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Biological control of *Fusarium solani* f. sp. *phaseoli* the causal agent of root rot of bean using *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01

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Abstract. Root rot, caused by *Fusarium solani* f. sp. *phaseoli,* is one of the main root diseases impacting production of common bean in Sri Lanka. Rhizobacteria were screened in dual Petri plate assays to select antagonistic strains against *F. solani* f. sp. *phaseoli*. *B. subtilis* CA32 effectively antagonized the pathogen. *T. harzianum* RU01 also showed the antagonistic activity. The efficacy of the *B. subtilis* CA32 and the *T. harzianum* RU01 were tested in greenhouse pot experiments against *F. solani* f. sp. *phaseoli.* Seed bacterization with *B. subtilis* CA32 and *T. harzianum* RU01 significantly protected bean seedlings from *F. solani* f. sp. *phaseoli* compared to the untreated control plants. Plant protection was more pronounced in *T. harzianum* RU01 treated plants than bacterized plants. Enhanced root growth was observed only *T. harzianum* RU01 treated plants, suggesting that the biotic modifications of the mycorrhizosphere as a result of colonization with *T. harzianum* RU01.

Keywords: Rhizobacteria, Biological control, bean, antagonism

1 Introduction

Root rot of common bean (*Phaseolus vulgaris* L.) is a soil-borne disease that is incited by several fungal pathogens including *Fusarium* spp., *Pythium* spp. and, *Rhizoctonia solani*. It occurs in all bean-growing areas of the world. Root rot caused by *F. solani* f. sp. *phaseoli* is a major concern in many bean growing areas in Sri Lanka leading to enormous crop losses. The pathogen is known to be very persistent in soil and capable of surviving in infested fields for very long period and is difficult to control. *Fusarium* root rot is characterized by reddish-brown lesions along the tap roots and lower hypocotyls. Diseased areas of the plant enlarge with age and gradually turn brown. Longitudinal cracks may develop in older lesions and the cortical tissues be discolored and decayed. Root rots are particularly severe under water-stress condition (Burke and Hall, 1991).

Disease management options include crop rotation, improving soil fertility levels, use of resistant cultivars, use of fungicides and biological control. The impetus for developing biological control agents has been the public perception of pesticide toxicity in the environment. Residue-free produce has become a valuable commodity. Biological control is

compatible with pesticide-free agriculture and the environment. Roots of plants support the growth of a complex of microorganisms that can have a profound effect on the growth and survival of the plant. Among these organisms, arbuscular mycorrhizal fungi (AMF) are known to improve the nutritional status of their host, provide alterations in the host's physiology and exudation from roots (Filton et al., 2006). There is accumulating evidence that AMF can reduce disease incidence and propagule number of several soil-borne pathogens including *Fusarium* (Filton *et al*., 2006). Apart from the AMF, other microbial biological control agents such as rhizospheric bacteria (Handelsman & Stabb, 1996) and certain saprophytic fungi (Steyaert *et al*., 2003) have been identified as promising candidates to combat with various deleterious soil-borne pathogens on different crops. Certain biological control agents protect plants under field condition and commercial formulations of these organisms are now available (Spadaro & Gullino, 2005). However, control of *F. solani* f. sp. *phaseoli* by using bacterial biological control agents and *Trichoderma* spp. do not exist. At present, there is very limited knowledge and experience regarding the biological control of soil-borne diseases in Sri Lanka. Therefore, the main objective of the current study was to screen rhizospheric bacteria and test *Trichoderma* isolate RU01 for *in vitro* antagonism against *F. solani* f. sp. *phaseoli* and evaluate their efficacy under greenhouse conditions in order to select potential biological control agents which could be used in the field.

2 Materials & Methods

Biological control agents used in this study

Bacterial cultures

Bacteria used in this study were isolated from *Capsicum annuum* L. rhizosphere as described in Abeysinghe, (2007). Bacterial isolates were maintained in 80% glycerol (v/v) at -80 ^{0}C. In order to culture these bacteria, a loopful of inoculum was streaked on CAA plates (Bactocassamino acid 5g (Difco, Detroit, MI), K_2HPO_4 0.18 g, $MgSO_4.7H_2O$ 0.25 g, 18 g of bactoagar, water 1 l). After incubation for 24 h at room temperature, single colonies were streaked on fresh CAA plates.

Trichoderma harzianum RU01

Trichoderma spp. was isolated from soil samples obtained from a commercial chili field at Angunukolapalasse by using dilution Petri plate technique on *Trichoderma* Selective Medium (Askew and Laing, 1993) and maintained on PDA. Identification was performed by using colony characteristics described in Gams & Bissett (1998). *T. harzianum* RU01 was selected for further studies because of its antagonistic activity against different fungal pathogens.

Screening of antagonistic bacteria against *F. solani* in Petri plate assay

All bacterial isolates were initially screened for the ability to inhibit fungal growth on CAA plates. Single colonies were selected and patched along the perimeters of plates and incubated overnight at 28 ^{o}C. The following day each plate was inoculated at the centre with

a 5 mm diameter plug of the growing fungus. The plates were incubated at 28^{o}C for 2 -3 days and observed for inhibition of fungal growth. The bacteria positive for antagonism were selected. Zones of inhibition indicated antifungal activity and the strains were ranked according to the inhibition zone. An inhibition zone was defined as the distance between the leading edge of the fungal growth and the closest edge of the bacteria. Inhibition was expressed relative to a control strain spotted on the same plate.

Interaction between *T. harzianum* and *F. solani* in Petri plate assay

Five millimeter-diameter discs of *T. harzianum* isolate were removed from the edge of colonies of 4-day old PDA cultures and placed on one side of a Petri dishes containing PDA medium. Similar dishes of *F. solani* isolate grown in the same manner were placed on the opposite side of Petri plates and made three replicates. Cultures were observed daily and recorded for antagonism or parasitism of *Trichoderma* isolate against *F. solani*.

Isolation of pathogen

Phaseolus vulgaris plants showing root rot were collected from commercial bean fields at Balangoda, in the Sabaragamuwa Province of Sri Lanka. Infected parts of the plants were excised with a sterile scalpel and, surface sterilized with 3% (w/w) NaOCl for 2 min. Sterilized pieces were washed twice with sterile water for 60s and, cut into small pieces (1 cm length) and transferred on to antibiotic amended PDA plates. Plates were incubated at room temperature for 48 h, and white mycelium growth from the infected stem pieces were transferred to new PDA plates. After incubation for 5 days, a single spore was isolated and cultured on new PDA plates. The pathogen was identified as *Fusarium solani f. sp. phaseoli*, based on the characteristics described by Booth (1977). Koch's postulates were demonstrated for the pathogen and confirmed as the causal agent of root rot of *P. vulgaris*.

Bacterial innoculum preparation for seed bacterization

Among the bacterial isolates the most antagonistic bacterial isolate, according to the *in vitro* Petri plate assay, *Bacillus subtilis* CA32 was selected for greenhouse pot experiments. Fresh cells were obtained from stock cultures stored at – 80 ^{o}C and grown in CAA broth overnight at room temperature in a shaker. From this 100 mL of CAA broth in a 250 mL flask was inoculated and incubated for 48 h at room temperature in a rotary shaker (100 round/min). The bacterial culture was centrifuged (6000 g for 10 min at 4 ^{o}C) and the supernatant was discarded. The cell pellet was resuspended in sterile 0.85% NaCl and centrifuged again under 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 spectrophotometricaly adjusted to 10^{8} CFU/mL and used for seed bacterization.

Inoculum production of *Trichoderma harzianum*

For seed inoculation, *Trichoderma* was grown on PDA in Petri plates for seven days at room temperature 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 and mixed (0.01%) Tween-20 as a wetting agent.

F. solani inoculum and experimental set-up

The pathogen inoculum was produced on potato dextrose agar (PDA). The plates were inoculated with an agar plug (5 mm in diameter) containing actively growing *F. solani* mycelium, and incubated under fluorescence for 10 days at room temperature. Spores were washed from the plates with sterile distilled water and the concentration was adjusted to 10^6 conidia/ml with a haemocytometer. Ten days after planting when the primary leaves were fully expanded, the best seedlings were selected by thinning to three plants per pot. Five milliliters of spore suspension was applied by pipette just below the collar region around the hypocotyls of each plant. Disease severity was assessed 21 days after inoculation. Seedlings were removed from the pots and excess soil clumps were removed by gently shaking and by dipping the roots in water. Roots were dried with a paper towel and rated immediately for symptoms of root rot. The severity of root rot was visually scored by assessing necrotic lesions on the roots and hypocotyls using a rating scale of 0-5 described according to Filion *et al*., (2003). Treatments consisting of 3 seedlings/pot and 5 replicates were included per treatment. The trial was conducted twice and the experiment was arranged in a completely randomized block design.

3 Results

Pathogen identification and characteristics

Colonies grown on PDA or CAA became brown 7 day after incubation, and produced macro and micro conidia. The conidia were approximately 0.4 mm diameter. The pathogenicity tests for the *F. solani* isolate was examined with different local bean cultivars. Based on the Koch's postulates *Fusarium* isolate was pathogenic to all tested local bean cultivars to different levels (data not shown).

Identification and characterization of *Trichoderma* spp.

Trichoderma spp. grew rapidly at room temperature on PDA. Cultures at first were white and cottony then turned to bright green, finally they became dark green. Chlamydospores were intercalary and/or terminal, globose and smooth walled. Single phialides arose laterally on the conidiophores in clusters. Therefore *Trichoderma* isolate was identified as *T. harzianum* and named as RU01.

In vitro antagonism tests

T. harzianum RU01 against *F. solani*

Antagonistic properties of *T. harzianum* RU01 was tested using dual Petri plate method. *Trichoderma* inhibited the mycelial growth of *F. solani* but could not overgrow the pathogen until 3 to 4 days. However, several days later *F. solani* over grew the *Trichoderma* mycelia. Furthermore, conidia production decreased compared to the control plates (data not

shown). *T. harzianum* hyphae coiled around hyphae of *F. solani* causing vacuolization and disintegration of *F. solani* hyphae indicating strong antagonistic activity of the *T. harzianum* isolate RU01.

Screening of rhizobacteria against *F. solani*

From the preliminary round of screening for antagonism in Petri plate assay, only four bacterial isolates were selected for further study. Among these isolates, *B. subtilis* CA32 was selected for greenhouse pot experiments because CA32 was the most antagonistic isolate in terms of inhibition zones in the plate assay (Table I).

Table 1: Mycelial inhibition of *F. solani* f. sp. *phaseoli* in CAA by bacterial isolates

Isolate	Mycelial inhibition mean (%)[x]
Bacillus subtilis CA32	55.50 [a]
Pseudomonas fluorescens CA05	32.00 [b]
Pseudomonas putida CA28	30.32 [b]
Bacillus subtilis CA16	28.70 [b]
Control	0 [c]

[X] *The percent mycelial inhibition data were analyzed using analysis of variance (ANOVA) and a mean separation was done by Fisher's least significant difference at P < 0.05. Three replication plates were included per bacterial isolates and the data were pooled from two separate experiments. Mycelial inhibition means with the same letter are not significantly different from each other (95% confident limit).*

Plant protection ability of biological control agents under greenhouse conditions

All bean seedlings grown in soil inoculated with a conidial suspension of *F. solani* f. sp. *phaseoli* showed red lesions on hypocotyls and tap roots characteristically distinctive of Fusarium root rot. In contrast, all plants from seeds bacterized with CA32 or treated with *T. harzianum* RU01 conidia showed significantly less number of lesions and low disease severity. The noninfested controls showed no symptoms. However, *T. harzianum* treated plants were well protected from the pathogen infection than CA32 treated plants (Table 2).

Table 2. Effect of application of *B. subtilis* CA32 and *T. harzianum* to the seeds of *P. vulgaris* on infection of *F. solani* f. sp. *phaseoli* in pot experiments

Treatment	Disease severity [x]		
	Trial I [y]	Trial II	Trial III
Healthy control	0 a	0 a	0 a
Disease control	4.2 b	4.5 b	3.8 b
B. Subtilis CA32	2.4 c	2.2 c	2.6 c
T. harzianum	1.6 c	1.8 c	2.0 c

[X] *Disease severity was assessed using a rating scale of 0-5, where 0 = no disease symptoms, 1 = slightly brown <50% surface discoloration of the hypocotyl, 2 = >50% surface discoloration, 3 =*

discolored hypocotyls and extensive root pruning, 4 = darkly discolored hypocotyls and root completely collapsed and severe root pruning, and 5 = dead or dying plant.

[Y] *Values followed by a different letter within columns are significantly different according to the LSD test at P<0.05 using Duncan's multiple range test.*

The length and fresh weight of root was significantly increased ($P < 0.05$) in plants colonized by *T. harizanum* than CA32 treated plants as compared with nontreated control seedlings (Table 3).

Table 3. Effect of biological control agents on *P. vulgaris* root development

Treatment	Growth parameters	
	Root length (cm)	Root fresh weight (g)
Healthy control	5.2±1.25 a[X]	9.25±1.25 a
Disease control	3.2±1.87 c	5.28±0.98 c
B. subtilis CA32	5.6±1.92 a	10.25±1.75 a
T. harzianum	9.8±2.12 b	15.35±2.02 b

[X] values are the means of three replicates ± standard error of the mean. Values followed by a different letter within columns are significantly different using Duncan's multiple range test ($P<0.05$).

4 Discussion

Although the interactions between many bacteria and fungi have been studied those involving *Fusarium solani* f. sp. *phaseoli* have received less attention. This study presents the data of screening of bacteria isolated from *C. annuum* rhizosphere and *Trichoderma harzianum* against *Fusarium solani* f. sp. *phaseoli*, one of the major causal agents of root rot of bean in Sri Lanka. Among 53 bacterial isolates only 4 isolates were able to antagonize *F. solani* in dual Petri plate assay. *B. subtilis* CA32 was the strongest antagonistic isolate (Table 1). More over, *Trichoderma harzianum* RU01 also inhibits the growth of *F. solani* and reduces the production of conidia indicating antagonistic properties of *T. harzianum* RU01 against the pathogen. However, spore production inhibition did not quantify. The *in vitro* culture of *F. solani* and *T. harzianum* RU01 in culture media led to a variety of interactions. *F. solani* growth was generally inhibited; the host cell contents disorganized and the hyphae were intensively parasitized by *T. harzianum* RU01. Similar reactions have been reported on other fungal pathogens (Hanson & Howell, 2004) but no reports on *F. solani*.

The results reported here suggest that the presence of either *B. subtilis* CA32 or *T. harzianum* RU01 in the rhizosphere significantly reduces the root rot caused by *F. solani* in bean plants. The reduction might be related to the decline of the population density of *F. solani* in soil and also due to alterations caused by *T. harzianum* RU01 in the *F. solani* hyphae as observed *in vitro*. However, the protection exerted by the *T. harzianum* RU01 against *F. solani* was pronounced than *B. subtilis* CA32. This difference may be due to the more than one mode of mechanisms exerted by the *T. harzianum* RU01 which may have an

additive effect in plant protection. Most likely the enhanced growth of root system by *T. harzianum* as evidenced by increased biomass may be positively acted in this respect. More over *T. harzianum* is a well-known producer of cell wall-degrading enzymes and the antibiotics thus could act synergistically with other mechanisms (Vinale *et al*., 2006).

In conclusion, the present study clearly demonstrated that antagonistic bacterial strain *B. subtilis* CA32 and *T. harzianum* RU01 can be used as biological control agents in order to protect bean plants from *F. solani* f. sp. *phaseoli* under greenhouse conditions. The combine use of these biocontrol agents and the evaluation of the biological control efficacy under field conditions are underway.

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