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Biocontrol of Fusarium root rot in beans by antagonistic *Trichoderma* fungi

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ABSTRACT
Common bean (*Phaseolus vulgaris*) is one of the most important economic crop which is attacked by serious diseases such as Fusarium root rot. In this study, the efficacy of *Trichoderma viride* and *T. koningii* were evaluated for the suppression of *Fusarium oxysporum* causing root rot of beans in vitro and disease control under greenhouse condition. Three food carriers - broken dehulled rice grain, sorghum seeds, and vermiculite were also evaluated for suitability as growth and delivery media for *Trichoderma* isolates as determined by the sporulation ability and root rot control respectively. In vitro studies resulted in effective suppression of *F. oxysporum* by the two *Trichoderma* isolates. Saprophytic growth on rice and sorghum was good at two weeks but poor on vermiculite. *T. viride* in half broken dehulled rice produced excellent bean root rot control as measured by disease severity at 7th week and was significantly ($P \leq 0.05$) better than standard seed dresser Murtano (20% Thiram and 20% Lindane); *T . koningii* and *T . viride* in sorghum, and *T. koningii* in rice had moderate disease control whilst the two *Trichoderma* isolates in vermiculite had poor disease control. There was some positive correlation between saprophytic growth of *Trichoderma* isolates in different carriers in the laboratory and their suppression of Fusarium root rot in the greenhouse. The results of this study indicates that potential exist for management of Fusarium root rot in beans by antagonistic *Trichoderma* isolates in their respective carriers and may form part of IPM for bean root rot.

Keywords**:** *Antagonistic Trichoderma isolates, Fusarium oxysporum, in vitro suppression, food carriers, bean root rot control.*

INTRODUCTION
Beans are extremely important pulse crop and food source of cheap protein in Kenya. In recent past it has become increasingly common for Kenya to import beans as annual domestic demands of 450,00 metric tones overwhelms production of 150,000-2000,000 tones harvested from about 250,000 hectares (Ministry of Agriculture, Republic of Kenya 2011). Diseases are the major constrains facing bean production in Kenya though other factors such as insect pests, soil infertility, environmental stress especially lack of enough moisture and lack of proper agronomical practices add to the very low production that averages 220-670 kg/ha (Acland 1971).

Among the important bean diseases, the root and stem rots caused by various species of soil borne fungi, bacteria and nematodes are considered to be the most economically important diseases of beans in Africa and

Latin America with yield reductions of as high as 86% reported in *Fusarium* infested fields under stressful conditions (Abawi and Pastor-corrales 1990). In Kenya Fusarium root rot is the most prevalent diseases in the bean growing areas of central and eastern Kenya with popular local varieties which includes Rose Coco, Canadian Wonder, Red Harricot, Mwitemania, Mwezi Moja and Wairimu being susceptible to the disease (Mutitu 1988 and Ministry of Agriculture, Republic of Kenya 2011). A serious bean root rot has been reported in Western Kenya since 1987 (Lianda M, Ministry of Agriculture, western province office, Kenya, personal communication 2000).

Options available for controlling root rots after planting are very limited and of questionable effectiveness (Abawi and Pastor-corrales 1990). Although there are many broad spectrum and highly specific soil fumigants such as methyl bromide that effectively control root rot pathogens and the diseases they cause, their use is limited by the high costs, and toxicity to man and environment (Nolling 1991**,** United Nations 2008). No resistant commercial varieties are available locally and crop rotation is not feasible due to land pressure (Mutitu 1988). The efficacy of the various chemical seed dressing fungicides in the market such as Melaxyl, Chloroneb, Captan among others in the control of Fusarium and other root rot diseases in beans are not sustainable perhaps due to resistance development resulting from multiple genera of the pathogens involved in most production locations and degradation after continued use (Abawi and Pastor Corrales 1990 and Nolling 1991). This underscores the need to intensify research on use of biocontrol strategy based on microbial antagonists and IPM for control of Fusarium root rot in beans. In vitro inhibition of many root rot pathogens and control of the disease they cause in the greenhouse and field by the various species of the antagonistic fungi *Trichoderma* has been reported by many researchers (Luban 2005, Ozbay and Newsman 2004, Ebtsum et al. 2009, Zahoor et al. 2012). This study was undertaken to evaluate the effectiveness of two *Trichoderma* isolates – *Trichoderma viride* and *Trichoderma Koningii* against *Fussarium oxysporum* causing root rot in beans in vitro and the disease control under greenhouse condition. The materials broken dehulled rice, sorghum and vermiculite were evaluated for suitability as *Trichoderma* food carriers for growth and delivery of the antagonists.

MATERIAL AND METHODS

Growth of *Fusarium oxysporum*

Fusarium Oxysporum was isolated from diseased bean plants with root rot symptoms sampled from infected fields in western Kenya. Five pure cultures were produced by growing the pathogen on PDA (potato dextrose agar) plates for one week at 20 ^{0}C. A spore suspension was prepared by flooding a pure *Fusarium oxysporum* cultures with sterile distilled water. The spores were dislodged with a bent glass rod and the contents passed through two layers of cheese cloth. The spore concentration was determined by use of hemacytometer.

Screening of antagonism

Trichoderma species known to be antagonistic to many fungal pathogens were obtained from Professor Mutitu EW of the department of plant Science and protection, university of Nairobi. The two isolates identified as *Trichoderma viride an*d *T. Koningii* were plated and purified in PDA at 20 ^{0}C and tested against the five pure *Fusarium oxysporum* isolates in the laboratory. Two methods of screening for antagonism adopted from Onkar and Sinclair (1986) were used.

i) Random sprinkling

From the pure cultures of *Trichoderma* isolates, 5mm discs were removed using a

cork borer and placed at four equidistant points, 1 cm from the periphery of a 9 cm diameter petri dish. The entire surface was flooded with *Fusarium oxysporum* spore suspension ($1x10^6$spores/ml) and excess drained off. This was repeated for each of the *Trichoderma* isolate against each of the five *Fusarium* isolates. Plates with *Fusarium* alone were used as control. Five replicates were made for each isolation and all plates incubated at room temperature ($20\pm2\ ^0$C). Observations were done daily up to 7th day. Diameters of each *Fusarium* isolate was taken at the 7th day and average for the five isolates calculated against each antagonist.

ii) Equidistant plating

Each of the potential antagonists was seeded in PDA at four sites 1cm from the plate periphery, and 5mm disc of each of the *Fusarium* isolates then at the centre of the PDA plate. Untreated control, replication incubation and assessment was as for method (i) above.

Growth and survival of *Trichoderma* isolates in three food carriers.

In this experiment, three food carriers were evaluated for suitability as growth media for the two *Trichoderma* isolates as determined by the growth and sporulation ability of the fungi. The three food carriers were half broken dehulled rice grains, sorghum seeds and vermiculite. The broken rice grain and sorghum seeds were each soaked in tap water in a beaker for 1 – 2 hours. Excess water was decanted and the grains autoclaved for 1 hour for 2 consecutive days at 121 ^{0}C and 15 psi. Vermiculite was only sterilized. After cooling, the grains were spread out in sterile metallic trays making a layer of about 3 cm thick. T*richoderma* spore suspension ($1x10^6$condia/ml) was uniformly sprayed on the surface using atomizer until they were wet after when the trays were covered with aluminum foil and incubated at room temperature. The experiment was carried in triplicate for each *Trichoderma* isolate and carrier. The inoculated substrate was stirred and mixed every other day using a sterile glass rod. Spore concentration per gram of carrier was determined and color of colonies noted on the 7th, 14th and 18th day after inoculation.

Enumeration of *Trichoderma* propagules in the carrier

The soil dilution method was used. Ten grams of *Trichoderma* colonized carrier was suspended in 90 ml of sterile distilled water and shaken for 30 minutes on a shaker at 500 rpm. While the suspension was still agitated, 10 ml was withdrawn and added to 90 ml of sterile water blank in a conical flask. The procedure was repeated up to 10^{-6} dilution. From each of the last 3 dilutions (10^{-4}, 10^{-5} and 10^{-6}) spore concentration was determined using a haemocytometer. The average spore counts for each dilution were multiplied by the reciprocal of the dilution to get the initial concentration.

Inoculation of bean plants in the greenhouse with antagonists grown on carriers.

The *Trichoderma* isolates grown on the carriers-broken dehulled rice, sorghum seeds and vermiculite previously treated in the laboratory were used. Steam sterilized soil allowed to cool for five days was placed in 20 cm diameter polythene sleeves. Spore suspension of each of the five *Fusarium oxysporum* isolates at a rate of 30000 conidia/g of soil (15 ml spore suspension of 10^6 spores/ml) was added to ½ kg of the sterilized soil, mixed and a portion of it used to inoculate the soils in 20 cm diameter polybags by mixing with the top layer (2-4 cm) of soil in each bag at the ration of 1: 3 (inoculated soil : uninoculated sterilized soil mixture). Composition (V/V) of soil mixture – Soil : Sand : Ballast : Manure=2:1:1:1. Four bean seeds of Rose Coco (GLP-2) variety were planted in each polythene sleeve and the experiment laid out in a completely randomized design (GRD) with

antagonists and carriers at two and three levels respectively making six treatment combinations. Bean seeds dressed with a chemical fungicide by trade name Murtano (Manufactured and supplied by Twiga chemical industries, P. O. Box 30172-00100 GPO Nairobi Kenya; email twiga-chem.com) which contains 20% Thiram (fungicide) and 20% Lindane (insect repellant) was used as standard check and the uninfested as the control. Five replicates were made. Procedure adopted from Onkar and Sinclair (1986).

Disease assessment

Disease severity and incidence were evaluated at the 7th week using the method by Campbell et al (1980). The bean plants were carefully uprooted and the roots gently washed in running tap water. The roots were examined for any visible infection. Root rot severity was determined by liner measurements of characteristics reddish brown lesions in the hypocotyls and roots, and their percentage infected portion calculated whilst the number and percentage of infected plants were used to determine infection incidence. Average disease severity and incidence data were calculated for each antagonist. Root rot assessment procedure adopted from Abawi and Pastor-corrales (1990).

Statistical data analysis

At the end of the laboratory and field experiments the data collected was analysed by One Way ANOVA using Gens tart 6th edition software and means separated using fisher's least significance different (LSD) procedure at $P \leq 0.05$. In case of zero values on the data of percent seedling survival, transformation to respective arc sine values was done before analysis.

RESULTS

Growth and survival of *Trichoderma spp* in food carriers

The growth and survival of the two isolates was affected by the type of carriers. The color of half broken dehulled rice and sorghum carriers changed assuming the characteristic greenish yellow and whitish color of the fungal isolates *T. viride* and *T. Koningii* respectively by the 14 days of incubation. Growth as determined by spore concentration per gram of carrier was found to increase with time after inoculation for the first ten days or so. Half broken dehulled rice grain proved to be excellent medium for growth and survival of both *Trichoderma* isolates; *T. viride* and *T. koningii* with 2.5x 10^7 and 1.4 x 10^7conidia/g respectively after 18 days. Sorghum seeds was moderately good with spore count for *T. viride* and *T. koningii* at $1.3x10^7$ and $2.2x10^7$ respectively. Growth on vermiculite was poor with spore concentration of $2.0x10^3$ and $1x10^3$ for *T. viride* and *T. koningii* respectively (Table 1).

Table 1. Mean number of *Trichoderma* conidia per gram of carrier at 7, 11, 14 and 18 days of incubation

Treatment	Incubation period (days)			
	7	11	14	18
T. viride in rice grain	55,500	2,000,000	25,000,000	25,000,000
T. koningii in rice grain	12,500	447,500	14,000,000	14,000,000
T. viride in sorghum seeds	47,200	47,667	13,333,333	13,000,000
T. koningii in sorghum seeds	606	95,000	22,000,000	22,000,000
T. viride in vermiculite	17	479	1,400	2,000
T. koningii in vermiculite	240	107	1,000	1,000

Values are means of three replicates.

Reduction of *Furarium* growth by *Trichoderma* isolates in vitro

There was nearly 100% inhibition of *Fusarium oxysporum* growth by antagonistic *T. viride* using the sprinkling method as observed by the 7th day after plating. Very poor growth of *Fusarium* with a reduction of 91% was observed in the presence of antagonist *T. koningii* (Table 2a). A similar trend was observed with the equidistant plating where percentage growth reduction by antagonistic *T. viride* and *T. koningii* was 98.2 % and 56.4% respectively (Table 2b). All the treatments were significantly (*P≤0.05*) different from the control.

Control of the bean root rot in the greenhouse using antagonists grown in carriers

The antagonist and the type of carrier used determined the level of the disease management in the greenhouse. The best control as determined by disease severity (%) at the 7th week was effected by *T. viride* in broken rice which had 32% root rot severity and significantly (P≤0.05) better than conventional fungicide Murtano and the uninfesated control. *T. viride* and *T. koningii* in sorghum had 59% and 51% respectively, and *T. koningii* in rice (56%) had moderate disease severity that was not significantly (P≤0.05) different from Murtano but significantly different from the control. *T. viride* and *T. koningii* in vermiculites gave poor disease control with 73% and 74% severity respectively and was not significantly (P≤0.05) different from both Murtano and the control. Mean lesion length and percent root rot incidence followed almost similar trends as those of disease severity with *T. viride* in rice giving the best results and antagonistic isolates in vermiculate the poorest (Table 3).

Table 2a and 2b. Inhibition of *Fusarium oxysporum* growth by antagonistic *Trichoderma* in culture.

2a.

Mode of treatment	Antagonist	Mean radial growth of *Fusarium* (cm)	% growth reduction
Sprinkling method	*T. koningii*	O.400a	91a
Sprinkling method	*T. viride*	0.004b	100ab
Untreated control	-	9.000c	0.00c

2b.

Mode of treatment	Antagonist	Mean radial growth of *Fusarium* (cm)	% growth reduction
Equidistant planting	*T. koningii*	1.90a	56.4a
Equidistant planting	*T. viride*	0.08b	98.2b
Untreated control	-	9.00c	0.0c

Values are means of five *F. oxysporum* isolates and five replicates. Values followed by the different letters of the alphabet are significantly different from each other at 5% (*P≤0.05*).

DISCUSSION

The inhibition of the *Fusarium oxysporum* growth by antagonistic *Trichoderma* isolates in dual culture assay suggested the production of inhibitory substance(s) by the antagonist which diffuses through the media causing growth and sporulation inhibition of the pathogen inoculums though other processes such as mycoparasitism and competition may be involved. Similar reports by Ahmand and Baker (1986), Cook and Baker (1983) and Elad et al. (1982) indicates that direct parasitism of *Trichoderma* on hyphae of other fungi, production of extracellular lytic enzymes for cell wall degradation and competition may play a major role in the control of soil borne plant pathogens by *Trichoderma* species. Haran et al (1996) reported differential expression of *Trichorderma harzianum*

chitinases during mycoparasitism. Hadar et al. (1979) found an isolate of *T. harzianum* which directly attacked mycelium of *Rhizoctonia solani*. *T. harzianum, T. koningii and T. viride* has been used against damping off caused by *Rhizoctonia* and *Pythium* in the laboratory, glasshouse and the field (Papavizas 1985).

The three food carriers evaluated for suitability as growth and delivery media as determined by the sporulation ability of the Culture tests through in vitro evaluation in the laboratory helps find potential antagonists but give no information on their activity in the field hence the need to look for an appropriate food base to be used for the delivery of *Trichoderma* isolates for field evaluation.

Trichoderma spp varied in their ability to support the antagonist growth and consequently activity on the root rot disease.

Table 3. Been root rot severity and incidence with antagonistic *Trichoderma viride* and *T. koningii* in different carriers.

Treatment	% root rot severity	% root rot incidence	Root rot severity (cm)
T. viride in rice grain	32c	15e	0.9d
T. koningii in sorghum seeds	51b	30d	2.4c
T. koningii in rice grain	56b	20e	2.7c
T. virid in sorghum seeds	59b	65b	2.9bc
T. viride in vermiculate	73a	45c	3.1b
T. koningii in vermiculate	74a	85a	3.2b
Murtano	60b	75ab	3.9a
Control	75a	95a	3.8a

Values are means of five replicates. Values followed by the different letters of the alphabet are significantly different from each other at 5% ($P \leq 0.05$).

Use of wheat bran-peat for application of *T. harzianum* as a seed coating for tomatoes and water melon was found to be effective against *F. oxysporum fsp. radicis-lycopersici* in tomatoes and *F . oxysporum fsp. niveum* on melon (Alex and Ilan 1993). Wells et al. (1972) found dehulled broken rice to be an excellent growth and delivery medium for *T. harzianum* and *T. Koningii* in the control of *Sclerotium rolfsii*.

The rapid suppression of the bean root rot disease by the applied antagonistic *Trichoderma* isolates in their respective carriers implied that the application rate used of 1:3 of antagonist infested carrier to pathogen inoculated planting soil mixture was appropriate for rapid growth and colonization of the media. This could had resulted in the antagonist being present in high concentration which increased the number of ‘’initial hits’’ of the pathogen propagules by antagonist. Van der Plank (1975) reported that continued infections and destruction of the pathogen might be expected to follow a pattern of infection foci, but with the amount of secondary spread of the hyperparasites among propagules being limited or non existence owing to greater distance between the propagules. Pathogen propagules tend to be more uniformly distributed in soil over time which increases their chance of contacting a host root but also their chance to escape an antagonist growing from other infested propagules. A model of Coy et al (1974) for the relationship between the nearest propagules uniformly distributed in soil indicates distances of 0.8 and 1.1 mm for 2,000 and 1,000 propagules per gram respectively. Applying the antagonist just as the inoculums is formed or released might help circumvent this advantage of escape otherwise gained by the pathogen when distributed more uniformly and sparsely in

the soil. Application of antagonists to the soil directly without a food base has been known to fail (Baker and Cook 1974, Mutitu 1988).

Reduction of the pathogenic fungi *Fusarium oxysporum* growth by the antagonist in vitro and suppression of the bean root rot disease caused by the pathogen under greenhouse condition demonstrated the ability of *Trichoderma* species to control diseases caused by the pathogen. Many workers have reported similar positive results involving use of *Trichoderma* isolates either singly or in combination with other strategies to control soil borne diseases. Luban (2005), and Ozbay and Newsman (2004) reported significant reduction of crown and root rot of Tomato disease incidence caused by *Fusarium oxysporum fsp radicis-lycopersici* by *Trichoderma spp* in the greenhouse in comparison to the check treatment. Ebtsum et al. (2009) reported suppression of *Fusarium solani* by a combination of *T. viride* and *Bacillus subtilis* in vitro and reduced infection of tomato by the pathogen in the field. Zahoor et al (2012) reported effective control of Fusarium root rot of Okra resulting in increased yield by use of *T. harzianum* and *T. viride* in combination with three fungicides Benlate, Ridomil and Dithane M-45.

A common feature of microbiological agents when compared with chemicals is the widespread resistance they encounter from the biotic environment and this perhaps explains the poor performance of the tested *Trichoderma* isolates in some carriers.

However, the vitro suppression of the bean root rot pathogen *Fusarium oxysporum* and effective control of the root rot disease it causes in beans by *T. viride* and *T. koningii* could be the dawn of a new era of effective management of the disease by microbial antagonism. Furthermore the control could be extended to other soilborne pathogens such as *Pythium* and root- knot nematode that together act to depress bean yield in most production locations.

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