: The highly antagonistic isolate of *Trichoderma* sp. grew rapidly at room temperature on PDA. The culture at first was white and cottony then turned to bright green, finally turned dark green. Chlamydospores were intercalary and/or terminal, globose and smooth walled. Single phialides arose laterally on the conidiophores in clusters Bissett (1991). Therefore, the *Trichoderma* sp. was identified as *T. harzianum* and designated as RU01 and selected for further experiments.

***In vitro* antagonism tests**

***R. solani* against *T. harzianum* RU01:** Antagonistic properties of *T. harzianum* RU01 was tested through dual Petri plate method. *T. harzianum* RU01 inhibited the mycelial growth of *R. solani* but could not overgrow pathogen's colony. However, several days later *R. solani* completely over grew by the *Trichoderma* mycelia. Furthermore, sclerotia formation did not observe compared to the control plates.

***B. subtilis* CA32 against *T. harzianum* RU01:** Antagonistic properties of *B. subtilis* CA32 were tested by using dual Petri plate assay. *B. subtilis* CA32 did not show any antagonism against *T. harzianum* RU01 isolate. Indeed after 7 days, the *Trichoderma* isolate was over grown the bacterial colony (data not shown).

***R. solani* against *B. subtilis* CA32:** Number of bacterial isolates was isolated from *C. annuum* rhizosphere according to colony characters appeared on CAA, KB and water agar plates. From the preliminary round of screening for antagonism in Petri plate assay, only four isolates were selected for further study based on the inhibition zone (Table 1). Among these isolates, *B. subtilis* CA32 was selected for greenhouse pot experiments because CA32 was the most antagonistic isolate in terms of the inhibition zone in the plate assay. The inhibition of the mycelial growth was permanent. No sclerotia were developed and the cytoplasm of the mycelium showed dramatic changes when observed under microscope (data not shown). Mycelium closest to the bacteria became yellow indicating some diffusible compounds from bacteria had reached this part of the mycelium (Fig. 1). Mycelial plugs obtained from the inhibition zone were not able to grow when transferred on to PDA plates thus indicating the fungi toxic nature of diffusible compound(s) produced by the bacterial strain CA32.

Table 1: Inhibitory activity of selected bacteria against *R. solani* on Petri plate assay

	Inhibition zone (mm)[a]		
Strains	CAA	KB	PDA
B. subtilis CA32	14.2±0.52	14.5±0.32	11.5±0.25
CA28	4.1±0.25	5.1±0.33	3.2±0.15
CA48	2.7±0.22	3.2±0.28	3.5±0.12
CA10	5.5±0.30	4.8±0.26	3.8±0.28

[a]Inhibition zone is the distance of inhibition between growth of the fungal and bacterial colonies on plates. The values are the mean of 9 replications from 2 experiments

Effect of antagonists on Rhizoctonia disease of chili and brinjal: All the biological control treatments showed variation in plant disease severity compared to inoculated control (Table 2). In most of the treatments, the disease severity was reduced significantly ($p = 0.05$) in most of the biocontrol treatments compared to disease control. Bacterial seed treatment alone significantly reduced the disease severity on both chili and brinjal where the disease severity ratings were 2.20 and 2.15 for chili and brinjal, respectively. When *T. harzianum* RU01 alone applied to seeds prior to sowing significant plant protection was not observed where disease severity rating was on average 4.25 and 4.80 for chili and brinjal, respectively after 30 days. However, when *T. harzianum* RU01 was added to the soil disease severity was significantly reduced on both crops. Values observed for disease severity was a little bit higher in this case compared to that of the bacterial treatment although were not significant in all cases (Table 2). On the other hand, no significant protection was observed when *B. subtilis* CA32 was added to soil and values observed for disease severity were also similar in this treatment with *T. harzianum* RU01 applied to seeds. It was noticed that maximum disease suppression was observed when combined application of antagonists was used. Combine application of biocontrol agents, seed coating with *B. subtilis* CA32 and soil application of *T. harzianum* RU01, resulted in the highest protection from *R. solani* in both crops where the lowest disease severity was recorded. However, combined seed application of *T. harzianum* RU01 with soil application of *B. subtilis* did not enhanced protection, being similar to that reached with seed application alone with *T. harzianum* RU01 (Table 2).

Fig. 1: *In vitro* antagonism of *R. solani* by *Bacillus subtilis* CA32

Table 2: Severity of root and collar rot (1-5) scale at 30 days old chili and brinjal seedlings when the different treatment consisting of different mode of application of *B. subtilis* CA32 and *T. harzianum* RU01

	Disease severity[a]	
Treatments	Chili	Brinjal
T_1	1.00a	1.00a
T_2	4.75d	4.85d
T_3	2.15b	2.20b
T_4	4.25d	4.80d
T_5	4.58d	4.20d
T_6	2.35b	2.55b
T_7	1.55a	1.45a
T_8	4.25d	4.65d

[a]The values representing disease severity are the means of 50 plants (data from three replicates were pooled and analyzed). The means followed by the same letters in each column are not significantly different accordingly to Duncan's multiple range test at $p = 0.05$

Population dynamics of *R. solani* in soil and the rhizosphere: Application of either *B. subtilis* CA32 or *T. harzianum* RU01 decreased population of the pathogen on the rhizosphere and in soil closer to the root system (bulk soil) respectively depending on the mode of application of the biological control agents. Application of *B. subtilis* CA32 alone on seeds significantly reduced the population of *R. solani* on roots (Table 3). However, *T. harzianum* RU01 significantly reduced the population of *R. solani* in bulk soil (Table 4). The strongest reduction of *R. solani* in both rhizosphere and bulk soil was observed when two antagonists were applied in combination *B. subtilis* CA32 and *T. harzianum* RU01 to seeds and soil respectively (Table 3, 4).

Table 3: *R. solani* population in the rhizosphere of chili and brinjal at day 30

Treatments	Chili	Brinjal	Chili	Brinjal
T_1	ND	ND	ND	ND
T_2	5.25a	5.50a	5.00a	5.28a
T_3	2.20bc	2.50b	-	-
T_4	-	-	4.50a	4.85a
T_5	4.95a	5.20a	-	-
T_6	-	-	2.20bc	2.00bc
T_7	1.25c	1.10c	1.28c	1.25c
T_8	4.25a	3.85a	4.20a	4.15a

ND: Not Detected. Values are means of three replicates. Data followed by the same letter(s) in a column are not significantly different from each other according to Duncan's multiple range test at $p = 0.05$

Table 4: *R. solani* population in the bulk soil of chili and brinjal at day 30

Treatments	Chili	Brinjal	Chili	Brinjal
T_1	ND	ND	ND	ND
T_2	7.25a	6.50a	6.00a	7.28a
T_3	6.20a	6.50a	-	-
T_4	-	-	4.90b	4.85b
T_5	5.95a	5.20a	-	-
T_6	-	-	1.20c	2.00c
T_7	2.25c	2.10c	2.28c	1.95c
T_8	5.25a	5.85a	5.20a	4.15b

ND: Not Detected. Values are means of three replicates. Data followed by the same letter(s) in a column are not significantly different from each other according to Duncan's multiple range test at $p = 0.05$

DISCUSSION

A few strategies have been suggested to reduce variability and enhanced the efficacy in biological control (Raupach and Kloepper, 1998; Guestsky *et al*., 2002). Among them applying several biocontrol agents simultaneously is one of them. Several reports have demonstrate the use of more than one biocontrol agents to control different plant pathogens in different crops (Sid Ahmed *et al*., 2003; Kim *et al*., 2008). In this study, two beneficial microorganisms *B. subtilis* CA32 and *T. harzianum* RU01 were tested for their ability to suppress damping-off of chili and brinjal caused by *R. solani* at the seed-bed stage under greenhouse conditions. *R. solani* strain used in the study significantly increased the disease severity. Results of the present investigation revealed potentiality of two biological control agents in reducing the disease severity in two Solanacious crops, chili and brinjal, caused by *R. solani* depending on their mode of applications.

Bacterization of seeds resulted significant plant protection but seed application of *T. harzianum* RU01 did not provide significant protection although *T. harzianum* RU01 antagonizes *R. solani in vitro*. On the other hand soil application of *B. subtilis* CA32 was not effective for protecting both crops. However, *T. harzianum* RU01 when applied to soil gave better protection than seed bacterization. This indicated the importance of the mode of application of biological control agents in order to achieve *in situ* biological control ability. Some other reports also demonstrate bacterization of different seeds with rhizobacteria and application of *Trichoderma* sp. to provide disease suppression in chili including *R. solani* as the challenging pathogen (Sid Ahmed *et al*., 2003; Rini and Sulochana, 2006) but to our knowledge no reports are available on brinjal and co-application of biocontrol agents in combination with *Bacillus* sp. and *Trichoderma* sp. isolates against *R. solani*.

In the event of antagonism it is essential that growing together of antagonistic and target organisms at the near vicinity or interact each organism at least by means of antagonistic compounds produced by the biocontrol agent with the target organism. In this context, the population of the biocontrol agent should be maintained in the rhizosphere at least at the threshold level (Raaijmakers *et al*., 1995). In some cases, the population of biocontrol agent in the rhizosphere and the biocontrol activity seems to be directly correlated (Weller, 1988). Therefore, one of the possible explanations for the difference observed in this study could be due to the unavailability of suitable level of biocontrol agent at the suitable time in order to suppress the pathogen development. The population density differences of the two biocontrol agents used in this study supports this hypothesis because *B. subtilis* CA32 maintained bacterial population around 10^6 cfu/plant but *T. harzianum* RU01 population in the rhizosphere on both crops was much lower when conidia added to seeds prior to sown (Table 5). Most probably seed bacterization with physiologically active bacteria would take the advantage of vigorous root colonization of bacteria that did not happen when application in soil. Moreover conidia application to the seeds would take some time to germinate and colonize throughout the root system. Therefore, bacteria would take the advantage over the fungal biocontrol agent when applied them as seed treatment. On the other hand soil application of bacteria could have been disappeared from the rhizosphere via percolation or exclude by the niche due to the microbial competition thereby deprived from the biocontrol ability.

Table 5: Plant root colonization ability of *B. subtilis* CA32 and *T. harzianum* RU01 on chili at different time point after application via different mode of application

	Log_{10} (CFU/g roots)					
	B. subtilis CA32			*T. harzianum* RU01		
	10	20	30	10	20	30
Treatments	days					
T_3	6.20b	9.55a	8.25a	ND	ND	ND
T_4	ND	ND	ND	4.25c	3.25c	4.50c
T_5	3.80c	3.20c	3.25c	ND	ND	ND
T_6	ND	ND	ND	6.25b	8.50a	9.75a
T_7	6.25b	9.25a	9.00a	6.50b	8.75a	9.25a
T_8	3.25c	3.50c	2.98c	4.25c	3.50c	3.80c

ND: Not Detected. Values are means of three replicates. Data followed by the same letter(s) in a column are not significantly different from each other according to Duncan's multiple range test at p = 0.05

Among the different strategies of application of biocontrol agents used in this study, seed bacterization with *B. subtilis* CA32 in combination with soil application of *T. harzianum* RU01 was found to be the most effective in terms of the lowest disease severity achieved by biocontrol agents. In this combination, apparently the bacterial biocontrol agent would be mainly responsible for rhizosphere protection while the fungal biocontrol agent responsible for providing the control effect in soil surrounding the root system rather than rhizosphere. To support this hypothesis the observation made this study on the population density of *R. solani* on rhizosphere and soil while adding bacteria and fungal biocontrol agent to seed and soil respectively alone and in combinations provides evidences to elucidate this effect. It has been observed that the number of *R. solani* propagules in the roots of the host plant is much lower when seeds were bacterized than that reached when added to the soil or also adding *T. harzianum* to seeds. At the same time, *R. solani* propagules in the bulk soil were much lower

when *T. harzianum* applied to soil than that reached when bacteria was also applied to the soil. This effect would work synergistically when two biocontrol agents are applied in the appropriate combination and thus resulted in a better plant protection.

The main conclusion of this study is that the biocontrol agents, *B. subtilis* CA32 and *T. harzianum* RU01, despite of their different nature, are synergistic and can be added together *via* different mode of applications to produce a greater effect on the pathogen. The *in vivo* assay confirmed that the antagonists are more effective in combination than when acting individually if applied in proper manner to protect chili and brinjal from root and collar rot caused by *R. solani*.

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