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Evaluation of arbuscular mycorrhizal fungus and *Trichoderma harzianum* against *Armillaria* species and growth response of *Dombeya torrida* seedlings

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Armillaria root rot is a fungal root rot caused by several different members of the genus *Armillaria*. It has an extremely broad host range and hundreds of trees and shrubs are susceptible to root rot to varying degrees. Arbuscular mycorrhizal fungi (AMF) and *Trichoderma harzianum* are known to affect plant growth and disease resistance through interaction with phytohormone synthesis or transport in the plant. In the present study, potential of AMF and *T. harzianum* were tested for the biocontrol of *Armillaria* species and growth response of *Dombeya torrida* seedlings. *Armillaria* species was isolated from a severely infected *Dombeya torrida* plant and mass cultivation was done in malt extract Agar. Each plant was inoculated after every 1 month of growth by placing four agar plugs from the 14-day *Armillaria* species. Inoculant of mycorrhizae comprised, hyphae and infected root systemsand *T. harzianum* were prepared according to manufacturer's instructions. Single inoculation of arbuscular mycorrhizal fungi did not significantly change shoot fresh weight compared with control plants, while inoculation with *T. harzianum* alone significantly increased shoot fresh weight compared with control plants. Plants inoculated with *T. harzianum* alone had significantly. The presence of *T. harzianum* increased arbuscular mycorrhizal fungi root colonization compared to plants inoculated with the arbuscular mycorrhizal fungi alone, co-inoculation with arbuscular mycorrhizal fungi and *T. harzianum* producing a higher percentage of colonization than any other treatment. The study clearly demonstrated that inoculation of AMF and *T. harzianum* either individually or in combination enhanced plant growth response of *Dombeya torrida* seedlings. The inhibitory effects of the *T. harzianum*and AMF should be evaluated against *Armillaria* species under field growth conditions in order to explore their whole potential as biocontrol agents suppressing Armillaria root rot in the forest ecosystems.

Keywords: Arbuscular mycorrhizal fungi, *Armillaria* root rot, *Armillaria* species, *Dombeya torrida, Rhizomorphs, Trichoderma harzianum*

INTRODUCTION

Armillaria is a genus of parasitic fungi that live on trees and woody shrubs. *Armillaria* belongs to Family Tricholomataceae, Order Agaricales, classes Basidiomycetes, phylum Basidiomycota, and Kingdom Fungi. (Agrios, 2005). *Armillaria* species differ in geographical distribution as well as in ecological behaviour. The genus *Armillaria* comprises about 40 species worldwide. There are exannulate and annulate

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species (Schmidt, 2006). Most species are heterothallic and have a tetrapolar mating system. *Armillaria mellea* has both homothallic and heterothallic populations (Baumgartner *et al*., 2011). Populations of *A. mellea* in Europe and North America are heterothallic and are primarily out-crossing (Baumgartner*et al*., 2010). Populations in Africa (Ethiopia, Kenya, and Tanzania) and Japan are homothallic and are recognized as subspecies *A. mellea* ssp*. africana* and *A. mellea* ssp*. nipponica* respectively (Baumgartner *et al*., 2012). China is the only location where both heterothallic and homothallic *A. mellea* are known to occur (Qin *et al*., 2007).

Armillaria occurs worldwide on a wide variety of hardwood and softwood plants including many stone fruit species such as peach (Cox and Scherm, 2006). Members of the fungal genus *Armillaria* occur in boreal, temperate, and tropical forests worldwide, and Armillaria root disease is known to occur on all continents except Antarctica (Westwood *et al*., 2012). Incidences of *Armillaria* infection are becoming more common across Kenya, and are thought to be favoured by forest management activity that creates stumps.This fungus usually attacks trees that have been stressed or weakened by some other factor such as drought or insect attack (FAO, 2007). Predicting and understanding causes of tree mortality is essential to effectively manage forests for timber production as well as ecosystem and wildlife values (Westwood *et al*., 2012).

The spread of Armillaria root rot in forest has been associated with logging stumps. When infected trees are removed, *Armillaria* species rapidly colonize the cambium of the entire root system and above ground portion of the remaining stump and may persist in this food base for up to 25 or more years. The disease spread when healthy roots of neighbouring trees contact the infected roots of diseased trees or stumps (Robinson, 2003). All *Armillaria* species can survive saprotrophically by degrading woody substrates and typically produce, in the soil or under the tree bark, highly differentiated filamentous aggregations named rhizomorphs (Tsykun *et al*., 2011). Rhizomorphs are among the most complex organs produced by fungi, consisting of a series of differentiated tissues each with distinctive hyphal orientation, size, and function. Extension growth of these invasive organs can occur through dry and nutrient-poor soils and rotting wood, and rhizomorphs can breach mechanical obstacles (Yafetto *et al*., 2009). Rhizomorphs produced by pathogenic species allow fungi to travel between host plants. This process has contributed to the well-publicized spread of *Armillaria* species over vast territories (Smith *et al*., 1992).

The use of antagonistic microorganisms to control plant pathogens has been investigated as an alternative control method. Species of *Trichoderma*, applied as treatment of seeds or soil, have been demonstrated to control the pathogen in a variety of cultures in the greenhouse and field studies (Gajera and Vakharia, 2010). The genus *Trichoderma* is known to thrive under diverse environmental conditions among billions of aggressive competitors (Nitta *et al*., 2012). Their ability to survive in different regions can be attributed to diversified metabolic capabilities and natural competitive aggression (Lopes *et al*., 2012). The beneficial effect of *Trichoderma* is due to a complex of different mechanisms such as: direct mycoparasitism, antibiotic production, nutrient and space competition, enhancement of plant resistance to pathogens and systemic induced or acquired plant resistance (López-Mondéjar *et al*., 2012).

Many BCAs, such as fungi, bacteria and viruses, are not only able to control the pathogens that cause plant disease, but are also able to promote plant growth and development. *Trichoderma*-based Biocontrol Agents (BCAs) have an ability to promote plant growth (Wijesingh e*et al*., 2011). Growth stimulation is evidenced by increases in biomass, productivity, stress resistance and increased nutrient absorption. Increased crop productivity associated with the presence of *Trichoderma* has been observed in a broad range of species, such as carnation, chrysanthemum, petunia, cucumber, eggplant, pea, pepper, radish, tobacco, tomato, lettuce, carrot, corn, poppy, cotton, millet, bean, cocoa and ornamental grasses (Hoyos-Carvaja l*et al*., 2009). *Trichoderma harzianum* has the ability to directly enhance root growth and plant development in the absence of pathogens and it has been suggested that this could be due to the production of some unidentified growth-regulating factors by the fungus (Sofo *et al*., 2012). Shukla *et al*. (2012) pointed out that root and shoot length was markedly increased in response to treatment with drought tolerant isolates of *Trichoderma*, prior to altering the water cycle. Vinale *et al*. (2008) suggested that the secondary metabolites such as auxin like compounds or auxin inducing substances by *Trichoderma* plant interaction might be a reason for the improved growth

MATERIALS AND METHODS

Source of Fungal Inoculants

Armillaria species initially isolated from a severely infected *Dombeya torrida* plant was kindly provided by Tea Research Institute. The species was isolated using Armillaria selective medium (ASM) and maintained on malt extract agar medium at room temperature.

Source of Biocontrol Agents

The inoculum of AMF and *T. harzianum* were provided by Homegrown (K) Limited-Naivasha. In order to obtain fresh active cultures of *Trichoderma* isolates for *in-vitro* test, isolates were sub-cultured on MEA plates and incubated at 25^{o}C for 7 days.

Source of Potting Medium and Host Plants

The dark brown loam soil collected from Sururu forest of Eastern Mau (2716 m above sea level, S 00º38.862 and E 036º01.467), was sieved (4 mm), mixed with river sand

and compost manure (1:1:1) and sterilized by autoclaving for 1 h at 121ºC twice, on two consecutive days (Toshihiro *et al*., 2004). *Dombeya torrida* was used as the host plant. The seeds of these plants were collected from Mau forest complex.

Experimental Design and Biological Treatments

The experiment was conducted to test the interaction between AMF and *T. harzianum*. The experiment investigated interaction effects of *T. harzianum* and AMF on fungal colonization, plant growth promotion and *Armillaria* species suppression. The experiment had treatments defined as. (1) Control (c) (no addition of *Armillaria* species or biocontrol inoculum); (2) *Armillaria* species (A); (3) *T. harzianum*(T); (4) Arbuscular mycorrhizae fungi (M); (5)*T. harzianum* and Arbuscular mycorrhizae fungi (T + M); (6)*T. harzianum*and *Armillaria* species (T+A); (7) Arbuscular mycorrhizae fungi and *Armillaria* species (M+A); (8) *T. harzianum* and Arbuscular mycorrhizae fungi and *Armillaria* species (T + M +A). Each experiments was conducted in trial one and trial two. Each treatment had four replications and the experiment was laid out in a completely randomized design.

Inoculation of Plants with Fungal Species

The experiment was conducted in glasshouse at the department of Biological Sciences, Egerton University. Inocula of mycorrhizal comprised 15 g of mycorrhizal inoculum (spores, hyphae and infected root systems) or 15 g of autoclaved inoculum plus 10 ml of 15g inoculum filtrate through a 25µm filter to correct the potential differences in microbial communities between mycorrhizal and non-mycorrhizal pots (Wu and Zou, 2010). Inoculum of *T. harzianum* comprising of powder formulation was prepared according to manufactures instruction. Sterile water (100 mL) was added to the 10g of the formulation and mixed with a sterile stirring rod. The resulting suspension was introduced to potted *D.torrida* seedlings. The inoculations of *Trichoderma* species were done at 0, 30, 60, 90, 120 and 150 days from the beginning of the experiment in order to maintain sufficient populations of the antagonist in the soils and hence to favour biological control, as suggested by Knudsen *et al*. (1991). *Armillaria*species inoculum was prepared by growing the culture on malt extract agar and incubating at 25ºC for 14 days. Each plant was inoculated after every 1 month of growth by placing four agar plugs from the 14-day *Armillaria* species (Baumgartner *et al*., 2010). The inoculum was introduced to the root zone of potted plants. The two fungal inocula, *Armillaria* species, and *T. harzianum* were applied singly or in combination according to the treatment.

Plant Growth

Selected tree seeds were surface sterilized by immersing them in a 1% solution of sodium hypochlorite for 5 min, rinsing in sterile distilled water and air drying on sterile filter paper (Coskuntuna and Ozer, 2008). The seeds were then incubated in water agar plates at 28^{o}C in the dark for two days. Five pre-germinated seeds of each *D. torrida* seedlings were planted in pot filled with potting medium mixed with fungal inoculant according to the treatment combination. After three weeks the seedlings were thinned to one seedling per pot. The experiment was laid out in a complete randomized design with eight treatments and four replicates. Final harvest was done after 28 weeks and the roots washed in sterile water.

Determination of Colony Forming Units of *Trichoderma harzianum*

The estimation of colony forming units (CFUs) of *T. harzianum* in the rhizoplane was determined using a slightly modified protocol used by Rosa and Herrera (2009). One gram fresh root tissue, previously disinfected in sodium hypochlorite solution for 6 min, was cut into pieces and transferred to test tubes containing 100 ml sterile distilled water, then serially diluted, and 0.1 ml of each dilution was finally plated on fresh *Trichoderma* selective medium (TSM) (Elad *et al*., 1981). The plates were incubated for 14 to 18 hours at 25ºC in the dark. There were five replicates for each plate. The population counts of *T. harzianum* colonies on each Petri dish were recorded and data expressed as colony forming units (CFUs) per gram fresh root.

***Armillaria* species assay**

At the end of the experiments plants were assayed for viability of *Armillaria* species using a slightly modified protocol used by Otieno *et al*. (2003). One gram fresh root tissue, previously disinfected in sodium hypochlorite solution for 6 min, was cut into pieces and transferred to Armillaria semi-selective medium. Inoculated Petri dishes were incubated at room temperature in the dark for at least 21 days.

Measurement of Plant Parameters

At the end of the experiment plant height, shoot and root fresh weights were measured. Roots were separated from the soil by washing in tap water.

Data Analysis

For all data (plant growth parameters and mycorrhizal parameters), treatments were compared using one-way analysis of variances (ANOVA) ($p<0.05$) using Gen stat. Data from the repeated trials were pooled and subjected to ANOVA and treatment means were compared by Fisher's least significant difference (LSD) test.

Table 1: The shoot and root fresh weight (g) and the shoot/root ratio of *Dombeya torrida* plants inoculated with *Trichoderma harzianum* and/or AMF 28 weeks after planting

Treatment	Plant height (cm)	Shoot fresh weight (g/plant)	Root fresh weight (g/plant)	Shoot/root ratio
C	53.38ab	12.25a	3.589a	3.376b
A	49.25a	12.38a	3.612a	3.389b
A+M	62.62bc	13.10a	4.050a	3.207b
M	69.25c	13.59a	3.750a	3.696b
A+T	71.75 cd	19.05b	8.425b	2.798ab
T	69.25 c	19.43b	6.616ab	2.960b
A+T+M	75.88cd	24.04bc	15.587c	1.707a
M+T	83.50d	26.42c	17.137c	1.702a

Data in the same column sharing a letter in common do not differ significantly (P<0.05) by the Fischer's least significant difference test.
Treatments
A-*Armillaria* species; C-control; M-mycorrhizae; T-*Trichoderma* species

RESULTS

Bio-inoculants (Arbuscular mycorrhizae fungi and *Trichoderma harzianum*), had pronounced effects on plant growth, and root colonization.

Interaction between AMF and *Trichoderma harzianum* under glasshouse Conditions

Plant shoot and root fresh weight

Growth parameters of *D. torrida* seedlings grown in soils inoculated with arbuscular mycorrhizal fungi and *T. harzianum* were variably affected when examined after 28 weeks. Single inoculation of arbuscular mycorrhizal fungi did not significantly change shoot fresh weight compared with control plants, while inoculation with *T. harzianum* alone significantly increased shoot fresh weight compared with control plants. Plants inoculated with *T. harzianum* alone had significantly increased shoot fresh weight compared to the plants inoculation with arbuscular mycorrhizal fungi. Plants co-inoculated with *T. harzianum* and arbuscular mycorrhizal fungi showed the highest fresh weights. Co-inoculation with arbuscular mycorrhizal fungi and *T. harzianum* significantly increased root and shoot fresh weights compared to plants inoculated with arbuscular mycorrhizal fungi alone or *T. harzianum* alone (Table 1). Inoculations with *T. harzianum* only resulted in better root weight compared to inoculation with arbuscular mycorrhizal fungi alone. On the other hand, fresh shoots of plants inoculated with arbuscular mycorrhizal fungi alone only were weightier than control plants. Although there were no significant changes in the shoot fresh weight due to inoculation of arbuscular mycorrhizal fungi, an increased shoot/root ratio was observed in arbuscular mycorrhizal fungi inoculated plants compared to control plants (Table 1). A significant interaction between the arbuscular mycorrhizal fungi and *T. harzianum* was observed regarding the shoot/root ratio. Combination of *T. harzianum* and mycorrhizal fungi resulted in higher increases of root weight than shoot weight as illustrated by the decrease in the shoot/root ratio. Moreover, smaller fresh shoot/root ratios were recorded in plants inoculated with *T. harzianum.*

Plant height

The other important parameters reflecting the seedling vigour of plants include the direct measurements of plant growth such as plant height. Although arbuscular mycorrhizal fungi inoculated plants had increased the plant height, the difference was not significant from the control. *Trichoderma harzianum* inoculants significantly increased the plant height compared to the arbuscular mycorrhizal fungi inoculated plants. Plant height was found to be enhanced by the combined inoculation of arbuscular mycorrhizal fungi and *T. harzianum* compared to individual inoculations. The highest plant height of 83.50cm was recorded in treatment which received combined inoculation of two groups of microorganisms (Table 1).

Root colonisation

A significant interaction between the biocontrol agents, arbuscular mycorrhizal fungi and *T. harzianum* was observed regarding arbuscular mycorrhizal fungi root colonization. The presence of *T. harzianum* increased arbuscular mycorrhizal fungi root colonization compared to plants inoculated with the arbuscular mycorrhizal fungi alone, co-inoculation with arbuscular mycorrhizal fungi and *T. harzianum* producing a higher percentage of colonization than any other treatment (Table 2). The number of CFUs in the assays performed ranged between 27200 and 48400. In non-inoculated treatments, there were no detectable levels of native *T. harzianum*. Significant differences in the number of *T. harzianum* colony forming units (CFUs) recovered from the roots were observed for the treatments involving co-inoculation with arbuscular mycorrhizal fungi, with respect to inoculation with *T. harzianum* alone (Table 2). Among all the treatments challenged with *Armillaria* species it was observed that there was no disease incidence 28 weeks after inoculation. When the roots were cultured *in-vitro*

Table 2: The AMF root colonization and *Trichoderma harzianum* CFUs 28 weeks after planting and Armillaria root rot incidence

Treatment	*T. harzianum* CFUs	AM root colonization	Armillaria root rot incidence
A	0.00a	0.00 a	0.00
C	0.00a	0.00 a	0.00
M	0.00a	43.20 b	0.00
M+A	0.00a	52.00 b	0.00
T+M+A	27.20b	80.00c	0.00
T+M	30.20b	84.00c	0.00
T+A	45.80c	0.00 a	0.00
T	48.40c	0.00a	0.00

Data in the same column sharing a letter in common do not differ significantly (P<0.05) by the Fischer's least significant difference test
Treatments
A-*Armillaria* species; C-control; M-mycorrhizae; T-*Trichoderma* species

using Armillaria selective medium, there was no growth of *Armillaria* species in the medium confirming the absence of the pathogen (Table 2).

DISCUSSION

Interaction between AMF and *Trichoderma harzianum*

This study clearly demonstrated that inoculation of *T. harzianum* either individually or in combination with AMF enhanced growth in *D. torrida* seedlings under glasshouse conditions. The combination of AMF and *T. harzianum* resulted in the greatest improvement in growth compared to uninoculated controls. Beneficial rhizosphere microorganisms that improve plant growth include arbuscular mycorrhizal fungi (AMF) and *T. harzianum*. AMF inoculation resulting in increased growth, yield and nutrient content of plants has been associated with increased accumulation of phosphorus, nitrogen and potassium from soil sources (Jacobsen, 1994; Erman *et al*., 2011). Brotman *et al.* (2010) stated that *Trichoderma* species can promote growth improvement of up to 300%.These results are in agreement with previous work that has demonstrated improvement of plant growth parameters by arbuscular mycorrhizae and other BCAs. Sukhada *et al*. (2011) showed that BCAs like arbuscular mycorrhizal fungi, *T. harzianum* and *P. fluorescens* improved plant growth parameters in papaya individually and in combination and also had a protective effect against the root pathogen *Phytophthora* under field conditions. Haggag and Abd-El latif (2001) found that the combined inoculation of *Glomusmosseae* and *T. harzianum* enhanced growth of geranium plants. Similarly, combined inoculation of *T. aureoviride* and *G. mosseae* had a synergistic effect on the growth of marigold plants (Calvet *et al*., 1993). Erman *et al*. (2011) also point out that whey, AMF and *Rhizobium* inoculation alone or in combination provided significantly increased yield and yield components over the control in chickpea.

The results of the current study may be explained by the different modes of action of *T. harzianum* and AMF fungi and their interaction in dual inoculated *D. torrida* seedlings. Co-inoculations of plant beneficial microorganisms in plant production systems may improve the efficacy of desired features in relation to biocontrol and plant growth promotion (Larsen *et al*., 2009). Growth improvement of plants could be due to the synergistic activity of *T. harzianum* and AMF on host plant. These beneficial microorganisms might have colonized around the root and increased the root biomass and helped to increase the availability of nutrients. In this regard, Martínez-Medina *et al*. (2011) observed a synergistic effect on AMF root colonization due to the interaction between *T. harzianum* and *Glomus constrictum* or *Glomus intraradices*. The synergistic effect produced by the interaction between *T. harzianum* and AMF in the current study could have been caused by a direct beneficial action of soluble exudates and volatile compounds produced by the saprophytic fungus as reported by Calvet *et al*. (1992). No negative interaction was observed in these results, in contrast to the results of Martinez *et al*. (2004). According to these authors, root and shoot weights of soybean were decreased by co-inoculation with *Trichoderma pseudokoningii* and *Gigaspora rosea.* Microbial inoculants are promising components for integrated solutions to agro-environmental problems because inoculants possess the capacity to promote plant growth, enhance nutrient availability and uptake, and support the health of plants (Adesemoye *et al*., 2009; Mader *et al*., 2011)

The current findings, further demonstrate that the growth of *T. harzianum* around *D. torrida* seedlings roots was suppressed by the presence of AMF. However, the presence of *T. harzianum* improved root colonization of *D. torrida* roots by AMF. McAllister *et al*. (1994) who studied the interactions between *G. mosseae* and several other saprophytic soil fungi found that the population of *Trichoderma koningii* and *Fusarium solani* in the rhizosphere of maize plants was decreased by *G. mosseae* when these saprobes were inoculated two weeks after the AMF, but not when they were inoculated at the same time as *G. mosseae.* Similarly, McAllister *et al*. (1995) also reported similar reduction of the population of a saprophytic fungus *Aspergillus niger* in the rhizosphere of maize and lettuce when *G. mosseae* was

established in the plants. Fracchia *et al*. (1998) observed antagonistic, synergistic and neutral interactions between *G. mosseae* and associated saprophytic fungi.

The suppression of *T. harzianum* growth observed in the current investigations could have been directly caused by the AM fungus and/or indirectly by AM symbiosis-mediated factors. The establishment of AMF symbiosis in plants is known to change physiological and biochemical properties of the host plant and these changes may alter the composition ofroot exudates which play a key role in the modification of the microbial population qualitatively and quantitatively in the mycorrhizosphere (Siddiqui and Pichtel, 2008). Moreover, direct competition between extraradical mycelia of the AMF and *T. harzianum* fungi for the colonization sites and nutrients could also have occurred. Green *et al*. (1999) who found the mutually inhibitory interaction between *T. harzianum* and the external mycelia of an AMF *Glomus intraradices* suggested that the competition for nutrients as the possible cause. Additionally, the nature and extent of arbuscular mycorrhizal associations appears a crucial factor influencing rhizosphere microbial communities (Veresoglou *et al*., 2012). Although the *T. harzianum* population was lower in the dual inoculated plants, the AMF colonization in roots was enhanced in compensation. Similarly, Calvet *et al*. (1992) observed a significant enhancement of *G. mosseae* growth by the presence of *T. harzianum in-vitro*.

Trichoderma isolates were able to colonize the root surface of *D. torrida* seedlings, characterized by the growth of the CFUs by all the root surface of *Trichoderma* inoculated *D. torrida* seedlings suggesting that the isolates used in this study are rhizosphere-competent strains. According to Fontenelle *et al*. (2011) the penetration process of *Trichoderma* species into the roots of plants is associated with the ability of the fungus to secrete an arsenal of hydrolytic enzymes which degrade the cell wall, including cellulase. These enzymes are capable of inducing defense mechanisms in plants, probably due to the ability to release fragments of cell wall in plants. According to Harman *et al*. (2004) the activation process of broad spectrum systemic resistance by *Trichoderma* species begins with the colonization of plant roots by this fungus. Shoresh and Harman (2008) also provided evidence that the existence of a relationship between increased growth and induced resistance in plants by root colonizing microorganisms such as *Trichoderma* species and non-pathogenic rhizobacteria. According to these authors, these effects could be mediated by different elicitors. Thus, the possibility of combining plant growth promotion and induction of disease resistance makes the use of *Trichoderma* species in disease control programs even more attractive.

CONCLUSIONS

The current study provides evidence that some *Trichoderma* isolates native to Kenya can be considered as strong candidates for bioinoculants that enhance plant growth. In this study *T. harzianum* was found to stimulate early stages of growth in *D. torrida* seedlings, potentially leading to the use of these strains as novel bioinoculants in agriculture and forestry. The study clearly demonstrated that dual inoculation of AMF and *T. harzianum*im proved root colonization in *D. torrida* seedlings under glasshouse conditions.

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