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Evaluation of Local Isolates of *Trichoderma* Spp. against Black Root Rot (*Fusarium solani*) on Faba Bean

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Abstract

Faba bean (*Vicia fabae L.*) is one of the most important pulse crops in Ethiopia and is now cultivated on large areas in many countries. In most growing areas, however, the production of the crop is constrained by several disease infections, including fungal diseases. Black root rot caused by *Fusarium solani* is the most destructive disease of faba bean. The antagonistic potentials of locally isolated *Trichoderma* spp. from rhizosphere soils of faba bean plants in the highlands of northeastern Ethiopia were evaluated against *F. solani*, responsible for black root rot. All isolates of *Trichoderma* spp. had strong biological control activity against *F. solani in vitro* as well as *in vivo* pot experiment. Under dual culture, the percentage of mycelial growth inhibition of *F. solani* by the *Trichoderma* ranged from 33.9 to 67.0%. The highest (67.0%) inhibition was obtained from isolate TS036, while the lowest (33.9%) by isolate TS015. Pathogen-inoculated faba bean plants grown in pots that were treated with antagonists had taller plant heights and biomass than the *Trichoderma* untreated control inoculated with *F. solani*. The *Trichoderma* isolates significantly reduced black root rot severity on faba bean seedlings with disease reduction ranging from 64.4 to 74.6% over control. Use of *Trichoderma* species can be a potential source of biological control agents for the management of black root rot in faba beans grown in the region. Hence, the potential *Trichoderma* isolates under field condition might used as a components in the integrated management of *F. solani* that caused faba bean black root rot in the highlands of northeastern Ethiopia.

Keywords: Antagonist; Biocontrol; *Fusarium solani*; Inhibition; Rhizosphere; *Trichoderma*

Introduction

Faba bean (*Vicia fabae L.*) is one of the most important pulse crops in the world and is used as a source of protein in human diet for substitute of animal protein, animal feed, generate incomes and improve soil fertility [1]. In Ethiopia, black root rot of faba bean caused by *Fusarium solani* is the most widespread and destructive disease in black clay soils, where water-logging is severe [2,3] with yield reduction of up to 45% [4]. A number of management options have been used in minimizing the effects. These include use of resistant varieties; application of cultural practices, such as crop rotation and furrow planting to drain out excess water [2,5,6].

Biological control of plant diseases is considered as one of the viable alternative methods to manage plant diseases [7,8]. Application of fungicides is not economical in the long term because they pollute the environment, leave harmful residues and can lead to the development of resistant strains of the pathogen with repeated use [9]. However, use of biological control is safe, nonhazardous for human, farm animals and avoids environmental pollution [7,10]. The application of biological controls using antagonistic microorganisms has proved to be successful for controlling various plant diseases in many countries [11]. Most of these studies were on the control of root and soil-borne plant pathogens and, to a lesser extent of foliar pathogens [12].

One of the most important biological control agents is the use of *Trichoderma* spp. that are most frequently isolated soil fungi and present in plant root ecosystems [12,13]. They colonize the root and rhizosphere of plant and suppress plant pathogens by different mechanisms, such as competition, mycoparasitism, antibiosis production and induced systemic resistance, improvement of the plant health by promote plant growth, and stimulation of root growth [13-17].

The antagonistic activity of the genus *Trichoderma* to *F. solani* has been widely demonstrated [18]. Application of *T. harzianum* as seed treatment significantly reduced the incidence of damping-off diseases of faba bean, lentil, and chickpea, when planted in a soil naturally contaminated with *Fusarium* spp. and *R. solani* [19]. *Trichoderma harzianum*, *T. koningii* and *T. viride* are reported to improve seedling emergence and health of runner bean (*Phseous coccineus*) when applied as seed treatments [20,21]. *Trichoderma harzianum* introduced to the rhizosphere of faba bean plants significantly reduced root rot incidence more than the fungicide Rizolex-T [22].

Little work has been done on *Trichoderma* spp. as antagonists in the control of faba bean black root rot in Ethiopia. So far, not much previous research has been done on the use of biological control agents for the control of black root rot of faba beans in Ethiopia. The strain of *Trichoderma viride*, tested in the laboratory, proved to be an effective antagonistic activity against *Fusarium solani*, black root rot of faba bean [23,24]. Therefore, the objective of the present study was to identify native *Trichoderma* species and to evaluate their antagonistic effects against *Fusarium solani* on faba bean.

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Materials and Methods

Isolation of *Trichoderma* spp.

Soil samples were collected from different healthy faba bean growing fields in the highlands of northeastern Ethiopia (Table 1). One hundred gram rhizosphere soil was collected into each sterile plastic bag and kept in the refrigerator at the Plant Pathology Laboratory, Haramaya University, for further analysis. Isolation of antagonistic *Trichoderma* spp. from rhizosphere soil was made using serial dilution technique [25]. Each composite soil sample was thoroughly mixed and pulverized by means of mortar and pestle, and passed through a 0.5 mm soil screen sieve before 1 g was suspended in 9 ml sterile distilled water. The suspensions were made homogeneous by agitation using a vortex mixer and further serial dilutions of 10^{-2}, 10^{-3} and 10^{-4}.

One milliliter of serially diluted suspension from each dilution was pipetted into potato dextrose agar (PDA) medium and the petri plates were thoroughly mixed by gently swirling in clockwise and anti-clockwise direction to uniformly spread the suspension. Isolates of *Trichoderma* colonies were picked for antagonism studies after incubating the plates at 25 ± 1°C for 48 h. and re-streaked on a new plate but of the same media to obtain pure colonies. Nineteen *Trichoderma* isolates were identified according to Kubicek and Harman, [26] based on their conidial morphology, color and texture, and growth characteristics and the isolates were confirmed to be *Trichoderma koningii* (17 isolates) and *Trichoderma viride* (2 isolates) (Table 1).

In vitro screening test

The antagonistic effects of each *Trichoderma* sp. against *F. solani* were tested using dual culture technique [10,27]. The tested isolates of *Trichoderma* spp. were grown on potato dextrose agar (PDA) medium at 20°C, for 6-days and used as inocula. Disks from each isolate of *Trichoderma* spp. (5 mm in diameter) were inoculated on PDA medium in one side in Petri plate and the opposite side was inoculated by *F. solani* inocula. The inoculated plate with *F. solani* only but without *Trichoderma* treatment was used as control. The treatments were replicated three times and arranged in a completely randomized design (CRD) and the experiment was repeated. Four and five days after incubation periods at 25 ± 2°C, data on growth inhibition zone and colony diameter were recorded for each plate. The radii of the fungal colony towards and away from the antagonistic colony were measured and the percentage growth inhibition was calculated [28] as follows:

$$\% \text{ Inhibition} = \frac{(R\text{-}r)}{R} \times 100$$

where, *R* is the maximum radius of the fungal colony away from the antagonist colony, and *r* is the radius of the fungal colony opposite the antagonist colony

Greenhouse test

On the bases of *in-vitro* results, nine *Trichoderma* isolates were tested to assess their effects on black root rot development on faba bean seedlings. *Trichoderma* isolates were grown on the plates and the spores were washed from the plates with sterile distilled water and the concentration was adjusted to 10^6 conidia ml^{-1}. Sterilized faba bean seeds of the susceptible faba bean variety CS-20DK were dipped into the suspension of each *Trichoderma* spp. for five hrs. Seeds inoculated with sterile water only (i.e. without antagonists) were used as control. The inoculated seeds (5 seeds per 15 cm diameter pot) were planted in sterile black soil (clay 70%: silt 16%: sand 14%) and kept in the greenhouse at 24-28°C during day and 15-20°C at night. After a week, each pot was artificially drenched with aggressive isolate of *F. solani* at a rate of 10^6 conidia ml^{-1}. The experiment was conducted in a randomized complete block design (RCBD) with three replications and the experiment was repeated.

One month after planting, plants were removed from the soil and the roots were washed with sterile distilled water and the root rot severity was scored by assessing necrotic lesions on the roots and hypocotyls using a rating scale of 0-4, where 0=hypocotyls and roots white and firm, no root pruning; 1=slightly brown or discolored hypocotyls and roots, slight root pruning; 2=moderately discolored hypocotyls and roots, extensive root pruning; 3=darkly discolored hypocotyls and roots, hypocotyls completely collapse or, severe root pruning; 4=dead or dying plant [29] (McFadden et al.), Based on the disease severity data, percentage of root rot suppression was calculated [30] as follows:

$$\% \text{ Suppression} = \frac{A\text{-}B}{A} \times 100$$

where, *A* is the disease severity exhibited in the root region due to *F. solani* alone and *B* is the disease severity exhibited in the root region after inoculation with both the pathogen and bacterial antagonists. Data of plant height and biomass was also recorded.

Data analyses

Statistical analyses were performed using General Linear Modeling (GLM) procedure of SAS® System for Windows Version 9.1 software [31]. Severity ratings were normalized before analysis using square root transformation with the formula $(X + 0.5)^{1/2}$, where *X* is the severity rating of root rot and 0.5 is constant number added [32]. The least significant difference (LSD) test at 5% level of significance was used to separate treatment means for each measured parameter.

Results

In vitro screening test

Isolates of *Trichoderma* species was found to occur on faba bean rhizospher soil in the three districts of northeastern highlands of Ethiopia (Table 2 and Apendix C3); however, the distribution of isolates was found to vary among the districts. Highest number of isolates was obtained from Jamma, followed by Woreillu and Delanta. The elevation of the sampled areas varied between 2551 and 3017 meters above sea level.

The efficacy of local *Trichoderma* isolates in inhibiting the mycelial growth of *F. solani* in dual culture was determined on PDA medium. Results of dual culture tests clearly showed that all the isolated

Isolates	*Trichoderma* species
TS004, TS007, TS015, TS018, TS019, TS022, TS025A, TS027, TS030, TS032, TS037, TS041A, TS047, TS050, TS058, TS064, TS090	*Trichoderma koningii*
TS010, TS036	*Trichoderma viride*

Table 1: Identification of the local *Trichoderma* isolates.

Districts	Altitude	Grid reference	Potential antagonistic *Tricoderma* isolates
Delanta	2885	50^{0}E, 12^{0}N	TS090
Jamma	2551-2621	52-53^{0}E, 11^{0}N	TS004, TS007, TS010, TS015, TS018, TS019, TS022, TS025A, TS027, TS030, TS032, TS036, TS037, TS041A,
Woreillu	2627-2725	54^{0}E, 11^{0}N	TS047, TS050, TS058, TS064,

Table 2: Occurrence of *Trichoderma* isolates on the soil rhizospher of faba bean plants in the highlands of northeastern Ethiopia.

Trichoderma spp. significantly ($P \leq 0.05$) inhibited the radial growth of *F. solani* at varying degrees (Table 3 and Figure 1). These *Trichoderma* isolates were able to inhibit the mycelial growth of *F. solani* by the range of 33.9 to 67%. Nine isolates significantly inhibited the colony mycelial growth of *F. solani* the most promising isolates resulting in more than 50% inhibition. Maximum inhibition zone (67.0%) was exhibited by the isolate TS036, followed by TS025A (65.9%) and TS050 (63%), while the lowest (33.9%) inhibition was due to the isolate TS015. Generally, the antagonists inhibited the mycelial growth of *F. solani* but could not overgrow the pathogen until three to four days. However, five days later, the *Trichoderma* overgrew the pathogen and wholly occupied the medium.

Greenhouse test

The application of antagonists reduced significantly ($P \leq 0.05$) the extent of black root rot infection in comparison to *F. solani* alone (without *Trichoderma* isolates) inoculated plants (Table 4). The effectiveness of the antagonists ranged from 69 to 74% disease suppression over the control. The maximum (74%) control or suppression of black root rot was observed in bean plants treated with isolate TS025 and TS050, while the lowest (64.4%) was with isolate TS058.

In the present study, all antagonists significantly enhanced the height and biomass of bean seedlings as compared to bean seedlings inoculated with *F. solani* alone (Table 4 and Figure 2). The maximum plant height was observed in plants treated with isolates TS036 (38.5 cm), TS019 (38.4 cm) and TS007 (37.9 cm) followed by isolates TS010 (36.8 cm), TS050 (36.7 cm), TS058 (36.6 cm) and TS022 (34.5 cm), while the shortest (33.4 cm) plant height was observed in plants treated with isolate TS025. *Trichoderma* treated seedlings increased in fresh biomass ranging from 24.1 to 40.5% over the control. Among the potential biological control agents in this study, isolates TS019, TS036, and TS010 resulted in 40.5, 37.8 and 37.4%, respectively, increased fresh biomass over control. The least reduction in plant fresh weight was observed in plants treated with TS047 (42.4 g). Better overall growth of seedlings indicated the efficiency of *Trichoderma* antagonists in controlling faba bean black root rot.

Isolates	*Trichoderma* radius (mm)	Inhibition (mm)	Inhibition (%)
TS004	49.7 ± 0.1b-d	19.3 ± 0.1a	40.2 ± 0.1f
TS007	52.3 ± 0.2ab	13.3 ± 0.1c	55.1 ± 0.2d
TS010	54.0 ± 0.1a	12.7 ± 0.2cd	60.5 ± 5.1b-d
TS015	50.3 ± 0.2bc	20.7 ± 0.1a	33.9 ± 2.7g
TS018	50.0 ± 0.2b-d	16.7 ± 0.2b	47.4 ± 5.0e
TS019	52.3 ± 0.2ab	11.3 ± 0.2d-f	61.4 ± 4.4a-c
TS022	50.0 ± 0.1b-d	12.3 ± 0.2c-e	60.7 ± 3.8b-d
TS025A	51.7 ± 0.2ab	10.7 ± 0.1ef	65.9 ± 1.2ab
TS027	47.0 ± 0.2de	19.3 ± 0.1a	34.8 ± 1.7fg
TS030	45.7 ± 0.5e	19.3 ± 0.2a	37.0 ± 2.8fg
TS032	47.0 ± 0.3de	20.0 ± 0.0a	34.8 ± 1.3fg
TS036	49.6 ± 0.1b-d	10.3 ± 0.1f	67.0 ± 1.6a
TS037	49.7 ± 0.1b-c	16.0 ± 0.1b	47.2 ± 3.5e
TS041A	48.0 ± 0.3c-e	17.0 ± 0.2b	40.1 ± 5.9f
TS047	51.3 ± 0.2ab	12.0 ± 0.1c-f	60.4 ± 3.2b-d
TS050	49.3 ± 0.2c-d	11.3 ± 0.1d-f	63.0 ± 0.5a-c
TS058	49.3 ± 0.1b-d	12.3 ± 0.1c-e	58.6 ± 5.8cd
TS064	49.7 ± 0.1b-d	19.6 ± 0.1a	34.3 ± 2.0fg
TS090	49.3 ± 0.1b-d	20.0 ± 0.0a	35.9 ± 5.1fg
Control	-	31.6	-
CV%	3.89	7.05	7.11

Values in parentheses are square root transformed $(x + 0.5)^{1/2}$ values.
Means within column followed by the same letter(s) are not significantly different from each other at 5% level of significance.

Table 3: Effects of *Trichoderma* isolates on the radial growth (inhibition) of *Fusarium solani*.

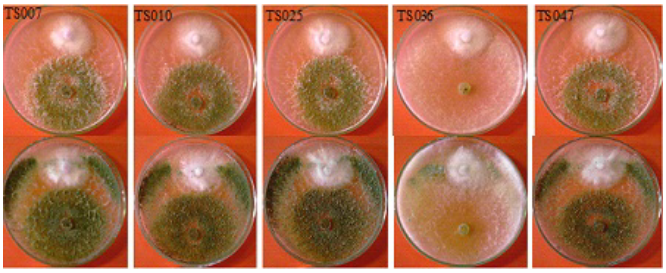

Figure 1: Differences *In vitro* antagonistic effect of selected *Trichoderma* isolates against *Fusarium solani* using dual culture method four (top) and five (bottom) days after inoculation. At the bottom of the plates overwhelming growth of *Trichoderma* isolate on *F. solani*, which indicates parasitic interaction. Heavy sporulation by *Trichoderma* is seen on and in vicinity of *F. solani* colony. Each perti dish has *F. solani* at the upper and *Trichoderma* at the lower side of the petri plate.

Isolate code	Disease severity (0-4 scale)	Disease suppression (%)	Plant height (cm)	Plant biomass	
				Fresh weight (g)	Dry weight (g)
TS007	1.20 (1.30)b	69.5	37.9 ± 3.2a	46.8 ± 7.4a-c	6.8 ± 1.6b
TS010	1.07 (1.23)b	72.8	36.8 ± 4.5ab	51.4 ± 3.7ab	7.7 ± 0.5ab
TS019	1.07 (1.23)b	72.8	38.4 ± 0.9a	54.1 ± 2.7a	8.5 ± 0.2a
TS022	1.06 (1.23)b	73.0	34.5 ± 1.6ab	47.1 ± 3.3a-c	7.0 ± 0.5b
TS025	1.00 (1.20)b	74.6	33.4 ± 2.4b	48.3 ± 3.7a-c	7.2 ± 0.5b
TS036	1.07 (1.23)b	72.8	38.5 ± 2.1a	51.8 ± 7.3ab	7.2 ± 0.8b
TS047	1.13 (1.27)b	71.3	33.5 ± 2.4b	42.4 ± 2.1c	6.9 ± 0.3b
TS050	1.00 (1.20)b	74.6	36.7 ± 1.6ab	49.5 ± 3.1a-c	7.3 ± 0.5b
TS058	1.40 (1.37)b	64.4	36.6 ± 1.5ab	45.5 ± 3.6bc	6.7 ± 0.4b
Control	3.93 (2.10)a	0.0	28.8 ± 1.9c	32.2 ± 2.9d	4.8 ± 0.6c
Mean	1.39		35.5	46.9	7.0
CV%	8.84		6.76	9.48	8.92

Values in parentheses are square root transformed $(x + 0.5)^{1/2}$ values.
Means within column followed by the same letter(s) are not significantly different from each other at 5% level of significance.

Table 4: Efficacies of potential *Trichoderma* isolates on faba bean black root rot under greenhouse condition.

Figure 2: Greenhouse pot experiment illustrating the efficacy of *Trichoderma* isolates in the suppression of black root rot infection in four weeks old faba bean seedlings. Faba bean plants inoculated with bioagents (A) showed better shoot and root biomass than plants treated with *F. solani* alone (B).

Discussion

Trichoderma is known antagonist of plant pathogens, and has been shown to be very efficient biological control agent of several soil-borne

plant pathogenic fungi [12,13,33]. *Trichoderma* is a good candidate for biological control due to the different modes of action that inhibit the growth of other fungi. The study was undertaken to determine the potentials of locally isolated antagonistic *Trichoderma* spp. that perform as biological control agents for the management of *F. solani*, which is responsible for black root rot on faba bean.

The results revealed that the isolates of *Trichoderma* spp., which were obtained from the rhizosphere soil of healthy bean plants, had effective biological control activity against *Fusarium solani* under *in vitro* and *in vivo* pot experiments. The potentiality of *Trichoderma* spp. as biological control agents of phytopathogenic fungi in several crops is well known, especially to *Fusarium solani* infection [34]. *T. harzianum*, *T. koningii* and *T. viride* protected the germinating bean seedlings against *Fusarium* spp. and *R. solani* infection [19,35].

Antagonistic capability of all *Trichoderma* isolates showed inhibitory effect against mycelial growth of *F. solani* in dual *in vitro* testing. The percentage of mycelial growth inhibition by the *Trichoderma* isolates against *F. solani* varied between 33.9 and 67.0%. Some isolates were highly inhibitory to *F. solani* growth, whereas others showed only lower activity. In the medium, the *Trichoderma* isolates grew much faster and suppressed the growth of *F. solani in vitro*. The competition mechanism of *Trichoderma* depends on their rapid growth rate limiting nutrients and space for *F. solani* and this may produce inhibition of *F. solani* growth up to 67%. Effective biological control agents inhibit the growth of the target organisms through their ability to grow much faster than the pathogenic fungi thus competing efficiently for space and nutrients [13]. Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens. Competition is effective when the pathogen conidia need exogenous nutrients for germination and germ-tube elongation [36].

A second mechanism of pathogen control that *Trichoderma* displayed was mycoparasitism. The mycoparasitic activity of the tested *Trichoderma* isolates was detected morphologically by subsequent profuse sporulation of *Trichoderma* and its ability to overgrow upon the mycelial growth of *F. solani* in culture which may indicate its ability to directly parasitize the pathogen. *Trichoderma* species exert biological control against fungal phytopathogens either indirectly by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms and antibiosis, or directly by mechanisms such as mycoparasitism [15,16,37].

The *Trichoderma* isolates, which showed high efficacy *in vitro* study, also significantly reduced the root rot severity in the greenhouse. The effective colonization of faba bean roots by *Trichoderma* isolates might have contributed to their capability to inhibit *F. solani* infection on faba bean roots. The effect of antagonist on the faba bean plant growth under pot condition revealed that faba bean seedlings grown in *Trichoderma*-treated soils had taller plant height and fresh weight than *F. solani* alone inoculated faba bean plants. The isolates significantly reduced black root rot severity on faba bean seedlings with disease reduction ranging from 64.4 to 74.6% over control. Application of the antagonist *Trichoderma* spp. as seed treatment significantly reduced the incidence of *Fusarium* spp. and *R. solani* in some leguminous crops [19,35], and grew readily along with the developing root system of the treated plants [35,37,38]. This might due to modification of the rooting system.

Conclusion

All antagonist species of *Trichoderma* isolates proved to be effective in controlling *Fusarium solani*, both under laboratory and pot conditions. The results indicate that the selected *Trichoderma* species could be potential sources of antagonistic agents for the management of black root rot on faba bean grown in the highlands of northeastern Ethiopia, where biological control agents can be used as one of the components in the integrated management of the disease. However, applying biological control agents in the field is influenced by many environmental, biological and physical factors. So, it desirable to evaluate further the biological control potential of the *Trichoderma* spp. under field conditions.

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