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EVALUATION OF LOCAL *TRICHODERMA* ISOLATES FOR THEIR EFFICIENCY IN BIOLOGICAL CONTROL OF *FUSARIUM OXYSPORUM* F. SP *PHASEOLI* IN COMMON BEAN

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Master of Science in Biotechnology

DECLARATION

This study is my original work and has not been presented for a degree in any other University.

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ABSTRACT

In Kenya common beans are one of the most important food crops widely grown in the country. The production trend of beans has been on the decline over the years mostly as a result of infectious diseases from fungal pathogens. One such pathogen is *Fusarium oxysporum* f. sp. *phaseoli* (FOP) which causes *Fusarium* wilt of beans. In the country yield losses from FOP have been estimated to be between 10% and 100%. The major control method of *Fusarium* wilt has been with chemical fungicides. However this has been reported to be ineffective under conditions that are conducive for the disease development. This therefore calls for development of alternative methods to control the pathogen. The strategy of integrated disease management (IDM) that incorporates use of biocontrol agents which are safe, easy to adopt and environmentally friendly is one such system that can contribute greatly towards management of this disease. Antagonistic fungus *Trichoderma* is one of the bio-control agents that can be developed for this purpose. The overall objective of the study was to evaluate local *Trichoderma* isolates for their virulence potential against *Fusarium oxysporum* f. sp. *phaseoli*. The focus of this study was to isolate *Trichoderma* from soil samples obtained from Embu Agricultural Training Center (ATC) farm, identify and to test them *in vitro* and *in vivo* for their ability in control of FOP. The molecular identification and characterization of the *Trichoderma* isolates was based on the ITS region. The ability of the isolates to antagonize FOP was evaluated *in vitro* using dual culture assays and through use of volatile and non-volatile metabolites produced by the isolates. The two local isolates *T. koningiopsis* (T18) and *T. viride* (T35) that showed the highest inhibitory effect against the pathogen at 28.56% and 27.42% respectively were used as seed treatment on bean variety mwezi moja and tested against the pathogen in the greenhouse experiment. The inhibition tests effects were analyzed using the analysis of variance (ANOVA) and the significance of the means was evaluated by Turkey HSD and Duncan's multiple range tests for *in vitro* experiments and greenhouse experiment respectively. The local isolates of *Trichoderma* were identified as; *Trichoderma hamatum* (T6), *Trichoderma koningiopsis* (T18), *Trichoderma hamatum* (T21), *Trichoderma viride* (T35), *Trichoderma spirale* (T41) and *Trichoderma asperellum* (ECO-T). In dual culture experiments the commercial ECO-T and the five local *Trichoderma* isolates showed

substantial inhibition of the growth of FOP. Use of non-volatile and volatile metabolites produced by the *Trichoderma* isolates also confirmed that there was production of inhibitory substances by all the isolates against FOP. In the greenhouse studies seeds inoculated with *Trichoderma* reduced infection of bean plants with FOP and resulted in increased plant growth relatively compared with untreated control. All the plants from seeds that were treated with *T. koningiopsis* (T18), *T. viride* (T35), ECO-T and the combination of *T. koningiopsis* (T18) + *T. viride* (T35) showed significant resistance against FOP in the greenhouse. *T. viride* (T35) achieved the highest reduction of post-emergence damping-off at 50%; *T. koningiopsis* (T18), ECO-T and the combination of *T. koningiopsis* (T18) + *T. viride* (T35) achieved reductions of 35%, 43.3% and 35% respectively. Combination of *T. koningiopsis* (T18) + *T. viride* (T35) achieved the highest increase in dry weight of shoots (680%) and dry weight of roots (166.6%) which were significantly different ($P \leq 0.05$) compared to *T. koningiopsis* (T18), *T. viride* (T35) and ECO-T with increases in dry weight of shoots (230%, 210%, 80% respectively) and dry weight of roots (33.3%, 133.3%, 33.33% respectively). The local *Trichoderma* isolates *T. koningiopsis* (T18) and *T. viride* (T35) are effective and promising biocontrol agents against FOP in beans.

Keywords: *Trichoderma,* Biocontrol**,** *Fusarium oxysporum.*

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ABBREVIATIONS AND SYMBOLS

Aib	Alpha-aminoisobutyric acid
AM	Arbuscular Mycorhiza
ANOVA	Analysis of variance
ATC	Agricultural Training Centre
BCAs	Biocontrol agents
BecA-ILRI	Biosciences eastern and central Africa-International Livestock Research Institute
BLAST	Basic Local Alignment Search Tool
bp	base pair
CCF	cell free culture filtrates
Cfu	Colony forming unit
CLA	Carnation Leaf Agar
CTAB	Hexacetyl-Trimethyl-Ammonium Bromide
DDW	Double Distilled Water
d.f	degrees of freedom
DNA	Deoxyribo nucleic Acid
dNTPs	Deoxy-nucleotide triphosphate
ECO-T	Commercial *Trichoderma harzianum*
EDTA	ethylenediamine tetraacetic acid
EF	Elongation Factor
FAO STAT	Food and Agricultural Organization Statistics
FOP	*Fusarium oxysporum phaseoli*
GDP	Gross Domestic Product
GlobalGAP®	Global Good Agricultural Practice
IDM	Integrated Disease Management
ITS1	internal transcribed spacer 1 region
ITS4	internal transcribed spacer 4 region
Kenya-GAP®	Kenyan Good Agricultural Practice
KOH	Potassium hydroxide

mg	Milligrams
Min	Minute
ml	millilitres
mm	millimeter
mM	millimolar
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NRP	Non- Ribosomal Peptide
PCNB	Pentachlorobenzene
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PTC	Peltier Thermal Cycler
rDNA	Ribosomal Deoxyribonucleic Acid
RNase	Ribonuclease
Rpm	revolution per minute
rRNA	Ribosomal Ribonucleic acid
SDW	Sterile distilled water
SNA	Spezieller Nahrstoffarmer Agar
Spp	Species
SPSS	Statistical Package for Social sciences
TAE	Tris Acetate Ethylenediamine Tetraacetic Acid
Taq	*Thermus aquaticus*
TE	Tris ethylenediamine tetraacetic acid
Tris-HCl	Tris hydrochloric acid
µl	Microlitre

CHAPTER ONE

1.0. INTRODUCTION

Agriculture is the mainstay of Kenya's economy and its growth and development is crucial for Kenya's overall economic and social development. Agriculture contributes directly to 26% of the Gross Domestic Product (GDP) and has indirect contribution of 25% to the GDP through manufacturing, distribution and service related sectors (Agricultural Sector Development Strategy 2010–2020). The Kenya's vision 2030 policy document identifies agriculture as one of the six key economic sectors expected to drive the economy to a projected 10% economic growth annually over the next two decades through promotion of innovative, commercially oriented and modern agriculture. Nevertheless the sector continues to face various challenges. Some of these constraints include low application of technology and innovation; high cost, adulteration and low application of key inputs such as fertilizers and agrochemicals; pests and diseases. Losses in agricultural production due to pests and diseases at pre- harvest and post-harvest levels remain extremely high. There is therefore need to diversify strategies for the control of pests and diseases for increased productivity in the agricultural sector.

Beans are one of the most important pulses and second to maize in Kenya as a food crop (Mwangombe *et al.*, 2008). It is also one of the important export crops, earning foreign exchange for the country. Bean production in Kenya is mainly undertaken by small scale farmers and has been characterized by low input production systems (Katungi *et al.*, 2009). In addition high human population pressure in the bean producing areas has resulted to intensification of land use characterized by reduced crop rotation and fallow periods and this is contributing to the decline in soil fertility and a buildup of inoculums of root rot pathogens (Buruchara *et al.*, 2003). As a result there has been a considerable up surge in the bean rot diseases.

These diseases are greatly contributing to the unsuccessful quality production of beans. Among the diseases is the *Fusarium* wilt which is caused by the fungus *Fusarium oxysporum* f. sp. *phaseoli* and is an economically important disease in bean production in Kenya.

The control of *Fusarium oxysporium* on beans has been through use of chemical fungicides which is often considered uneconomical and for most small scale farmers this is an option beyond their reach. Continuous chemical usage also has negative impact on the environment and has potential effect of generating strains that are resistant to the fungicides in use. Since introduction of systemic fungicides globally in the early 1970s, farmers are increasingly confronted with pathogens' resistance to available chemical compounds due to misuse or abuse in their employment (Tahia *et al*., 2004). Globally these concerns have resulted in greater restrictions on chemical pesticide use in agricultural production. In the recent times quality assurance standards e.g. the GlobalGAP® and Kenyan version Kenya-GAP® are being implemented to minimize detrimental environmental impacts of farming operations, reducing the use of chemical inputs and ensuring a responsible approach to worker health and safety as well as animal welfare" (FPEAK, 2007). For a sustainable development of production of beans, it is important to develop environmentally friendly methods for reducing fungicide usage in the management of bean diseases. Biological control is one such promising alternative to an extended use of pesticides (Chet *et al*., 2006), which are often expensive and accumulate in plants or soil, having adverse effects on humans.

So far in the developed countries, bio-control agents are being employed especially in the integrated disease management strategies for crop productivity improvement as they are safe, easy to adapt and environmentally safe relative to agrochemicals; however in Africa this is not being fully exploited especially by the small scale farmers. One of the bio-control agents which is being utilized is the *Trichoderma* species and a few of the isolates are commercially available. In Kenya the commercially available strain ECO-T (*Trichoderma harzianum* strain k.d) is from South Africa, manufactured by plant health products (PTY) Ltd., South Africa.

Antagonistic *Trichoderma* strains have been successfully applied as biological control agents (BCAs), against soil-borne *Fusarium* wilt (Kucuk and Kivan 2002; Tahia *et al.*, 2004). It has been demonstrated that *Trichoderma* spp. mycoparasitize the hyphae and resting structures of plant pathogens *in vitro* and also in natural soil (Papavizas.1985). However, most *Trichoderma* strains are more efficient for control of some pathogens than others, and may be largely ineffective against some fungi (Shelton, 2012). Available reports indicate that *Trichoderma* species are common soil inhabitants with a wide spread occurrence. Therefore exploitation of this potential and development of the local strains would make them readily available for use against *Fusarium* wilt.

The focus of this study was to isolate *Trichoderma* from soil samples obtained from Embu Agricultural Training Center (ATC) farm, identify and to test them *in vitro* and *in vivo* for their ability in control of *Fusarim oxysporum phaseoli*. The *in vivo* experiments were performed in the greenhouse using the commercial ECO-T and two local isolates that showed the greatest inhibition *in vitro* against *Fusarium oxysporum.* The identification of effective local *Trichoderma* strains and the studies of their activities against *Fusarium oxysporum* could facilitate the development of improved strains of these bio-control agents. The improved strains would be employed in effective sustainable management of the bean *Fusarium* wilt disease in Kenya. The overall objective of the study was to evaluate local *Trichoderma* isolates for their virulence potential against *Fusarium oxysporum* f. sp. *phaseoli*.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1.0. Production of Beans in Kenya

Field beans are one of the most important food crops cultivated in Kenya. It is ranked number two after maize (Mwangombe *et al*., 2008) as a food crop in the country. It is widely grown in the country and is the major source of affordable dietary proteins for the majority of the Kenyan population (Obogoya *et al*., 2009). Beans have also been known to reduce diseases such as cancers, diabetes or coronary heart disease when consumed on regular basis (Katungi *et al*., 2009). In the year 2010 the country had a total of 689,377 hectares under the field beans and realized a production of 390,598 tons (FAO STAT. 2012). The production trend of beans has been on the decline over the years (Figure 2.1) mostly as a result of infectious diseases from fungal pathogens.

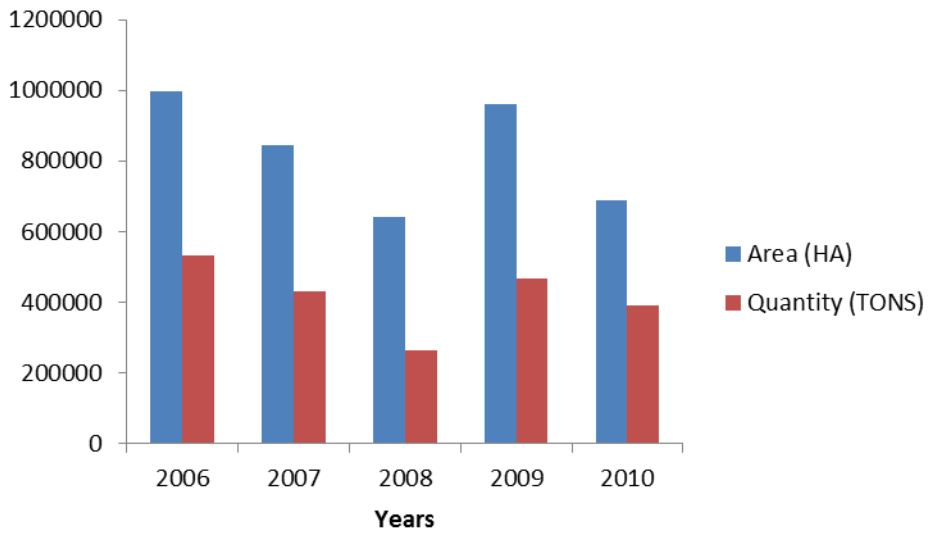

Figure 2.1. Production trends of beans in Kenya 2006-2010 (Kenya)
Source: FAOSTAT | © FAO Statistics Division 2012 | 18 May 2012
Source: FAO stat at www.fao.org

In Kenya high human population pressure in the bean producing areas has resulted in intensified land use and therefore reduced crop rotation and fallow periods thus contributing to low soil fertility and build up in inoculums of soil borne disease pathogens (Buruchara *et al.,* 2003). And because the crop is grown mainly by the small scale farmers, the production is characterized by low input production systems (Katungi *et al*., 2009). The above scenarios continue to favor the problem of fungal disease infection in beans. One such pathogen is *Fusarium oxysporum Phaseoli* (FOP) which causes *Fusarium* wilt of beans. This disease is widespread and of economic importance in bean production all over the world. It has adverse effect on bean production with severely infected fields recording loses of up to 50% (Estevez *et al*., 2002). In Kenya, the disease has been reported in many bean growing areas with yield losses estimated between 10% and 100% (Mwangombe *et al*., 2008).

2.2.0. *Fusarium* wilt disease of beans

Fusarium oxysporum, is ubiquitous phytopathogen causing root rot, vascular wilt and damping off diseases in many plant species. The fungus grows principally inside the vascular elements causing discoloration and browning of the affected areas. Once it invades the xylem vessels, its mycelium and in some cases it produces toxins which result in wilting symptoms in some plants and yellowing of leaves and shoots in others. Under unfavourable conditions and in the absence of a host the fungus produces chlamydospores in the soil (Obongoya *et al*., 2009). The chlamydospores are resistant to adverse physical and chemical conditions and therefore the pathogen is very persistent in the soils and can survive in infested fields for a very long time. This makes it one of the most difficult pathogens to control. Its soil borne nature makes the use of fungicides highly impracticable.

There are many *formae speciales* of *Fusarium* o*xysporum* and each *forma specialis* (f. sp.) can usually only cause wilt disease on one plant host species. *Fusarium* wilt of beans is an important disease in Kenya and it is caused by *Fusarium oxysporum* f. sp. *phaseoli.* This pathogen exhibits high variability in cultural characteristics, Mwangombe *et al*., (2008) therefore poses challenges at breeding for *Fusarium* wilt resistant varieties.

The disease is being controlled by several methods such as: use of chemical fungicides, cultural control methods, biological control; particularly by use of species of *Trichoderma* and *Gliocladium* (Butt *et al*., 2001).). Although control of *Fusarium* wilt through use of chemical fungicide has been the major control method in use, the soil borne nature of the causal pathogen requires intensive chemical application which makes it very costly. Secondly control of *Fusarium* spp. with fungicides has been reported to be ineffective under conditions that are conducive for the disease development. This therefore calls for development of alternative methods to control the pathogen. The strategy of integrated disease management (IDM) that incorporates use of bio-control agents which are safe, easy to adopt and environmentally safe is one such system that can contribute greatly towards management of this disease. Antagonistic fungus *Trichoderma* is one of the bio-control agents that can be developed for this purpose.

2.3.0. Study of *Trichoderma* spp. as Bio-control Agents

The *Trichoderma* spp. have been recognized as agents for bio-control of plant diseases with several species being extensively studied for their biological control effects against plant pathogens of the fungal origin. The discoveries of the antifungal abilities of these beneficial micro-organisms date back to the 1930s (Chet *et al*., 2006; Schubert *et al.,* 2008). Since then there have been a lot of effort towards their utilization as plant disease bio-control agents (Schubert *et al.,* 2008). Numerous studies have confirmed that *Trichoderma* spp. can directly affect mycelium or survival propagules of other fungi through production of toxic secondary metabolites, formation of specialized structures and secretion of cell wall degrading enzymes. To date many *Trichoderma* strains have been identified as potential bio-control agents of plant pathogenic fungi on crops such as beans (*Phaseoli vulgaris*), strawberries (*Fragaria vesca)*, peas *(Pisum sativum)*, cucumber *(Cucumis sativus),* Tomatoes *(solanum nigrum),* radish *(Raphanus sativum*), sugar beets *(Beta vulgaris)* and cotton *(Gossypium hirsutum L)* (Nusret and Steven, 2004*). Trichoderma* affects most soil borne and foliar plant pathogens *(*Agosin, *et al.,* 1997; Kucuk and Kivan, 2002; Nusret, and Steven, 2004*)* which are responsible for major crop losses in agricultural production, some of which include: *Phytophthora, Pythium, Botritis, Armillaria, Fusarium, Rhizoctonia, Sclerotinia, Verticillium, Phoma* (Monte, 2001).

Since bio-control agents (BCAs) are living organisms, their activities depend on the different conditions of physiochemical environment which they are subjected to. Because of this, it is sometimes unpredictable the extent to which different *Trichoderma* strains achieve bio-control. In addition, there are isolates belonging to *Trichoderma* species which are unable to act as effective BCAs (Hermosa *et al*., 2000). It is important that the genetic diversity of strains within *Trichoderma* spp. and their mechanisms of bio-control is understood to facilitate improved application of the different strains as BCAs. Past research indicate that *Trichoderma* spp. have evolved various mechanisms that they utilize to attack other fungi and to enhance plant and root growth. These include:-

Aggressive competitiveness (Harman *et al*., 2004); *Trichoderma* grow very fast, quickly occupying free space and colonize substrates in order to use free nutrients and this is important in their suppressive activity against the growth of soil borne pathogens. Secondly; the *Trichoderma* spp. possess the ability to grow alongside the developing root system of the plants, in addition it is rhizosphere competent, this further enhances its ability as a biocontrol agent against soil borne pathogens (Ahmad and baker, 1987). Biocontrol by competition has been demonstrated during the biocontrol of *Fusarium oxysporum* by *T. harzianum* through competition for Rhizosphere colonization and nutrients and the best control is achieved with diminishing nutrient concentration (Lorito, 1994). Competitive activity alone does not explain the biocontrol activity of the *Trichoderma*, more often it is accompanied by other antagonistic events such as parasitism, antibiosis or resistance induction in the host plant (Sivan and chet, 1989).

Mycoparasitism; this process was demonstrated in dual experiments that were conducted on pathogen interactions where *Trichoderma* attacks the pathogen by coiling its hyphae around the pathogen's hyphae and degrade cell walls of the pathogen by the secretion of lytic enzymes (Nusret and Steven, 2004). A detailed observation of the parasitic process of *Trichoderma* against a pathogen has shown that this interaction is specific and not merely a contact response.

Once *Trichoderma* recognizes signals from a fungal target organism, it grows towards it by chemostatic hyphal branching; it then either grows alongside the hyphae of the pathogen or coils around it (Harman *et al*., 2004). The result of the interaction is loss of turgor and collapse of cells. This comes about due to the different lytic enzymes such as chitinase, glucanase and pectinase secreted by *Trichoderma* and are of key importance in this process (Tahia *et al.,* 2004).

Induced resistance, this is caused in plants where the *Trichoderma* in association with the plant roots activates the plant's own defense mechanism against any potential attack from phytopathogens including fungi, bacteria and viruses (Vinale *et al*., 2006). It has been shown that when bean plants are grown in soils amended with *Trichoderma harzianum*, plants showed significantly reduced disease symptoms from *Botrytis cinera* and *Colletrotichum lindemuthianum* and this was as a result of *Trichoderma* -mediated induced resistance (Meyer *et al*., 1998).

Antibiosis, antibiotic secondary metabolites Produced by *Trichoderma* spp. play a key role in the processes of induction of resistance in plants and direct mycoparasitism of plant pathogenic fungi. *Trichoderma* spp. are well-known producers of secondary metabolites with antibiotic activity (Schuhmacher *et al.,* 2007; Vinale *et al*., 2008). These secondary metabolites are important natural products used to inhibit microbial growth and are produced during microbial development and sporulation (Vinale *et al*., 2008; Vinale *et al*., 2009). The spectrum of secondary metabolites secreted by *Trichoderma* is species and strain-dependent and includes volatile and nonvolatile antifungal substances (Vinale *et al*., 2009).

Trichoderma spp. produce in abundance antifungal secondary metabolites such as peptaibols. This is a group of amphiphilic non-ribosomal peptide (NRP) antibiotics, which are rich in non-proteinogenic amino acids such as alpha-aminoisobutyric acid (Aib) as well as a modified N-terminus and C-terminus amino alcohol. In fungal culture samples Peptaibols often occur as species-specific micro-heterogeneous mixtures of peptides of up to 20 amino acid residues with very similar chemical structures. The biological and physio-chemical properties exhibited by Peptaibols and Peptaibiotics are; formation of pores in bilayer lipid membranes, antibacterial, antifungal and sometimes antiviral activities and may elicit plant resistance to pathogens (Szekeres *et al*., 2005). This is achieved by inducing metabolic activities like ethylene emission

and biosynthesis of volatile compounds. Due to their bioactive properties, these compounds can be target compounds for the development of bio-control agents and also for the study of the interactions between *Trichoderma*, pathogenic fungi and plants. Literature study performed by Stoppacher *et al*., (2008), shows that, in total, about 780 different peptaibiotics have been described to date, with 317 sequences listed in the 'Peptaibol Database'.
Other important secondary metabolites of *Trichoderma* spp. include- antibiotic metabolites like pyrones, polyketides, terpenoids and metabolites derived from alpha amino acids (Butt *et al*., 2001).

2.4.0. Application of *Trichoderma* as Biocontrol Agents (BCAs)

Intensive use of agrochemicals has been known as a cause to environmental pollutions in the world. Agrochemicals have been recognized as factors in contamination of groundwater and also impact negatively on a wide range of organisms once they enter into the food chains. This has resulted for a need to significantly reduce their usage in agricultural production systems. The use of biological control strategies is an option to limit the agrochemicals usage. Fungi of the genus *Trichoderma* are bio-control agents (BCAs) that are successfully used as bio-pesticides worldwide, and many species are well known producers of secondary metabolites with antibiotic activity. In fact more than half of the commercial bio-control preparations are *Trichoderma* or *Gliocladium* based (Butt *et al*., 2001). Some of the *Trichoderma* species that are being used as biocontrol agents against plant pathogenic fungi include; *T. virens, T. harzianum* and *T. atroviride.*

Commercial *Trichoderma viren* preparations are being used in the control of *Rhizoctonia solani and pythium* spp. in vegetable and ornamental plant seedlings in green houses. *Trichoderma harzianum* strain 1295-22 (T-22) as a conidial formulation is applied as seed dressing in crops such as maize, beans and cotton to protect them from damping off diseases and other root rot diseases caused by *Fusarium* and *Rhizoctonia. Trichoderma* are widely used bio-control agents because they occur widely worldwide, they are relatively easy to produce and have low toxicity. The other application of *Trichoderma* is in biodegradation of toxic compounds, they are able to degrade hydrocarbons, chlorophenolic compounds, polysaccharides, xenobiotic herbicides,

fungicides and pesticides including DDT and other phenolic compounds. They recover rapidly after addition of sublethal doses of some of these compounds (Tahia *et al*., 2004). This is possible because *Trichoderma* spp. possess rhizosphere competency and natural resistance to these toxic compounds. However, biological control agents such as *Trichoderma* are affected by abiotic and biotic factors such as; weather, disease pressure and competition from the indigenous microflora and thus presents inconsistency in performance. The other possible setback to *Trichoderma* use is also its possible adverse effect on arbuscular mycorhizal (AM) fungi (Nusret and Steven, 2004). The AM fungi are beneficial component of plant-soil systems where through symbiosis they benefit the plants by enhancing their uptake of low mobility minerals and nitrogen (Nusret and Steven, 2004).

2.5.0. Problem Statement

The production of beans in Kenya is mainly by small scale farmers and continues to be characterized by low input production systems (Katungi *et al*., 2009). In addition high human population pressure in the bean producing areas of Kenya has resulted to intensification of land use characterized by reduced crop rotation and fallow periods and this is contributing to the decline in soil fertility and a build-up of inoculums of root rot pathogens (Buruchara *et al.*, 2003). Among the major root rot pathogens contributing to the unsuccessful quality production of beans is the *Fusarium oxysporum* f. sp. *Phaseoli.* This fungus is a major bean rot pathogen which causes *Fusarium* wilt, an economically important disease in bean production in Kenya causing damage to beans of between 10-100%. Destruction by this pathogen results into overwhelming losses to the farmers due to reduced crop productivity hence reduced market prices. At the same time such a loss impacts greatly to food insecurity among the Kenya population whose majority depend on legumes and mostly beans as the major source of affordable dietary proteins.

2.6.0. Justification

Control of *Fusarium* wilt through use of chemical fungicide is costly as it requires intensive chemical application due to the soil borne nature of the causal pathogen. Such treatment can only be applicable to large scale production and are obviously uneconomical for small scale farmers. Furthermore control of *Fusarium* spp. with fungicides has been reported to be ineffective under conditions that are conducive for the disease development (Jegathambigai *et al.,* 2009). This therefore calls for development of alternative methods to control the pathogen. The strategy of integrated disease management (IDM) that incorporate use of bio-control agents which are safe to use, easy to adopt and environmentally friendly is one such system that can contribute greatly to management of this disease. Antagonistic fungus *Trichoderma* is one of the bio-control agents that can be developed for this purpose (Jegathambigai *et al*., 2009). The different species or strains of *Trichoderma* show specificity against various pathogens with each strain possessing the ability to control only one plant disease (Papavizas, 1985). In addition, there are isolates belonging to *Trichoderma* species which are unable to act as effective BCAs (Hermosa *et al*., 2000). It therefore makes it necessary to undertake studies to determine which specific species of *Trichoderma* is effective on *Fusarium oxysporum phaseoli*. Furthermore it is also important that strains that are adapted to local conditions are developed for effective management of this pathogen (Handelsman and Stabb, 1996).

In Kenya local strains of *Trichoderma* have not been fully exploited for bio-control of *Fusarium oxysporum phaseoli* (FOP) in bean production. To facilitate the effective use of *Trichoderma* as bio-control agents against FOP, knowledge on their antagonistic behavior is essential and therefore, laboratory and field bioassays will help identify the most antagonistic/virulent, ecologically fit strains. Precise identification, detailed understanding of the *Trichoderma* isolates modes of action and their limitations are essential to allow for selection of appropriate and effective strains against *Fusarium oxysporum phaseoli*.

2.7.0. Research Hypothesis.

2.7.1.0. Null hypothesis.

Local *Trichoderma* species are not virulent against *Fusarium oxysporum* f.sp. *phaseoli.*

2.7.2.0. Alternative hypothesis.

Local *Trichoderma* species are virulent against *Fusarium oxysporum* f.sp. *phaseoli.*

2.7.3.0. Overall objective

To evaluate local *Trichoderma* isolates for their virulence potential against *Fusarium oxysporum* f. sp. *phaseoli.*

2.7.4.0. Specific objectives

1. To isolate and identify pathogenic *Fusarium oxysporum* f. sp. *phaseoli* from roots of beans.
2. To isolate, and identify local *Trichoderma* isolates from the soil.
3. To screen isolates of *Trichoderma* spp. *in vitro* for their effectiveness in controlling growth of *Fusarium oxysporum* f. sp. *phaseoli.*
4. To test the ability of *Trichoderma* isolates *in vivo* for control of *Fusarium oxysporum* f. sp. *phaseoli* in the greenhouse.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1.0. Plant Materials and Soil Samples

The diseased bean plants for isolation of *Fusarium oxysporum* f. sp. *phaseoli* were obtained from Embu, Agricultural Training Center. The plants were uprooted, cleaned and placed in moist polythene bags to enhance fungal growth (Jegathambigai *et al*., 2009).

The bean seed variety mwezi moja for the greenhouse experiment was purchased from Kenya Seed Company.

The soil samples for isolation of *Trichoderma* were obtained from the Embu Agricultural Training Centre (ATC) farm. The soil samples were obtained from a field with crop showing *Fusarium* wilt symptoms. The soil was sampled randomly from a 1 acre plot, this was achieved through a diagonal transect across the plot. Six points were marked at equal distance along the transect and from these points soils were sampled where 3m and 6m radius circles were drawn around each sampling point and 4 soil samples were cored from the 3m radius and another 8 samples from the 6m radius at a depth of 0-20cm. A total of 60 soil samples were randomly collected. *Trichoderma* are saprophytic in nature and commonly present in the soil's top horizons where high densities of mycelium can be recovered (Bourguignon 2008). The soil samples were collected and transported in paper bags. Samples were kept at 2-5^{0}C in the laboratory to reduce microbial activity.

3.2.0. Media Preparation

3.2.1.0. Potato Dextrose Agar plates

This was prepared as per the standard method described by Poinars and Thomas together with manufacturer's instructions for the media preparation. 39g of potato dextrose agar (PDA) was suspended in 1 litre of distilled water and stirred until completely dissolved. The mixture was then autoclaved at 121^0C and 15 psi (pressure) for 15 minutes. The agar was left to cool to 45-50^0C, antibiotics (streptomycin 50 mg/L, and cyclosporine 10 mg/L) were added to make a selective media. 20ml of this was poured into sterilized disposable Petri dishes under aseptic condition and left to cool and solidify. This was then used in the fungal culturing experiments.

3.2.2.0. Potato Dextrose Broth

Potato dextrose broth (PDB) was prepared by adding 24 grams of potato dextrose broth to one litre of distilled water. The media was mixed thoroughly by stirring until completely dissolved and distributed into 250 conical flasks, covered with cotton wool and aluminum foil and autoclaved at 121^0C, 15 lb pressure for 15 minutes. It was then allowed to cool to 45-50^0C after which antibiotics (streptomycin 50 mg/L, and cyclosporine 10 mg/L) were added. This was then used in the fungal culturing experiments for production of mycelia.

3.2.3.0. Water agar plates

Two percent agar was prepared in distilled water. The mixture was then autoclaved at 121^0C and 15psi (pressure) for 15 minutes. The agar was left to cool to 45-50^0C, antibiotics (streptomycin 50 mg/L, and cyclosporine 10 mg/L) were added to make a selective media. 20ml was poured into sterilized disposable Petri dishes under aseptic condition and left to cool and solidify. This was then used in the fungal culturing experiments.

3.2.4.0. Spezieller Nahrstoffarmer Agar (SNA)

The SNA was prepared as per the standard method described by Nirenberg:1976 (Nelson *et al*., 1983); 23.4g of a mixture of Agar, KH_2PO_4, KNO_3, $MgSO_4.7H_2O$, KCL, Glucose and Sucrose was added to one litre of distilled water mixed and autoclaved at 121^0C for 15 minutes. The agar was left to cool to 45-50^0C, antibiotics (streptomycin 50 mg/L, and cyclosporine 10 mg/L) were added to make a selective media. 20ml was poured into sterilized disposable Petri dishes under aseptic condition and left to cool and solidify. This was then used in the fungal culturing experiments.

3.2.5.0. Penta- Chloro-Nitro-Benzene (PCNB) agar Media, PPA/Narsh Snyder Medium

The basal medium in one litre of water was prepared with 37.5g of a mixture of agar, peptone, $KH2PO_4$, $MgSO_4.7H_2O$ and PCNB. This was autoclaved and cooled to 55^0C; antibiotics (streptomycin 50 mg/L, and cyclosporine 10 mg/L) were added to make a selective media. 20ml was poured into sterilized disposable Petri dishes under aseptic condition and left to cool and solidify. This was then used in the fungal culturing experiments.

3.3.0. Isolation and Identification of *Fusarium oxysporum phaseoli* (FOP)

3.3.1.0. Isolation procedure for FOP

Isolation of *Fusarium oxysporum* was performed by direct plating (Nur *et al*., 2011; Samson *et al*., 2010). *Fusarium oxysporum* was isolated from naturally infected bean plants obtained from Embu, Agricultural Training Center. The plants were uprooted, cleaned and placed in moist polythene bags to enhance fungal growth (Jegathambigai *et al*., 2009). The stem and root were cut into 5mm pieces and then sterilized with 0.5% sodium hypochlorite solution for 5 minutes and rinsed with sterilized water. The pieces were dried with sterile filter paper and directly plated (three pieces per plate) on penta-chloro-nitro-benzene (PCNB) medium and incubated for 10 days at 25-26^0C.

The PCNB was prepared as per procedure outlined in Section 3.2.5.0. The grown colonies were then sub cultured on PDA. The PDA was prepared as per the procedure outlined in Section 3.2.1.0. Pure cultures were obtained using a single spore technique (Choi *et al*., 1999; Leslie and Summerell, 2006).

3.3.2.0. Single spore isolation of FOP

The single spore isolation was carried out as per the procedure described by Choi *et al*., (1999). Seven day old fungal culture on PDA was removed from the substrate surface using fine forceps and broken open in sterilized water in order to provide a spore suspension. The homogenized suspension was then transferred with sterilized pipette onto the surface of water agar petri plates. The agar plates were then incubated at 25^0C for 12-24 hours after which the germinated spores were picked with a sterilized inoculation needle and transferred to PDA plates and incubated at 25^0C. Cultures were then checked for any contamination and the uncontaminated cultures were then picked for further experiments.

3.3.3.0. Morphlogical identification of FOP

Further sub culturing of the prospective isolates from single spore cultures was done on PDA, Spezieller Nahrstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA). The SNA was prepared as per procedure outlined in Section 3.2.4.0. PDA cultures were incubated at 25ºC under normal light while CLA and SNA cultures were incubated at 25ºC under near UV light for four weeks. Morphological identification of isolates was done using the criteria of Gerlach and Nirenberg (1982): (Nelson *et al*., 1983).

3.3.4.0. DNA extraction, amplification and sequencing of FOP

The *Fusarium oxysporum phaseoli* was grown on potato dextrose agar petri plates at 25^0C for 7 days. Four fungal plugs from the actively growing margin of the *Fusarium* culture were then obtained and inoculated in 100 ml potato dextrose broth (PDB) and cultures maintained in the dark.

The cultures were agitated for 7 days on a rotary shaker at 70 rpm while maintained at room temperature. Mycelia from 100 ml PDB cultures were collected by filtration and washed with distilled water. The DNA extraction was done according to the procedure adapted from Sambrook *et al*., (1989). "This is as outlined in Section 3.4.4.1.0.**"**

Standard 25 µL PCR reactions were employed for each isolate to amplify a section of the elongation factor gene using primers EF1 (forward primer, 5'-ATGGGTAAGGARGACAAGAC-3' and EF2 (Rev primer, 5'-GGA RGT ACC AGT SAT CAT GTT-3'), (O'Donnell *et al*., 1998). The primers were designed by BIONEER. The PCR reaction contained 10–100 ng/µl genomic DNA, 10x PCR buffer, 10 mM dNTPs mix, 50 mM $MgCl_2$, 5U/µ1 *Taq* DNA polymerase, 10µM EF1 and 10µM EF2. The reactions were incubated in the thermal cycler starting with 1 minute of denaturation at 94^0C. This was followed by 36 cycles consisting of 1 minute at 94^0C, 1 minute at 51^0C and 3 minutes at 72^0C. One cycle for 5 minutes at 72^0C was conducted after the 36 cycles.

3.4.0. Isolation and Identification of *Trichoderma*

Trichoderma isolates were obtained from soil samples collected from Embu Agricultural Training Centre (ATC) in field with *Fusarium* wilt symptoms. The soil samples were obtained as outline in "section 3.1.0" at a depth of 0-20cm. *Trichoderma* are saprophytic in nature and are commonly present in the soil's top horizons where high densities of mycelium can be recovered (Bourguignon 2008). The soil samples were collected and transported in paper bags. Samples were kept at 2-5^0C in the laboratory to reduce microbial activity.

3.4.1.0. Isolation procedure for *Trichoderma*

Isolation of *Trichoderma* spp. was carried out by soil dilution plating (Rahman *et al.,* 2011; Samson *et al*., 2010). One gram of each soil sample was suspended in 9ml of autoclaved distilled water in 20ml universal bottles and mixed by vortexing for 1minute. After which aliquots of 1 ml of the supernatant from each bottle was diluted with 9ml of sterile distilled water to attain a tenfold dilution. The dilutions were done successively to 10^{-3}. Approximately 1ml aliquot from the three dilutions (10^{-1}, 10^{-2}, 10^{-3}) of the above soil suspensions were cultured on PDA using spread plate method and incubated at 28^0C for 7 days and examined for colonies that resembled *Trichoderma* spp. morphology. The PDA was prepared as per procedure outlined in Section 3.2.1.0. Single colonies picked from the PDA plates were cultured on separate PDA plates and incubated at 28^0C for 7 days. Further purification was achieved through single spore isolation.

3.4.2.0. Single spore isolation for *Trichoderma*

For the purpose of DNA analysis for *Trichoderma* species isolates, there was need for further purification of the *Trichoderma* colonies through single spore isolation. This was achieved as per the procedure described by Choi *et al*., (1999). Seven day old fungal culture on PDA was removed from the substrate surface using fine forceps and broken open in sterilized water in order to provide a spore suspension. The homogenized suspension was then transferred with sterilized pipette onto the surface of water agar petri plates. The agar plates were then incubated at 25^0C for 12-24 hours after which the germinated spores were picked with a sterilized inoculation needle and transferred to PDA plates and incubated at 25^0C. Cultures were then checked for any contamination and the uncontaminated cultures were then picked for further experiments.

3.4.3.0. Morphological identification of *Trichoderma* isolates

Morphological identifications of the isolates was performed based on the identification keys of the genus *Trichoderma* (Chaverri *et al*., 2003).

5mm disc of culture from actively growing colonies (7 days old) from single spores cultures of *Trichoderma* isolates were sub-cultured on selective PDA media prepared as per the procedure outlined in Section 3.2.1.0. The cultures were then incubated at: 20^0 C, 25^0 C and 35^0 C. relatively few species grow well at 35^0 C: growth at this temperature is an important taxonomic criterion. Resulting colonies were examined for the characteristic structures. This was achieved by slide preparations and examination through the light microscope by mounting the culture in lacto phenol cotton blue and for size measurements in KOH and water as mounting fluids. The 95% confidence intervals of conidia, conidiophores and phialides were derived from measurements of 15 structures.

3. 4.4.0. Molecular characterization of *Trichoderma* isolates

3.4.4.1.0. DNA extraction from the *Trichoderma* isolates

From all the *Trichoderma* isolates five mycelia discs derived from colony margins of 7 day old cultures were inoculated in 100 ml potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks. The procedure outlined in Section 3.2.2.0 was used to prepare the PDB and the cultures were maintained in the dark. The cultures were agitated for 7 days on a rotary shaker at 70 rpm while maintained at room temperature. Mycelia from 100 ml PDB cultures were collected by filtration over a funnel using suction and washed with distilled water. The DNA extraction was done for each isolate according to the procedure adapted from Sambrook *et al*., (1989). Approximately 50 mg of mycelium were transferred to a 1.5ml eppendorf tubes. The mycelium was suspended in 600 µl CTAB lysis buffer containing: 0.1M NaCl, 0.5M Tris-HCl, (pH8.0), 50mM EDTA and 2% CTAB, 0.2% mercaptoethanol and 0.1mg/ml proteinase k. Two glass beads for crushing of cell walls were also added. The tubes were then placed into a mixer mill 301 (Retsch) and homogenized for 2 ½ minutes at maximum speed.

The tubes were incubated in a water bath for 1 hour at 65^0C. 600µl of phenol were added and the suspension mixed by vortexing gently for 10 seconds. Then, the tubes were centrifuged for 20 minutes at 14,000 rpm. After centrifugation, the top aqueous layer was transferred to new tubes and the extraction procedure was repeated. 600µl of phenol: chloroform was added to each sample; the samples were then vortexed briefly, and centrifuged for 10 minutes in a micro centrifuge. The aqueous layer was transferred to a new tube and extracted again with chloroform. The samples were mixed and centrifuged for 10 minutes at 14,000 rpm. The supernatant was transferred to new eppendorf tubes, and 60µl of 3M sodium acetate was added to the recovered aqueous solution and mixed by vortexing.
The DNA was then precipitated from the solution with 750µl of ice cold ethanol by incubating the samples in a freezer at -20^0C overnight.

Samples were then centrifuged at 4°C for 10 minutes at 14,000 rpm. The pellets were washed twice with cold 70% ethanol, air-dried, and then re-suspended in 80ul TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). To remove any RNA contamination, 5µl (1mg/ml) RNase was added to the DNA solution and incubated for 5 hours at room temperature. The quality and concentration of the DNA was determined by running 5µl of the DNA on 1% agarose gel stained with ethidium bromide in 1X TAE buffer, at 80V for 45 minutes (Płaza *et al.*, 2004). The visualization was done on UV light and photographed using the UVR TRANSILLUMINATOR apparatus. The initial quality of the obtained DNA was estimated by comparing with the molecular marker on the agarose gel. The DNA sample was sent to ILRI for the actual determination of the concentration.

3.4.4.2.0. PCR amplification and DNA sequences of *Trichoderma* isolates

Polymerase chain reaction (PCR) was performed and the nuclear rDNA region spanning the ITS1, ITS2 and 5.8S rRNA gene was amplified for each of the *Trichoderma* isolates.
The primers ITS1F (5'- TCCGTAGGTGAACCTGCGG -3') and ITS4R (5'- TCCTCCGCTTATTGATATGC-3'): (White *et al.*, 1990) were used for the PCR. The sequences were designed by BIONEER.

The PCR was carried out with PTC-100 peltier thermal cycler - using the following program: PCR reactions were performed in a total volume of 25 µl, containing 10–100 ng/µl genomic DNA, 10x PCR buffer, 10 mM dNTPs mix, 50 mM $MgCl_2$, 5U/µ1 *Taq* DNA polymerase, 10µM ITS1 and 10µM ITS4. The reactions were incubated in the thermal cycler starting with 1 minute of denaturation at 94^0C. This was followed by 36 cycles consisting of 1 minute at 94^0C, 1 minute at 51^0C and 3 minutes at 72^0C. One cycle for 5 minutes at 72^0C was conducted after the 36 cycles.

3.4.4.3.0. PCR products analysis

The PCR products were analyzed by loading 5 µl of the reaction mixture in 2% (wt/vol) agarose gel/ 1x Tris-Acetic-EDTA; pH 8.0 (TAE) followed by electrophoresis in 1% agarose gel in 1 × TAE buffer containing 1µl of ethidium bromide at 80V for 45 minutes. A 1-kb DNA ladder and 100bp ladder were used as molecular size markers for comparison. The ethidium bromide-stained gels were visualized on UV light and photographed using the 2UV Transilluminator*(UVP) from the school of biological sciences University of Nairobi. The PCR products were purified using the Qiagen QIAquick PCR Purification kit. The purified PCR products were then sent to BecA-ILRI Hub Services SegoliP (Kenya) for sequencing.

3.4.4.4.0. Analysis of sequence data

The sequences of all the isolates were edited and assembled using MEGA 5.05 software.
For species identification the ITS1 sequences from the 5 local *Trichoderma* isolates and the commercial *Trichoderma* ECO-T were submitted to GenBank using trichOKEY (http://www.isth.info/tools/molkey) (Druzhinina *et al.,* 2005: Roberts *et al.,* 2010). For phylogenetic analysis of the isolates the sequences alignment was done by Clustal W and manually edited using MEGA 5.05 program and the phylogenetic tree was constructed using the same program (Tamura *et al.,* 2011). Neighbor joining method was used to estimate the relationships among the isolates sequences data (Roberts *et al.,* 2010).

3.5.0. *In Vitro* Evaluation of Antagonism of *Trichoderma* spp. against FOP

The *Trichoderma* isolates growth inhibition against *Fusarium oxysporum* was done in dual cultures and through use of volatile and non-volatile metabolites.

3.5.1.0. Slide culture interactions

Sterilized wet filter paper was placed in petri dishes and on the filter paper a sterile microscope slide containing a thin layer of PDA (1x1 cm) was placed. From actively growing plate cultures of each *Trichoderma* isolate and the *Fusarium oxysporum*, an agar plug was obtained and inoculated at the edge of the PDA on opposite sides; a cover slip was then placed on the slide-cultures, and incubated for 5 days at 25° C. Microscopic observations were performed where the cover slip was transferred to another microscope slide, lactophenol-cotton blue was used to stain the fungi. The point of interaction between the two fungi was observed for presence of mycelia penetration or cell wall degradation or disintegration.

3.5.2.0. Dual plate cultures

The dual plate culture experiments were performed as per the techniques developed by Morton and Stroube (Mishra.*et al*., 2011). Mycelial plugs (5 mm diameter) from the edge of 7 day old cultures of each of the *Trichoderma* isolates and the pathogen (*Fusarium oxysporum*) were placed opposite each other on petri plates containing sterilized PDA. The experiments were done in triplicates. In the control petri plates, sterile agar blocks were placed opposite the *Fusarium* plugs instead of the *Trichoderma* isolates. The plates were incubated at 25 ± 1^{0}C for one week. Measurements of the growth of: *Trichoderma* mycelia towards the pathogen, the pathogen towards *Trichoderma* and the pathogen towards agar plug were done daily for up to 7 days after inoculation. The percent inhibition of the pathogen's radial growth was calculated in relation to growth of the controls as follows:

$L = [(C - T)/C] \times 100$; L - Inhibition of radial mycelial growth of pathogen;

C - Radial growth measurement of the pathogen in control; T - Radial growth of the pathogen in the presence of *Trichoderma* isolates as per method of Edington *et al.* (1971): (Hajieghrari *et al*., 2008).

3.5.3.0. Production of inhibitory substances by *Trichoderma* isolates.

3.5.3.1.0. Production of extracellular metabolites of *Trichoderma* isolates

The effect of non-volatile substances produced by the local *Trichoderma* isolates against the pathogen was studied using the methods described by Dennis and Webster (1971): (Tonsmo and Dennis 1977). The culture filtrate was prepared by growing *Trichoderma* spp. in potato dextrose broth (PDB) for 15 days at room temperature (28±2ºC). The mycelial mat of the fungus was removed from the broth and centrifuged at 10,000rpm for 15 min. The supernatant was collected and was passed through bacteria proof filter (Millipore, 0.22 μm) and used for further study. The effect of extracellular metabolites of *Trichoderma* isolates on the radial growth of pathogen was determined by the addition of cell free culture filtrates (CCF) on agar medium. The filtrates were added to pre cooled potato dextrose agar medium at final concentration of 5, 10, and 20 % (v/v) before pouring into petri plates. Each plate was inoculated with 5 mm mycelia disc of the pathogen cut from the advancing margins of plate culture grown on PDA (Mishra *et al*., 2011). The inoculated plates were incubated at 25 ± 1^{0}C. The colony diameter in each concentration were measured daily for 5 days.

The pathogen inoculated on PDA medium without any culture filtrate served as control.
Pathogen growth inhibition was expressed as the percentage of radial growth inhibition relative to the control as follows:

$L = [(C - T)/C] \times 100$

L - Inhibition of radial mycelial growth of pathogen;

C - Radial growth measurement of the pathogen in control;

T - Radial growth of the pathogen in the presence of *Trichoderma* extracellular metabolites

The extracellular metabolites were obtained in three different cultures

a. *Trichoderma* cultures in the presence of viable pathogen

Co-cultures of *Trichoderma* and *Fusarium oxysporium* f. sp. *phaseoli* pathogen were prepared. Four 5 mm diameter plugs of each *Trichoderma* isolate, and pathogen (*Fusarium oxysporium* f. sp. *phaseoli.)* obtained from actively growing margins of PDA cultures, were co-inoculated into 500-ml conical flasks containing 100 ml sterile one-fifth strength potato dextrose broth (PDB). The stationary cultures were incubated for 15 days at 25^0C. The CCF was harvested as per the procedure outlined in Section 3.6.3.2.0.

b. *Trichoderma* culture alone

Four 5 mm diameter plugs of each *Trichoderma* isolate were obtained from actively growing margins of PDA cultures, inoculated into 500-ml conical flasks containing 100 ml sterile one-fifth strength potato dextrose broth (PDB). The stationary cultures were incubated for 15 days at 25^0C.

The CCF was harvested as per the procedure outlined in Section 3.6.3.2.0.

c. *Trichoderma* cultures in the presence of nonviable pathogen biomasses

Cultures of *Trichoderma* and *Fusarium oxysporium* f. sp. *phaseoli* cell wall were prepared. Four 5 mm diameter plugs of each *Trichoderma* isolate were obtained from actively growing margins of PDA cultures, inoculated into 500-ml conical flasks containing 100 ml sterile one-fifth strength potato dextrose broth (PDB). Purification of cell walls of the pathogen was carried out as described by Jean Marie Francois (2007). The FOP cell wall was purified as per the procedure outlined in Section 3.6.3.2.0.d.

The purified cell walls were ground to fine powder under liquid nitrogen and added to the broth at a concentration of 10 g/ l. The stationary cultures were incubated for 15 days at 25^0C. The CCF was harvested as per the procedure outlined in Section 3.6.3.2.0.

d. Extraction of *Fusarium oxysporum* cell wall

To determine the extent of production of the antimicrobial metabolites by *Trichoderma* in the presence of non-viable pathogen material; *Fusarium* cell wall was extracted from the pathogen using the protocols described by Jean Marie Francois (2007). *Fusarium oxysporium* was grown on potato dextrose broth for 7 days at 25°C. The mycelial biomass was harvested and centrifuged for 5 minutes at 1000g. The resulting pellet was washed with sterile water and centrifuged at 1000g for 5 minutes. Then suspended in sterile water, centrifuged at full speed and supernatant removed. The cell pellet was re-suspended in Tris EDTA Buffer (TE) and glass beads added. The cells were then broken open by vortexing the suspension vigorously for six 20 seconds periods with 30 seconds intervals on ice. The suspension was then centrifuged and the supernatant transferred to falcon tubes. The beads were washed 5 times with TE by vortexing and the resulting suspensions pooled together with the supernatant in the falcon tubes. This was then centrifuged at full speed for 15 minutes. The resulting pellet was dried in the oven overnight. This was then used as the nonviable biomass that was cultured with *Trichoderma* for the production of the extracellular metabolites.

The pathogen inoculated on PDA medium without any culture filtrate served as control.

All experiments in this study were done in triplicate and data represented as the mean value.

3.5.3.2.0. Production of volatile metabolites

The *Trichoderma* isolates were evaluated for production of volatile inhibitory substances *in vitro* following the method of Dennis and Webster (1971): (Tonsmo and Dennis 1977). Five millimeter disc of *Trichoderma* from the edges of 7 day old cultures were inoculated centrally in PDA petri plates and incubated at 25^0C. After 5 days *Fusarium oxysporum* plugs from the edge of 7 day old cultures was inoculated on fresh PDA and the lids of the petri plates inoculated with the *Trichoderma* were replaced with the culture of *Fusarium oxysporum.*

The plates were fixed with cello tape and incubated for 7 days.

The control was set with plates inoculated with *Fusarium oxysporum* but inverted over petri plates containing only PDA. Growth of the pathogen was measured after 5 days of incubation and the percent inhibition was calculated as explained below:

PI=[(C-T)/C]*100

PI- Percent inhibition

C- Radial growth of pathogen in control plates

T- Radial growth of pathogen in dual culture

3.6.0. Green House Experiment to Test the Ability of *Trichoderma* spp. in Control of FOP

The commercial *Trichoderma* ECO-T and two local *Trichoderma* isolates [*T. koningiopsis* (T18), *T. viride* (T35)] that showed fast colony growth and best inhibition effect on colony growth of the *Fusarium* oxysporum f.sp. *phaseoli in vitro;* were compared in their ability in controlling the pathogen on beans grown in the green house. The trials were carried out in the greenhouse at the University of Nairobi, Chiromo campus.

The soil infestation with *Fusarium oxysporum* was carried out with 20 ml of fungal suspensions containing $5x10^6$cfu/ml; this was added to 1kg of sterilized soil before planting (Nashwa *et al.*, 2008).

The *Trichoderma* spp. were applied as seed coating at the rate of 8×10^8 CFU (Aziz *et al.*, 1997). The seeds were inoculated with *Trichoderma* spp. [*T. koningiopsis* (T18), *T. viride* (T35), ECO-T and combined (T18 + T35)]. This was performed as per the procedure described below: Mwezi Moja bean variety seeds (Simlaw seeds) were surface-sterilized by dipping the seeds in 2.5% sodium hypochlorite solution for 3 minutes, and then rinsed several times with sterilized water (Meki *et al.*, 2011). The coating with the inoculums was achieved by using powdered inoculum and 40% gum Arabic (FAO/NifTAL. 1984). The control treatments were coated only with 40% gum arabic. The treated beans seeds were spread to dry in the hood before being planted.

Six treatments were prepared for this experiment which included; *T. koningiopsis* (T35), *T. viride* (T35), ECO-T, combined (P18 + P35) bean seed inoculations planted in pathogen infested soils, positive control and negative control. Positive control consisted of non-inoculated beans seeds planted in pathogen infested soil and negative control consisted of non-inoculated beans seeds planted in non-infested soil. Experiments were set up with plastic pots each containing 1 kg of sterilized soil infested with *Fusarium oxysporum*; five bean seeds were sown in each pot. The experiments consisted of three replicates per each treatment set in a completely randomized design.

The conidial concentration was determined using a colony-forming unit (cfu) assay (Whipps *et al*., 1989) with 1 g of treated seed. Aliquots (1 ml) of each dilution were spread over the surface of *Trichoderma* selective medium. The number of cfu per seed was calculated after 7 days of incubation at 25°C (McLean *et al.*, 2005).

3.6.1.0. Experimental design for the greenhouse experiment

The experimental design used was completely randomized design (CRD) with each treatment replicated three times (Plate 3.1.).
Five bean seeds were sown in each pot, and three pots were used as replicates for each treatment.

Plate 3.1. *Trichoderma* treatments experiment set up in the green house showing the plant pots (A) planting time and (B) 12 days after sowing.

3.6.2.0. Investigation of effects of *Trichoderma* isolates on control of FOP in the green house

To investigate the effects of *Trichoderma* on the control of *Fusarium oxysporum* f. sp. *phaseoli in vivo* the following were assessed:-

The disease severity assessment of the bean plants was done as follows: damping-off incidence of beans at pre and post-emergence stages were calculated after 11 and 15 days, respectively. At pre-emergence the damping-off percentage was as per the number of non-emerged seeds in relation to the number of sown seeds, while post-emergence percentage was based on the number of plants showing disease symptoms out of the number of emerged seedlings (Nashwa *et al.*, 2008).

The growth of the beans was assessed by the following parameters: plant height, root length, number of leaves, fresh and dry weights of the shoots and roots. This was done 2, 3 and 4 weeks after sowing. Plants were sampled randomly from each treatment, with 3 replicates per each treatment.

3.7.0. Data Analysis

The inhibition tests effects were analyzed using analysis of variance (ANOVA). Differences among the means of effects of *Trichoderma* isolates against FOP were evaluated for significance according to turkey HSD test ($P<0.05$) and Duncans multiple range test was used for the greenhouse experiment. Data processing was performed using SPSS (Jegathambigai *et al*., 2009)

CHAPTER FOUR

4.0. R E S U L T S

4.1.0. The species of the *Fusarium* isolates

4.1.1.0. Morphology of the *Fusarium* isolates

Two distinct *Fusarium oxysporun* f. sp. *phaseoli* isolates were obtained from the roots and stems of the bean plants. The isolates exhibited a slow growth on PDA plates reaching up to between 5.1 to 5.7 cm in diameter 7 days after incubation at 20^0 C (Plate 4.2, Table 4.1). The microscopic features on CLA (Plate 4.3) and SNA (Plate 4.4), presented by the isolates were; macroconidia which were elongate, curved, septate and slightly foot shaped, the microconidia produced were cylindrical, non septate and abundant. The two isolates were morphological identified as *Fusarium oxysporum* in the section *Elegans* as per the keys to *Fusarium* species (Nelson *et al*., 1983).

Table 4.1. Characteristics of the *Fusarium* isolates by morphological features

	Isolate 1[*Fusarium* 1(F1)]	**Isolate2 ([*Fusarium* 2 (F2)]**
Color of Colony on PDA	White aerial mycelium, purple undersurface	White aerial mycelium, purple undersurface
Diameter length on PDA(cm) after 7 days	5.7cm	5.1cm
Type of macroconidia µm (CLA)	Elongate, curved, three septate and with a slight foot shaped end cell	Elongate, curved, three septate and with a slight foot shaped end cell
Size of macroconidia µm (CLA)	2.4423 x0.394 µm	2.61x0.4 µm
Type of microconidia µm (SNA)	Cylindrical, non septate, Abundant	Cylindrical, non septate, Abundant
Size of microconidia µm (SNA)	0.818x0.236 µm	0.811x0.2 µm
Type of clamydospores	Both terminal and intercalary clamydospores, smooth walled	Both terminal and intercalary clamydospores, smooth walled
Phialides	Short and non septate	Short and non septate

SNA: Spezieller Nahrstoffamer Agar; CLA: carnation leaves on water agar; PDA:potato dextrose agar

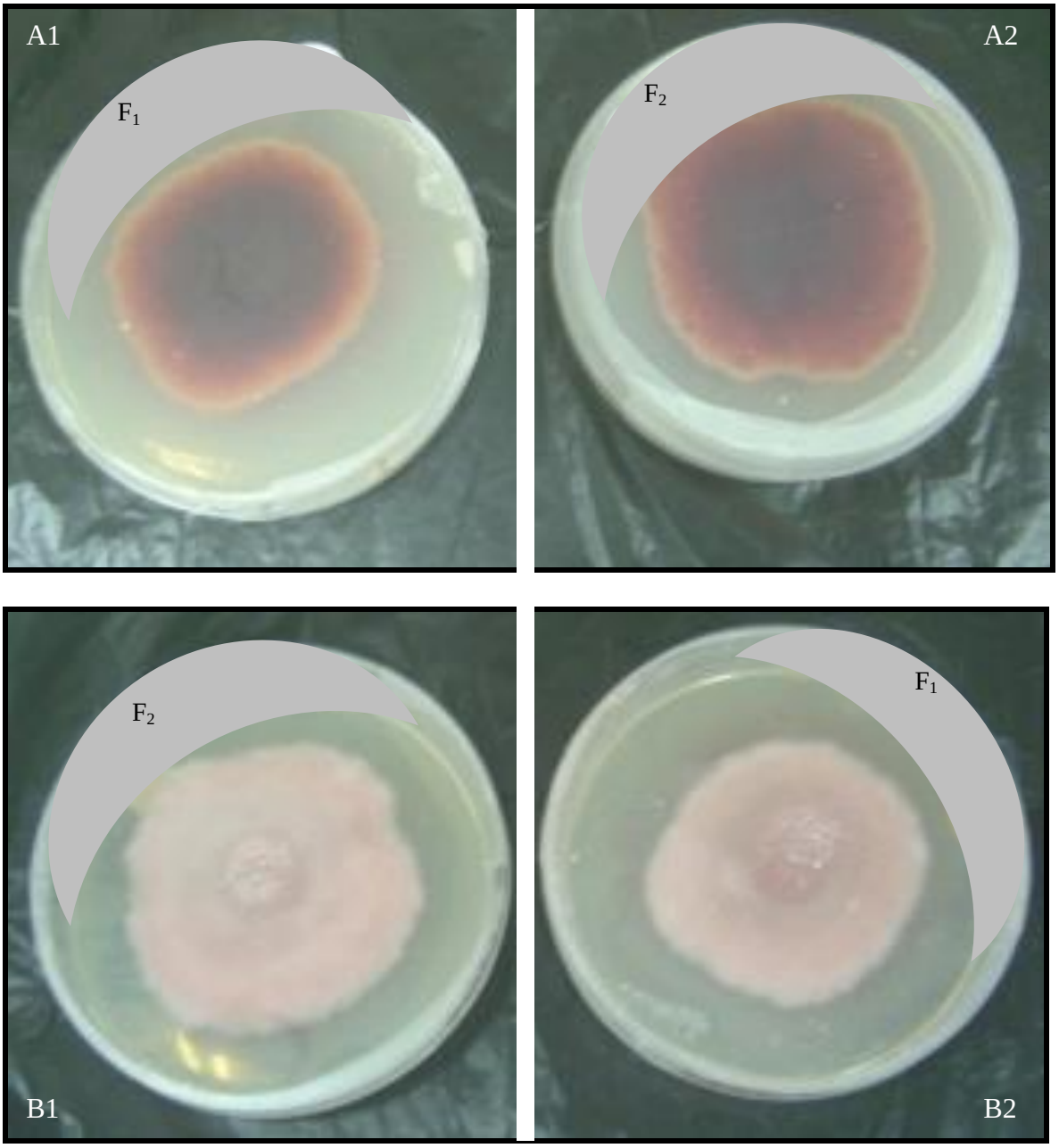

Plate 4.2: Morphologies of *Fusarium oxysporum* isolates on potatoe dextrose agar (PDA) culture plates 7 days after incubation A1, A2: bottom views B1, B2; top views

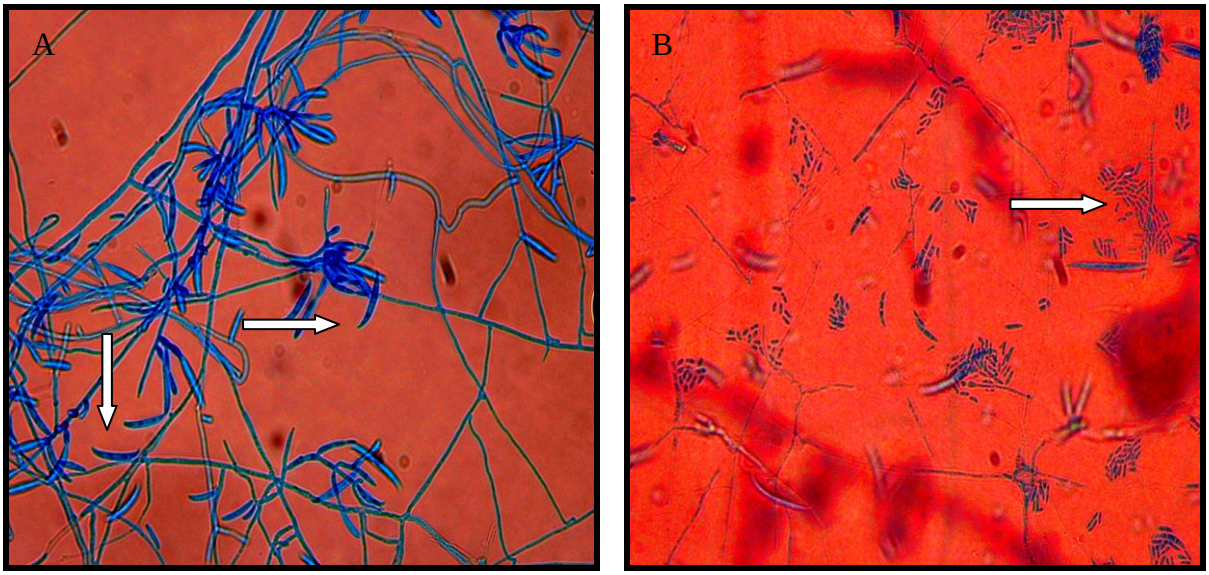

Plate 4.3: Microscopic features *Fusarium oxysporum* on carnation leaves on water agar (CLA) A: macroconidia B: microconidia (Magnification X400)

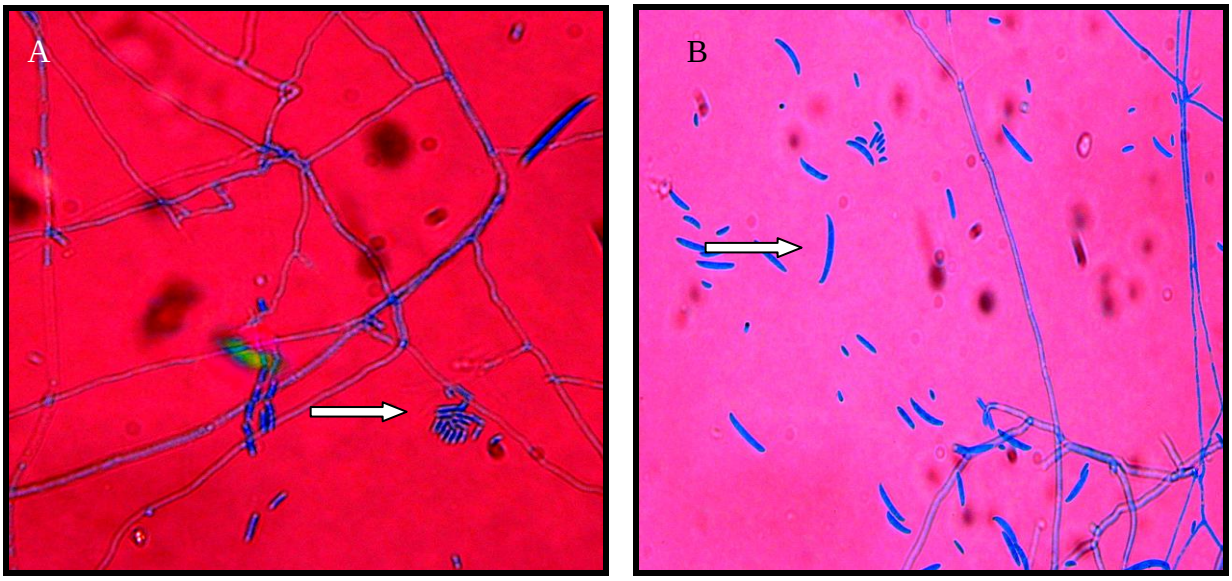

Plate 4.4. Microscopic features of *Fusarium oxysporum* on Spezieller Nahrstoffamer Agar (SNA) A: microconidia B: macroconidia (MagnificationX400)

4.1.2.0. Molecular characteristics of *Fusarium oxysporum*

4.1.2.1. DNA, PCR products and DNA sequences

From both the *Fusarium oxysporum* isolates DNA was successfully extracted and was visualized on UV light and photographed using the UVR TRANSILLUMINATOR apparatus. The amplification of a section of the elongation factor gene using primers EF1 (forward primer;5'-ATGGGTAAGGARGACAAGAC-3'and EF2 Rev (5'-GGA RGT ACC AGT SAT CAT GTT-3') (O'Donnell *et al.*, 1998) produced an amplicon in the size range of 700bp (Plate 4.5.).

The amplified region was successfully sequenced and using BLAST with the sequences deposited in NCBI GenBank the *Fusarium* isolates were identified as *Fusarium oxysporum* **(**gi|93009171|gb|DQ465933.1) (Altschul *et al.*, 1997).

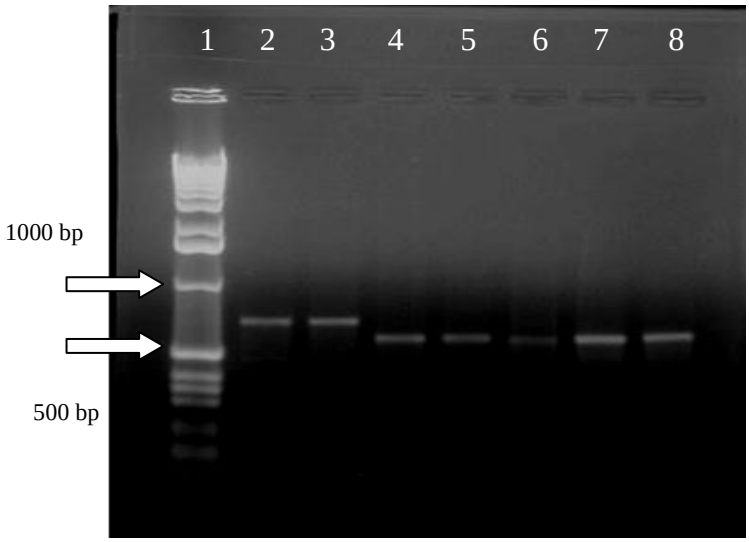

Plate 4.5. PCR amplified section of the elongation factor gene of *Fusarium oxysporum* (lane 1: Molecular Marker, lane 2*: Fusarium oxysporum* (F1), lane 3: *Fusarium oxysporum* (F2)

And PCR amplified ITS region of the *Trichoderma* isolates (lane 4: *T. hamatum* (T6), lane 5: *T. koningiopsis*, lane 6: *T. hamatum* (T21), lane 7: *T. viride*, lane 8: ECO-T)

4.2.0. Species of *Trichoderma* Isolates

4.2.1.0. Morphological characteristics of *Trichoderma* isolates

The *Trichoderma* isolates that were obtained from the 60 soil samples collected showed five morphologies that were distinct. In total 15 isolates were obtained and grouped into one of the five morphologies hereby named T6, T18, T21, T35 and T41 as described below. The isolates were maintained on PDA plates. The bio-control strain ECO-T was used as a reference control. The isolates morphological characteristics are presented on PDA plates and by microscopic features (Plates 4.6-4.11).

Morphology 1 - T6

The colony growth was moderately rapid, attaining a diameter of 6.5 cm on agar plate after 5 days incubation at 20^0C. The mycelium was uniform and white in faint concentric rings (Plate 4. 6 A, B). The isolates did not cause discoloration of the medium (Plate 4. 6 C). Conidiophores showed irregular branching. Conidia were 3.4 x 2.9µm (Plate 4. 6 D).

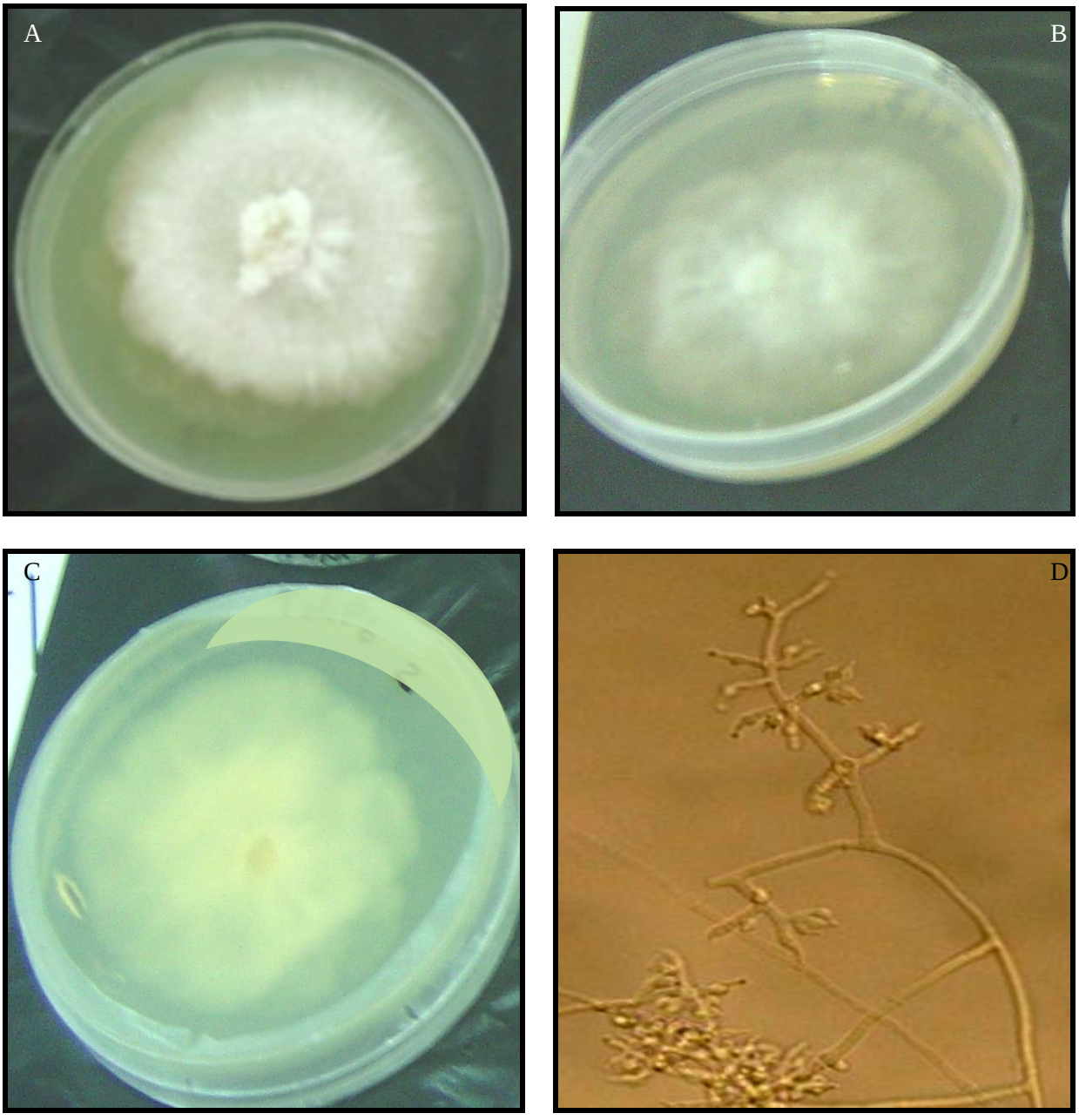

Plate 4.6. *Trichoderma hamatum* (T6). A & B: Top view, Colonies on PDA. C: Bottom view Colonies on PDA. D: conidiophores with conidia (magnification X400)

Morphology 2 - T18

The colony growth was fast attaining a diameter of 8.0 cm on agar plate after 5 days incubation at 20^0C. The mycelium was white in 2 concentric rings; Green conidia were formed within the centre of the colony (Plate 4.7 A, B). The isolates did not cause discoloration of the medium (Plate 4.7 C). Conidia measured 5.7 x 4.2µm (Plate 4.7 D).

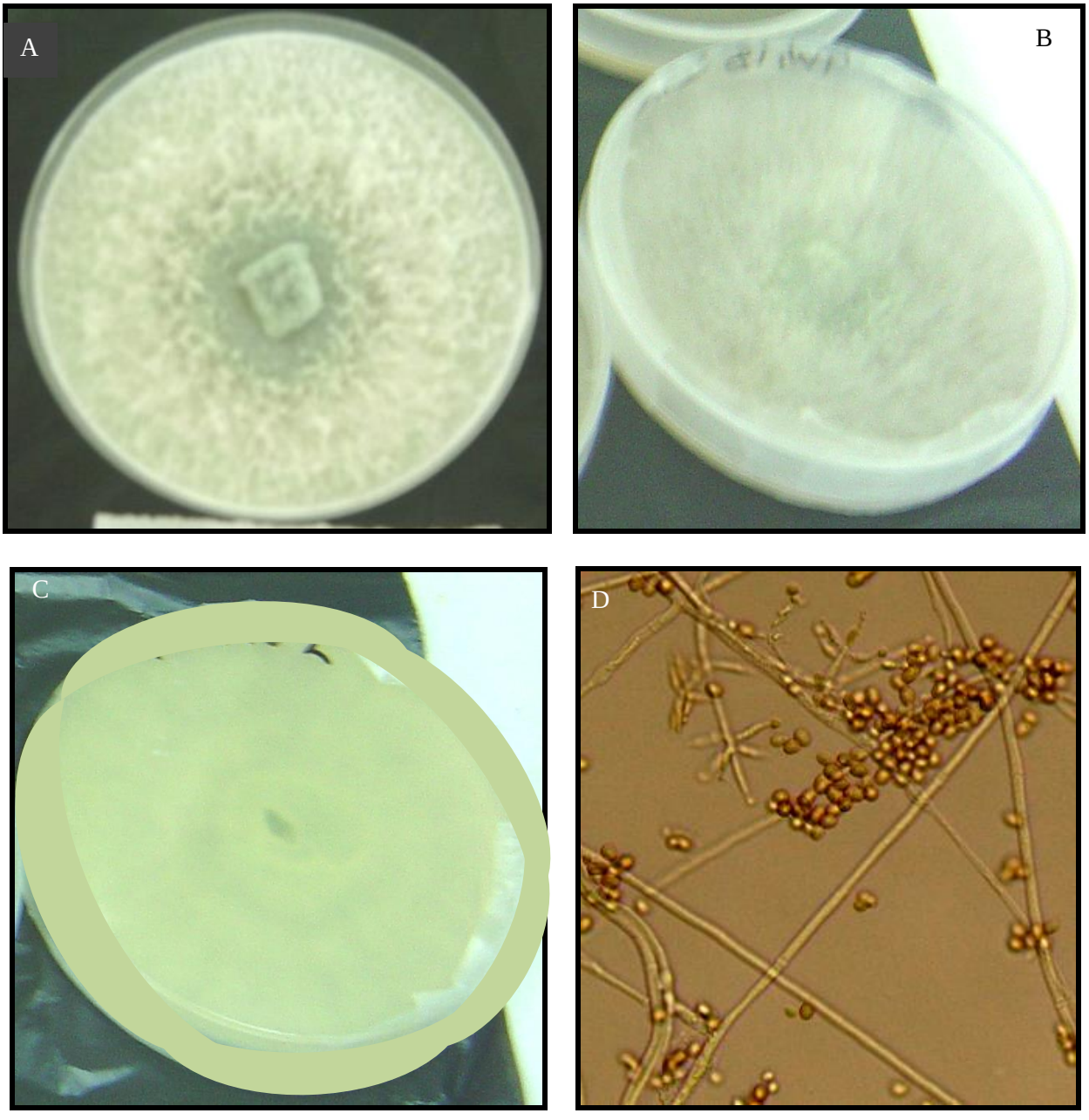

Plate 4.7. *Trichoderma koningiopsis* (T18). A & B: Top views Colonies on PDA. C: Bottom view Colonies on PDA. D: conidiophores with conidia (magnification X400)

Morphology 3 - T21

The colony growth was slow attaining a diameter of 6.6 cm on agar plate after 5 days incubation at 20^0C. The mycelium was white in faint concentric rings (Plate 4.8 A,B). The isolates did not cause discoloration of the medium (Plate 4.8 C). The conidia measured 3.7 x 2.3µm (Plate 4.8 D).

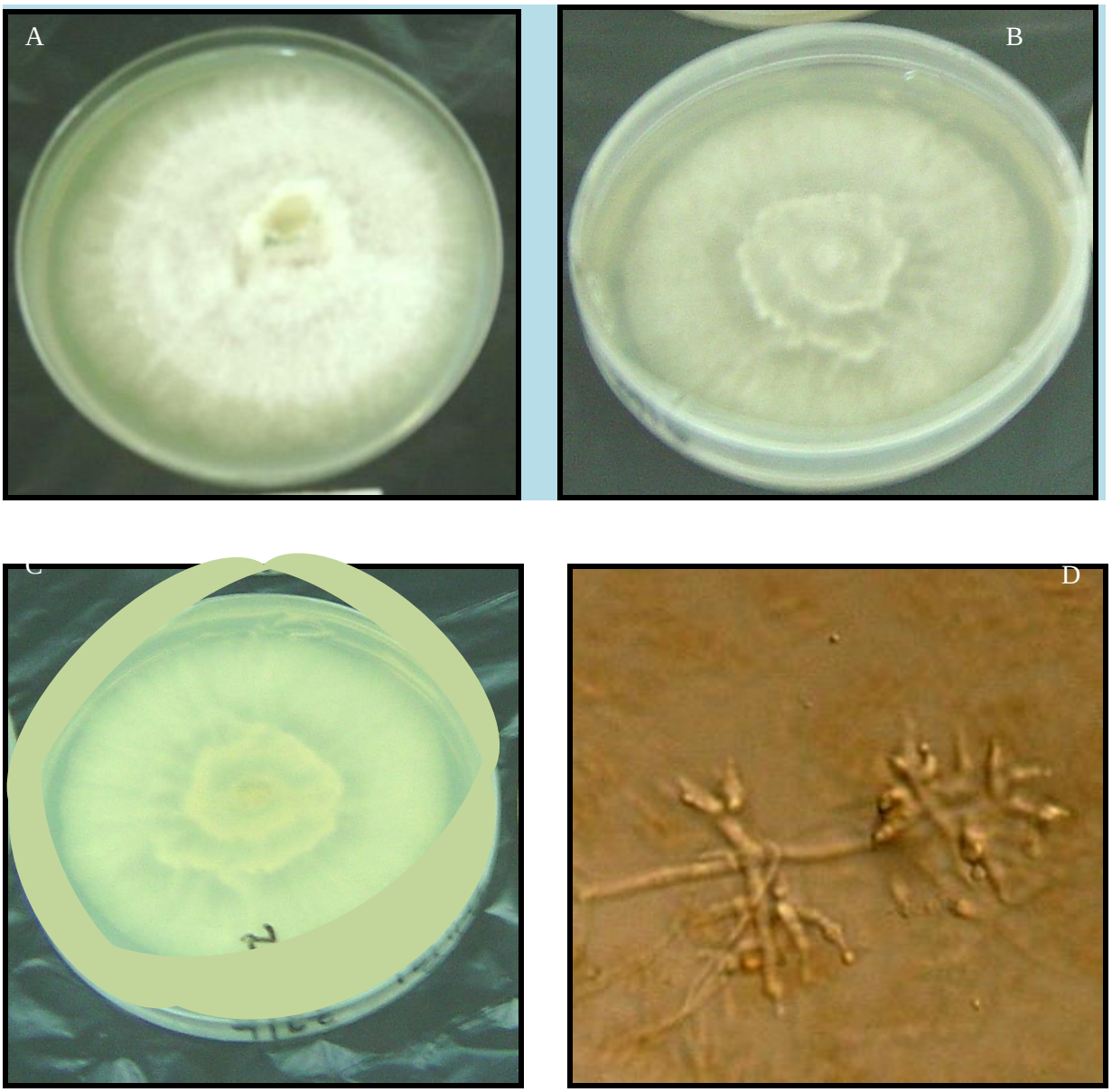

Plate 4.8. *Trichoderma hamatum* (T21). A & B: Top views, Colonies on PDA. C: Bottom view, Colonies on PDA. D: conidiophores with conidia (magnification X400)

Morphology 4 - T35

The colony growth was fast attaining a diameter of 7.3 cm on agar plate after 5 days incubation at 20^0C. The mycelium was white in 2 concentric rings; Green conidia was formed within the centre of the colony (Plate 4.9 A, B).The isolates did not cause discoloration of the medium (Plate 4.9 C).The conidia measured 4.3 x 4µm (Plate 4.9 D).

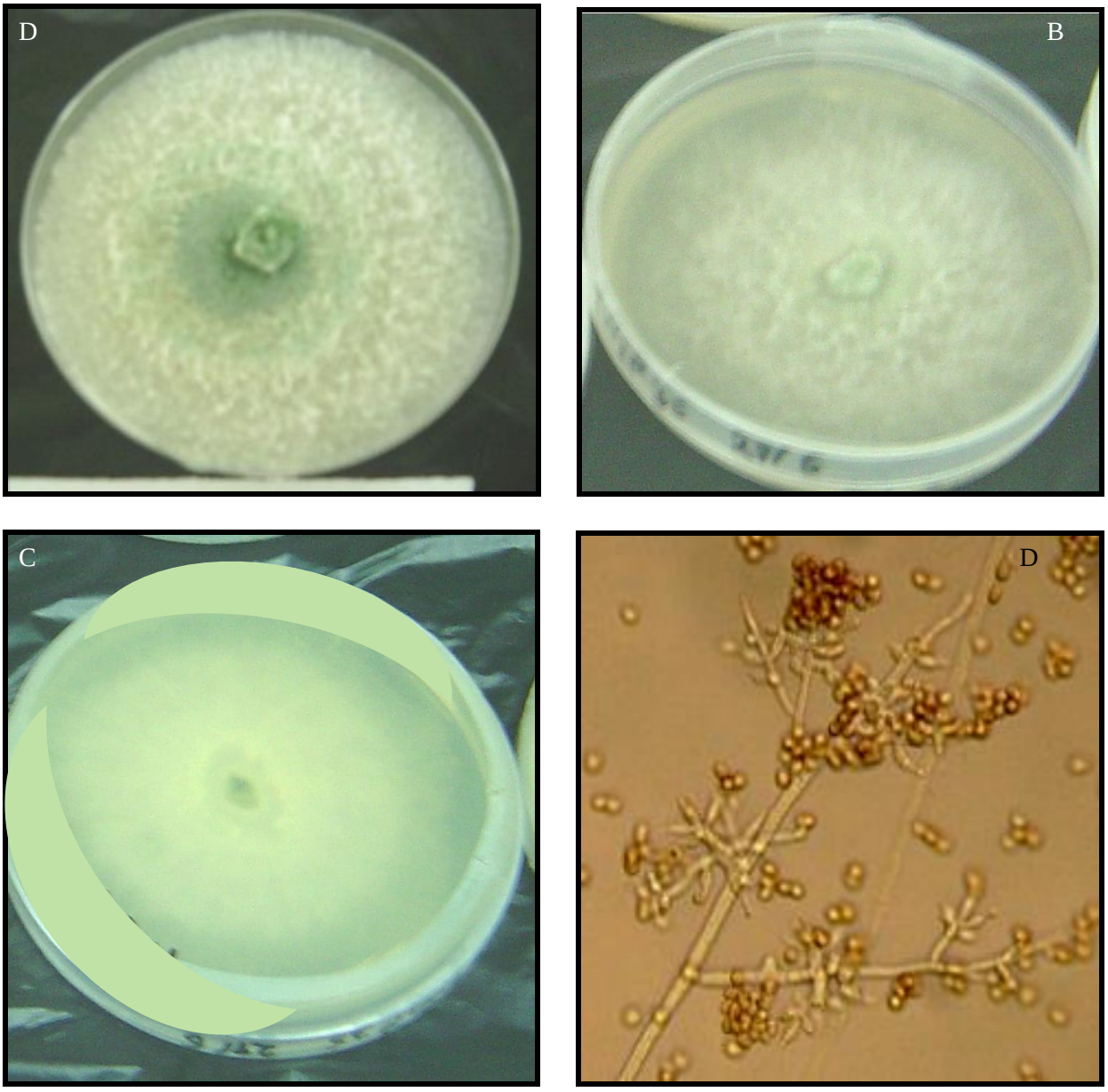

Plate 4.9. *Trichoderma viride* (T35 A & B: Top views, Colonies on PDA. C: Bottom view, Colonies on PDA. D: conidiophores with conidia (magnification X400)

Morphology 5 - T41

The colony growth was slow attaining a diameter of 5.8 cm on agar plate after 5 days incubation at 20^0C. The mycelium was white with faint concentric rings (Plate 4.10 A,B). The isolates produced a yellow colour on the bottom side (Plate 4.10 C). The conidia measured 3.2 x 2.3µm (Plate 4.10 D).

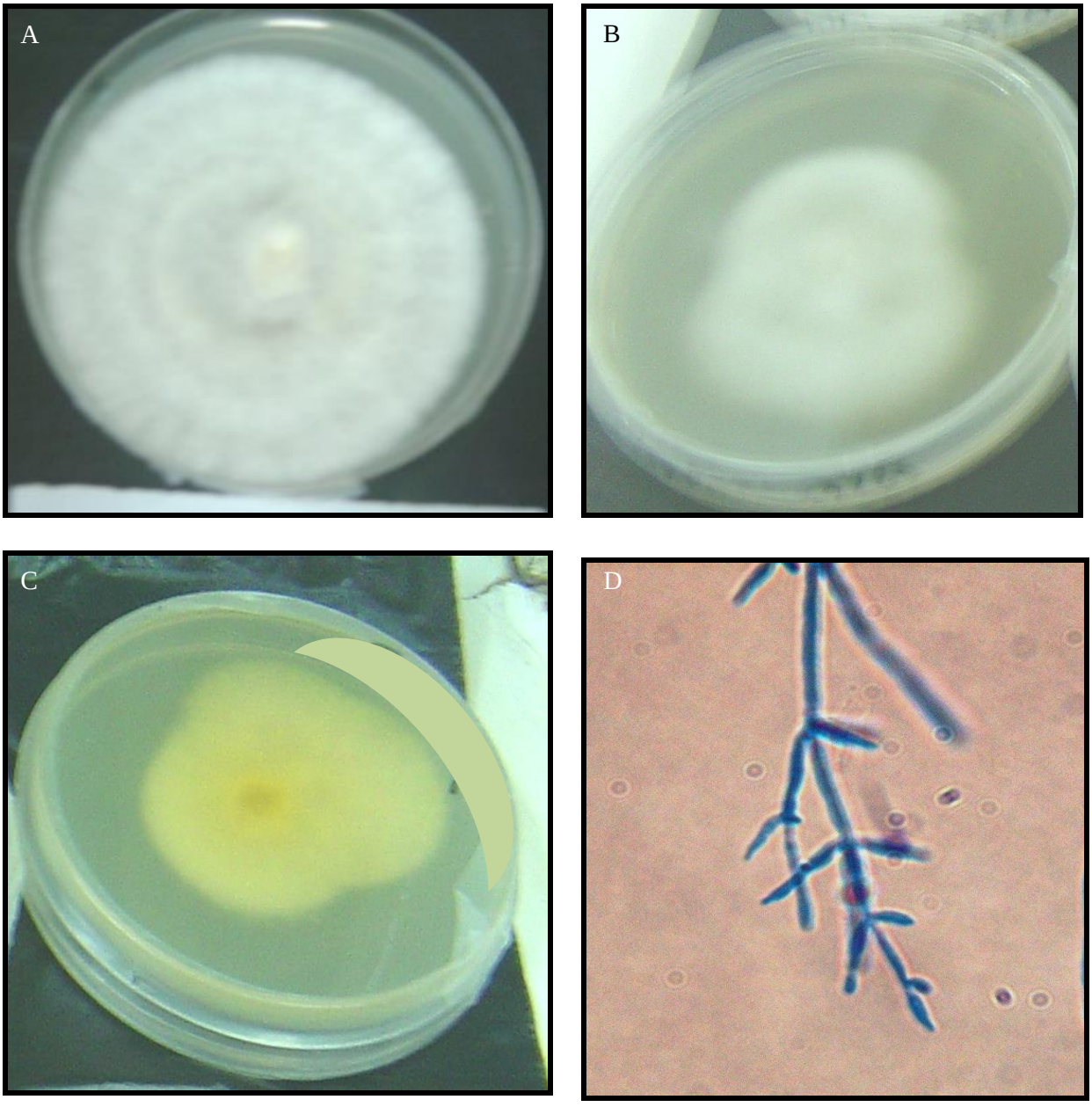

Plate 4.10. *Trichoderma spirale* (T41). A & B: Top views, Colonies on PDA. C: Bottom view, Colonies on PDA. D: conidiophores with conidia (magnification X1000)

Morphology of the commercial biocontrol agent - ECO-T

The colony growth was fast attaining a diameter of 8.0 cm on agar plate after 5 days incubation at 20^0C. The mycelium was white in 5 concentric rings; Green conidia were formed on 3 concentric rings of the colony (Plate 4. 11 A, B). The isolates did not cause discoloration of the medium (Plate 4. 11 C). The conidia measured 3.7 x 2.3µm (Plate 4. 11 D).

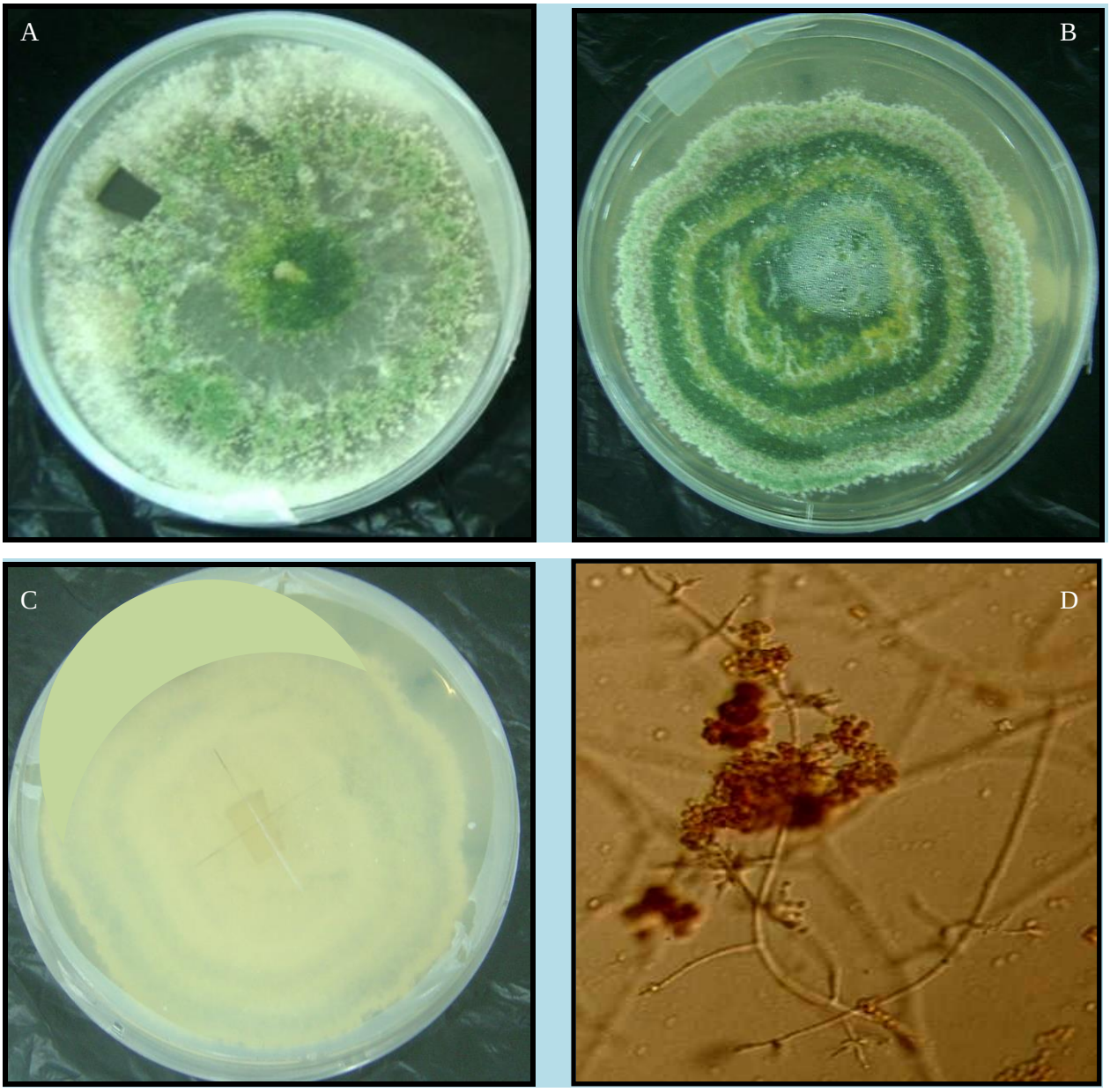

Plate 4.11. *Trichoderma asperellum* (ECO-T). A & B: Top views, Colonies on PDA. C: Bottom view, Colonies on PDA. D: conidiophores with conidia (magnification X400)

4.2.2.0. Molecular characteristics of the *Trichoderma* isolates

4.2.2.1.0. DNA, PCR products and DNA sequences of the *Trichoderma* isolates

From all the *Trichoderma* isolates DNA was successfully extracted and was visualized on UV light and photographed using the UVR TRANSILLUMINATOR apparatus**.**

The amplified nuclear rDNA region spanning the ITS1, ITS2 and 5.8S rRNA gene for each of the *Trichoderma* isolates with the primers ITS1F (5'- TCCGTAGGTGAACCTGCGG -3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3'),(White *et al.*, 1990) yielded amplicons in the size range of 600bp (Plate 4.12)

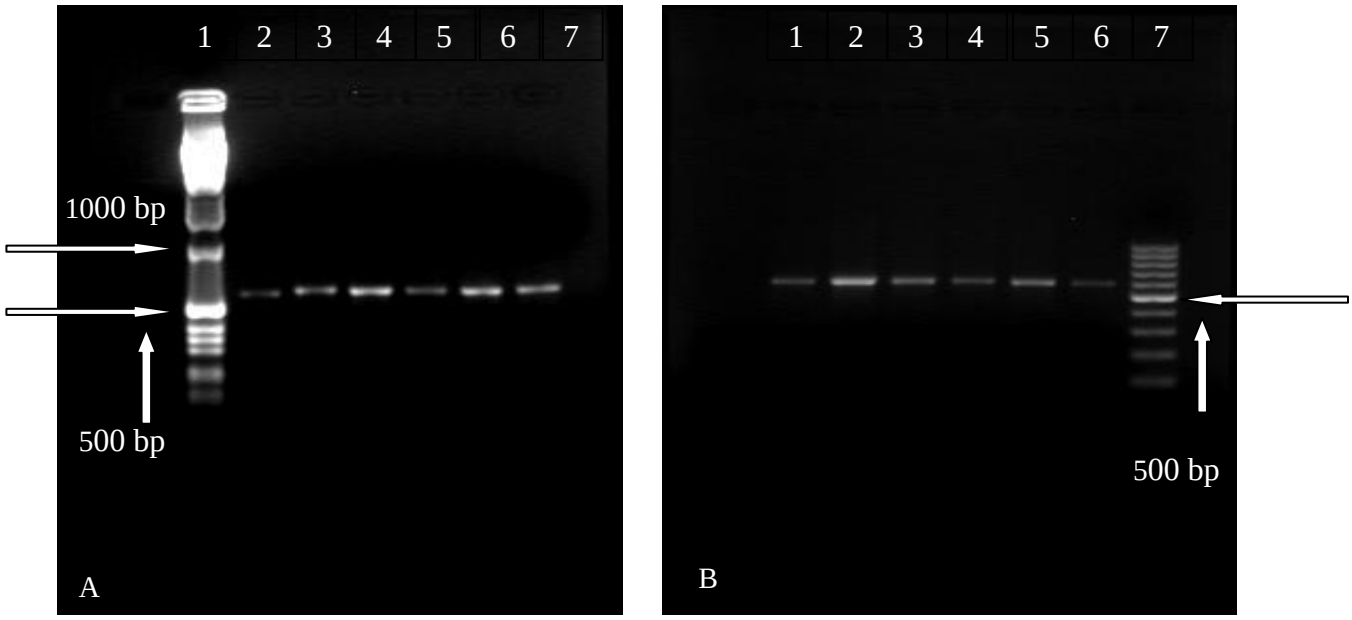

Plate 4.12. PCR amplified rDNA gene of *Trichoderma* isolates

A: lane 1- 1kbp ladder, lane 2-ECO-T, lane 3-*T.spirale*, lane 4-*T.viride*, lane 5- *T. hamatum* (T21), lane 6- *T. koningiopsis* lane 7- *T. hamatum* (T6);

B: lane 1- ECO-T, lane 2- *T.spirale*, lane 3- *T.viride*, lane 4- *T. hamatum* (T21), lane 5- *T. koningiopsis* , lane 6- *T. hamatum* (T6);, lane 7-100bp Ladder

4.2.2.4.0. Molecular Phylogeny

4.2.2.4.1 Sequences of the *Trichoderma* isolates

The amplicon obtained of the nuclear rDNA region spanning the ITS1, ITS2 and 5.8S rRNA gene of the *Trichoderma* isolates was in the size range of 600bp (Plate 4.12.).The Aligned Sequences for all the *Trichoderma* isolates were compared with the sequences available in GenBank using trichOKEY (http://www.isth.info/tools/molkey) (Druzhinina *et al* 2005: Roberts *et al.*, 2010) and results that were obtained were as below (Table 4.2).

Table 4.2. Sequencing results of *Trichoderma* isolates

Isolate morphology	***Trichoderma* spp.**	**Isthdb (TrichOkey)**	**Maximum identity**	**Reference**
T6	*Trichoderma hamatum*	isth|114|T.hamatum|ITS1 and 2|DAOM167057	97%	Altschul *et al* 1997
T18	*Trichoderma koningiopsis*	isth|911|T.koningiopsis|ITS1 and 2|GJS93-20	100%	Altschul *et al* 1997
T21	*Trichoderma hamatum*	>isth|114|T.hamatum|ITS1 and 2|DAOM167057	97%	Altschul *et al* 1997
T35	*Trichoderma viride*	gi|307239067|gb|HM776435.1|	97%	Altschul *et al* 1997
T41	*Trichoderma spirale*	isth|86|T.spirale|ITS1 and 2|CBS346.93	97%	Altschul *et al* 1997
ECO-T	*Trichoderma asperellum*	isth|116|T.asperellum|ITS1 and 2|CBS433.97	99%	Altschul *et al* 1997

4.2.2.4.2. Phylogenetic tree of the *Trichoderma* isolates

The phylogenetic tree obtained by sequence analysis of ITS1 of the local isolates and the commercial *Trichoderma* bio-control agent ECO-T divided the isolates into 2 major branches (Figure 4.2). The *Fusarium oxysporum* used in this study was used as the out group. Neighbor joining method was used to estimate the relationships among the isolates sequences data (Saitou *et al.*, 1987).

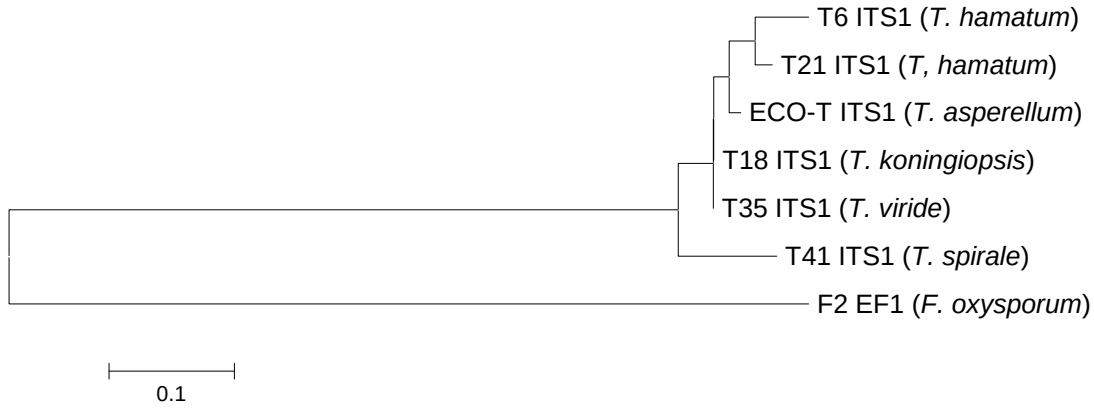

Figure 4.2. The phylogenetic relationships of the *Trichoderma* isolates inferred by analysis of their ITS1 sequences

4.3.0. Effects *In Vitro* of *Trichoderma* Isolates against *Fusarium oxysporum phaseoli* (FOP)

4.3.1.0. Effects in Dual plate cultures

Growth inhibition of the pathogen by all the *Trichoderma* isolates was evident from the sixth day of incubation except for isolates *T. hamatum* (T21) and *T. spirale* (T41) where the inhibition was not significant compared with the control. Inhibition by the commercial bio-control agent ECO-T (*T. asperellum*) at 19.91% against the pathogen was not significantly different (p=0.05) from inhibition by local isolates *T. viride* (T35), *T. koningiopsis* (T18) and *T. hamatum* (T6) which caused 21.3% ,13.43% and 10.65 % inhibition on day six respectively. Generally for all the

Trichoderma isolates the inhibition effect increased with time. Highest inhibition by day 8 was caused by the commercial bio-control agent ECO-T at 33.0% followed by the local isolates T18 (29.9%), T35 (27%), T6 (17.17%) and T21 (9.78%) (Table 4.3)..

From the PDA cultures of plugs obtained from the points where the pathogen and the antagonists interacted in the dual plate cultures (Plate 4.13), only the *Trichoderma* colonies were obtained except for isolate *T. spirale* (T41), where both the pathogen and antagonist grew for all the three plugs that were cultured (Plate 4.14)

From these results, ECO-T, *T. koningiopsis* (T18) and *T. viride* (T35) which gave the highest inhibitory effects against the pathogen were used in the greenhouse experiment (Table 4.3).

Table 4.3. Effects of *Trichoderma* isolates on growth of *Fusarium oxysporum* f.sp. *phaseoli* (FOP) in dual plate assay.

***Trichoderma* isolates**		**% Inhibition on growth of *Fusarium oxysporum***				
		Day4	**Day5**	**Day6**	**Day7**	**Day8**
T. hamatum (T6)		13.89±1.88^{bc}	15.60±2.26^{b}	10.65±1.23^{abc}	18.03±4.70^{ab}	17.17±7.08^{ab}
T. koningiopsis (T18)		16.39±2.17^{bc}	16.43±1.80^{b}	13.43±2.00^{bc}	28.56±5.63^{b}	29.97±1.78^{c}
T. hamatum (T21)		-4.17±4.64^{ab}	4.17±2.15^{ab}	3.70±2.37^{ab}	10.17±0.52^{ab}	9.78±2.22^{ab}
T. viride (T35)		16.40±4.42^{bc}	16.43±6.18^{b}	21.3±4.42^{c}	27.42±4.29^{b}	24.58±4.31^{ab}
T. spirale (T41)		-15.0±7.88^{a}	-6.67±3.88^{a}	0.93±2.84^{a}	13.56±4.08^{ab}	6.73±1.75^{ab}
T. asperellum (ECO-T)		20.56±5.69^{c}	15.6±4.71^{b}	19.91±1.27^{c}	31.29±10.35^{b}	33.00±13.61^{c}
Control		0±0^{abc}	0±0^{ab}	0±0^{a}	0±0^{a}	0±0^{a}
Statistics	F_{st}	8.65	7.22	13.31	4.64	4.09
	df	14	14	14	14	14
	P-value	0.000	0.001	0.000	0.008	0.014

Means in the same column for each treatment followed by same letter(s) are not significantly different according to Tukey HSD. p= 0.05).

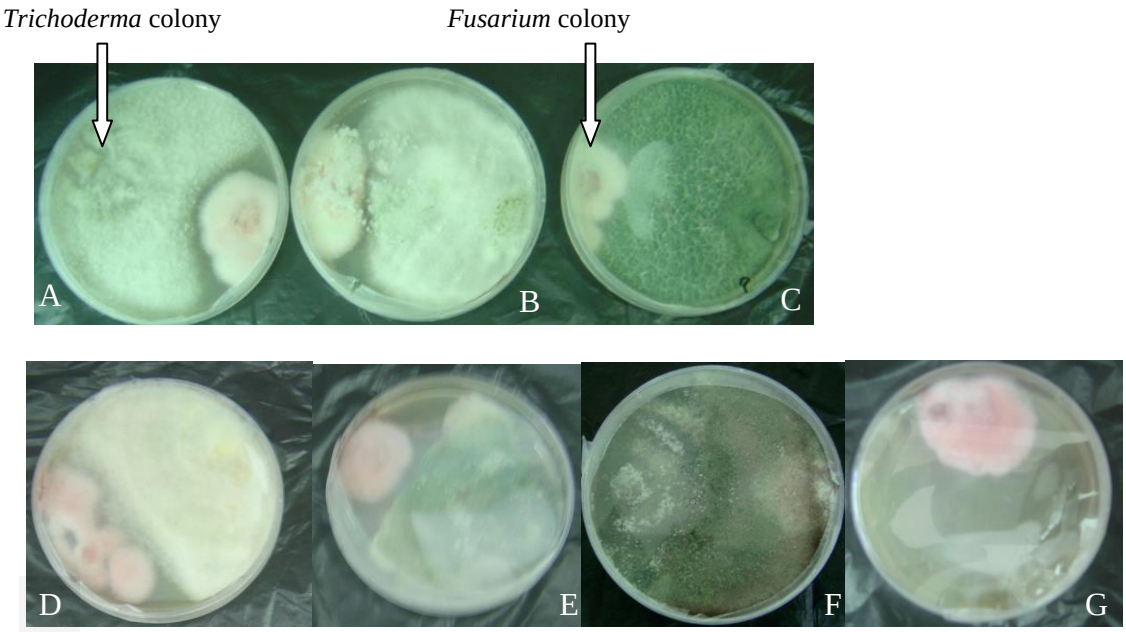

Plate 4.13. Colonies of dual cultures of *Trichoderma* isolates and *Fusarium oxysporum* f. sp. *Phaseoli* (FOP*)*.
A*: T. hamatum* (T21) + FOP, B*: T. hamatum* (T6) + FOP, C*: T. koningiopsis* + FOP,
D*: T. spirale* + FOP, *E: T. viride* + FOP, *F:* ECO-T+ FOP, G*:* Control.

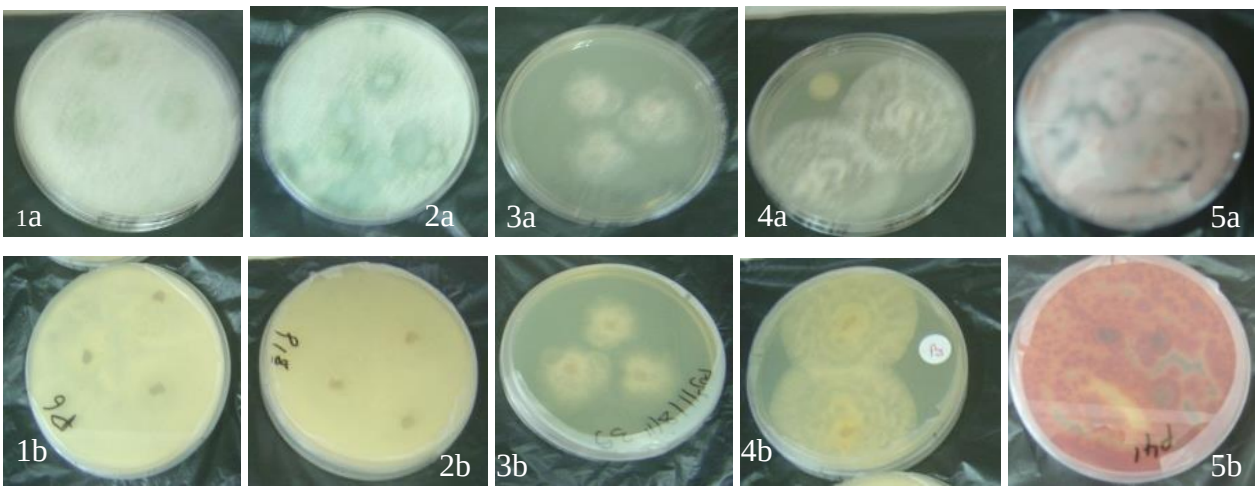

Plate 4.14. Cultures of colonies obtained at point of interaction of *Trichoderma* and *Fusarium oxysporum* from the dual plate experiments.

((a) top and (b) bottom views). 1a&1b: *T. hamatum* (T6); 2a&2b: *T. koningiopsis* (T18); 3a&3b: *T. viride* (T35); 4a&4b:*T. hamatum* (T21); 5a&5b: *T. spirale* (T41)

4.3.2.0. Effect of non-volatile metabolites from *Trichoderma* isolates on FOP growth

The results from the experiments with non-volatile metabolites obtained from different culture conditions; sole *Trichoderma* cultures, *Trichoderma* co-cultured with viable pathogen and *Trichoderma* co-cultured with non-viable pathogen showed that there was production of inhibitory substances that influenced the growth of *Fusarium* (Table 4.4, 4.5, 4.6). The extent of growth inhibition varied according to the *Trichoderma* isolate and the condition of the culture where the non-volatile metabolite was obtained. From non-volatile metabolites obtained from the sole *Trichoderma* cultures, *Trichoderma* isolate *T. koningiopsis* (T18) achieved the highest growth inhibition of FOP at 47.1%, followed by *T. viride* (T35) at 44.5%, *T. asperellum* (ECO-T) at 34.16%, T. spirale (T41) at 15.15% and *T. hamatum* (T6) at 16.89% with least inhibition of the pathogen growth achieved by *T. hamatum* (T21) at 13.6% (Figure 4.3 and Table 4.4).

From non-volatile metabolites obtained from *Trichoderma* co-cultured with viable FOP, isolate *T. viride* (T35) achieved the highest inhibition at 38.92% followed by ECO-T at 36.33%, *T. koningiopsis* (T18) at 34.96%, *T. hamatum* (T21) at 23.36%, *T. hamatum* (T6) at 22.65% and *T. spirale* (T41) at 12.14% (Figure 4.4 and Table 4.5).

From non-volatile metabolites obtained from co-cultures of *Trichoderma* isolates and non-viable FOP, none of the isolates achieved any significant growth inhibition against the FOP (Figure 4.5 and Table 4.6).

These results were obtained at metabolite concentration 25% on day 5 of incubation.

From these results Figure (4.6) effect of non-volatile metabolites obtained from co-cultures of each *Trichoderma* isolate i.e. *T. hamatum* (T6) , *T. koningiopsis* (T18), *T. hamatum* (T21) *T. viride* (T35), ECO-T and viable FOP was not significantly different from that obtained from cultures of sole *Trichoderma.* Non-volatile metabolites from co-cultures of *Trichoderma* isolates and non-viable FOP did not achieve a significant inhibitory effect against *Fusarium*. This shows that co-culturing of *Trichoderma* and nonviable FOP seemed to result to lower yields of non-volatile metabolites inhibitory against *Fusarium* (Fig.4.6)

Table 4.4. Effect of non-volatile metabolites obtained from cultures of each *Trichoderma* isolate, on *Fusarium oxysporum* f. sp. *phaseoli*

***Trichoderma* isolate**		**Non-volatile metabolites in increasing concentrations and the %inhibition on growth of FOP (Mean±SE)**					
		5%		10%		25%	
		D4	D5	D4	D5	D4	D5
T6		13.65^{abc}±3.89	13.33^{abc}±6.01	8^{ab}±3.00	10.91^{ab}±5.01	5.91^{a}±5.14	16.89^{abc}±10.37
T18		20.6^{bc}±3.65	30.83^{bc}±3.00	34.26^{c}±5.52	43.17^{c}±5.99	37.96^{c}±3.20	47.1^{d}±3.24
T21		4.5^{ab}±1.42	9.17^{ab}±2.20	7^{a}±5.35	12.65^{ab}±5.18	5.29^{a}±3.14	13.60^{ab}±7.30
T35		28.81^{c}±7.93	35.22^{c}±11.05	35.42^{c}±5.31	43.75^{c}±1.91	39.33^{c}±4.61	44.5^{cd}±4.77
T41		11.68^{abc}±4.30	14.17^{abc}±3.00	11.33^{ab}±6.33	15.24^{ab}±5.62	14.17^{ab}±3.00	15.15^{ab}±2.76
ECO-T		27.11^{c}±0.85	26.67^{bc}±1.67	30.77^{bc}±4.45	32.33^{bc}±5.04	31.84^{bc}±6.38	34.16^{bcd}±5.45
CONT		0^{a}±0	0^{a}±0	0^{a}±0	0^{a}±0	0^{a}±0	0^{a}±0
TOTAL		15.20±2.60	18.48±3.10	18.11±3.41	22.58±3.84	19.21±3.70	24.48±4.08
Statistics	F_{st}	7.68	12.3	9.86	13.87	26.14	9.423
	df	20	20	20	20	20	20
	P-value	0.001	0.000	0.000	0.000	0.000	0.000

* Means in the same column for each treatment followed by same letter(s) are not significantly different according to Tukey HSD. p= 0.05.

Source of the metabolites: T6-*T. hamatam*, T18-*T. koningiopsis*, T21-*T. hamatam*, T35-*T.viride*, T41-*T. spirale*, ECO-T- T.*asperellum*

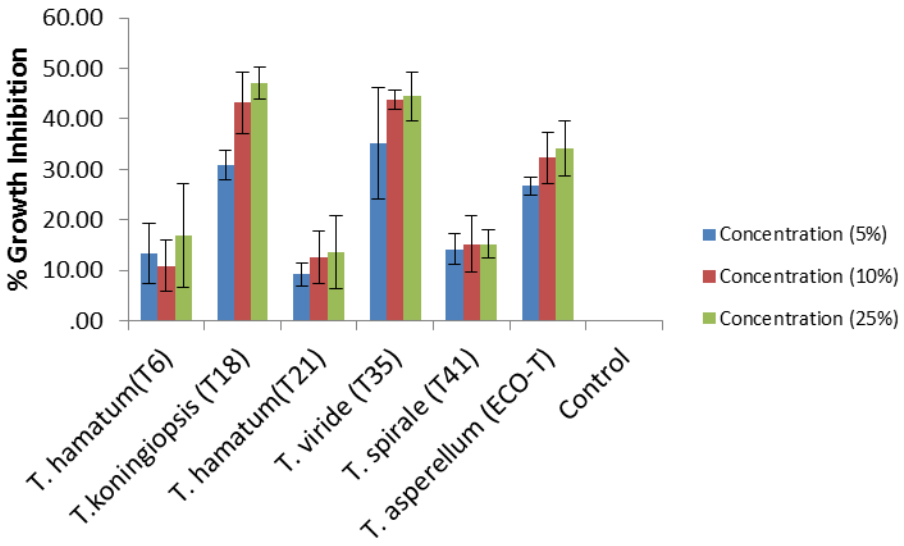

Figure 4.3. Effect of non-volatile metabolites obtained from cultures of each *Trichoderma* isolate, on *Fusarium oxysporum*. Error bars represent standard errors of the means.

Table 4.5. Effect of non-volatile metabolites obtained from co-cultures of each *Trichoderma* isolate and viable FOP, against *Fusarium oxysporum phaseoli*

Trichoderma isolate		Non-volatile metabolites in increasing concentrations and the %inhibition on growth of FOP (Mean±SE)								
		5%			10%			25%		
		D3	D4	D5	D3	D4	D5	D3	D4	D5
T6		12.48^{a} ^{b}±1.67	13.05^{bc}±2.95	13.46^{b}±1.47	7.02^{ab}±1.75	12.0^{ab}±4.62	8.92^{abc}+1.68	17.44^{ab}±3.84	20.93^{c}±1.36	22.65^{bc}±1.79
T18		12.48^{a} ^{b}±1.67	14.10^{bc}±1.16	17.21^{bc}±0.72	7.02^{ab}±1.75	14.83^{b}±2.59	18.69^{abc}±6.05	20.06^{ab}±2.53	30.78^{d}±0.88	34.96^{cde}±3.39
T21		8.78^{a}±4.64	9.05^{b}±1.50	3.74^{a}±0.76	17.54^{b}±6.33	18.33^{b}±1.67	17.65^{abc}±3.62	12.31^{ab}±2.71	18.10^{bc}±3.39	23.36^{bcd}±1.10
T35		14.43^{b} ±4.94	19.29^{c}±0.71	19.09^{c}±1.08	14.04^{ab}±3.51	10.83^{ab}±1.42	29.80^{c}±9.07	27.16^{c}±6.63	32.56^{d}±1.70	38.92^{e}±1.87
T41		14.23^{a} ^{b}±1.56	15.43^{bc}±2.38	15.25^{bc}±1.87	5.26^{ab}±3.04	7.83^{ab}±2.32	5.68^{ab}±0.3	10.93^{ab}±3.85	9.86^{b}±2.21	12.14^{ab}±4.16
ECO-T					10.53^{ab}±3.04	16.0^{b}±2.31	25.50^{bc}±2.25	27.16^{c}±6.62	37.60^{d}±2.39	36.33^{de}±3.88
Cont		0^{a}±0	0^{a}±0	0^{a}±0	0^{a}±0	0^{a}±0	0^{a}±0	0^{a}±0	0^{a}±0	0^{a}±0
Total		10.40±1.59		11.46±1.76	8.77±1.60	11.40±1.49	15.18±2.64	16.44±2.43	21.40±2.84	24.05±3.07
Statistics	F_{st}	3.391	21.43	48.11	3.303	4.188	7.639	5.912	62.61	32.654
	df	17	17	17	20	20	20	20	20	20
	P-value	0.039	0.000	0.000	0.031	0.013	0.001	0.003	0.000	0.000

* Means in the same column for each treatment followed by same letter(s) are not significantly different according to Tukey HSD. p= 0.05).

Source of the metabolites:T6-*T. hamatam*, T18-*T. koningiopsis*, T21-*T. hamatam*, T35-*T.viride*, T41-*T. spirale*, ECO-T- *T.asperellum*

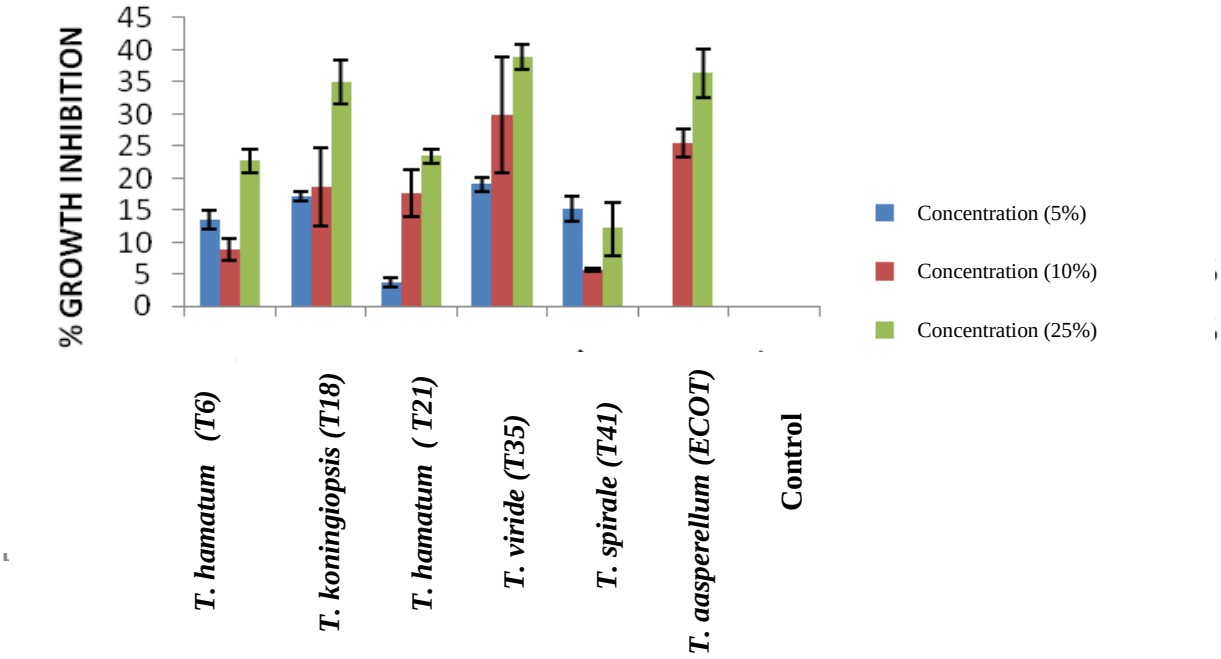

Figure 4.4. Effect of non-volatile metabolites obtained from co-cultures of each *Trichoderma* isolate and viable FOP, against *Fusarium oxysporum phaseoli.* Error bars represent standard errors of the means.

Table 4.6. Effect of non-volatile metabolites obtained from co-cultures of each *Trichoderma* isolate and non-viable FOP, against *Fusarium oxysporum phaseoli*

***Trichoderma* isolate**	**Non-volatile metabolites in increasing concentrations and the %inhibition on growth of FOP (Mean±SE)**								
	5%			10%			25%		
	D3	D4	D5	D3	D4	D5	D3	D4	D5
T6	-3.11^{ns}±8.93	4.85^{ns}±1.45	5.42^{ns}±1.55	-11.09^{ab}±8.52	-1.66^{ns}±11.32	3.66^{ab}±4.54	6.70^{ns}±4.01	12.96^{ns}±4.06	17.90^{b}±1.67
T18	-22.52±6.44	-9.86±7.17	-8.96±5.78	-21.88^{a}±6.12	-6.15±8.96	-1.99^{a}±3.97	-7.20±6.10	4.44±9.45	9.91^{ab}±3.69
T21	-22.21±12.74	-10.04±9.1	-3.92±7.89	-14.20^{ab}±6.15	-1.63±1.90	-0.15^{ab}±3.32	-19.92±12.15	-0.69±4.90	3.51^{ab}±3.03
T35	6.36±6.81	13.02±1.90	11.19±4.10	10.76^{b}±3.91	14.30±6.20	15.90^{b}±2.54	-3.28±4.81	6.38±5.73	10.55^{ab}±2.30
T41	-3.92±5.46	6.93±2.44	6.82±3.41	-21.07^{a}±10.28	-1.33±5.14	0.61^{ab}±5.06	-23.84±13.09	-9.14±5.68	-2.63^{a}±7.34
ECO-T	-21.23±17.33	-8.13±8.92	-6.02±13.94	-4.07^{ab}±4.41	2.90±5.46	8.25^{ab}±1.92	-8.51±10.46	3.21±12.78	13.08^{ab}±4.03
Control	0.0±0.0	0.0±0.0	0.0±0.0	0.0^{ab}±0.0	0.0±0.0	0.0^{ab}±0.0	0.0±0.0	0.0±0.0	0.0^{ab}±0.0
TOTAL	-9.52±3.97	-0.46±2.61	0.65±2.66	-8.78±3.18	0.92±2.49	3.75±1.70	-8.01±3.49	2.45±2.67	7.47±1.95

* Means in the same column for each treatment followed by same letter(s) are not significantly different according to Tukey HSD. p= 0.05).

Source of metabolites;T6-*T. hamatam*, T18-*T. koningiopsis*, T21-*T. hamatam*, T35-*T.viride*, T41-*T. spirale*, ECO-T- *T. .asperellum*

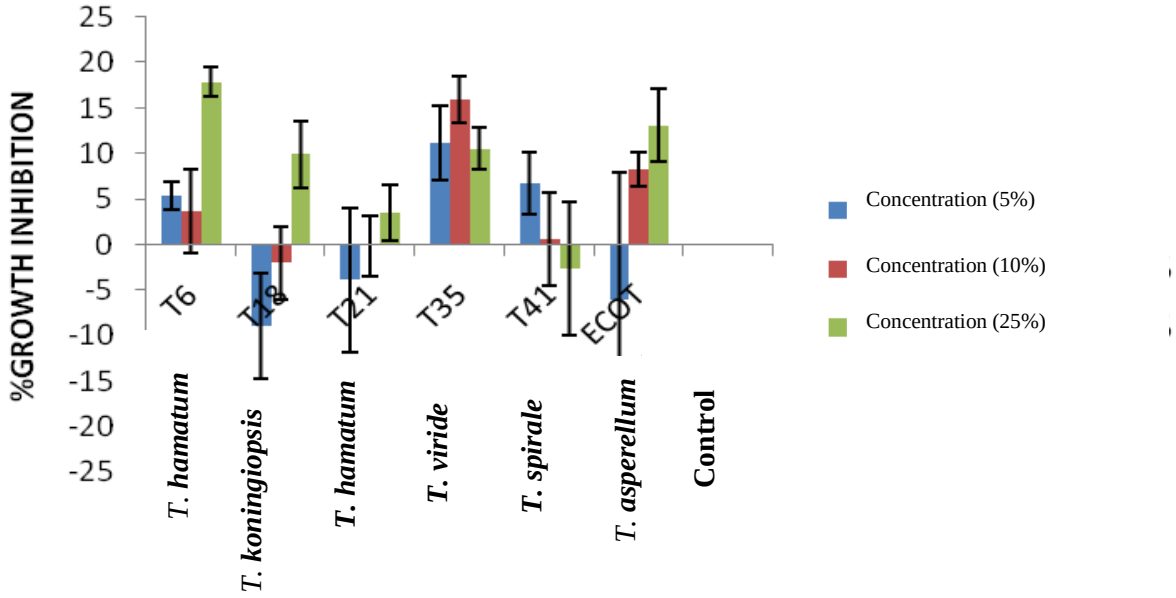

Figure 4.5. Effect of non-volatile metabolites obtained from co-cultures of each *Trichoderma* isolate and non-viable FOP, against *Fusarium oxysporum phaseoli.* Error bars represent standard errors of the means

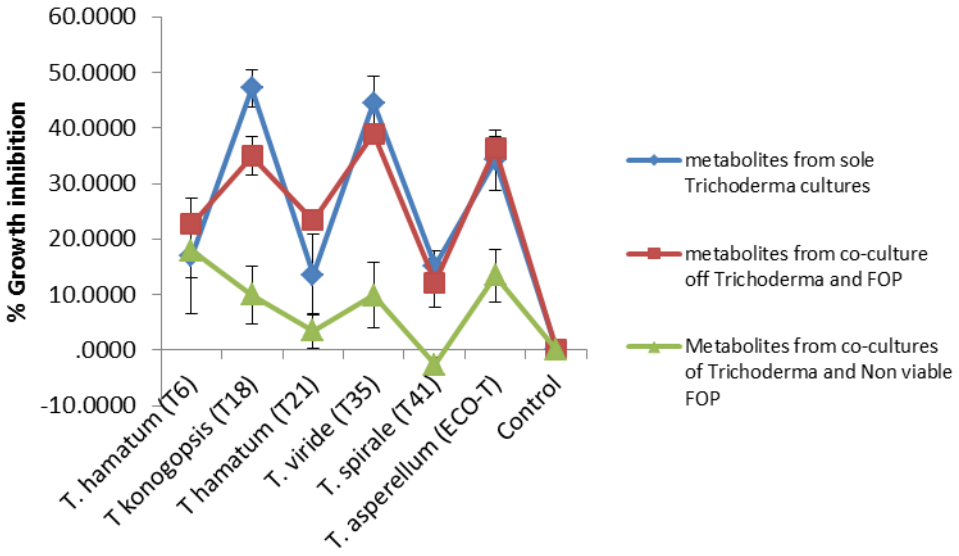

Figure 4.6. Effect of the non-volatile metabolites from the *Trichoderma* isolates obtained from the three different culture conditions, against FOP. Error bars represent standard errors of the means.

4.3.3.0. Effects of volatile metabolites from *Trichoderma* isolates against *Fusarium oxysporum phaseoli* growth

Trichoderma isolates produced volatile metabolites that significantly inhibited the growth of *Fusarium oxysporum* f. sp. *phaseoli.* The volatile metabolites from the different isolates showed significant differences in the inhibition against the pathogen. The commercial ECO-T gave the highest inhibition at 22.59% followed by isolates *T. viride* (T35) and *T. koningiopsis* (T18) at 15.30% and 13.59%, then *T. hamatum* (T21) at 13.62% *T. hamatum* (T6) at 12.09% and lastly *T. spirale* (T41) at 5.83% after 6 days of incubation (Table 4.7). The inhibitory effect of the volatile metabolites from the isolates increased with time but after day 6, except for *T. koningiopsis* (T18), there was a decrease in the inhibitory effect on the pathogen.

Table 4.7. Effect of volatile metabolites obtained from *Trichoderma* isolates against *Fusarium oxysporum phaseoli*

***Trichoderma* isolate**		**Volatile metabolites % inhibition (Mean±SE)**		
		D5	D6	D7
T. hamatum (T6)		8.44^{ab}±2.11	12.09^{abc}±1.61	11.46^{bc}±0.80
T. koningiopsis (T18)		10.01^{b}±0.25	13.59^{bc}±0.37	14.62^{c}±0.89
T. hamatum (T21)		11.26^{bc}±3.54	13.62^{bc}±1.17	11.03^{bc}±1.59
T. viride (T35)		7.30^{ab}±1.63	15.30^{bc}±5.30	14.84^{c}±4.17
T. spirale (T41)		6.57^{ab}±2.44	5.83^{ab}±1.42	2.22^{ab}±2.22
T. asperellum (ECO-T)		20.61^{c}±1.77	22.59^{c}±3.27	19.67^{c}±1.74
CONTROL		0^{a}±0	0^{a}±0	0^{a}±0
TOTAL		9.17±1.44	11.86±1.69	10.55±1.60
Statistics	F_{st}	9.346	8.064	11.93
	df	20	20	20
	P-value	0.000	0.001	0.000

Means in the same column for each treatment followed by same letter(s) are not significantly different according to Tukey HSD. p= 0.05).

4.4.0. *In Vivo* Antagonism of *Trichoderma* Isolates against FOP in the Green House

4.4.1.0. Effect on Pre-emergence and post emergence damping off

There was a significant difference between *T. viride* and control on pre-emergence disease severity. However the treatments [*T. asperellum* (ECO-T), *T. Koningiopsis* and (*T. Koningiopsis + T. viride)*] had no significant effect on the pre-emergence disease severity compared to the control (Table 4.8). The *Trichoderma* isolates were effective in reducing the post-emergence damping-off compared with positive control (Table 4.9). Treatment with isolate *T. viride* (T35) reduced damping off incidence in beans in the green house by 50% compared to the untreated control. The rest of the treatments; *T. koningiopsis*, ECO-T and (*T. konongiopsis+ T. viride*) reduced the damping off incidence by 35%, 43.33% and 35% respectively. This result was obtained 15 days after the plants were sown.

Table 4.8. Effects of the *Trichoderma* treatments on pre-emergence disease severity of the beans in the green house

Treatment		**Sown seeds**	**Emerged seeds**	**Non emerged seeds**	**% pre-emergence disease incidence (Mean±SE)**
T koningiopsis (T18)		5	4.666667	0.333333	6.67 ± 6.67^{ab}
T. viride (T35)		5	5	0	0.0 ± 0^{a}
T. asperellum (ECO-T)		5	4.666667	0.333333	6.67 ± 6.67^{ab}
T koningiopsis+ T. viride		5	4.666667	0.333333	6.67 ± 6.67^{ab}
Negative Control		5	5	0	0.0 ± 0^{a}
Positive Control		5	4	1	20 ± 0^{b}
Statistics	Fst				2.4
	df				17
	P value				0.099

Means in the same column for each treatment followed by same letter(s) are not significantly different according to Duncans multiple range test p= 0.05.

Disease severity assessment (pre-emergence) incidence was based on number of non-emerged seeds in relation to number of seeds sown (day 11 after sowing)

Table 4.9. Effects of the *Trichoderma* treatments on post-emergence disease severity of the beans in the green house

Treatment		Emerged Seedlings	Diseased Seedlings	%Disease Severity (Mean±SE)
T koningiopsis (T18)		4.67	0.67	15.0 ± 7.64^{b}
T. viride (T35)		5	0	0.0 ± 0.0^{b}
T. asperellum (ECO-T)		4.67	0.33	6.67 ± 6.67^{b}
T koningiopsis+ T. viride		4.67	0.67	15.0 ± 7.64^{b}
Negative Control		5	1	$20.0^{b}\pm20.0^{b}$
Positive Control		4.67	1.67	50 ± 0.0^{a}
Statistics	F_{st}			3.20
	df			17
	P value			0.046

Means in the same column for each treatment followed by same letter(s) are not significantly different according to Duncans test p= 0.05).

Disease severity assessment (post-emergence) incidence was based on number of diseased seedlings in relation to number of emerged seedlings (day 15 after sowing)

4.4.2.0. Effect of *Trichoderma* treatments on the growth of the beans in soil infested with *Fusarium oxysporum* f. sp. *phaseoli* in the greenhouse.

The *Trichoderma* treatments boosted the growth and productivity of the bean variety mwezi moja in the *Fusarium* infested soil in the green house compared with the untreated control (Table 4.10, Table 4.11 and Plate 4.15).The extent of growth and productivity promotion varied according to treatment and this was observed at four weeks after planting (Plates 4.15 and 4.16). The treatment that consisted of the combination of [*T. koningiopsis* (T18) + *T. viride* (T35)] as inoculum gave the highest growth in terms of the plant height and number of leaves followed by single inoculations of *T. koningiopsis* (T18) and *T. viride* (T35) and the lowest effect was from the commercial *Trichoderma* ECO-T. The same trend was also observed for top fresh/dry weights and dry root weights (Figure 4.7) where there were significant differences with the positive control and the combination of [*T. koningiopsis* (T18) + *T. viride* (T35)] gave the highest weights and ECO-T gave the lowest weights (Table 4.11).

Table 4.10. The effects of the *Trichoderma* treatments on the growth of beans in the green house

Treatment		Plant height mm (Mean±SE)		Root length mm (Mean±SE)		Number of leaves per plant (Mean±SE)		Length of longest leaf mm (Mean±SE)	
		3weeks	4weeks	3weeks	4weeks	3weeks	4weeks	3weeks	4weeks
T. konongiopsis		24.8±6.33 ^{ns}	32.1±3.46 ^{cd}	11.97±0.52^{ns}	12.77±0.93^{ns}	4.0±2.0 ^{ns}	6.0±2.65^{ab}	8.37±0.57 ^{ns}	6.83±0.90 ^{ab}
T. viride		25.9±8.07 ^{ns}	31.83±2.89 ^{cd}	15.57±1.73 ^{ns}	10.73±0.96 ^{ns}	5.0±1.73^{ns}	8.0±0.0^{b}	7.07±1.67 ^{ns}	6.63±0.32 ^{ab}
ECO-T		21.57±1.03 ^{ns}	19.07±1.54 ^{ab}	15.33±1.42^{ns}	13.67±2.96^{ns}	5.0±0.0^{ns}	4.0±1.0^{ab}	7.33±1.33 ^{ns}	5.63±0.52 ^{a}
T. konongiopsis+ *T. viride*		24.53±6.63 ^{ns}	35.4±1.25 ^{d}	13.37±2.92^{ns}	14.57±2.0 ^{ns}	6.0±1.0 ^{ns}	13.0±1.0^{c}	6.83±0.38	9.67^{c}±0.60
Negative Cont.		19.97±0.09 ^{ns}	25.0±2.65^{bc}	13.87±0.24^{ns}	12.3±2.12 ^{ns}	5.0±0.0 ^{ns}	8.0±1.73^{b}	6.57±0.54 ^{ns}	8.47±1.48 ^{bc}
Positive Cont.		18.0±4.52 ^{ns}	12.9±2.64^{a}	13.27±2.28 ^{ns}	9.5±1.32 ^{ns}	3.0±1.0 ^{ns}	3.0±1.0^{a}	5.27±1.27 ^{ns}	4.13±0.63 ^{a}
statistics	F_{st}	0.341	11.951	0.582	1.012	0.711	5.908	0.890	5.631
	df	17	17	17	17	17	17	17	17
	P value	0.879	0.000	0.713	0.452	0.627	0.006	0.517	0.007

Means in the same column for each treatment followed by same letter(s) are not significantly different according to Duncan's multiple range test p= 0.05.

Table 4.11. Effects of *Trichoderma* treatments on the productivity of beans in the greenhouse.

Treatment		Fresh top weight gm (Mean±SE)		Dry top weight gm (Mean±SE)		Fresh root weight gm (Mean±SE)		Dry root weight gm (Mean±SE)	
		3weeks	4weeks	3weeks	4weeks	3weeks	4weeks	3weeks	4weeks
T. konongiopsis		2.50±0.58 ns	3.09±0.87 bc	0.27±0.05 ns	0.33±0.11 ab	0.33±0.06 ab	0.14±0.01 ns	0.04±0.005 ab	0.04±0.01 ab
T. viride		2.98±0.89 ns	3.05±0.52 bc	0.33±0.09	0.31±0.05 ab	0.45±0.04 b	0.26±0.11 ns	0.07[b]±0.021	0.07±0.02 ab
ECO-T		2.58±0.60 ns	1.58±0.19 ab	0.25±0.05 ns	0.18±0.02 ab	0.22±0.08 a	0.12±0.08 ns	0.04±0.010 ab	0.04±0.01 ab
T. konongiopsis+ *T. viride*		2.56±0.30 ns	4.70±0.78 c	0.26±0.05 ns	0.78±0.09 c	0.20±0.04 a	0.21±0.02	0.03±0.004 ab	0.08±0.01 b
Negative Cont.		2.02±0.37 ns	2.71±0.51 b	0.22±0.04 ns	0.46±0.14 b	0.21±0.01[a]	0.28±0.09 ns	0.03±0.002 a	0.06±0.01 ab
Positive Cont.		1.25±0.41 ns	0.85±0.13 a	0.18±0.03 ns	0.10±0.02 a	0.22±0.06 a	0.16±0.12 ns	0.03±0.007 a	0.03±0.01 a
statistics	F_{st}	1.181	5.471	0.805	8.094	3.515	0.617	1.757	2.264
	df	17	17	17	17	17	17	17	17
	P value	0.374	0.007	0.568	0.002	0.035	0.690	0.196	0.114

Means in the same column for each treatment followed by same letter(s) are not significantly different according to Duncans multiple range test p≤ 0.05.

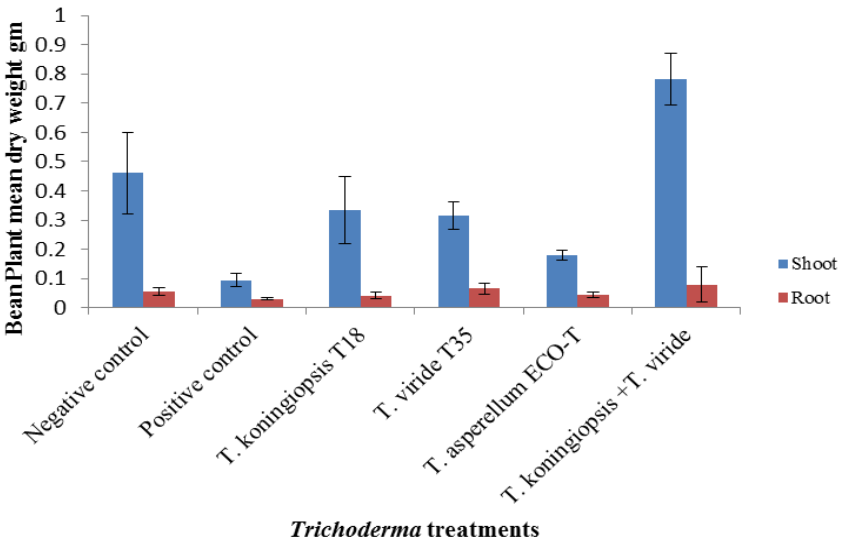

Figure 4.7. Effects of *Trichoderma* treatments on mass of shoots and roots. Error bars represent standard errors of the means.

Plate 4.15. Effect of Trichoderma treatments on growth Bean plants. A: ECO-T, B: *T. viride* (T35), C: *T. koningiopsis* (T18), D: *T. koningiopsis* + *T. viride*, E: Positive Control, F: Negative Control.

Plate 4.16. Effects of *Trichoderma* treatments on the bean plants

A: *T. koningiopsis* + *T. viride*, B: ECO-T, C and D: *T. viride*, E: *T. koningiopsis*,

F: Positive Control, G: Negative Control.

CHAPTER FIVE

5.0. DISCUSSIONS

Four *Trichoderma* species were obtained from the area of study. This result is in concurrence to earlier research that have reported that *Trichoderma* species are cosmopolitan fungi, that occur widely worldwide and are frequently present in all types of soil, manure and decaying plant tissues (Rahman *et al*., 2011).

Local *Trichoderma* isolates showed potential in controlling of *Fusarium oxysporum* f. sp. *phaseoli* both *in vitro* and *in vivo* but with varying degrees of inhibition amongst the isolates. Competition, mycoparasitism and antibiosis resulting from production of secondary metabolites were the main mechanisms observed in the effect of *Trichoderma* against the pathogen. This finding is similar to other research that has reported that *Trichoderma* uses several mechanisms in controlling soil pathogens (Vinale *et al*., 2008). This study also agrees with reports that have suggested that different strains of *Trichoderma* control every pathogenic fungus. However, most *Trichoderma* strains are more efficient for control of some pathogens than others, and may be largely ineffective against some fungi (Shelton, 2012).

5.1.0. Species of the *Trichoderma* isolates

The molecular characterization was based on the ITS region where the nuclear rDNA region spanning the ITS1, ITS2 and 5.8S rRNA gene was amplified for each of the *Trichoderma* isolates. The amplicon obtained of this region for each of the isolates was in the size range of 600bp (Plate 4.12.). This result is similar to those obtained in previous studies on identification, characterization and genetic variability of the *Trichoderma* isolates where the amplification of the rDNA region of *Trichoderma* isolates yielded amplicons in the size range of 600pb (Chakraborty *et al.,* 2010). Use of the ITS region is a recognized method for *Trichoderma* species identification; the *Trichoderma* species specific PCR primers, ITS1 and ITS4 provide a fast and accurate tool for identification and characterization of *Trichoderma* spp. The analysis of the sequences of ITS-1, the 5.8S rDNA and ITS-2 regions has greatly improved the identification of *Trichoderma* species (Hermosa *et al.,* 2004; Kubicek *et al.,* 2003).

From the molecular identification, the isolates belonged to two major sections of *Trichoderma*; section *Trichoderma* which included *(T. koningiopsis ,T viride , T. hamatum ,T asperellum*) and section "*Pachybasium* B" (*T spirale*) (Druzhinina and Kubicek 2005). The phylogenetic analysis based on ITS sequences Figure (4.2.) in this study produced a phylogenetic relationship among the *Trichoderma* isolates that is consistent with the phylogenetic relationships of these two sections as presented in phylogenetic relationships of the currently recognized sections and clades in the genus *Trichoderma* (Druzhinina and Kubicek 2005)**.**

5.2.0. Antagonistic Activity of *Trichoderma* Isolates against *Fusarium Oxysporum*

Results from this study showed that all the local isolates used in this experiment exhibited potential antagonism against *Fusarium oxysporum* through competition, mycoparasitism, volatile metabolites and antibiosis. However the different local *Trichoderma* isolates possess different abilities in their inhibitory activities against *Fusarium oxysporum.*

In this study the results from the dual plate cultures showed that different species of *Trichoderma* have differing abilities in the bio-control of *Fusarium oxysporum* f. sp. *phaseoli*. From the cultures of plugs obtained from the points of interaction between each isolate and the pathogen, the absence of the mycelial growth of the pathogen showed its total elimination**.** This outcome is similar to earlier experiments that have demonstrated that *Trichoderma* spp. mycoparasitize the hyphae and resting structures of plant pathogens *in vitro* and also in natural soil (Papavizas 1985). However, most *Trichoderma* strains are more efficient for control of some pathogens than others, and may be largely ineffective against some fungi (Shelton, 2012).

In the experiments with the non-volatile metabolites results obtained demonstrated significant differences in inhibitory effects of the non-volatile metabolites obtained from the different *Trichoderma* species. The inhibitory effect obtained with non-volatile metabolites from the co-culture of viable pathogen + *Trichoderma* was not significantly different from the case where the non-volatile metabolites were obtained from sole *Trichoderma* culture. The non-volatile metabolites obtained from co-culture of *Trichoderma* + non-viable pathogen gave no significant inhibitory effect for all the isolates.

From the results there is an indication that the amount of secondary metabolites produced by the *Trichoderma* against *Fusarium oxysporum* depends on the strain or species used and the condition of the culture.

These results are consistent with many studies that have confirmed that *Trichoderma* spp. can directly affect mycelium or survival propagules of other fungi through production of toxic secondary metabolites, formation of specialized structures and secretion of cell wall degrading enzymes. *Trichoderma* species have been shown to produce peptaibols, which are important for its antagonistic activity and represent a potential alternative to conventional (Szekeres *et al*., 2005). It has been proven that *Trichoderma* has the ability to produce diffusible (nonvolatile) substances toxic to other fungi *in vitro* and even in organic substrates in soil (Papavizas, 1985). In addition it has been reported that although some antibiotics may be the major factor for the bio-control activity for a given strain, it may not be the case for others (Harman 2000; Vinale *et al*., 2006).

In the volatile experiments results support the fact; that volatile metabolites from *Trichoderma* are important in bio-control. Studies by Dubey and Suresh (2006), reported similar findings where it was shown that *Trichoderma virens, Trichoderma viride* and *Trichoderma harzianum* produced volatile metabolites that were inhibitory to growth of *Fusarium* f. sp. *ciceris.*

The *in vivo* experiments that were performed in the greenhouse using the commercial ECO-T and 2 local isolates *T. koningiopsis* (T18) and *T. viride* (T35) showed that the *Trichoderma* isolates were effective in reducing the post-emergence damping-off compared with control. The *Trichoderma* treatments in this study boosted the growth and productivity of the bean variety mwezi moja in the *Fusarium* infested soil compared with the untreated control. This means that the seed inoculation with the *Trichoderma* prevented any serious infection of the bean plants with the *Fusarium* pathogen. The extent of growth and productivity promotion varied according to treatment and this was observed at four weeks after sowing. The present study showed that the *Trichoderma* isolates; *T. koningiopsis* (T18) and *T. viride* (T35) are effective in controlling *Fusarium oxysporum* f. sp. *phaseoli* in the greenhouse. Their ability to control this pathogen seemed to be enhanced when they were used as a combination.

These results are consistent with those obtained from the studies performed by Nashwa *et al.*, (2008), which indicated that formulation of *Trichoderma* spp. treatments not only suppressed both damping-off and wilt diseases but also enhanced green yield of bean plants compared to infected control. Further reports from the same study indicated that formulations of *Trichoderma* spp. were effective in controlling *Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *phaseoli*, the causal agents of bean damping-off and wilt diseases respectively, under greenhouse and in artificially infested field conditions (Nashwa *et al.,* 2008).

5.3.0. Conclusion

From these results there is evidence that the *Fusarium* wilt of beans can be managed by the local *Trichoderma* isolates *T. viride* (T35), *T. koningiopsis* (T18) effectively. This makes the possibility of further exploitation of these local isolates as bio control agents in bean *Fusarium* wilt disease management system.

The *Trichoderma* isolates could be used either as living antagonists or based on bioactive compounds from the secondary metabolites. Co-culture of *Trichoderma*+viable *Fusarium* can be used to further enhance the yields of *Trichoderma* metabolites under the right optimum condition of elicitation to obtain the bioactive compounds for development of biopesticides against *Fusarium oxysporum.*f. sp. *phaseoli.*

In a practical biological control situation it is therefore important to characterize, define and select an efficient bio-control *Trichoderma* species for a target pathogen because the effectiveness or non-effectiveness is a function of target and the functions required against the pathogen (Grondona *et al*., 1979).

5.4.0. Recommendations

Fusariun oxysporum f. sp. *phaseoli* is a major bean rot pathogen which causes *Fusarium* wilt, an economically important disease in bean production in Kenya causing damage to beans of between 10-100%. Destruction by this pathogen results into overwhelming losses to the farmers due to reduced crop productivity hence reduced market prices. The search for and development of effective methods for control of this pathogen will contribute a great deal to the objective of attainment of food security among the small scale farmers.
The strategy of integrated disease management (IDM) that incorporate use of bio-control agents which are safe to use, easy to adopt and environmentally friendly is one such system that can contribute greatly to management of this disease.

While *in vitro* experiments are important in the initial stages of selection of *Trichoderma* species that can act as bio-control agents through different synergistic mechanisms against the *Fusarium oxysporum* f. sp. *phaseoli*; it is always difficult to extrapolate the bio-control activity of a given strain from the laboratory to natural environments. *Trichoderma* ecology and biological control activity are greatly influenced by soil properties. In agricultural systems the soil properties are affected by farming practices such as ploughing, irrigation, liming and fertiliser and pesticide applications. Future work should focus on; further evaluation of the local isolates [*T. koningiopsis* (T18) and *T. viride* (T35)] under field conditions against *Fusarium oxysporum* f. sp. *phaseoli* to determine their effectiveness. It is also important to determine the farming practices that would inhibit or enhance the antagonistic activity of the *Trichoderma* species to realize their optimum bio-control ability.

Further studies involving isolation and characterization of the main inhibitory secondary metabolites obtained from culture filtrates of the isolates used in this study and their production during antagonistic interaction with the *Fusarium oxysporum* f. sp *phaseoli* need to be done in order to identify the active ingredients responsible for inhibition of the pathogen with a view of packaging it as a fungicide..

5.5.0. Significance and Impact of the Study

The results from this study could be used to develop bio-control strategy against *Fusarium* oxysporum f. sp. *phaseoli*. This could have a significant beneficial impact on the management of *Fusarium* wilt disease in beans.

Availability of local *Trichoderma* isolates that are effective against this pathogen would provide a source of readily available bio-control agents for use against *Fusarium* wilt of beans and as a result the Kenyan smallscale farmers will realize improved productivity of beans.

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7.0. APPENDICES

APPENDIX 1: MEDIA AND BUFFERS

SNA: Spezieller Nahrstoffarmer agar

Agar,	20.0g
KH_2PO_4,	1.0g
KNO_3,	1.0g
$MgSO_4.7H_2O$,	0.5g
KCL,	0.5g
Glucose,	0.2g
Sucrose,	0.2g
Distilled water	1000ml

Peptone PCNB agar Media (PPA/Narsh Snyder Medium)

Agar,	20.0g
peptone,	15.0g
$KH2PO_4$,	1.0g
$MgSO_4.7H_2O$	0.5g
PCNB	1.0g
Distilled water	1000ml

Ethylenediamine Tetraacetic Acid (EDTA 0.5M PH 8.0)

EDTA	186.12g
NaOH	20g

Volume topped to 1000ml with distilled water

TRIS HCL(PH 8.0)

1M Tris base	121g
1M HCL	35ml

Volume topped to 1000ml with distilled water

TRIS EDTA(TE)

1M Tris HCL	10ML
0.5M EDTA	2ML

Volume topped up to 1000ML with distilled water

50X Tris-Acetate EDTA buffer (TAE)

Tris base	242g
Acetic acid	57.2ML
EDTA	100ML

Volume topped up to 1000ML with distilled water

APPENDIX 2: *TRICHODERMA* ISOLATION AND IDENTIFICATION

A2.1. Preparation of slides for size measurements for identification of *Trichoderma* isolates

After 7 days microscope preparations were made from pustules where there were still white conidia before they over matured. A small amount of material from the edge of each *Trichoderma* colony was placed in a drop of 3% KOH on a slide. The KOH wets the conidia and allows conidiophores to spread; it was then replaced with water after the preparation had been made. Before placing a cover slip on the preparation the hyphae and conidiophores were spread apart using insect pins. With the cover slip in place the preparation was flooded with water and the water drawn out by applying a small piece of tissue to the edge of the cover slip. The examination was done using a light microscope.

A 2.2 Trichoderma DNA extraction results

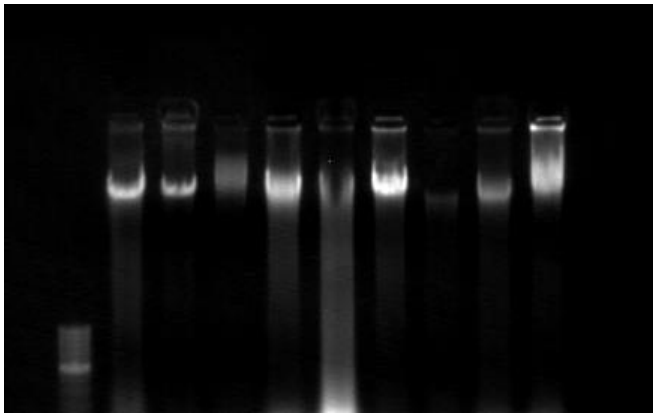

Figure A 2.2.1 1% agarose gel showing the the DNA of the *Fusarium* and *Trichoderma* isolates lane 1- LAD; lane 2-F2; lane 3-F1; lane 4-ECOT; lane 5- T4; lane 6-T35; lane7 – T21; lane 8- T21; lane 9-T18; lane 10-T6,

A 2.3 *Trichoderma* isolates sequences

T. harmatum (T6): Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, sequence (482bp).

AGGGCCTCCGGGGGTCACCACCCAACCCATGTGACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCC
CCGGGTGCGTAAAAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCC
CTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATT
TCAACCCTCGAACCCCTCCGGGGGGATCGGCGTTGGGGATCGGGACCCCTCACCGGGTGCCGGCCCTGG
AGATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCTGTAGTGTGCAGAATGCGCACCAGGAGCGCG
GCGG

T. koningiopsis (T18): Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, sequence (579bp).

CTGGCTCGGAGGTTGCTCTCCCTACCCATGTGACCATACCAAACTGTTGCCTCGGCGGGGTCACGCCCC
GGGTGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGGACCAACCAAACTCTTTCTGTAGTCCCCT
CGCGGACGTTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTC
TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTT
CAACCCTCGAACCCCTCCGGGGGGTCGGCGTTGGGGATCGGGAACCCCTAAGACGGGATCCCGGCCCC
GAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGC
GGCGCGTCCACGTCCGTAAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGC
TGAACTTAAGCATATCAATAAGCGGAAGAAAA

T. harmatum (T21): Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, sequence (575bp).

TTGGGGTCGAGGTTATCTCCCAACCCCTGTGACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCG
GGTGCGTAAAAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTACTCCCCTC
GCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTCTT
GGTTCTGGCATCAATGAAGAACGCAACGAAATGCGATAAGTAATGTGAATTGCATAATTCAGTGAATC
ATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCA
ACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCCTCACCGGGTGCCGGCCCTGAAAT
ACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCCCAATATTTTGCTCAACTCGCACCGGGAGCGCGGCGCG
TCCACGTCAGTAAAACACCCAACTTCTGAAATGTTAACCTCGGAACAGGTAGGAATACCCGCTGAACT
TAAGCTATCAATAAACGGAAAAATGTA

***T. viride* (T35): Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, sequence (581bp).**

TTGCCTTCCGAGTTCTCTCCCAACCCATGTGACCATACCAAACTGTTGCCTCGGCGGGGTCACGCCCCG
GGTGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGGACCAACCAAACTCTTTCTGTAGTCCCCTC
GCGGACGTTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTCT
TGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTC
AACCCTCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGGAACCCCTAAGACGGGATCCCGGCCCCG
AAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCG
GCGCGTCCACGTCCGTAAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCT
GAACTTAAGCATATCAATAAGCGGGAGGAAAATG

***T. spirale* (T41): Internal transcribed spacer 1, 5.8S ribosomal RNA gene,**

and internal transcribed spacer 2, sequence (600bp).

TGGGGGTTCCGACGTTCTCTCCCTGCCCATGTGACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCC
CCGGGTGCGTCGCATCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCTAAAACTCTTTTGTATACCC
CCTCGCGGGTTTTTTATATCTGAGCCATCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACT
TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCAGGCATGCC
TGTCCGAACGTCATTTAAACCCTCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGCCCTTTACGGGA
CCGGCCCCGAAATACAGTGGCGGACTCGGCGCAGCCTCTCCTGCGCAGACTTTGCACACTCGCGTCGG
GAGCGCGGCGCGTACATTGTGGTAAATCAACCACTTTCTGAATTGTTGTCGTCGGATCGGGGAGGAAT
ACCCGATTGAGCTGAAGCATATGAATTGGACGGAGGAAAAATGGTGGGGTGG

***T. asperellum* (ECOT): Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, sequence (578bp).**

TTGGCTTCGGGGGTTCTCTCCCAACCCATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCC
CGGGTGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCC
TCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTC
TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTT
CAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCCTCACACGGGTGCCGGCCCCTA
AATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGG
CGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTG
AACTTAAGCATATCAATAAGCGGAGGAAAA

***Fusarium oxysporum* (F2): Elongation Factor gene sequence (690bp).**

CGACCTTTACCGGTCGTCGGGCCCGTCGACTCTGGCAGTCGACCACTGTGAGTACTCTCCTGGACGATG
AGCATATCTGCCATCGTGATCCCGACCAAGTCTGGCGGGGTATTTCTCAAAATAAAAATAGTGGACTCGT
TTCCCAAAACGGTCCCTTTGACTAACCAGGCGGTGGGATCGACAAGCGAACCATCCAGAAATTTCAGAA
AGTTTATCCCTTTCCCTTTCGACCCGCCGCCTTTGCCCATCGATTCCCCTTGCAATTCAAACGTGCCCTGC
ACCCCGCTCGAGAACCAAAATTTTTGCAAATGAACCGAATTTTTTTGGGGGGGGACTTTACCCCCCCCTT
GAACGAAGGAAGCGTTTGCCCTCTTTTCCTGCTCACAACCTCAATGAGTGCATTGTCACGTGTCAAGCA
GCGACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGTTCCTTCAAGTACGCCTGGGTTCT
TGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATCGATATTGCTCTCTGGAAGTTCGAGACTCC
TCGCTACTATGTCACCGTCATTGGTATGTTGTCGCTCATGCCTCATCCTACTTCCTCTCAGAAACACATAA
CTCACACACTCCCGGTCACACTGAGTTCTCCTCGAAAAATGATATCAGTGGCCCCCAACAAGG

APPENDIX 3: GREENHOUSE EXPERIMENT TO TEST THE ANTAGONISTIC ACTIVITY OF *TRICHODERMA* AGAINST *FUSARIUM OXYSPORUM*

Conidial concentration of the *Trichoderma* inoculum used for the bean seeds

The conidial concentration was determined using a colony-forming unit (cfu) assay (Whipps *et al*., 1989) with 1 g of treated seed. Aliquots (1 mL) of each dilution were spread over the surface of *Trichoderma* Selective Medium– The number of cfu per seed was calculated after 7 days of incubation at 25°C (K. L. McLean *et al.*,2005).

Table A3.1 The experimental design used for the greenhouse experiment was completely randomized design (CRD) with each treatment replicated three times

(untreated seeds) + non infested soil	Seed treatment {T18+T35} + Fusarium Infested soil	Seed treatment (T35) + Fusarium Infested soil	Untreated seed + Fusarium Infested soil	Seed Treatment(T18) + Fusarium Infested soil	Seed treatment(ECOT) + Fusarium Infested soil
Seed treatment (T35) + Fusarium Infested soil	Untreated seed + Fusarium Infested soil	(untreated seeds) + non infested soil	Seed Treatment(T18) + Fusarium Infested soil	Seed treatment(ECOT) + Fusarium Infested soil	Seed treatment {T18+T35} + Fusarium Infested soil
Seed Treatment(T18) + Fusarium Infested soil	Seed treatment(ECOT) + Fusarium Infested soil	Seed treatment (T35) + Fusarium Infested soil	Seed treatment {T18+T35} + Fusarium Infested soil	Untreated seed + Fusarium Infested soil	(untreated seeds) + non infested soil

Table A3.2 Pre-Emergence disease severity investigation results from green house experiment

TREATMENT	D11(number of emerged seedlings)			%INCIDENCE of disease in each replicate			
	R1	R2	R3	R1	R2	R3	MEAN
T35	5	5	5	0	0	0	0
T18	5	4	5	0	20	0	6.666667
ECOT	4	5	5	20	0	0	6.666667
MIX	4	5	5	20.00	0	0	6.666667
Cont1	5	5	5	0	0	0	0
Cont2	4	4	4	20	20	20	20

Each replication was sown with 5 seeds

Table A3.4 Post Emergence disease severity investigation results from the green house experiment

Treatment		D15			MEAN
		R1	R2	R3	
T35	E	5	5	5	5
	D	0	0	0	0
	% SEVERITY	0	0	0	0
T18	E	5	4	5	4.666667
	D	1	1	0	0.666667
	% SEVERITY	20	25	0	15
ECOT	E	4	5	5	4.666667
	D	0	1	0	0.333333
	% SEVERITY	0	20	0	6.666667
MIX	E	4	5	5	4.666667
	D	1	1	0	0.666667
	% SEVERITY	25	20	0	15
Cont1(negative control)	E	5	5	5	5
	D	3	0	0	1
	% SEVERITY	60	0	0	20
Cont2 (positive control)	E	4	4	4	4
	D	2	2	2	1.666667
	% SEVERITY	50	50	50	50

E- emerged seedlings

D- Disease seedling

Table A3.5 Soil analysis results

					Cmol/kg					ppm				
Lab No	pH Water	pH 0.01M $CaCL_2$	%c	%N	K	Na	Ca	Mg	CEC	Cu	Fe	Mn	Zn	P
13	6.88	5.86	0.87	0.08	1	0.6	6.05	1.58	16.2	1.5	29	100	2	7

APPENDIX 4: COMPLETE STATISTICAL ANALYSIS TABLES

A 4.1. Antagonist tests on culture plates

A 4.1.1 Dual plate cultures

Table A4.1 Analysis of Variance of antagonistic activity of *Trichoderma* treatments against *Fusarium oxysporum*

		Sum of Squares	df	Mean Square	F	Sig.
inhibD4	Between Groups	3196.214	6	532.702	8.650	.000
	Within Groups	862.211	14	61.587		
	Total	4058.425	20			
inhibday5	Between Groups	1641.120	6	273.520	7.222	.001
	Within Groups	530.205	14	37.872		
	Total	2171.325	20			
inhibday6	Between Groups	1379.883	6	229.981	13.307	.000
	Within Groups	241.958	14	17.283		
	Total	1621.841	20			
inhibday7	Between Groups	2341.736	6	390.289	4.639	.008
	Within Groups	1177.960	14	84.140		
	Total	3519.697	20			
inhibday8	Between Groups	2783.243	6	463.874	4.085	.014
	Within Groups	1589.870	14	113.562		
	Total	4373.113	20			

A4. 1.2 Effect of non-volatile metabolites from *Trichoderma* isolates on *Fusarium* growth

Table A4.2 . Analysis of Variance (ANOVA) of non-volatile metabolites from *Trichoderma* isolates in the antagonistic activity against *Fusarium oxysporum* 5 days after incubation The treatments consisted of non-volatile metabolites obtained from 6 *trichoderma* isolates (T6, T18, T21, T35, T41, ECO-T and control) in sole culture.

Analysis		Sum of Squares	df	Mean Square	F	Sig.
Percent inhibition of non-volatile metabolite obtained from sole *Trichoderma* culture on the growth of *Fusarium oxysporum*	Between Groups	5605.811	6	934.302	9.423	.000
	Within Groups	1388.170	14	99.155		
	Total	6993.981	20			

Table A4.3. Analysis of Variance (ANOVA) of non-volatile metabolites from *Trichoderma* isolates in the antagonistic activity against *Fusarium oxysporum* 5 days after incubation. The treatments consisted of non-volatile metabolites obtained from 6 *Trichoderma* isolates (T6, T18, T21, T35, T41, ECO-T and control) from each *Trichoderma* and viable pathogen cultures.

Analysis		Sum of Squares	df	Mean Square	F	Sig.
Percent inhibition of non-volatile metabolite obtained from co-culture of *Trichoderma* and viable pathogen on the growth of *Fusarium oxysporum*	Between Groups	3641.349	6	606.892	32.654	.000
	Within Groups	260.194	14	18.585		
	Total	3901.543	20			

Table A4.4. Analysis of Variance (ANOVA) of non-volatile metabolites from *Trichoderma* isolates in the antagonistic activity against *Fusarium oxysporum* 5 days after incubation. The treatments consisted of non-volatile metabolites obtained from 6 *Trichoderma* isolates (T6, T18, T21, T35, T41, ECO-T and control) from each *Trichoderma* and non-viable pathogen cultures.

Analysis		Sum of Squares	df	Mean Square	F	Sig.
percent age inhibition of non -volatile metabolites from *Trichoderma* isolates and non viable pathogen against fusarium oxysporum	Between Groups	988.096	6	164.683	3.212	.034
	Within Groups	717.699	14	51.264		
	Total	1705.794	20			

A4. 1.3 Effect of volatile metabolites from *Trichoderma* isolates on *Fusarium* growth

Table A4.5. . Analysis of Variance (ANOVA) of volatile metabolites from *Trichoderma* isolates in the antagonistic activity against *Fusarium oxysporum* 5 days after incubation.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
percentinhibD5	Between Groups	692.490	6	115.415	9.346	.000
	Within Groups	172.889	14	12.349		
	Total	865.380	20			
percentinhibD6	Between Groups	930.391	6	155.065	8.064	.001
	Within Groups	269.208	14	19.229		
	Total	1199.599	20			
percentinhibD7	Between Groups	899.646	6	149.941	11.930	.000
	Within Groups	175.958	14	12.568		
	Total	1075.604	20			

A4.1.4. Green house experiment

Table A4.6.One-way Analysis of Variance (ANOVA) of height (cm) of Bean plants 27 days after sowing in soil infested *Fusarium oxysporum.* where the seeds were inoculated with five different *Trichoderma* treatments
The treatments consisted of two control treatments with no *Trichoderma* inoculation which were:- positive control in non -infested soil and negative control in FOP infested soil; four other treatments with *Trichoderma* isolates were T35, T18, ECO-T and (T35+T18)

Analysis		Sum of Squares	df	Mean Square	F	Sig.
HEIGHT OF SEEDLING IN CM 27 DAYS AFTER SOWING	Between Groups	1140.792	5	228.158	11.951	.000
	Within Groups	229.093	12	19.091		
	Total	1369.885	17			
NUMBER OF LEAVES DEVELOPED BY THE BEAN PLANT 27 DAYS AFTER SOWING	Between Groups	192.000	5	38.400	5.908	.006
	Within Groups	78.000	12	6.500		
	Total	270.000	17			

Table A4.7. One-way Analysis of Variance (ANOVA) of the mean above ground dry weight (g) of Bean plants after 27 days growth in soil infested *Fusarium oxysporum,* where the seeds were inoculated with five different *Trichoderma* treatments
The treatments consisted of two control treatments with no *Trichoderma* inoculation which were :- positive control in non -infested soil and negative control in FOP infested soil; four other treatments with *Trichoderma* isolates were T35, T18, ECO-T and (T35+T18)

Analysis		Sum of Squares	df	Mean Square	F	Sig.
Dry top weight of bean plants	Between Groups	.881	5	.176	8.094	.002
	Within Groups	.261	12	.022		
	Total	1.142	17			

Table A4.8. One-way Analysis of Variance (ANOVA) of the mean above ground dry weight (g) of Bean plants after 27 days growth in soil infested *Fusarium oxysporum.* where the seeds were inoculated with five different *Trichoderma* treatments.
The treatments consisted of two control treatments with no *Trichoderma* inoculation which were:- positive control in non -infested soil and negative control in FOP infested soil; four other treatments with *Trichoderma* isolates were T35, T18, ECO-T and (T35+T18)

analysis		Sum of Squares	df	Mean Square	F	Sig.
Dry root weight of bean plants	Between Groups	.005	5	.001	2.264	.114
	Within Groups	.005	12	.000		
	Total	.010	17			

NB: d.f. = degrees of freedom; s.s. = sum of the squares; m.s. = mean square;