# CHARACTRIZATION AND DETERMINATION OF EFFICACY OF LOCAL *TRICHODERMA* ISOLATES AS A BIOCONTROL AGENT (BCA) ON *FUSARIUM WILT ON* BEANS (PHASEOLUS VULGARIS L).

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## DECLARATION

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

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## DEDICATION

This work is dedicated to my family; my husband Emmans Otadoh, my son Absalom and daughters Joyce and Caren who supported and encouraged me to soldier on accommodating my busy schedules. This work is also dedicated to my late mother Mrs. Caren Omoro who has been my role model as a courageous woman of determination throughout my life.

## ABSTRACT

Recent studies show *Trichoderma* species are not only parasites of fungal plant pathogens but also can enhance plant growth and development. Heavy applications of agrochemicals in soils in Embu County due to dependence on inorganic fertilizers and pesticides have potential to cause negative effects on environment leading to decrease of food production in agriculture. A study was conducted to evaluate virulence efficacy of local *Trichoderma* isolates as Biocontrol Agent (BCA) on soil pathogenic fungi. Isolates of *Trichoderma koningii, Trichoderma asperellum, Trichoderma atroviride, Trichoderma reesei, and Trichoderma harzianum* from Embu soils were evaluated for their ability to inhibit the mycelial growth of pathogenic fungi *Fusarium oxysporum* f.sp. *phaseoli*., in culture. *Trichoderma spp.* were also evaluated for the suppression of *Fusarium* oxysporum f.sp. phaseoli., under greenhouse condition. *Trichoderma spp.* were further investigated for their ability to promote plant growth and development of maize and bean seedlings in Embu County in Kenya. Subsequently, genetic characteristics of the five *Trichoderma* isolates were analyzed to confirm bioefficacy activities in relation to their molecular similarity.

*Trichoderma* isolates significantly ($p < 0.01$) reduced the mycelial growth of the pathogen. Growth suppression by *Trichoderma reesei* against *Fusarium oxysporum* f. sp. *Phaseoli*., had the highest effect in inhibition of mycelial growth (60.0%) with p-value

(0.018), followed by *Trichoderma koningii* (55.2%) with (p=0.014) deploying antibiosis and mycoparasitism mechanisms. Differences were observed with treatment of *Trichoderma reesei* combined Manure recording the highest with seed emergence germination index of 1.88, followed by *Trichoderma koningii* combined *Fusarium oxysporum* (1.86). The application of inoculum with *Trichoderma koningii* combined *Fusarium oxysporum* (6.7%) and *Trichoderma reesei* combined Manure (6.7%) gave the highest reduction in disease incidence of *Fusarium* wilt on bean plants. Treatments with manure increased the shoot height by 36.6 mm followed by inoculation of *Trichoderma koningii* + Manure (35.2) and *Trichoderma koningii* (34.7). Effects were highly significant at $p < 0{,}001$ with f- value of 4.716. The highest rate of seedling emergence was 84% for maize seeds coated with the *Trichoderma* inoculum and planted in soil amended with manure. Similarly, differences were observed with Manure + *Trichoderma* treatment of 74.40% and Manure (74.00%) recording the highest germination rates followed by both *Trichoderma* treatments with both TSP/CAN and Mavuno (69.60%). This study demonstrated that local isolates of *Trichoderma reesei* and *Trichoderma koningii* were identical in their biocontrol characteristics as well as potential for use as biological control agents to protect bean plants from *Fusarium oxysporum* f.sp. *phaseoli.,*

Keywords: *Trichoderma, Biocontrol, Fusarium oxysporum.*

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| POPs | Persistent Organic Pollutants |
| AM | Arbascular Mycorrhizal |
| BCA | Biocontrol Agents |
| TSP | Triple Superphosphate |
| CAN | Calcium Ammonium Nitrate |
| ITS1 | Internal transcribed spacer |
| PCR | Polymerase Chain Reaction |
| rDNA | Ribosomal DNA |
| OC | Organochloride |
| GDP | Gross Domestic Product |
| MEA | Malt Extract Agar (MEA) |
| CMA | Corn Meal Agar |
| PDA | Potato Dextrose Agar |
| CAVS | College of Agriculture and Veterinary medicine |
| ATC | Agricultural Training Collage |

# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Background

Agrochemicals are the result of modern technology that depends on inorganic fertilizers and pesticides. Their applications are looked upon as a vehicle for improved crop production technology (Menale, 2009) despite being a costly input (Govinda, 2014). Globally, agriculture has to double food production by 2050 in order to feed the world's growing population (Cooper *et al.,* 2007) and at the same time reduce its reliance on inorganic fertilizers and pesticides (Vadakattu, 2012). It is now recognized that overuse of high input technologies and agricultural practices tend to degrade natural resources (Beddington, 2009) that may lead to immediate and long-term effects as reported by Savci, (2012) and Govinda, (2014).

Plant diseases caused by pathogenic fungi play a major role in the decrease of food production in agriculture, which often result in significant yield losses of agricultural crops. *Fusarium oxysporum,* found in its many pathogenic forms, is the most damaging species of the genus where in plants are concerned (Alwathnani, *et al*, 2011). Recently a number of new disease reports on *Fusarium* have been submitted to the literature pool on agricultural research (Felgueiras *et al*., 2010). Fusarium wilts caused by pathogenic forma specials of the soil inhabiting fungus, *F. oxysporum* can cause severe loses in a wide variety of crop plants (Larkin and Fravel, 1998). *F.oxysporum* f sp. *phaseoli,* Kendr. and Snyder is responsible for wilt disease of *Phaseolus vulgaris* L. (common bean); this pathogen causes wilting and early death of

the plants, which can cause extensive damage to the crop. Crop loss has been reported in South America and Africa (Muriungi, 2013). The most effective method in preventing fusarium wilt is chemical fungicides. However, the application of chemical fungicide has been known to cause various environmental Geiger *et al.,* 2010) and health problems.

### 1.1.1 Biocontrol of soil pathogenic fungi

Biological control methods involving use of natural antagonists of plant pathogens have been suggested in several studies as a safe alternative to chemical methods (Harman *et al,*. 2010). Biological control of plant diseases is a result of many different types of interaction among microorganisms and can occur through different mechanisms, which are generally classified as parasitism, antibiosis, competition, lytic enzymes, and induced resistance (Pal, 2006). Several microorganisms have been tested and proven to possess antagonistic properties against plant pathogenic fungi that are common in farmers' fields.

Other beneficial influences of microorganisms on plant growth include nitrogen fixation, acquisition and uptake of major nutrients, promotion of shoot and root growth, disease control or suppression and improved soil structure. Some of the commonly promoted and used beneficial microorganisms in agriculture worldwide include Trichoderma species (Vadakattu, 2012).

### 1.1.2 *Trichoderma* species in increased plant growth and development

*Trichoderma* are free-living fungi that are common in soil and root ecosystems. They occur worldwide and are easily isolated from soil and other forms of plant organic matter (Reza, 2013). *Trichoderma* spp. are effective biocontrol agents for several soil-borne fungal plant pathogens including including *Fusarium* species (Hermosa *et al*., 2012). *Trichoderma species* are now recognized as alternative agents for the control of plant disease and for their ability to increase plant growth and development. The biocontrol exercised by *Trichoderma* can occur by means of several antagonistic mechanisms such as nutrient competition, antibiotic production, and mycoparasitism (Saba *et al.,* 2014)

Molecular characterization of the potential biocontrol agents using Internal Transcribe Spacer-Polymerase Chain Reaction (ITS-PCR), helps to verify their identity in relation to biocontrol dynamics within geographical races (Mathu K. A, 2011).

Embu County, which was the area of study, has experienced high intensification of farming in attempt to improve productivity in agriculture. Management practices used in crop production is characterized by intensive management of agro-ecosystems and the use of high-level inputs such as manure, fertilizers and pesticides for maize, beans, tea, coffee and irrigated horticultural crops (Mutsotso *et al.,* 2011). In this study, tests were conducted in the laboratory, greenhouse and in the field to evaluate local *Trichoderma* isolates for efficacy, plant disease control and growth on maize and bean plants in order to identify a potential fungal biocontrol agent for the cropping systems in Embu.

# CHAPTER TWO

## 2.0. LITERATURE REVIEW

### 2.1 Economic impact of fungal disease on modern Agriculture

Plant diseases play a direct role in the destruction of natural resources in agriculture (Oerke C. E, 2005); in particular soil borne pathogens cause important losses in crops of economic importance. For instance, *Fusarium* head blight (FHD) is a significant disease of small grain cereals throughout the world. Up to seventeen (17) causal organisms have been associated with the disease (Muthomi *et al.*, 2007*). Fusarium spp., namely, F. avenaceum, F. culmorum, F graminearum* are the most species associated with cereal diseases (Kriel, 2006). A new strain of stem rust which is a major fungal disease of wheat has emerged in East Africa. Inoculation trials in Kenya with *Fusarium graminearum* on wheat varieties have shown yield reduction ranging between 23% and 57% (Muthomi *et al.,* 2002a). *Fusarium* infection can also lead to mycotoxin contamination of grain thus threatening the health of human and livestock (Heier, 2005). Contaminations at different level of Fusarium and mycotoxins have also been found in wheat grains from different parts of the country (Oerke C. E, 2005).

Kale and cabbage are two of the most important vegetables for the local economy in Kenya and other East African countries, but often, entire harvests are wiped out by black rot, a seed-borne disease that penetrates the leaves causing yellow lesions. The causal organism for sugarcane smut is a fungus *Ustilago scitaminea* which occurs wherever sugarcane is grown in Kenya, and is spread by spores, infested seed-cane and infested soil. Yield losses reported in Kibos area were established at between 21 to 38% in plant cane in commercial cultivars under field conditions (Nzioki

*et al.,* 2006). In passion fruits, incidences of dieback wilt disease caused by *Fusarium oxysporum* ranging between 0% - 33% were found to be the most important disease at farm levels. Fungi isolated from stems affected by dieback belonged to *Fusarium* spp. and *Phytophthora nicotianae* var *parasitica*. Early wilting disease in crops caused by *Fusarium* spp., a soil borne pathogenic fungi considered as one of the constraint responsible for 50-100% crop losses resulting in low productivity of most crops (Haware and Nene, 1982, 1980).

Bean (*Phaseolus vulgaris* L.) is one of the most important food legumes for direct human consumption in the world. The main pathogen responsible for wilt incidence of bean is *Fusarium oxysporum* f.sp. *phaseoli*., Under favourable conditions, the entire bean crop may be destroyed (Abawi, 1989). Yield losses in severely infested areas may be as high as 50%. Root rot severely constrains bean production in Kenya especially where soil fertility is low and bean production is intensive (Otsyula *et al.,* 1998; CIAT, 1992).

Various methods for controlling such diseases have been investigated including the use of resistant varieties (Brisa *et al.,* 2007), chemical control, cultural practices (Punja *et al.,* 1986), plant volatile compounds (Geiger *et al.,* 2010) plant extracts (and biological control particularly with species of *Trichoderma* (Ristaino *et al.,* 1994). Control of pathogens by chemicals is often uneconomical and has negative environmental impacts and development of fungicidal resistance variants while alternative use of resistant varieties is constrained by their unavailability. Farmers have often responded by applying heavy treatments of soils with pesticides and fungicides in an attempt to control soil borne diseases. While it may seem the logical course of action, chemical intervention only make things worse over time, with negative impact on human health and the environment.

## 2.2 Agrochemical impact on modern agriculture

The surveys by Geiger *et al., (*2010) demonstrated that, despite decades of European policy to ban harmful pesticides, their applications continue to cause disastrous consequences in biodiversity while reducing the biological control potentials. As observed by Savci, (2012), intensive management of agro-ecosystems to increase yields and attempts to reduce fungal diseases has led to decreased soil biodiversity, increased plant diseases, reduction of crop productivity, resulting in detrimental effects on crops of subsistence and economic importance. Wide spread distribution of several phytopathogenic fungi, such as *Phytophthora*, *Botrytis*, *Rhizoctonia* and *Fusarium*, during the last few years (Tahia,2004) can be attributed to changes introduced in farming. As Inbar and Hadar, (1997) noted, not only do crops in the fields are prey to fungal infections but also stored foods. Beans, an important legume commonly cultivated by smallholder farmers is susceptible to many diseases caused by fungi, bacteria and viruses. An increased susceptibility of the bean crop to soil-borne diseases has been enhanced by intensification of land use and declining soil fertility.

In Kenya losses in beans alone due to soil pathogens are estimated to be 60% of production due to *Rhizoctonia*, *Fusarium* damping off. Other notable losses in agriculture are caused by diseases such as *Phytophthora* spp., which are responsible for heart rot of pineapple, blight of potato and tomato and fruit rots. Similarly, *Rhizoctonia* spp. causes leaf blight in maize and head rot of cabbage. A combination of diseases like *Pythium*, *Phytophthora*, *Rhizoctonia*, *Sclerotium rolfsii* and *Fusarium* spp. also causes seedling blight and damping-off which infect the seedling during the germination, pre-emergence or post-emergence phases of seedling establishment.

Furthermore, modern agriculture is highly dependent on chemical pesticides and fungicides to control plant pathogens in agriculture. Although pesticides may be considered an immediate solution to increasing agricultural productivity and controlling pests that endanger crop production (Oerke, 2004; Rose, 2005), their effects have proved to be costly to

farmers in developing countries. The high costs of environmentally sound agrochemicals have caused farmers to continue to use proscribed chemicals in export crops thus increasing the chances of reduced crop quality.

### 2.3 Impact of agrochemicals on human and the environment

Agrochemicals have adversely affected human and the environment in various ways. According to Arktar, (2009), most new pesticides are broad spectrum compounds which act upon a range of pests rather than specific target organisms, whereas fungicides of broad spectrum produce undesirable consequences on non-target organisms (miller, 2004) thus destroying the natural in-built protection of the ecosystem. Further, heavy treatment of soils with pesticides, fungicides, nematicides and insecticides are known to cause persistence in the environment. In some instances, their application often extends to other areas not intended for their use.

Many pesticides are not easily degradable; depending on their chemical properties they can enter the organism, bioaccumulate in food chains and consequently influence human health. Excess pesticides may find their way into aquatic ecosystems where they contaminate ground water or accumulate in the soil, as well as pollute the atmosphere (Anasco *et al.*, 2010). This has raised public concerns on human and animal health implications. These concerns are supported by studies showing that persistent organic compounds accumulate in the tissues of most living organisms, poisoning humans and various forms of wildlife (Coat *et al.*, 2006).

A study carried out by researchers in the Caribbean island indicated that 800,000 inhabitants are at a risk of an environmental disaster with far-reaching ecological, economic and social impacts due to usage of persistent bio-accumulative pesticides. Concentrations detected in fishes are among the highest ever reported in the literature, these

have also been detected in other wildlife organisms (Coat *et al.,* 2006). Furthermore, it has been reported that the island has one of the highest prostate cancer rates in the world with increasing congenital malformation and birth defects in children.

Since the introduction of systemic fungicides globally in the early 1970s, farmers are increasingly confronted with pathogens resistant to available chemical compounds due to misuse or abuse in their employment (Oerke, 2004; Rose, 2005). Notable examples are the resistance and loss of efficacy to Thiabendazole used for control of *Fusarium* that causes dry rot and silver scurf, and Ridomil used for control of late blight, pink rot. More recently, the early blight fungus has become less sensitive to some fungicides. In Kenya, Permethrin and DDT resistance have been noted in control of insect parasites (Ranson, 2000).

### 2.4 Beneficial microorganisms for sustainable agriculture

Beneficial soil microorganisms influence on ecosystems (Shahid *et al.,* 2014) and plant growth include, nitrogen fixation, acquisition and uptake of major nutrients, promotion of shoot and root growth, disease control, suppression and improved soil structure (Chakraborty *et al.,* 2010). Among the commonly promoted and used beneficial microorganisms in agriculture worldwide as observed by Vadakattu, (2012) are *Rhizobia*, Mycorrhizae, *Azospirillum*, *Bacillus*, *Pseudomonas*, *Trichoderma*, *Streptomyces* species.

Bacteria, fungi and actinobacteria can act as biocontrol agents against root diseases Whipps, (2001) and their inoculants formulation used commercially to control diseases in agricultural and horticultural crops. Independent analyses as documented by Gupta, *et al*. (2011) and Kloepper *et al*., (2004) indicate the involvement of a diverse range of

microorganisms involved in reducing pathogen inoculum and infection, plant growth promotion and induction systemic resistance.

For instance beneficial microorganisms such as Bacillus *spp, Microbacteria spp, Pseudomonas brassicacearum, Pantoea* agglomerans have been associated with reduced pathogen inoculums through competition parasitism and predation. Organisms such as *Exiguobacterium acetylicum, Pseudomonas fluorescens ,Streptoverticillium sp., Streptomyces spp, Trichoderma spp., Penicillium griseofulvum* (Vadakattu, 2012) have been linked with reduced infection through general antibiosis, disrupt growth of pathogen from source to root, prevent access to infection sites, metabolize or disrupt plant-microbe, induced systemic resistance (Baily *et al.,* 1998) and general antibiosis signaling (Fox *et al*., 2007). Other benefits include plant growth promotion (Saba, 2014) by production of secondary roots, and improved nutrient availability (Richardson, *et al*. (2011).

Among the above organisms, *Trichoderma* strains are also associated with plant roots and root ecosystems. Their strains are defined as plant symbiont opportunistic avirulent organisms, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi and to produce compounds that stimulate growth and plant defense mechanisms (Harman *et al*., (2004).

### 2.5 *Trichoderma* as Biological control agent

Biological control is now emerging as a possible alternative for control of pests and diseases of crops with no detrimental environmental impact as a mitigation measure on the chemical impact (Nakkeeran *et al.,* 2002) on the soils due to intensified cultivation. As described by Benitez, (2004). *Trichoderma* is an anaerobic, facultative fungus found

naturally throughout the world in a large number of agricultural soils especially in organic matter or decomposing plant matter. There are more than 30 species of this microorganism with beneficial effects for agriculture and other fields (Benitez, (2004).

Its high tolerance to extreme environmental conditions and habitat, where fungi are the cause of various diseases, makes *Trichoderma* an efficient agent of control. It can survive in media with high levels of pesticides and other chemicals (Oerke, 2002).

It is proven that *Trichoderma* produces substances that stimulate plant growth and development (Vinale *et al*., 2008; Mustafa *et al*., 2009). It produces a large number of enzymes, induced by the presence of phytopathogenic fungi (Sharma, 2012) making it able to colonize the root surface and rhizosphere from the treated seeds in the soil protecting them from fungal diseases, so that the plants achieve faster growth than seedlings not treated with this microorganism (Saba, 2014).

### 2.6 Biocontrol mechanisms of *Trichoderma*

As documented by Sharma *et al.,* 2012, biocontrol by *Trichoderma* spp. have evolved numerous mechanisms acting synergistically in attacking other fungi and enhancing plant and root growth. These mechanisms include competition for space and nutrients, mycoparasitism, production of inhibitory compounds, inactivation of the pathogen's enzymes (Roco and Perez, 2001) and induced resistance.

### 2.6.1 Competition for nutrients and living space by *Trichoderma*

*Trichoderma* spp. have a rapid growth rate, persistent conidia and a broad spectrum of substrate utilization which makes them very efficient in the competition for nutrients and living space (Hjeljord and Tronsmo, 1998) causing starvation and thus resulting in biological control of fungal phytopathogens (Benitez *et al*., 2004). Some *Trichoderma* BCAs produce highly efficient siderophores that chelate iron, or resist metabolites that impede spore germination (fungistasis), kill the cells (antibiosis) or modify the rhizosphere by acidifying the soil to stop the growth of other fungi. This has been demonstrated in *Trichoderma harzianum* which controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients, with biocontrol becoming more effective as the nutrient concentration decreases (Lorito, 1994).

### 2.6.2. Direct attack and destruction of the pathogens by *Trichoderma*

Another area of intensive study is direct interaction between *Trichoderma* spp. and the pathogen through mycoparasitism and antagonism. Research results demonstrate that *Trichoderma* strains can detect other fungi and grow straight towards them. They remotely sense the pathogenic conidia prior to expression of hydrolytic, cell wall-degrading enzymes (Kullnig *et al*., 2000), when in contact with the fungi. *Trichoderma* spp. then attach to the host, coil around its hyphae and form appressoria on the host surface (Rocha-Ramirez *et al*., 2002). *Trichoderma* spp. penetrates the host cell wall by a synergistic action of the hydrolytic enzymes and antibiotics as demonstrated by Szekeres *et al*., (2005). The invasion of the pathogenic fungi is achieved through the appressoria sites, where holes are produced in the host cell wall, providing a direct entry of *Trichoderma* hyphae into the lumen of the target fungus (Inbar and Chet, 1992).

### 2.6.3. Promotion of plant beneficial processes by *Trichoderma*

Positive effects on plants and enhancement of plant growth by *Trichoderma* has been known for many years. *Trichoderma* spp. are known to cause an increase in plant growth and root development (biofertilization) and stimulate plant-defense mechanisms (Harman *et al*., 2004). Being free-living fungi as well as highly interactive in root, soil and foliar environments, they have the ability to promote plant growth by enhancing production of plant hormones, improving mineral uptake, increasing availability of biogenic elements as well as by releasing nutrients from the soil and organic matter (Kucuk and Kivanc, 2003). Some *Trichoderma* strains were shown to establish robust and long-lasting colonization of root surfaces and to penetrate into the epidermis. Colonization of the younger roots by the fungus have a dual effect of increasing efficient nutrient uptake by the plant while controlling soil borne plant pathogens. Harman, (2004) has demonstrated that root colonization by *Trichoderma* strains frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients. He established that crop productivity in fields can increase up to 300% after the addition of *Trichoderma hamatum* or *Trichoderma koningii*. Stimulation of plant growth by *Trichoderma* spp. has been reported for various plant species, including bean, carnation, cucumber, lettuce, maize, pepper and tomato, both under greenhouse and field conditions (Bailey and Lumsden, 1998).

### 2.6.4. Induced systemic and localized resistance by *Trichoderma*

*Trichoderma* spp., are 'rhizosphere competent' and can grow rapidly when inoculated in the soil. . They are capable of inducing systemic resistance which is characterized by the occurrence of disease control in the plant at a site distant

from the location of *Trichoderma.* The enzymes that degrade polysaccharides are designative for their broad spectrum of substrate utilization and their ubiquitous occurrence in nature. The enzymes stimulate the production of low-molecular weight compounds that have antimicrobial activity which are normally produced by plants in response to an attack by pathogens (Harman *et al*., 2004). Furthermore, they possess a wide spectrum of proteases which help them in the defense of their habitats and the competition for nutrients with other microorganisms (Muherjee, 2011). *Trichoderma* spp., are naturally resistant to many toxic compounds and are able to degrade hydrocarbons, chlorophenolic compounds, polysaccharides and the xenobiotic herbicides, fungicides and pesticides including DDT as the strains can recover very rapidly after the addition of sub-lethal doses of some compounds used in agriculture (Harman and Kubicek, 1998; Harman *et al*., 2004). Since different species in this genus are now being used as commercial (Sharma, 2012; Vadakattu, 2012) source of several enzymes and biofungicides as well as growth promoters, it is important that these species or isolates are identified correctly because the exhibited traits are species specific (Geiger *et al.,* 2010). Internal Transcribed Spacer-Polymerase Chain Reaction (ITS-PCR) helps to determine their diversity and identification.

### 2.6.5. Infection of maize (*Zea mays* L.) and beans (*Phaseolus vulgaris* L.) by *Fusarium* species

*Fusarium* species are important plant pathogens that attack a wide range of plant species ((Saremi, 2000; Bentley *et al.*, 2006; Bockus *et al.,* 2007)) including many important crops such as maize and beans. Studies indicate that the most frequently isolated fungi were those from genus *Fusarium* represented by *F. culmorum, F. oxysporum, F. solani* and *F. sporotrichioides.*

Maize (*Zea mays* L.) is a cereal crop grown throughout the word. Maize plays an important role in people's diet due to its ease of cultivation and adaptability to different agro-ecological zones, versatile food uses and storage characteristics (Fandohan, 2003). As much as insects may be considered a major cause of maize grain losses (Gwinner *et al.*, 1996), fungi are also important (Samson, 1991; Orsi *et al.,* 2000) being ranked second as the cause of deterioration and loss of maize (Ominski *et al.,* 1994). Yield losses caused by fungi ranging between 80% -100% have been reported in several studies (Kossou *et al.,* 1993; Burke *et al.,* 1991) in farmers maize crop. Maize contamination by fungi causes seed rot and seedling blight of maize, root rot, stalk rot and ear rot (Shurtleff, 1984; Marasas *et al.* 1984; Smith and White 1988). Fungal attacks also renders grains unfit for human consumption by quality reduction and nutritional value by mycotoxin production (Bokus *et al.,* 2007; Seremi *et al.,* 2011).

*Fusarium* root rot is a soil-borne pathogens causing root rots on bean (*Phaseolus vulgaris L.*). *Fusarium oxysporum* f. sp. *Phaseoli.,* being the causal agent of Fusarium wilt and root rot are found ( Patkowaska *et al.,* 2007) wherever beans are grown.

In Kenya, garden beans are the most widely grown legume and second most important food crop (Gethi *et al.,* 1997). It is predominantly grown by small- scale farmers commonly intercropped with Maize (*Zea mays*) and other food crops (Wortmann *et al*., 1998). Apart from providing families the cheapest source of protein, bean harvests lead to increased household incomes from sales. Bean root rot disease caused by a number of soil-borne pathogenic fungi is some of the major causes of low yields (Nderitu *et al.,* 1997). The disease has been reported in many bean growing areas including western Kenya (Otsyula and Ajanga 1994; Muriungi 1997), Embu and Taita hills (Nderitu *et al*., 1997), where yield losses are estimated to between 10 and 100% (Nderitu *et al.,* 1997).

Biological control can be applied to reduce instances of plant diseases caused by *Fusarium* spp., because they attack or compete with the pathogenic fungi (Mishra, 2013). Among fungi, the most widely used biofungicides are *Trichoderma* sp. (Mukherjee *et al*., 2013) because they have specific advantages over synthetic fungicides, including fewer non-target and environmental effects, efficacy against fungicide-resistant pathogens and reduced resistance development (Matroudi *et al*., 2009; Harman *et al.*, 2004; Eziashi., *et al* 2007).

### 2.6.6. Choice of study topic

Considering the literature review *Trichoderma* species have been widely studied for their biocontrol ability. However, their use as biocontrol agents in agriculture is limited due to the unpredictable efficiency which is affected by biotic and abiotic factors in the soil. Embu soil and roots samples were collected from the rhizosphere during harvesting. When assessed for inoculum density the soil samples demonstrated high incidence of infection by *Fusarium* spp. Root rot, a soil borne disease caused by *Fusarium oxysporum* f. sp. *phaseoli.,* is one of the main root diseases impacting production of common beans and is a major concern in many bean growing areas including Embu county . Several phytopathogenic species of *Fusarium* are also found to be associated with maize including *Fusarium verticillioides, Fusarium proliferatum*, *Fusarium graminearum* and *Fusarium anthophilum.* This study intends to evaluate local *Trichoderma* spp., for their ability to control *Fusarium oxysporum* f. sp. *phaseoli.,*and to promote seed germination and development in greenhouse and growth of maize and bean seedlings grown in the field at Embu County, Kenya. Furthermore, *Trichoderma* spp. includes an abundance of strains occupying one ecological niche which may differ in their antagonistic properties and efficacy as biocontrol agents. It is difficult to predict the degree of synergism and their behavior in a natural soil and root ecosystem and therefore the need to establish their genetic diversity.

### 2.6.7. Purpose of study

The purpose of this study is to screen, evaluate and test locally sourced isolates of *Trichoderma* spp. for biocontrol and ability to promote seed germination and growth of maize and bean seedlings grown in the field at Embu County, Kenya. Further, this study will attempt to confirm similarity of the five local *Trichoderma* species within their clade in relation to their ability to antagonize *Fusarium oxysporum* f. sp. *phaseoli.,* using Internal Transcribed Spacer-Polymerase Chain Reaction (ITS-PCR

### 2.6.8. Statement of the problem

Embu County, which is the area of study, has experienced high intensification of farming. Management practices used in crop production is characterized by intensive management of agro-ecosystems and the use of high-level inputs such as manure, fertilizers and pesticides for maize, beans, tea, coffee and irrigated horticultural crops. This has led to detrimental effects on crops of economic importance due to insurgence of soil pathogens, plant diseases and reduction of crop productivity.

### 2.6.9. Justification

Most African countries economies, including Kenya, depend on agriculture. In Africa, farming is the most important economic activity with 60% to 80% of the population engaged in farming. On the average, in Kenya the agricultural sector contributes about 24 percent of the GDP and 65 percent of total export earnings. Agriculture and related agro-industries accounts for over 51%. Above all, the sector employs over 80 percent of Kenya's workforce which has since increased from 18 percent in 2003 to 19 percent in 2007, thus contributing to poverty reduction. The Vision 2030 strategy identifies agriculture as one of the six (6) key economic pillars expected to drive the economy to a projected 10

percent growth annually over the next two decades. This goal will be realized by the promotion of innovative, commercially oriented and modern agriculture (Vision 2030).

The need to maintain soil health for sustainable crop productivity therefore cannot be underscored. Embu County, which is the area of study, is characterized by intensive management of agro-ecosystems and the use of high level inputs such as fertilizers and pesticides for food and cash crops. However, low yields have been experienced in the last couple of years. Garden beans are the most widely grown legume and often intercropped with maize (*Zea mays*). Yield losses due to *Fusarium* spp. are generally estimated to be 80%, (Sharma *et al.*, (2006). As observed by Haggag *et al.*, (2001), *Trichoderma* was effective in controlling *Fusarium* root rot and nematode disease complex in sunflower. *Trichoderma* also enhanced seed germination (Mukhtar, 2008). *Trichoderma strains* have been used as biocontrol agents with ability to; colonize the soil or parts of the plant as it occupy a physical space and avoiding the multiplication of the pathogens. It produces cell wall degrading enzymes against the pathogens; it can produce antibiotics that can kill the pathogens. More importantly it, promotes the plant development while inducing the defensive mechanisms of the plant (Harman *et al*., 2004).

Indiscriminate and imbalanced use of chemical fertilizers, especially nitrogen along with chemical pesticides and unavailability of organic manures has led to considerable reduction in soil health. ( Dharmendra *et al*., 2013. Findings on the alternative to disease management in the region will go a long way to improving soil health and ensuring sustainable management of agriculture. This study was therefore undertaken with the following hypothesis and objectives.

## 2.7 Hypothesis

The ability of *Trichoderma* to control *Fusarium* spp. varies considerably and it is possible to improve its biological control efficiency by the selection of isolates with high antagonistic potential and adapted to local ecological area. Therefore local isolates of *Trichoderma* spp., have potential for use as biological control agents to protect maize and bean plants from wilt as well as growth development in Embu sites.

### 2.7.1. General objective

To identify the most virulent strain among local *Trichoderma* isolates and establish its possible usage for control of *Fusarium oxysporum* f. sp. *phaseoli.*, a soil borne pathogenic fungi in Embu.

### 2.7.2. Specific objectives

1. To identify the most virulent strain of *Trichoderma* from the five local isolates for potential fungal biocontrol activity on *Fusarium oxysporum* f. sp. *Phaseoli.,*
2. To evaluate the antagonistic and biocontrol ability of the local *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *Phaseoli.,* in greenhouse and field tests.
3. To determine genetic diversity of the evaluated strains of the local *Trichoderma* strains using molecular ITS markers.

# CHAPTER THREE

## 3.0. MATERIALS AND METHODS

### 3.1 Description of study site

This study was carried out in Embu County located within the Mount Kenya region (Figure 1.0). The main land use systems are natural forest, tea, coffee, mixed small-scale cultivation of food crops, dairy cattle rearing and semi-extensive livestock production. The area of study is characterized by intensive management of agro-ecosystems through use of high level inputs such as fertilizers and pesticides for food and cash crops. Beans (*Phaseolus vulgaris* L.) are a widely grown legume and often intercropped (Okoth *et al*., 2010) with maize (*Zea mays*) and yield losses by *Fusarium* spp. are estimated to be 80% (Thung and Rao, 1999). *Trichoderma* species are very promising biocontrol agents which have been used commercially against a range of phtytopathogenic fungi such as *Fusarium*, *Pythium*, and *Rhizoctonia* (Mausam *et al*., 2007).

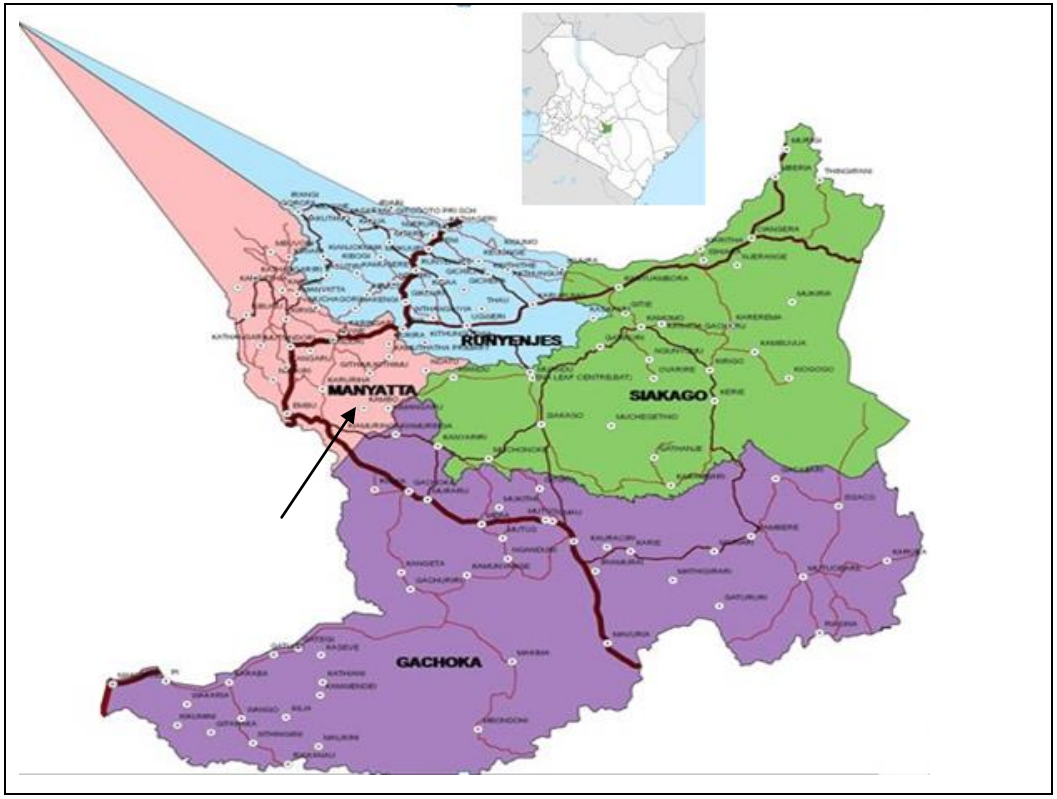



**Figure 3.1: Map of Kenya showing location of Embu County along the slopes of Mt. Kenya and the area of study shown by the arrow.**

### 3.2 Isolation of Trichoderma and Fusarium species

*Trichoderma* species evaluated in this study were obtained from a culture collection at the School of biological sciences, University of Nairobi, following a study (Okoth *et al.,* 2007) on the diversity, abundance and spatial distribution of *Trichoderma* species over the different land use systems in Embu. Pure cultures were obtained from sub-cultured fungi of three samples per isolate of five (5) *Trichoderma* isolates and one (1) *Fusarium* isolate from a total of 18 samples of Embu collections.

Diseased bean plant tissues showing symptoms of *Fusarium* wilt were obtained from the University of Nairobi farm at College of Agriculture and Veterinary Sciences (CAVS). *Fusarium oxysporum* f. sp. *phaseoli.,* isolate was obtained from samples of seedlings of bean plant tissues with wilting. Roots were washed with distilled water and using a sterilized sharp blade cut into 5-10 mm long sections. The pieces were surface disinfected for two minutes in 0.1% sodium hypochlorite and rinsed in several changes of distilled water, dried on sterile filter paper before plating on potato dextrose agar (PDA) containing 0.2% streptomycin and incubated at 30C. Fungi grown from the root pieces were then sub-cultured.

Single spore of each fungal isolates were obtained by using the technique of Manandhar *et al*., (1995). Petri dish containing fungi were flooded with distilled water and serial dilution made. Five ml of suspension (100 spores/ml) were added to water agar (2gm of agar in 100ml) and marked out using a grid drawn on the base of each Petri dish. The plates were incubated for 12 hours and single spores were sub-cultured onto fresh PDA.

The microscopic characteristics and cultural characteristics were confirmed with the description in Booth, (1971) for *Fusarium oxysporum* f. sp. *phaseoli.,* and Rifai (1969); Gams and Bisset, (1998) for genus *Trichoderma* isolates of

*Trichoderma koningii, Trichoderma asperellum, Trichoderma atroviride, Trichoderma reesei, and Trichoderma harzianum*. Cultures were maintained on PDA slants at 4°C.

### 3.2.1. Antagonism of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *Phaseoli.*,on dual culture

*Trichoderma* species were evaluated for the most antagonistic activity against *Fusarium oxysporum* f. sp. *phaseoli.*, following the dual culture technique (Odebode and Sobowale, 2001; Skidmore and Dickinson, 1976; Morton and Strouble 1995) by the dual culture technique. The highest rating antagonistic isolates were then selected for further studies.

Mycelial discs 5 mm in diameter exercised from the edge of an actively growing antagonist (7 days old) and the pathogen was cultured on the opposite ends of a Petri dish equidistant from the periphery. A Completely Randomized Block experiment design was used with three replicates for each isolate. In control Petri dish, a sterile agar disc was inoculated on the opposite side of pathogen in place of antagonist. Inoculated plates were incubated at 25°C for 5 days. Colony growth of both biocontrol agents and pathogen were observed constantly and the radial growth of the pathogen recorded daily up to day eight of inoculation. After the incubation period, radial growth of pathogens was measured and the percentage inhibition of average radial growth was calculated to the control as follows;

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

Where;

L is the percentage inhibition of radial mycelia growth,

C is the radial growth of the pathogen in the control in cm; while

T is radial growth of the pathogen in the presence of *Trichoderma* species in mm (Edington *et al*., 1971).

Mycelia samples from the interaction region were collected after 4, 5, 6 and 8 days after inoculation and processed for electron microscopy.

The scoring of *Trichoderma* isolates of paired culture were categorized as effective based on their ability to overgrow and inhibit the growth of the pathogens by giving them a score as per modified Bell's scale ( Bell *et al*., 1982), as follows; Class 1 = the antagonist completely overgrown the pathogen (100 % overgrowth). Class 2 = the antagonist overgrown at least 3/4th of pathogen surface (75% overgrowth). Class 3 = the antagonist colonized on half of the growth of the pathogen (50% overgrowth). Class 4 = the pathogen and the antagonist locked at the point of contact. Class 5 = the pathogen overgrown the mycoparasite.

**3.2.2. Examination of antagonistic and mycoparasitic mechanisms:**

The hyphal interactions between the antagonists and the test pathogen were studied from the dual culture plate technique. Each isolate of *Trichoderma* species were examined for both antibiotic and mycoparasitic activities against *Fusarium oxysporum* f. sp. *phaseoli*. Antibiosis was observed directly by light microscopy of paired cultures while mycoparasitism was observed in paired cultures of the pathogen.

To observe the antagonist interaction, a thin film of agar, was prepared by pouring approximately 1 ul molten PDA on a sterile glass slide. Each agar-covered slide was inoculated with an agar disc colonized by *Fusarium oxysporum* f. sp. *phaseoli.,* placed in the centre of the slide. Two days later each end was inoculated with an agar disc colonized by *Trichoderma* species. Slides were placed on moist sterile tissue paper in sealed Petri dishes and examined four, six and eight days later. One to three drops of 70% ethanol were added to the surface of the colonized agar, followed by two to three drops of lactophenol cotton blue. The slides were covered with cover slips before being examined under the light microscope. Hyphal interactions including coiling, parallel hyphal growth, appressoria formation and direct penetration between *Fusarium oxysporum* f. sp. *Phaseoli.,* and *Trichoderma* species were examined under light microscope. Freeze-dried slides were examined using Scanning Electron Microscope (SEM).

### 3.2.3 Conidial measurements *Trichoderma* and *Fusarium* species of spores

The mycelium preparations were from the evaluated isolates *Trichoderma* species earlier confirmed for virulence against *Fusarium oxysporum* f. sp. *phaseoli*. The inocula, both from *Trichoderma* and *Fusarium* species were prepared according to Guarro *et al*., (1998) by flooding the surface of the agar slants of the isolates with sterile distilled water and scraping the sporulated aerial mycelium with a loop. The suspensions obtained were then filtered through sterile gauze to remove the excess hyphae, and spores counted with a haemocytometer.

### 3.2.4. *Trichoderma* conidial preparation and application in green house

The number of colony forming units for each *Trichoderma* spp., were divided by the volume plated and multiplied by total dilution used, to get the number of colony forming units per milliliter. Conidial suspension was adjusted to 5 $x10^8$ colony forming units (cfu) for *Trichoderma koningii* and 5 $x10^7$ colony forming units (cfu) for *Trichoderma*

*reesei* (Xixuan *et al*., 2011). The same protocol was used to prepare inoculum for *Fusarium* oxysporum f. sp. *Phaseoli.,* and colonies adjusted to approx 2 x$10^6$ colony forming units (cfu).

Based on the Biocontrol virulence such as antifungal, activity, mycoparasitism and growth promotion, two *Trichoderma* isolates namely *Trichoderma koningii* and *Trichoderma reesei* previously evaluated for virulence against *Fusarium oxysporum* f. sp. *phaseoli.,* were used in the experiment conducted in the greenhouse at the Department of Biological Sciences, University of Nairobi. The experiment investigated the potential of the two *Trichoderma isolates in* the control of *Fusarium* wilt, and plant growth promoting *in* common bean (*Phaseolus vulgaris* L.) seedling. Soil used was collected at Kabete Campus farm. The soils had been previously steam sterilized soil in a drum at 121°C for 1 hr on three successive days and allowed to cool before use. Pots were also sterilized using 70% ethanol before filling with soil.

### 3.2.5. Determination of *Fusarium* root wilt control and plant growth of bean seedling using *Trichoderma* species in the greenhouse

The efficacy of *Trichoderma* on *Fusarium* wilt control and growth promotion in bean plant was evaluated in pot culture experiment laid in Randomized block designs replicated four times. The pots (10 cm diameter) containing 1kg of sterilized peat soils were infested with 20 mycelial plugs of 5 x$10^8$ colony forming units (cfu) for *Trichoderma koningii* and 5 x$10^7$ colony forming units (cfu) for *Trichoderma reesei* (Mausam, 2007). The pathogenic fungus *Fusarium* oxysporum f. sp. *Phaseoli.,* was applied at 10 mycelial plugs of 2 x$10^6$ colony forming units (cfu). Manure was applied at 102 g per pot while fertilizer was applied at 10g per pot. The treatments were; (1) control with un-inoculated soils, (2) *Fusarium* oxysporum f. sp. *Phaseoli.,* infested soil at (3) *Trichoderma koningii* + *Fusarium* oxysporum f. sp.

*Phaseoli.,* (4) Manure (5) Mavuno (6) *Trichoderma koningii*+ Mavuno (7) *Trichoderma koningii*+ Manure *(8) Trichoderma koningii* (9) *Trichoderma reesei* (10) *Trichoderma reesei* + *Fusarium* oxysporum f. sp. *Phaseoli.,* (11) *Trichoderma reesei*+ mavuno (12) *Trichoderma reesei* + Manure (Table 3.2). Two seeds of common Beans (Phaseolus vulgaris L) plant were sown in each pot (Ghildiya, 2008) and watered daily. The greenhouse temperatures of were maintained between 25°C and 26°C.

**Table 3.1: *Trichoderma* treatments on soil**

| Code | *Trichoderma* treatments | Application per 1kg of soil in pots (10 cm diameter |
|---|---|---|
| T1 | Control | 1kg of sterilized peat soils |
| T2 | *Fusarium* oxysporum f. sp. *Phaseoli* | 10 mycelial plugs of 2 x$10^6$ colony forming units (cfu) |
| T3 | *Trichoderma koningii* + *Fusarium* oxysporum f. sp. *Phaseoli* | 20 mycelial plugs of 5 x$10^8$ colony forming units (cfu) +10 mycelial plugs of 2 x$10^6$ colony forming units (cfu) |
| T4 | Manure | 102 g |
| T5 | Mavuno | 10g |
| T6 | *Trichoderma koningii*+ Mavuno | 20 mycelial plugs of 5 x$10^8$ colony forming units (cfu) +10g |
| T7 | *Trichoderma koningii*+ Manure | 20 mycelial plugs of 5 x$10^8$ colony forming units (cfu) + |
| T8 | *Trichoderma koningii* | 20 mycelial plugs of 5 x$10^8$ colony forming units (cfu) |
| T9 | *Trichoderma reesei* | 20 mycelial plugs of 5 x$10^7$ colony forming units (cfu) |

| | | |
|---|---|---|
| T10 | *Trichoderma reesei* + *Fusarium* oxysporum f. sp. *Phaseoli* | 20 mycelial plugs of 5 x$10^7$ colony forming units (cfu) +10 mycelial plugs of 2 x$10^6$ colony forming units (cfu) |
| T11 | *Trichoderma reesei*+ mavuno | 20 mycelial plugs of 5 x$10^7$ colony forming units (cfu) +10g |
| T12 | *Trichoderma reesei* + Manure | 20 mycelial plugs of 5 x$10^7$ colony forming units (cfu)+ 102 g |

### 3.2.6. Determination of germination of *Trichoderma* treated seedlings under greenhouse

Germination emergence count was done 14 days after planting. Germination emergence count was made by counting the number of inoculated seed that germinated per pot. Germination of inoculated seeds was compared with untreated control and germination rate of seeds recorded. Data were analyzed using an Analysis of Variance (ANOVA). Germination percentage and germination index of bean seeds were calculated according to Irum Mukhtar, (2008) as follows;

$$\text{Germination (\%)} = \frac{\text{Number of germinated seed} \times 100}{\text{Total number of seeds}}$$

Bean plants were harvested after 14 days of planting (DAP) and assayed as the total percentage of plants showing any wilt symptoms due to the pathogen (necrotic lesions on roots and hypocotyls) according to Abeysinghe, (2007) using a rating scale of 0-5 described according to Filion *et al.,* (2003). Bean seedlings samples were gently uprooted using a

spade, washed in running water and dried up using paper towels. Data on Shoot Height, Fresh root weight and Fresh shoot weight were collected after harvesting the bean plants.

### 3.2.7. Application of *Trichoderma harzianum* inoculums in field tests

Indigenous *Trichoderma harzianum* strain previously tested in Embu for biological control against *Fusarium species* (Okoth *et al*., 2007; Siameto *et al*., 2011) was evaluated for ability to promote seed germination and growth in maize and beans plants in this study. Inoculums were mass produced according to Subash *et al.*, 2014 by growing the mycelia of the *Trichoderma harzianum* isolate in 100g of sorghum seeds boiled for 20 to 25 minutes to soften grains to 25%. The water was drained and sorghum seed spread to cool down and to decrease the moisture content. Calcium carbonate (2g) was added to every 100gm of dried sorghum seed to remove excess moisture and transferred to polythene bags and autoclaved at 121°C for 15 minutes. After cooling, the sorghum seeds were aseptically inoculated with *Trichoderma harzianum* mats grown in liquid culture and incubated at room temperature for 5-7 days. After incubation period, contents of the flasks were transferred to plastic plates under sterile conditions. The inoculum was left to air dry then mixed in a blender to become powder and kept in a plastic container at room temperature until ready for use in the field.

The formulation was used as a dry seed treatment where 2g of the formulation was mixed with 1 kg of seeds in a carrier medium of 4ml of gum Arabica solution (30g/300ml). The seeds of maize and beans were surface-sterilized by rinsing thoroughly in sterile distilled water, rolled with the formulation and planted immediately. Seeds treated with sterile distilled water served as control. Seeds of maize and beans coated with *Trichoderma harzianum* inoculum were tested

for its ability to promote seed germination and growth of maize and bean seedlings grown in the field at Embu County, Kenya.

### 3.2.8. Field test effect of *Trichoderma* spp. on seed germination and growth

An indigenous strain of *Trichoderma harzianum* was tested for its ability to promote seed germination and growth of maize and bean seedlings grown in the field. The trial was carried out for three seasons with the following treatments; two types of fertilizers, cow manure, and *Trichoderma* seed coat.

Field trials were done at Agriculture Training College (ATC) within Embu Town. The experiment was laid out at the ATC in a Randomized Complete Block Design (RCBD) with treatments replicated 5 times. These treatments were further replicated on farm on 12 split plots, 6 in each location, to nullify the effect of heterogeneity of farms. The farms were 500m apart to avoid auto-correlation (Groupe and Theriault Consultants, 1984). Each treatment was a stretch of 5 x 10m. The test crops were maize intercropped with beans. The maize type was hybrid (H516) with spacing of 90 x 30cm and planting done with two seeds per hole. Bean type was *Mwezi moja* with spacing of 75 x 25 cm and planted two seeds per hole. The treatments were Triple Superphosphate combined with Calcium Ammonium Nitrate (TSP + CAN), Cow manure, *Trichoderma* seed coating and Mavuno which is a blend of fertilizers containing 11 nutrients including Nitrogen (N½) 10%, Phosphorous ($P_2O_5$) 26%, Potassium ($K_2O$) 10%, Sulphur ($SO_4$) 4%, Calcium (CaO ) 10%, Magnesium (MgO) 4%, and appropriate additions of other Trace Elements like: Zinc, Copper, Molybdenum, Boron and Manganese (Table 3.3). The fertilizers were added by broadcasting during planting and top dressing of CAN, TSP and Mavuno done after first round of weeding. Planting was done during the long rains which occur

between March and May, and short rains between October and December. The on station experiments were researcher managed while on farm trials were farmer managed.

**Table 3.2: *Trichoderma* treatment on seeds**

| **Treatment** | **Rates of application** |
|---|---|
| TSP+CAN | 50 kg P/ha<br>100 Kg N/ha |
| Mavuno | 50 Kg P/ha<br>100 Kg N/ha |
| Cow manure | 10 Tones/ha |
| *Trichoderma harzianum* | Seed coat |
| Control | Nil |

KEY:

- Triple Superphosphate (TSP) fertilizer (44-52% $P_2O_{5)}$.
- Calcium ammonium nitrate (CAN) contains 27% N

Seedlings were counted 14 days after emergence. From each hole, one seedling was dug out using a spade and the following parameters measured; plant shoot/stem height was measured from soil surface to apical buds using a ruler. Stem/root caliper width measurement were also recorded.

### 3.3 Molecular characterization of local *Trichoderma* isolates

#### 3.3.1 Extraction of Genomic DNA

Each *Trichoderma* isolates were grown in 10 ml of potato dextrose-yeast broth for 8-10 days at room temperature with rotary shaking at 150-170 r.p.m. Mycelia were harvested by filtration through filter paper and washed with distilled water. Genomic DNA was extracted using the method of Raeder and Broda (1985) with some modification. Genomic DNA was isolated from 5 local *Trichoderma* isolate.

A total of 1g of lyophilized mycelia of each isolate was re-suspended in 500µl of TE lysis buffer (50mM Tris-Hcl (pH 8.0), 50mM EDTA. (1:1 TE50), 1 M NaCl,, 100µl of 10% SDS, and 20µl of 1 mg/ml Proteinase K). Samples were mixed by vortexing and incubated at 37ºC for 45 min. DNA was extracted using 600µl of phenol: chloroform: isoamyl alcohol (24:25:1) and mixed by inversion for 2 minutes then centrifuged at 14000r.p.m for 15 minutes. The top aqueous layer was removed and added to a new tube. The above step was repeated. A total of 600µl of chloroform was added to the resultant supernatant and centrifuged at 14000 r.p.m. for 15 minutes and the supernatant transferred to a clean tube. Precipitation was done by adding 60µl of 3M sodium acetate and 800µl of chilled absolute ethanol and inverted gently

a few minutes. The samples were incubated overnight at -20°C. The samples were then centrifuged at 14,000 r.p.m. for 30 minutes and supernatant was discarded. DNA pellets were then washed with 1 ml of 70 % ethanol. The DNA pellet was again centrifuged at 14,000r.p.m for 5 minutes and supernatant discarded and left on the bench for 15 minutes to dry. The pellets were resuspended in 50µl DNA elution buffer (10 mM Tris–HCl (pH 8), 1 mM EDTA). The nucleic acid dissolved in TE buffer were treated with 3 µl of RNase (20 mg/ml), incubated at 37ºC, and stored at –20ºC until use.

### 3.3.2 Quantitative and qualitative estimation of Genomic DNA from *Trichoderma* isolates

DNA was quantified by spectrophotometric measurement of UV absorption at 260 nm. DNA was also quantified by means of 0.8% agarose gel electrophoresis by loading 5µl of total DNA solution followed by ethidium bromide visualization using a 1 kbp DNA ladder as DNA size markers.

### 3.3.3 PCR amplification of ITS region of *Trichoderma* isolates

All isolates of *Trichoderma* were taken up for ITS-PCR amplification. Fragments of the ribosomal DNA (rDNA) containing the internal transcribed spacer (ITS) regions, ITS1(5'- TCT GTA GGT GAA CCT GCG G) and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC) were amplified and sequenced as described by Zhang *et al*., (2005). Primers ITS1 and ITS4 were synthesized by Microsynth AG Switzerland. The PCR reagents were supplied by Bioneer AccuPower® HotStart PCR PreMix. Genomic DNA was amplified by mixing the template DNA (30 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction amplifications was performed on each sample in a total volume of 20µl reaction mix containing 1x PCR Buffer (200 mM Tris-HCl, (pH 8.0); 500 mM

KCl), 1 unit of HotStart DNA Polymerase, 200 µM of each dNTP, 0.5 µM reverse and forward primers and 1µl of 30ng template DNA. The Polymerase Chain Reaction (PCR) cycling parameters were programmed as follows;

Initial denaturing temp 95°C for 5minutes, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 minute, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes in a GeneAmp PCR System 9700 (USA) thermal cycler. The total number of cycles was 35, with the final extension of 72°C for 10 min. Amplified products (5 µl) were size separated on 1.8 % agarose gel containing TE buffer TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) stained with GelRed and visualized by gel image system and gels photographed using Scion Pro software UV light camera. A 100-bp DNA ladder (*Fermentas*, South Africa) was used as molecular size markers

### 3.3.4 Nucleotide sequencing and *In silico* analysis

Prior to sequencing, PCR products were purified with QIAquick® PCR Purification Kit (Qiagen Inc., Valencia, California) according to the manufacturer's specifications. The sequencing of the the rDNA region, PCR product was carried out by automated Sanger DNA sequencing with fluorescent terminators using sequencer model 3730 (Applied Biosystems) at the Biosciences Eastern and Central Africa- ILRI Hub. Related sequences were searched using BLAST programme from the Gen-Bank database (http://www.ncbi.nlm.nih.gov/blast/) (Tamura *et al.,* 2011). The multiple sequence alignment and pairwise alignment were made using CLUSTAL W 1.6. Phylogenetic inference was performed by UPGMA method. Bootsrap tests after 1000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained. The phylogenetics analysis was conducted in MEGA 4 as described by Tamura *et al*., (2011). Each *Trichoderma* isolate was sequenced in both directions using primers ITS1 and ITS4.

### 3.3.5 Statistical analysis

Analysis of variance test were done to establish the effect of *Trichoderma* seed coat and soil amendments on the rate of germination and seedling establishment of maize and beans in completely randomized design. The sequenced PCR product of internal transcribed spacer (ITS) region of the rDNA was aligned with ex-type strain sequences from NCBI GenBank and established fungal genetic similarity following the Clustal W algorithm. The phylogenetics analysis was completed using MEGA package (version 4.01) (Tamura *et al*., 2007). Phylogenetic inference was performed by UPGMA method. Bootsrap tests with 1000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained. The phylogenetics analysis was conducted in MEGA 4 as described by Tamura *et al*., (2011).

# CHAPTER FOUR

## 4.0. RESULTS

### 4.1 Mycelial growth inhibition of *Fusarium oxysporum* f. sp. *Phaseoli.,* by *Trichoderma Reesei* and *T. koningii on* culture plates.

All *Trichoderma* isolates that were screened inhibited growth of *Fusarium oxysporum* f. sp. *Phaseoli.,* ($p \leq 0.01$) as their biocontrol potency increased with time. At 6 days after inoculation all the biocontrol agents exhibited strong (2) to very strong antagonism (3) on an assessment scale of 0 to 5 (Bell *et al.,* 1982). A strong growth inhibition was observed with *Trichoderma Reesei* and *T. koningii* antagonists with a score of 2 overgrowths (75%) of the pathogen out of 0-5 score rating (Table 4.3). These two antagonists were more effective against *Fusarium oxysporum* f. sp. *Phaseoli.,* compared to the other *Trichoderma* strains tested as shown in Plate 4.1. These results confirm earlier findings by Muriungi *et al*., (2013) showing nearly 90-100% inhibition of *Fusarium oxysporum* growth by antagonistic *Trichoderma.*

**Table 4.3: *Trichoderma* antagonism score**

| *Trichoderma* isolates | Daily mean growth measurements (mm) | | | | Antagonism score (0-5) |
|---|---|---|---|---|---|
| *Days* | 4 | 5 | 6 | 7 | |
| *Trichoderma atroviride* | 1.3 | 3.1 | 5.2 | 5.3 | 3 |
| *Trichoderma reesei* | 1.3 | 3.4 | 5.6 | 5.7 | 2 |
| Trichoderma *harzianum* | 1.3 | 2.9 | 4.5 | 5.5 | 4 |
| *Trichoderma koningii* | 1.2 | 3.2 | 5.0 | 5.6 | 2 |
| *Trichoderma asperellum* | 1.0 | 2.6 | 4.9 | 5.3 | 4 |
| Control *Fusarium* | 0.6 | 1.2 | 1.6 | 1.8 | - |

Antagonism scored as per modified Bell's scale (Bell *et al*., 1982), where 1=*Trichoderma* completely overgrew pathogen (100% overgrowth), 2= *Trichoderma* overgrew at least two-thirds pathogen (75% overgrowth), 3=*Trichoderma* colonizes half of the pathogen (50% overgrowth), 4= *Trichoderma* and the pathogen locked at contact point after inoculation. 5= Pathogen overgrew *Trichoderma* (25% inhibition)

### 4.1.1. Degree of antagonism of *Fusarium oxysporum* f. sp. *Phaseoli.*, by *Trichoderma Reesei* and *T. koningii on* culture plates.

The five *Trichoderma* isolates tested *in vitro* were effective in suppressing *Fusarium oxysporum* f. sp. *phaseoli.,* ($p \leq 0.05$). Growth suppression by *Trichoderma reesei* against *Fusarium oxysporum* f. sp. *Phaseoli.,* had the highest effect

in inhibition of mycelial growth (60.0%) with p-value (0.018), followed by *Trichoderma koningii* (55.2%) with (p=0.014). *Trichoderma atroviride* (52.8%) and *Trichoderma asperellum* (50.4%) showed moderate inhibition. The isolate of *Trichoderma harzianum* (48.0%) showed the lowest inhibitory effect to *Fusarium oxysporum* f. sp. *Phaseoli.*, as shown in Table 4.4. According to these results, *Trichoderma Reesei* and *T. koningii* were used in subsequent experiment.

**Table 4.4: Percentage inhibition of mycelial growth**

| Treatment | *Trichoderma* | *Fusarium* | Control *Fusarium* | Control *Trichoderma* | percentage inhibition |
|---|---|---|---|---|---|
| *Trichoderma koningii* | 4.2 | 1.4 | 1.6 | 5.0 | 55.2 |
| *Trichoderma reesei* | 4.4 | 1.3 | 1.6 | 5.6 | 60.0 |
| *Trichoderma asperellum* | 4.1 | 1.5 | 1.6 | 4.9 | 50.4 |
| *Trichoderma atroviride* | 4.2 | 1.4 | 1.6 | 5.2 | 52.8 |
| *Trichoderma harzianum* | 3.7 | 1.5 | 1.6 | 4.5 | 48.0 |

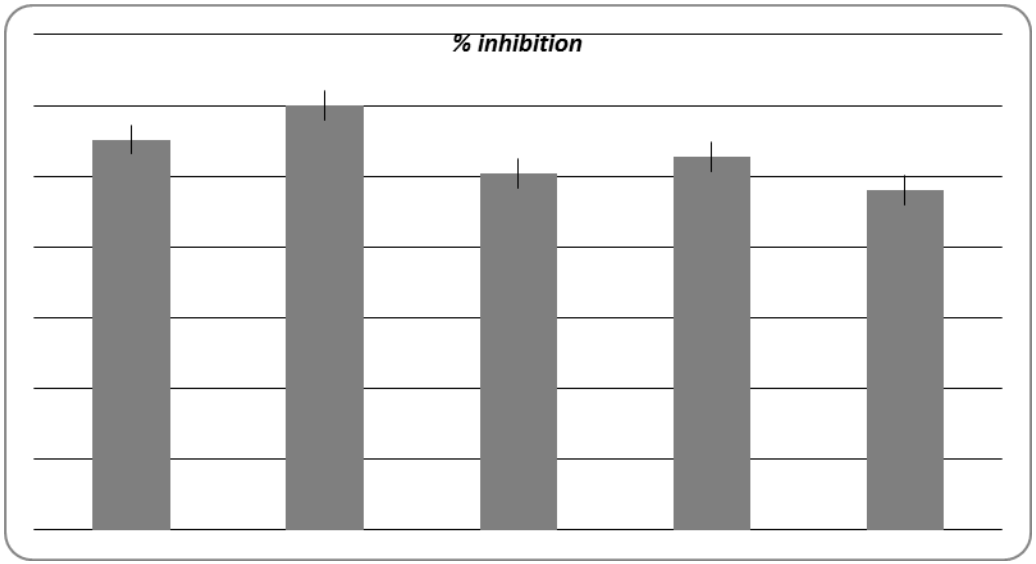
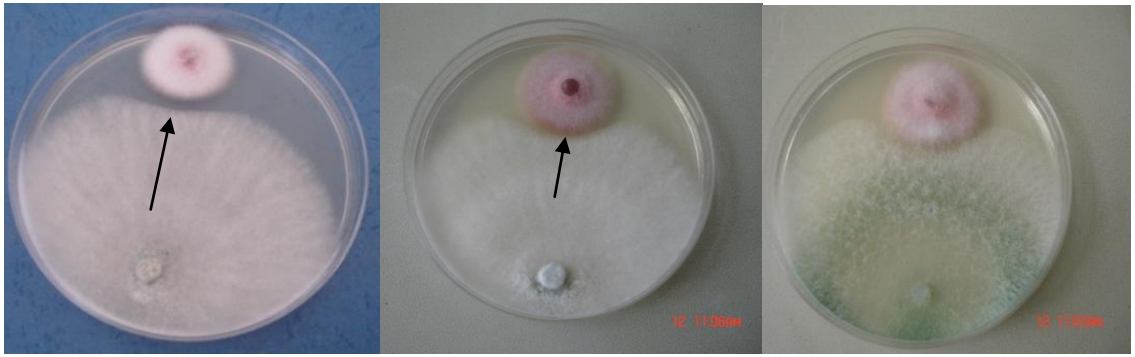



**Figure 4.2: Percentage inhibition of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *phaseoli.*, on day 4 of inoculation *in vitro***

### 4.1.2. Inhibition features of growth of *Fusarium oxysporum* f. sp. *Phaseoli.*, in the presence of *Trichoderma Reesei* and *T. koningii on* culture plates.

Observations were made on the growth of the pathogen and the presence of an inhibition zone that might develop between the two colonies of *Trichoderma* isolates. All *Trichoderma* antagonistic interactions on *Fusarium oxysporum* f. sp. *Phaseoli.*, reduced the radial mycelia . The common mode of action observed was antibiosis and mycoparasitism appearing in paired inoculated plates as an inhibition zone. Contact between *Fusarium* fungi and *Trichoderma* isolates occurred but the ability to overgrow and to parasitize the mycelia of the pathogenic fungi was highly dependent on the antagonistic potential and growth rate of each *Trichoderma isolate* as observed by Schubert, (2008). Synergistic mode

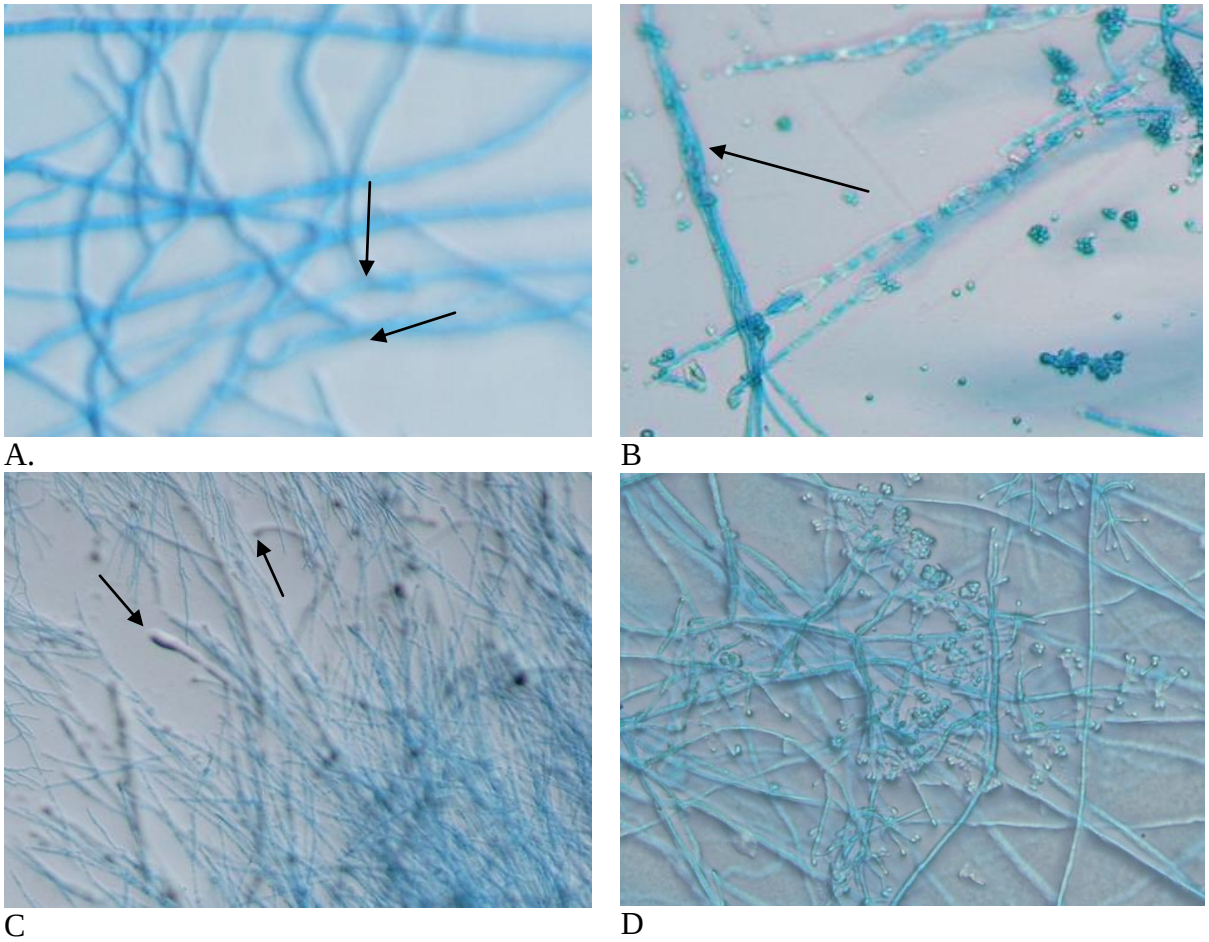
of action of inhibitory properties of *Trichoderma* isolates were discernible when assessed on the 6th day of inoculation (Plate 4.1).



| | | |
|---|---|---|
| Mycoparasitism (direction of arrow) mode of action between *Fusarium oxysporum* f. sp. *Phaseoli.*, (Pink colony) and *Trichoderma Reesei* | Antibiosis mode of action by *Trichoderma Reesei* (white colony) on *Fusarium oxysporum* f. sp. *Phaseoli.*, | *Trichoderma Reesei* colonizes *Fusarium oxysporum* f. sp. *Phaseoli* |

**Plates 4.1: Plate confrontational tests showing mode of action of *Fusarium oxysporum* f. sp. *Phaseoli.*, in the presence of *Trichoderma Reesei* at day 6**

Lysis of *Fusarium oxysporum* f. sp. *Phaseoli.*, hyphae was associated with *Trichoderma* isolates. The isolates induced abnormal stunted, highly branched hyphal tips and swollen hyphae at the edge of *Fusarium oxysporum* f. sp. *Phaseoli.*, colonies (Plate 4.2 C) leading to inhibition of pathogen growth. Mycoparasitism was observed as coiling (Plate 4.2 B), penetration by *Trichoderma* (Plate 4.2 A), and sporulation and disintegration of pathogenic fungus hyphae (Plate 4.2 D).




**Plates 4.2: Light Microscope slide pictures showing inhibition features of *Trichoderma Reesei* on mycelia of *Fusarium oxysporum* f. sp. *Phaseoli.***

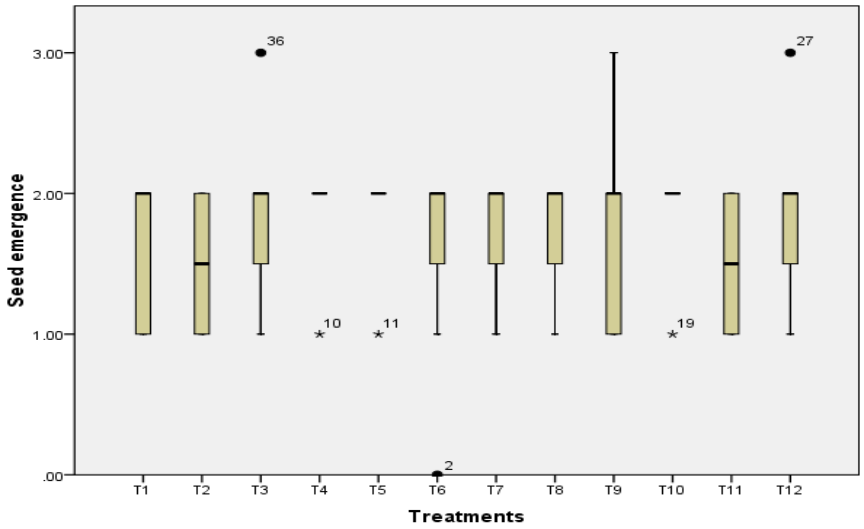
### 4.1.3. Bean seedlings improvement on germination by Trichoderma isolates at 14 days after emergence

In this study the two isolates of *Trichoderma reesei* and *Trichoderma koningii that* were found antagonistic to *Fusarium oxysporum f. sp. phaseoli.,* were further tested individually for their effect on bean seedlings improvement and germination. Seed emergence percentages of bean seedlings planted in soils amended differently are shown in Table 4.5. The effects were highly significant at a $p<0.05$ (Figure ) the highest seedlings emergence was 93% for seed treated with *Trichoderma reesei,* followed by 81% for soils treated with *Trichoderma koningii* and soils treated with manure. Soils treated with mavuno recorded the least germination rate (56%) and were comparable to soils amended with *Fusarium oxysporum* f. sp. *Phaseoli.,* (69%) and control (56%).

**Table 4.5: Effect of treatments with *Trichoderma* species on bean seedling germination in greenhouse**

| Code | *Trichoderma treatments* | Germination index | Germination percentage |
|---|---|---|---|
| T1 | Control | 1.57±0.20 | 69% |
| T2 | *Fusarium oxysporum* | 1.5±0.22 | 56% |
| T3 | *Trichoderma koningii* + *Fusarium* | 1.86±0.26 | 81% |
| T4 | Manure | 1.86±0.14 | 81% |
| T5 | Mavuno | 1.8±0.20 | 56% |
| T6 | *Trichoderma koningii*+ Mavuno | 1.57±0.30 | 68% |
| T7 | *Trichoderma koningii*+ Manure | 1.71±0.18 | 75% |
| T8 | *Trichoderma koningii* | 1.71±0.18 | 75% |
| T9 | *Trichoderma reesei* | 1.71±0.29 | 75% |
| T10 | *Trichoderma reesei* + *Fusarium O.* | 1.88±0.13 | 93% |
| T11 | *Trichoderma reesei*+ mavuno | 1.5±0.19 | 75% |
| T12 | *Trichoderma reesei* + Manure | 1.88±0.23 | 93% |

Treatment of bean seedlings with *Trichoderma* did not influence rate of germination significantly (f value of 1.613). All treatments were able to score between 25% to 50% rate of germination. Differences were observed with treatment of *Trichoderma reesei* + Manure recording the highest with seed emergence germination index of 1.88 followed by *Trichoderma koningii* + *Fusarium oxysporum* (1.86) and manure soil treatments alone with an index of 1.86 (Figure 4.3). Control plots and those treated with *Fusarium oxysporum*, scored the least (1.5).

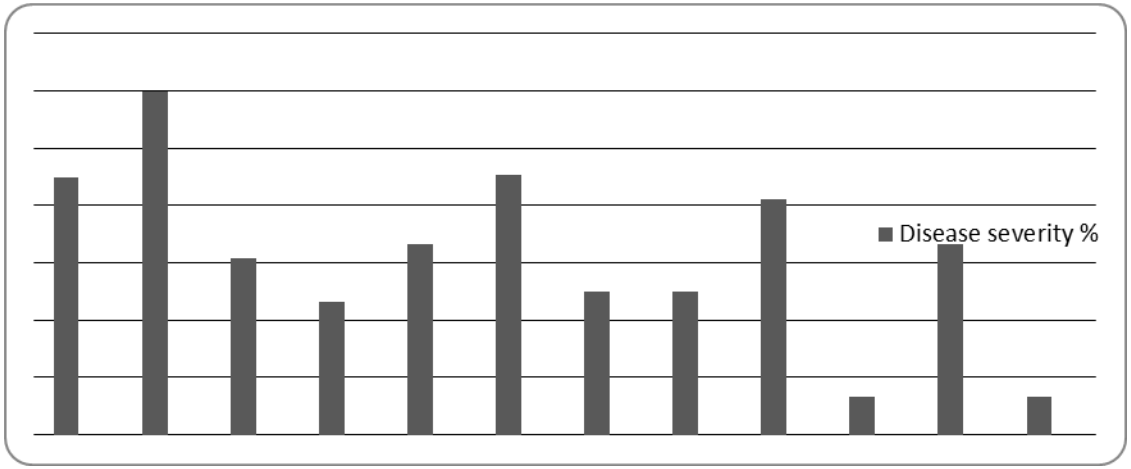



T1=Control, T2=*Fusarium oxysporum,* T3=*Trichoderma koningii* + *Fusarium oxysporum,* T4= Manure, T5= Mavuno, T6= *Trichoderma koningii*+ Mavuno, T7=*Trichoderma koningii*+ Manure, T8=*Trichoderma koningii,* T9= *Trichoderma reesei* , T10= *Trichoderma koningii* + *Fusarium oxysporum,* T11=*Trichoderma reesei*+ mavuno, T12=*Trichoderma reesei* + Manure

**Figure 4.3: Effect of soil treatment with innoculum of *Trichoderma species* on incidence of *Fusariun* wilt diseases of bean under greenhouse conditions:**

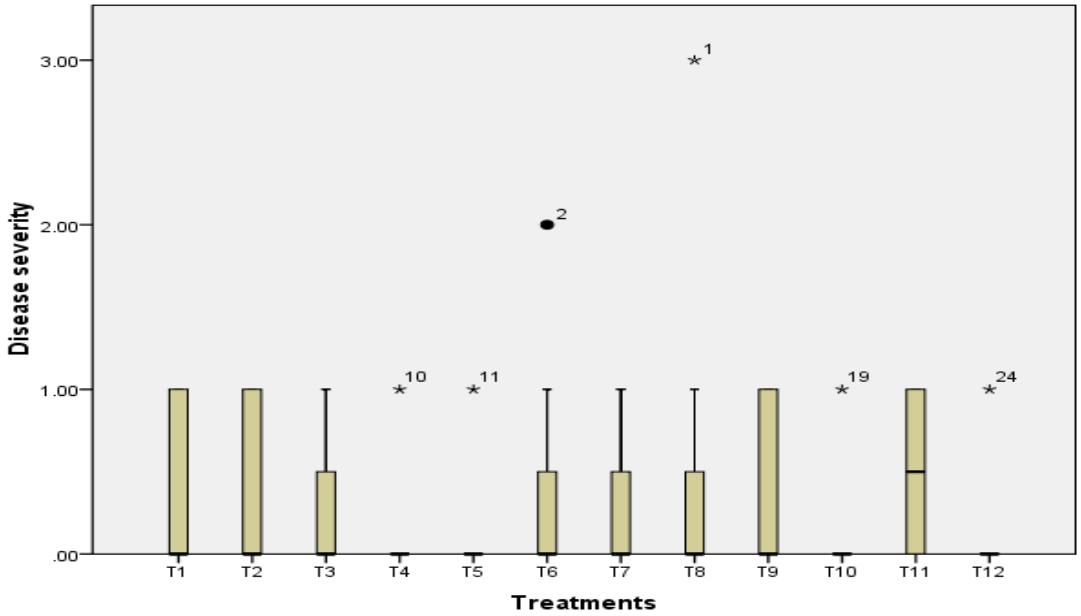
The applications of *Trichoderma* isolates under greenhouse conditions reduced the incidences of *Fusarium* wilt. The application of inoculum with *Trichoderma koningii+ Fusarium oxysporum* (6.7%) and *Trichoderma reesei* + Manure *(6.7%)* to soils at the time of planting gave the highest reduction in disease incidence of *Fusarium* wilt on bean plants. The lowest percentage of disease incidence was obtained by application of *Fusarium oxysporum (60%)* followed by *Trichoderma koningii*+ Mavuno (45%) and control (45%) to infested soil (Figure 4.4 ).

In comparison, bean plants from soil treatments with *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *phaseoli.,* conidia at the time of planting significantly reduced post emergence *Fusarium* wilt disease caused by *Fusarium oxysporum* f. sp. *Phaseoli.,* by 25% to 50% compared to the untreated control at lowest percentage . Application of mavuno alone or manure alone did not show any significant difference in reducing disease severity.




T1=Control, T2=*Fusarium oxysporum*, T3=*Trichoderma koningii + Fusarium oxysporum*, T4= Manure, T5= Mavuno, T6= *Trichoderma koningii*+ Mavuno, T7=*Trichoderma koningii*+ Manure, T8=*Trichoderma koningii*, T9= *Trichoderma reesei* , T10= *Trichoderma koningii + Fusarium oxysporum*, T11=*Trichoderma reesei*+ mavuno, T12=*Trichoderma reesei* + Manure

**Figure 4.4: Effect of *Trichoderma species innoculation* on incidence of *Fusariun* wilt diseases of bean seedlings**




T1=Control, T2=*Fusarium oxysporum*, T3=*Trichoderma koningii* + *Fusarium oxysporum*, T4= Manure, T5= Mavuno, T6= *Trichoderma koningii*+ Mavuno, T7=*Trichoderma koningii*+ Manure, T8=*Trichoderma koningii*, T9= *Trichoderma reesei* , T10= *Trichoderma koningii* + *Fusarium oxysporum*, T11=*Trichoderma reesei*+ mavuno, T12=*Trichoderma reesei* + Manure

**Figure 4.5: Effect of inoculums treatment of *Trichoderma species* on growth of bean seedlings under greenhouse conditions:**

The results for the growth parameters of bean plants are presented in Figure 4.5. The results indicate that there were no significant differences between the *Trichoderma* isolates and the control in terms of root length, shoot length and total fresh weight. However, treatment with manure increased the shoot height by 36.6 mm followed by inoculation of *Trichoderma koningii* + Manure (35.2) and *Trichoderma koningii* (34.7) while inoculation of Mavuno (10.2) *Trichoderma reesei*+ mavuno (11.7) recorded the least growth. Treatment with *Trichoderma reesei* increased the fresh root weight by 1.58g followed by inoculation of *Trichoderma koningii* (1.39). Treatments with Mavuno (0.31) and inoculation with *Trichoderma reesei*+ mavuno (0.34) recorded the least growth. Although Treatments with *Trichoderma reesei* (4.9), *Fusarium oxysporum* (4.8) and *Trichoderma koningii* (4.6) caused higher yield than the infected control (4.5), no significant differences fresh shoot weight were found between them. Treatments with *Trichoderma reesei* (4.9), *Fusarium oxysporum* (4.8) and *Trichoderma koningii* (4.6) were comparable to the Control (4.5). Treatments with Mavuno (2.3) and inoculation with *Trichoderma koningii*+ Mavuno (2.7) recorded the least growth.

**Table 4.6: Growth parameters of bean plant**

| Code | *Trichoderma* Treatments | Shoot height (mm) | Fresh weight root (g) | Fresh weight shoot (g) | Disease severity (%) |
|---|---|---|---|---|---|
| T1 | Control | 29.95±4.67 | 1.05±0.20 | 4.59±0.34 | 0.43±0.20 |
| T2 | *Fusarium oxysporum* | 29.78±2.56 | 1.41± 0.23 | 4.88±0.59 | 0.33±0.21 |
| T3 | *Trichoderma koningii* + *Fusarium oxysporum* | 30.81±2.53 | 1.29±0.19 | 4.07±0.30 | 0.29±0.18 |
| T4 | Manure | 36.36±3.37 | 1.27±0.11 | 3.80±0.25 | 0.14±0.14 |
| T5 | Mavuno | 10.22±2.51 | 0.31±0.12 | 2.38±0.62 | 0.2±0.20 |
| T6 | *Trichoderma koningii*+ Mavuno | 13.19±1.65 | 0.56±0.20 | 2.73±0.28 | 0.42 ±0.3 |
| T7 | *Trichoderma koningii*+ Manure | 35.21±5.44 | 1.1±0.15 | 4.44±0.51 | 0.29±0.18 |
| T8 | *Trichoderma koningii* | 34.72±2.11 | 1.39±0.18 | 4.65±0.29 | 0.57±0.43 |
| T9 | *Trichoderma reesei* | 33.98± 2.52 | 1.58±0.35 | 4.91±103 | 0.43±0.20 |
| T10 | *Trichoderma reesei* + *Fusarium oxysporum* | 33.61±1.78 | 1.23±0.10 | 3.64±0.41 | 0.13±0.13 |
| T11 | *Trichoderma reesei*+ mavuno | 11.79±1.17 | 0.34±0.12 | 2.9±0.41 | 0.5±0.19 |
| T12 | *Trichoderma reesei* + Manure | 27.00±3.08 | 1.20±0.21 | 4.43± 0.43 | 0.13±0.13 |

All bean seedlings grown in soil inoculated with a conidial suspension of *Fusarium oxysporum* f. sp. *phaseoli.,* showed red lesions on hypocotyls and tap roots characteristically distinctive of *Fusarium* root rot. There was reduced root mass with loss of lateral root branching as shown in plate 4.3a. Plants infected with *Fusarium* root rot displayed stunted and

reduced root systems. Plants treated with *Trichoderma reesei* and *Trichoderma koningii* singly and the control with no treatment showed no signs of root wilt (4.3b)

a. Root infected by *F. oxyspurum* f. sp. *phaseoli.,*

b. Healthy control plant

**Plates 4.3: Symptoms of root rot infected common bean plant.**

Observed symptoms of root rot infected common bean plant (Plate 4.3a) and a healthy control plant (Plate 4.3b) at three weeks after soil inoculation; Lateral roots showed brown discolorations and reduced root mass in infected plant

### 4.1.4. Stimulative effect of *Trichoderma* spp. on seed germination of maize seedlings in field experiments

In addition to greenhouse studies, field experiments were performed with a highly antagonistic *Trichoderma* strain to enhance and to complete the greenhouse investigations (Schubert *et al*., 2008a). Germination rates of maize planted in soils amended differently are shown in Table 4.6. The effects were highly significant at $p < 0{,}001$ with f- value of 4.716. The highest rate of seedling emergence was 84% for seeds coated with the *Trichoderma* inoculums and planted

in soil amended with manure followed by 75.94 % for seeds planted in soil amended with manure, followed by 73.0% for seeds coated with *Trichoderma* and planted in soils amended with Mavuno fertilizer. Plots treated with *Trichoderma,* Mavuno, TSP and CAN without the seed coat recorded the least germination rate (70.94%).

**Stimulative effect of *Trichoderma* spp. on seed germination of bean seedlings**

Treatment of bean seedlings with *Trichoderma* did not influence rate of germination significantly (p = 0.687; f-value = 0.683) though differences were observed with Manure + *Trichoderma* treatment of 74.40% and Manure (74.00%) recording the highest germination rates followed by both *Trichoderma* treatments with both TSP/CAN and Mavuno ( 69.60%). Control plots and those treated with *Trichoderma* seed coat alone (62.40%) scored the least (Table 4.7).

Due to the complexity of soil ecology, coupled with year-to-year fluctuations in climate conditions, treatments with microbials are often inconsistent (Quarles N 1993). The inconsistency noted in this study may be attributed to the unpredictable weather conditions in the field at the time of these trials. The above results can be linked to the fact that *Trichoderma* capability to synthesize antagonistic compounds (proteins, enzymes, and antibiotics) and micro-nutrients (vitamins, hormones, and minerals) to enhance biocontrol activity was limited by the environmental conditions (Amsellem N. K *et al*., 1999).

**Table 4.7: Germination improvement by *Trichoderma* harzianum on maize and beans seedlings at fourteen days after emergence**

| | % seed germination after 2 weeks | |
|---|---|---|
| **Treatment** | **Beans** | **Maize** |
| **Control** | 58.00 ±4.90c | 56.80 ± 3.89 a |
| ***Trichoderma harzianum*** | 62.40 ± 5.27d | 70.80 ± 6.09 c |
| **TSP/CAN** | 68.00 ± 4.60c | 70.94 ± 6.14 c |
| **Mavuno** | 68.50 ± 4.43c | 72.40 ± 7 .12 d |
| **Mavuno + *Trichoderma harzianum*** | 69.60 ± 3.49b | 73.00 ± 4.72 b |
| **TSP/CAN + *Trichoderma harzianum*** | 69.60 ±7.22d | 74.33 ± 4.59 b |
| **Manure** | 74.00 ± 2.63a | 75.94 ± 4.63 b |
| **Manure + *Trichoderma harzianum*** | 74.40 ± 0.75a | 84.00 ± 2.01 a |
| **LSD0.05** | 11.11 | 12.25 |

### 4.1.5. Effect of Trichoderma and soil fertility amendment on maize growth

All the fertilizers and inoculum had positive effect on maize growth. The variables that were measured were all greater for the treatment compared to the control. This difference was highly significant at $p<0.0001$ with f value of 13.149, 12.748, 7.612, and 17.115 for root collar diameter, root length, stem diameter and stem length respectively (Table 4.8). Effect of *Trichoderma* on maize plant development was obvious with plots treated with a combination of TSP + CAN

+ *Trichoderma* recording the largest root and stem diameter and stem length, followed by TSP + Mavuno+ *Trichoderma* fertilizers as depicted in Table 4.8, Maize combination treatments of all the fertilizers performed better when combined with *Trichoderma* inoculum than when applied singly. Control plots recorded the smallest size of plants. Among the fertilizers manure performed the least close to control and Turky's HSD grouped it with control.

**Table 4.8: Growth effect of *Trichoderma harzianum* and soil amendment on maize root and stem**

| | | Root Diameter (mm) | | Root Length (mm) | | Stem Diameter (mm) | | Stem Length (mm) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Std. Error of Mean | Mean | Std. Error of Mean | Mean | Std. Error of Mean | Mean | Std. Error of Mean |
| Control | 54 | 0.14 a* | 0.005 | 9.78 a* | 0.139 | 0.34 a* | 0.026 | 13.31 a* | 0.433 |
| Manure | 54 | 0.15 a | 0.004 | 10.04 ab | 0.103 | 0.35 a | 0.027 | 14.76 ab | 0.363 |
| Manure + *Trichoderma* | 43 | 0.16 abc | 0.005 | 10.96 bcd | 0.204 | 0.46 a | 0.045 | 15.06 ab | 0.495 |
| Mavuno | 54 | 0.18 bc | 0.005 | 11.29 cd | 0.223 | 0.47 a | 0.048 | 16.78 c | 0.467 |
| Mavuno + *Trichoderma* | 47 | 0.18 c | 0.007 | 11.89 d | 0.410 | 0.47 a | 0.039 | 16.64 c | 0.460 |
| *Trichoderma* | 45 | 0.16 abc | 0.005 | 10.30 abc | 0.205 | 0.36 a | 0.025 | 14.47 ab | 0.494 |
| TSP+CAN | 54 | 0.19 cd | 0.005 | 11.69 d | 0.169 | 0.42 a | 0.032 | 17.80 c | 0.420 |
| TSP+CAN + *Trichoderma* | 15 | 0.21 d | 0.011 | 11.29 cd | 0.357 | 0.81 a | 0.092 | 20.82 d | 0.683 |
| Total | 366 | 0.17 | 0.002 | 0.86 | 0.090 | 0.4250 | 0.01429 | 15.78 | 0.188 |

***Figures followed by the same letter are not significantly different according to Tukey's Honestly Significant Difference (HSD) test**

### 4.1.6. Effect of Trichoderma and soil fertility amendment on bean growth

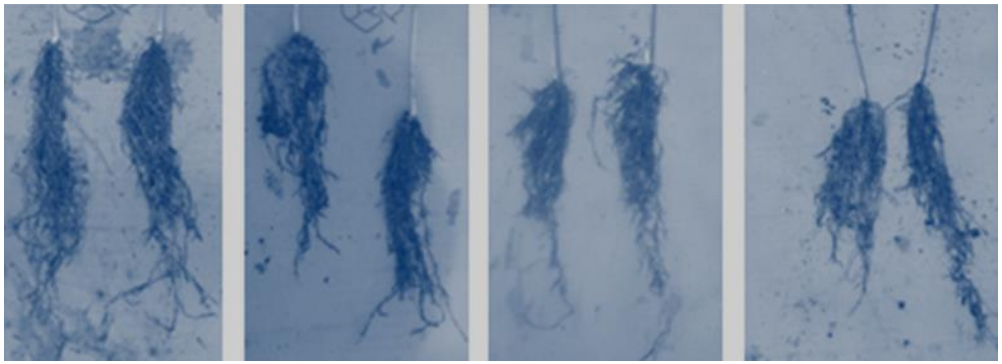
The effect of soil amendments on bean growth was highly significant at $p<0.0001$ with f value of 9.023, 3.805, 8.378 and 15.564 for root collar and length, stem diameter and length respectively. Plots treated with a combination of TSP and CAN record the highest values of root size and stem diameter followed by TSP+CAN+ *Trichoderma* combined, Mavuno + *Trichoderma.* The least was Mavuno as shown in Table 4.9. However, TSP+CAN performed best for beans Tukey's HSD grouped the performance of TSP+CAN as equal to TSP+CAN+ *Trichoderma*, Mavuno+ *Trichoderma* and Manure+ *Trichoderma.*

**Table 4.9: Growth effect of *Trichoderma harzianum* and soil amendment on bean root and stem**

| | | Root collar (mm) | | Root length (mm) | | Stem diameter (mm) | | Stem length (mm) | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | N | Mean | Std. Error of Mean | Mean (Mm) | Std. Error of Mean | Mean | Std. Error of Mean | Mean (Mm) | Std. Error of Mean |
| Control | 53 | 0.11 a* | 0.001 | 9.49 a* | 0.147 | 0.17 a* | 0.004 | 9.29 a* | 0.327 |
| Manure | 53 | 0.12 abc | 0.001 | 9.66 ab | 0.169 | 0.20 ab | 0.007 | 11.04 a | 0.265 |
| Manure + *Trichoderma* | 41 | 0.12 abcd | 0.002 | 9.87 ab | 0.201 | 0.21 b | 0.008 | 12.41 ac | 0.291 |
| Mavuno | 54 | 0.12 bc | 0.002 | 10.24 ab | 0.130 | 0.22 b | 0.007 | 12.59 c | 0.281 |
| Mavuno + *Trichoderma* | 47 | 0.12 cd | 0.003 | 10.35 b | 0.240 | 0.22 b | 0.007 | 12.28 bc | 0.298 |
| *Trichoderma* | 45 | 0.11 ab | 0.001 | 9.854 ab | 0.164 | 0.20 ab | 0.007 | 11.62 bc | 0.167 |
| TSP+CAN | 51 | 0.13 d | 0.003 | 10.46 b | 0.195 | 0.23 b | 0.006 | 11.64 bc | 0.322 |
| TSP+CAN + *Trichoderma* | 17 | 0.12 cd | 0.004 | 10.08 ab | 0.310 | 0.23 b | 0.007 | 12.94 c | 0.355 |
| Total | 361 | 0.12 | 0.001 | 9.99 | 0.068 | 0.21 | 0.003 | 11.58 | 0.119 |

***Figures followed by the same letter are not significantly different according to Tukey's Honestly Significant Difference (HSD) test.**

*Trichoderma* increased lateral root growth of both maize and beans (Plate 4.3). The increase was more pronounced in seedlings grown in plots treated with combination of fertilizer. As observed in this study, the efficiency of biocontrol inoculants when combined with manure and fertilizer had a positive and synergistic effect on maize yield the application of TSP/CAN combined with *Trichoderma* enhanced root growth in beans (Plate 4.4)



**TSP/CAN+ *Trichoderma*** **TSP/CAN** **Control**
***Trichoderma harzianum***

**Plates 4.4: Effect of *Trichoderma* on root growth of bean seedlings**

### 4.1.7. DNA purity and quality

Ratio of OD260/OD280 is determined to assess if the DNA sample is intact. The DNA ratio of absorbance 260/280nm (1.8-1.9 for pure DNA preparations) depicted in figure 4.6 provided an estimate of the purity of the DNA of a sample taken from *Trichoderma* isolates at 257 nm in this study, and therefore suitable for gene expression measurements.

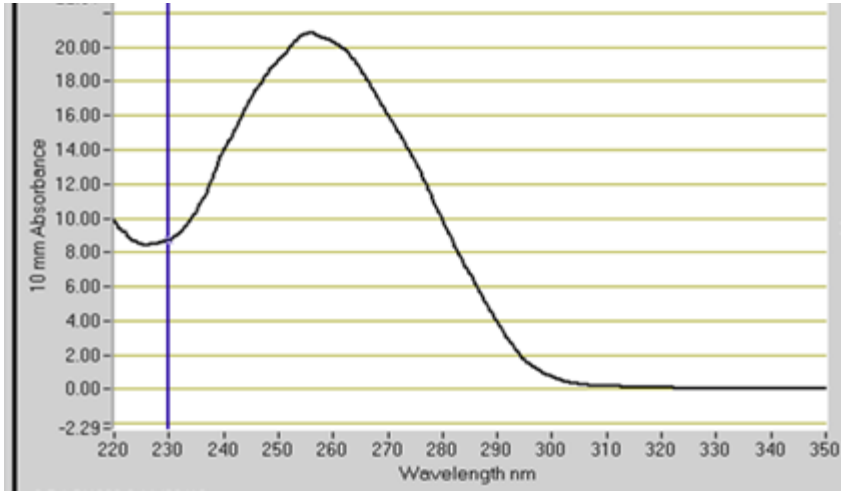
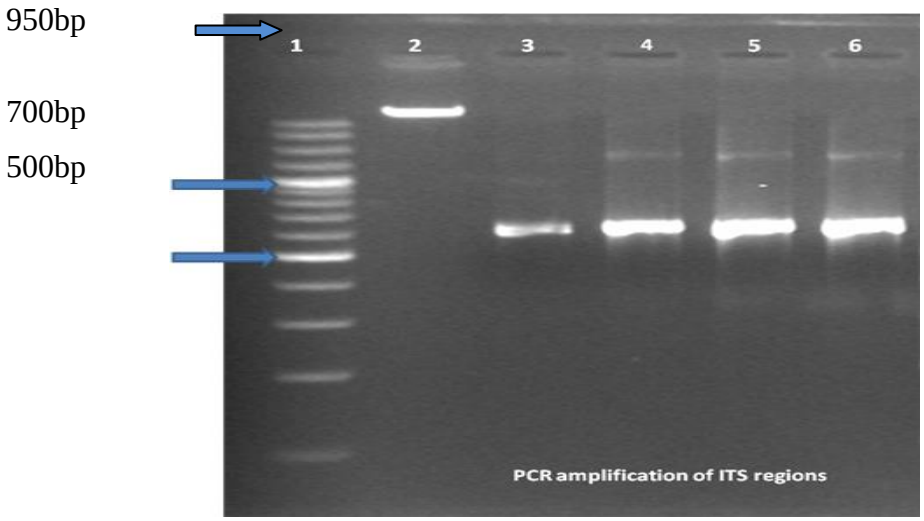



**Figure 4.6: DNA purity analysis and quantification by NanoDrop spectrophotometer**

### 4.1.8 Internal transcribed spacer

The universal primers ITS 1 and ITS 4 were used to amplify the internal transcribed spacer regions of rDNA yielding products of approximately 600bp as estimated by agarose gel electrophoresis as observed by Kumar *et al*., (2011). The universal primers ITS1: 5'- TCT GTA GGT GAA CCT GCG G and ITS2: 5'- TCC TCC GCT TAT TGA TAT GC, and rbp2 primers were used to amplify the internal transcribed spacer regions of rDNA of 5 isolates of *Trichoderma* species and compared with marker DNA bands (Plate 4.5.1). The ribosomal DNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. rDNAs are highly stable and exhibits a mosaic conserved and diverse regions within the genome. The DNA bands indicated the presence of genomic DNA intact band size for *Trichoderma asperellum* (Lane 3), *Trichoderma koningii* (lane 4), *Trichoderma harziunum* (Lane 5) and *Trichoderma atroviride* (Lane 6) in the range of 550bp and *Trichoderma reesei*,

DNA band size in the higher range of 950bp as estimated by agarose gel electrophoresis (Plate 4.5). The ITS PCR was able to detect polymorphism at ITS region of rDNA among the five *Trichoderma* isolates. However, the rbp2 primers was not able to detect the *T. reesei*, DNA band, and therefore only ITS- PCR products were analyzed (Plate 4.5).




**Plates 4.5: PCR amplification of ITS region of *Trichoderma* isolates.**

Lane 1: 1kb DNA ladder, lane2: isolate *T. reesei*, lane 3*: isolate T. asperellum*, lane 4*: isolate T. koningii*, Lane 5: *isolates T. harziunum*, Lane 6*: Isolate T. atroviride*

1 >1_*T.koningii*_ITS_ITS1

GGGTCGAGGGTACGGTTCTAGCGAGCGTACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCTGAAATGGCCCCGCCGGGGGCTCTCAGCCCCGGGCCCGCCTACTGGA
GACACCTCGAACTTTGTCTGATCTAGTGAAGTCTGAATTGATTGTATCGCAATCAGTTAAAACTTTCAAAAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGA
TAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTT
GTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCG
CTTGCCGAACGCAAATCAATCTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

2 >2_*T.resseei*_ITS_ITS1

GGTCGGGTCACTCCAACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGAAAGGCCACCGGACCAAGGCGCCCGCCGGAGGACAACCCTAAATTTTTT
GTATACCCCCTCGCGGGTTTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACC
CTCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGCCCTCCCTTAGCGGGGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCA
TCGGGAGCGCGGCGCGTCCACAGCCGTTAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGGCGGAGGAA

3 >3_*T.asperellum*_ITS_ITS1

GTCGGTCTCTCCAACCCATGTGACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCGGGCCCAAAGCCCACCGGACCAAGGCGCCCGCCGGAGGACCAACCCTTACTATTTTTGTA
TACCCCCTCGCGGGTTTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAAAACGCA
GCGAAATGCGATAAGTAATGTGAATTGCAAAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTC
GAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGCCCTCCCTTAGCGGGGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCG
GGAGCGCGGCGCGTCCACAGCCGTTAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCCGGAGGAA

3 >4_*T.harzianum*_ITS_ITS1

GTCGGGTCACTCCAACCCAATGTGACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCGGGCGTGAAAGGCACCGGACCAAGGCGCCCGCCGGAGGACCAACCCTTAATTTTTT
GTATACCCCCTCGCGGGTTTTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAAC
CCTCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGCCCTCCCTTAGCGGGTGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCG
CATCGGGAGCGCGGCGCGTCCACAGCCGTTAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCCGGAGGAA

5 >5_*T.atroviride*_ITS_ITS1

GTGGGTCAAGCTTCACTCCAACCCAATGTGACGTTACCAAACTGTTCGCCTCGGCGGGATCTCTGCCCCGGGCGGGAGTGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCAAAA
CACTTTTTGTATACCCCCTCGCGGGTTTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCA
TTTCAACCCTCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGCCCTCCCTTAGCGGGGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCA
CACTCGCATCGGGAGCGCGGCGCGTCCACAGCCGTTAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

**Figure 4.7: Sequence products of *Trichoderma koningii* (1), *Trichoderma Reesei* (2), *Trichoderma asperellum* (3), *Trichoderma harzianum* (4), *Trichoderma atroviride* (5) amplified by ITS1 and ITS4**

### 4.1.9. Sequence alignment

The amplified PCR products was eluded and sequenced by automated sequencer which encompass ITS 1, ITS 4 and 5.8s rDNA gene. The sequences (Figure 4.7) obtained were subjected to BLAST search for its identity and confirmation. The multiple nucleotide alignment of ITS regions was analyzed using BioEdit programme. There was substantial disparity in length of ITS sequences between *Trichoderma reesei, Trichoderma harziunum* and *Trichoderma asperellum* (199 bp) and *Trichoderma koningii* and *Trichoderma atroviride* (181 bp) isolates. Isolates of *Trichoderma reesei, Trichoderma harziunum* and *Trichoderma asperellum* showed 100% homology in nucleotide sequence. Conversely *Trichoderma koningii* and *Trichoderma atroviride* isolates showed nucleotide divergence of 2.2% in ITS 1 and 2.26% in ITS 4 region.

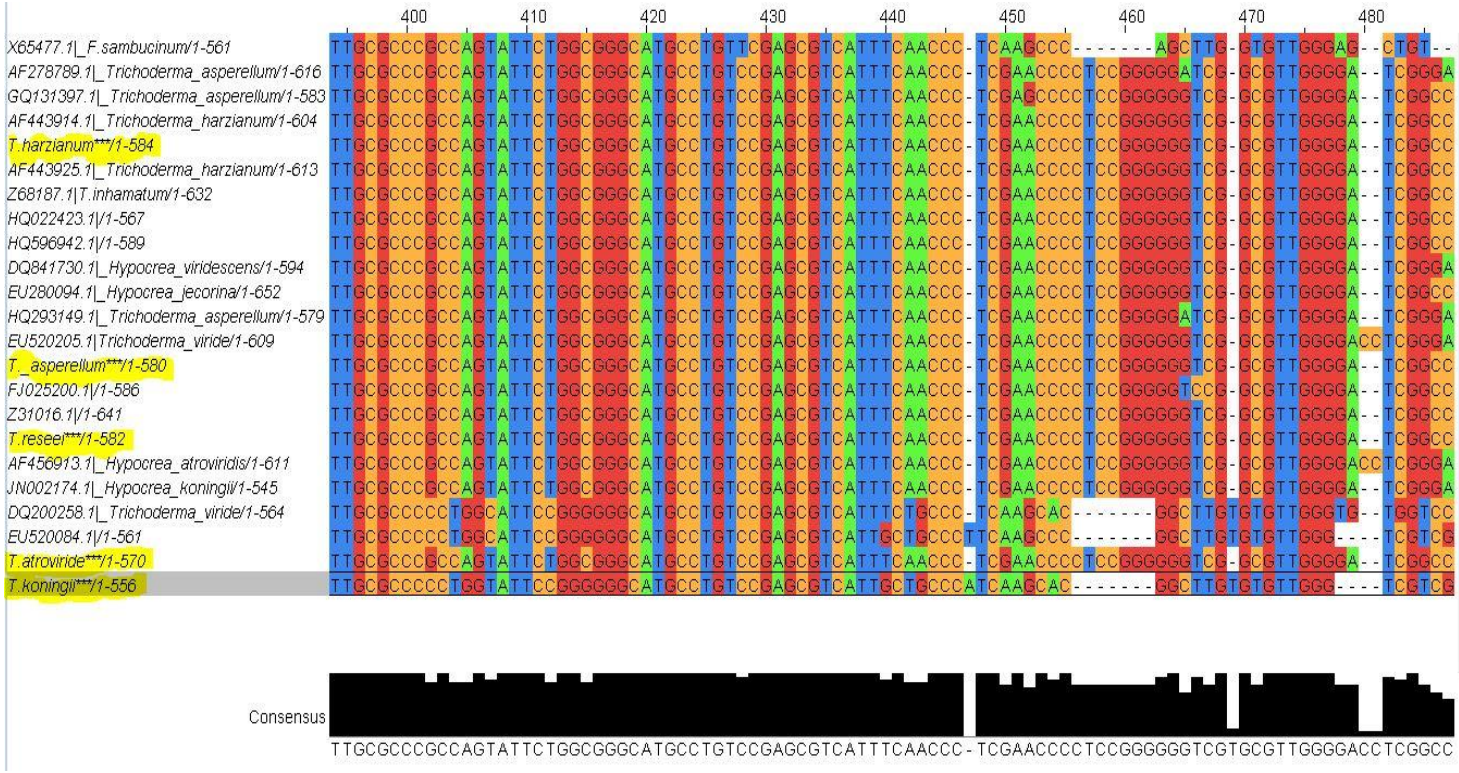



**Figure 4.8: BLAST sequence**

## 4.2 Phylogenetic analysis

To elucidate the genetic closeness of the *Trichoderma* isolates a phylogenetic tree was constructed based on sequence analysis of ITS 1 and ITS 4 regions using the neighbour-joining method using ClustalW 1.6 version ( Figure 4.8 ). A random sequence was used as an out-group to demonstrate the situation of the root. Bootstrap analysis of ITS 1 region with 1000 bootstrap replication demonstrated two main clusters. Isolates of *Trichoderma harzianum* formed one group which supported with a bootstrap value of 99.9%. One cluster represents *Trichoderma atroviride* and *Trichoderma koningii* isolates (clade C) and other cluster represents *Trichoderma asperellum* (clade A). The cluster of *Trichoderma asperellum* divided into subgroup *Trichoderma Reesei* and *Trichoderma harzianum isolates*, similar to the work conducted by Maymon *et al*., (2004). The bootstrap analysis of ITS 4 region also showed similar results. A phylogenetic tree generated from Neighbor-Joining analysis with 1000 replicates was identical in tree topology with a higher bootstrap value of 87-99% thus confirming phylogenetic relationships as shown in Figure 4.9.

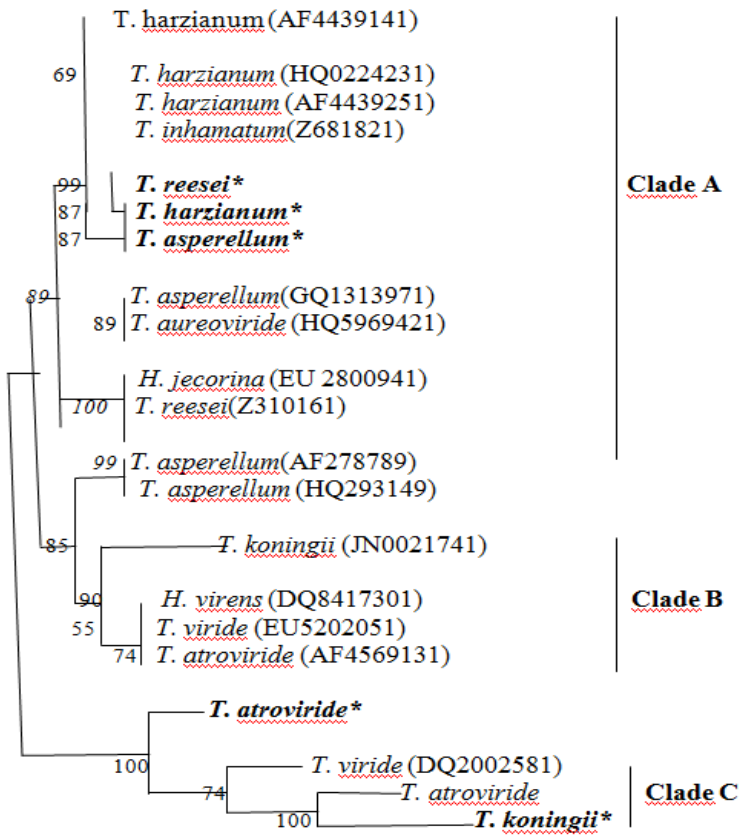



**Figure 4.9: Phylogram showing the phylogenetic relationships of Trichoderma species from NCBI**

*Isolates from this study bolded
*Bar indicates a 5% sequence divergence

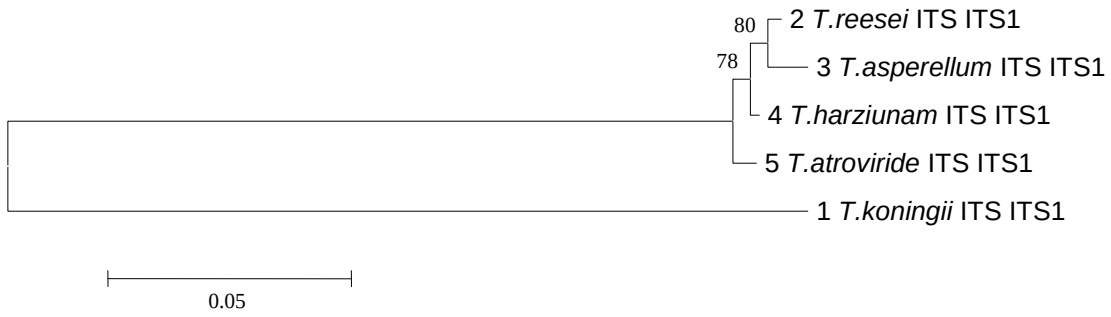



**Figure 4.10: Similarity coefficient of *T. koningii, T. asperellum, T. atroviride, T. reesei, and T. harziunum*, isolates based on ITS1 analysis**

# CHAPTER FIVE

## 5.0. DISCUSSION

### 5.1 Effect on mycelia growth of Fusarium oxysporum f. sp. phaseoli. by Trichoderma spp., on dual culture

*Trichoderma* spp. are widely used in agriculture as biocontrol agents because of their ability to reduce the incidence of disease caused by plant pathogenic fungi, particularly many common soil borne pathogens [21-29]. In present work, the dual culture antagonistic test of five *Trichoderma* isolates against pathogen *Fusarium oxysporum* f. sp. *phaseoli*, a causative agent of *Fusarium* wilt causing root rot in beans confirmed the mycoparasitic antagonism of *Trichoderma.* The five *Trichoderma* isolates tested *in vitro* were effective in suppressing *Fusarium oxysporum* f. sp. *phaseoli*., ($p \leq 0.05$). Similar results were observed by Sharma *et al* (2011); Barakat *et al*., (2006; 2005).

The results of dual culture revealed the maximum (60.0%) growth inhibition of test pathogen with *Trichoderma reesei* followed by *Trichoderma koningii (*55.2%) both with a score of 2 overgrowth (75%) as shown in Figure 2. Further, mycoparasitism of antagonists were observed at day 6 (Figure 4.2 A,B,C,D ). Pattern of growth inhibition of test fungus was observed with *Trichoderma atroviride* (52.8%) and *Trichoderma asperellum* (50.4) antagonists showed moderate inhibition. The isolate of *Trichoderma harzianum* (48.0) showed the lowest inhibitory effect to *Fusarium oxysporum* f. sp. *Phaseoli.*

These results was in confirmation with the finding of Melo and Faull [30], who reported that the *T. koningi* and *T. harzianum* were found to be effective in reducing the radial growth of *R. solani*, *T. koningi* strains produced toxic metabolites with strong activity against *R. solani,* inhibiting the mycelial growth.

Ramezani [31] also documented that *T. harzianum* significantly inhibited the growth of *M. phaseolina. T. viride* and *T. harzianum* had a greater inhibition on *M. phaseolina* than *T. hamatum*. This study showed *Trichoderma reesei* and *Trichoderma koningii* have a better growth inhibition of *Fusarium oxysporum* f. sp. *Phaseoli.,* compared to *Trichoderma atroviride, Trichoderma asperellum* and *Trichoderma harzianum*. *Trichoderma* spp., are known to act through several mechanisms such as hyperparasitism, inhibition and antibiosis. Shalini and Kotasthane (2006) screened seventeen *Trichoderma* strains against *R. solani in vitro*. All strains including *T. harzianum, T. viride* and *T. aureoviride* were inhibited the growth of *R. solani.*

There are several mechanisms involved in *Trichoderma* antagonism namely antibiosis whereby the antagonic fungus shows production of antibiotics, competition for nutrients. In case of mycoparasitism, *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1,3 glucanases and proteases(Gajera *et al.,* 2012). Mycoparasitism involves morphological changes, such as coiling and formation of appressorium- like structures, which serve to penetrate the host. The first contact between *Trichoderma* spp. and pathogen *Fusarium oxysporum* f. sp. *Phaseoli.,* occurred after 4 to 5 days of inoculations, followed by growth inhibition. Differential antagonistic activity has even been observed for various *Trichoderma* spp. which demonstrates semi-specificity in the interaction of *Trichoderma* with its host. The results on light microscopic in this study revealed that *Trichoderma reesei* showed effectively coiling on pathogen *Fusarium oxysporum* f. sp. *Phaseoli*.at day 6 and may start the parasitism earlier during antagonism compared to other *Trichoderma* spp. Similar results have been reported for *T. harzianum* against *Crinipellis perniciosa, Sclerotium rolfsii* and *Rhizoctonia cerealis*.

Sharma, (2011) reported that *T. harzianum* were capable of overgrowing and degrading *Rhizoctonia solani* and *M. phaseolina* mycelia, coiling around the

hyphae with formation of apressoria and hook-like structures. The interaction between five isolates of *Trichoderma spp.,* and the soil-borne plant pathogen *Fusarium oxysporum* f. sp. *Phaseoli.,* was studied using light microscopy which showed efficient coiling process followed by a substantial production of hydrolytic enzymes and abnormal stunted, highly branched hyphal tips and swollen hyphae. Howell, (2003) examined *Trichoderma* that attaches to the pathogen with cell-wall carbohydrates that bind to pathogen lectins. Because of the skeleton of pathogenic fungi cell walls contains chitin, glucan and proteins, enzymes that hydrolyze these components have to be present in a successful antagonist in order to play a significant role in cell wall lysis of the pathogen . In this study, Lysis of *Fusarium oxysporum* f. sp. *Phaseoli.,* hyphae was associated with *Trichoderma reesei* isolate. Filamentous fungal cell wall also contains lipids and proteins. It therefore expected that antagonistic fungi synthesized proteases that may act on the host cell wall. Similar observations were reported by Karmal, (2008) and Haran *et al*., (1996).

### 5.1.1. *Trichoderma* inoculums in controlling *Fusarium* wilt and growth development in bean seedling

Biological control of soil borne plant pathogens is a potential alternative to the use of chemical pesticides, which have already been proved harmful to the environment. There is a growing demand for sound, biologically based pest management practices. Recent surveys of both conventional and organic growers indicated an interest in using biocontrol products (Rzewnicki, 2000; Van Arsdall and Frantz, 2001).

This study was undertaken to determine the potential of locally isolated antagonist fungi to act as biocontrol agent for the management of *F. oxysporum* f sp. *phaseoli* responsible for wilt disease of common beans. The applications of *Trichoderma* isolates under greenhouse conditions reduced the incidences of

*Fusarium* wilt. The application of inoculum with *Trichoderma koningii*+ *Fusarium oxysporum* (6.7%) and *Trichoderma reesei* + Manure *(6.7%)* to soils at the time of planting gave the highest reduction in disease incidence of *Fusarium* wilt on bean plants. The lowest percentage of disease incidence was obtained by application of *Fusarium oxysporum (60%)* followed by *Trichoderma koningii*+ Mavuno (45%) and control (45%) to infested soil (Table ). The results revealed that bean plants from soil treatments with *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *phaseoli.,* conidia at the time of planting significantly reduced post emergence *Fusarium* wilt disease caused by *Fusarium oxysporum* f. sp. *Phaseoli.,* by 25% to 50% compared to the untreated control. Species of *Trichoderma* have been reported as effective colonizers of roots and growth inhibitors of *Fusarium oxysporum* f. sp. *Phaseoli*., possibly be due to fungistatic effect (Cook and Baker, 1983). This ability might be attributed to the secretion of antibiotics by the fungi or other inhibitory substances produced by the antagonists (Howell, 1998; Mondal *et al*., 2000; Vey *et al*., 2001; Landreau *et al*., 2002; Yan *et al*., 2006). The degree of effectiveness varies according to the nature, quality, and quantity of antibiotics/inhibitory substances secreted by the antagonists (Harman, 1998; Kubicek *et al*., 2001; Woo *et al*., 2006; Singh, 2006).

All *Trichoderma* antagonists tested increased the percent seed germination. Differences were observed with treatment of *Trichoderma reesei* + Manure recording the highest with seed emergence germination index of 1.88 followed by *Trichoderma koningii + Fusarium oxysporum* (1.86) and manure soil treatments alone (1.86) (Figure 4.6). Control plots and those treated with *Fusarium oxysporum,* scored the least (1.5). Pieta *et al*. (2003) observed that *Trichoderma harzianum, Trichoderma koningii* and *Trichoderma viride*, used as seed dressing, improved the seedling emergence and health of common bean. Similarly, seeds of common bean were dressed, prior to sowing; with conidia of *Trichoderma*

*harzianum* protected the germinating seedlings and plants against infection by *Fusarium* spp. soil borne pathogenic fungi (Pieta and Pastucha, 2004).

Results of the effect of antagonist on the bean plant growth under pot condition revealed that seedlings grown in antagonist fungi treated soils improved plant height and fresh weight. Treatment with manure increased the shoot height by 36.6 mm followed by inoculation of *Trichoderma koningii* + Manure and *Trichoderma koningii*. Treatment with *Trichoderma reesei* increased the fresh root weight followed by inoculation of *Trichoderma koningii*. Although Treatments with *Trichoderma reesei*, *Fusarium oxysporum* and *Trichoderma koningii* caused higher yield than the infected control , no significant differences fresh shoot weight were found between them.

Investigations suggest that the increased growth response caused by *Trichoderma* isolates may be through modification of the rooting system (Chao *et al*., 1986; Ahmad and Baker, 1987). *Trichoderma* species added to the soil or applied as seed treatments; grow readily along with the developing root system of the treated plant (Harman, 2006; Howell *et al*., 2000). It is well known that *Trichoderma* can parasitize fungal pathogens and produce antibiotics, besides the fungus have many positive effects on plants: increased growth and yield, increased nutrient uptake, increased fertilizer utilization efficiency, increased percentage and rate of seed germination and induced systemic resistance to plant diseases (Harman *et al*., 2004; Harman, 2006). A study carried by Yedidia *et al*. (1999) reported that *Trichoderma harzianum* inoculation improved the uptake of nutrients by the plant at a very early growth stage. Results indicated that all antagonist species significantly reduced the disease incidence in green house pot conditions. These results agreed with Abou-Zeid *et al*. (2003), Pieta and Pastucha (2004), Abd- El-Khair *et al*. (2011) and Otadoh *et al*. (2011). They reported that *Trichoderma album, T. hamatum, T. harzianum, T. koningii, Trichoderma reesei* and *T. viride*

protected the germinating bean seedlings against *Fusarium* spp. and *R. solani* infection.

Some recent studies indicated that these fungi can induce systemic resistance in plants, thus increasing the plant defense response to diverse pathogen attack (Harman *et al.*, 2004; Woo *et al*., 2006; Lorito *et al*., 2010). Alwathnani *et al*., 2011 has similarly demonstrated in his study that *P. citrinum, T. viride* and *T. harzianum* have potential to be used as a biological control agent to protect bean plants from *F. oxysporum* f. sp. *phaseoli.* However, antagonist fungi with the highest level of biocontrol *in vitro* may not perform as well *in vivo* since environmental conditions and competition with other microorganisms are much more restrictive recommending further evaluated in field condition. The present study demonstrated that *Trichoderma reesei* and *Trichoderma koningii* have potential to be used as a biological control agent to protect bean plants from *F. oxysporum* f. sp. *phaseoli.*

### 5.1.2. Effect of *Trichoderma* inoculums on growth of maize and bean seedling in the field

Recently, considerable efforts have been undertaken to study plant growth promotional activity of *Trichoderma* spp., (Hoyos-Carvajal *et al*., 2009; Harman *et al*., 2004; Lo and Lin 2002. The findings in this study indicates that the effects on germination rates of maize planted in soils amended differently were highly significant at $p < 0.001$ with f- value of 4.716. The highest rate of seedling emergence was observed in maize (82.7%) for seeds treated with *Trichoderma* inoculums, however, treatment of bean seedlings with *Trichoderma* did not influence rate of germination significantly ($p = 0.687$; f-value = 0.683). This can be attributed to the effects of climatic conditions on microbiological organisms ( Quarles., 1993) coupled with unpredictable weather experienced in the field during the trials. The environmental conditions can affect *Trichoderma* capability

to synthesize antagonistic compounds and micro-nutrients (Amsellem., *et al*., 1999). Next germination effect for both maize (82.2%) and beans (74.9%) were Positive for seeds coated with the inoculums and planted in soil amended with manure, followed by seeds coated with *Trichoderma* and planted in soils amended with Mavuno fertilizer. Plots treated with TSP and CAN without the seed coat for both maize and beans record the least germination rate. All the fertilizers and inoculum had positive effect on maize growth, though TSP+CAN performed the best for beans. *Trichoderma* have been reported to promoted growth of primary root length and root branching in maize and beans by inducing lateral root growth (Casimiro *et al.*, 2001; Hexon *et al.*, 2009). As for bean performance under fertilizer stress, it has been reported that Trichoderma treatment partially restored the vigour of seedling under different levels of salinity stress (Matsouri *et al*., 2010). The variables that were measured were all greater for the treatment compared to the control. This difference was highly significant for both maize and beans root collar diameter, root length, stem diameter and stem length. Growth effect for both maize ($p<0.0001$) and bean ($p<0.0001$) plants of *Trichoderma* development was obvious with plots treated with TSP and CAN with *Trichoderma* recording the largest root and stem diameter and stem length followed by TSP and lastly Mavuno combined with *Trichoderma* fertilizers. All the fertilizers performed better when combined with *Trichoderma* inoculums than when applied singly. Control plots recorded the smallest size of plants. Among the fertilizers, manure singly performed the poorest. The positive influence of *Trichoderma* on root system architecture would therefore relate to increased yield of plants. *Trichoderma* enhanced root biomass production and increased root hair development has also been reported by Bjorkman *et al.*, (1998) and Harman *et al.*, (2004b).

### 5.1.3. Molecular characterization of local *Trichoderma* isolates

This study was able to demonstrate that the molecular markers could not establish any variation within *Trichoderma* isolates instead grouped all the isolates into two

closely related clusters. The sequence identity matrix of ITS 1 and ITS4 region showed 100% homology among the five local isolates of *Trichoderma*. None of the markers were able to identify distinct variation among *Trichoderma* isolates. It is prominent that the local isolates exhibited less variation in their antagonism due to their genetic similarity. It has been observed by Hermorsa *et al*., (2000) that ITS markers were able to locate the biocontrol strains of certain species within the complex *Trichoderma atroviride* and *Trichoderma koningii*. Therefore it can be interpreted that these isolates taken for the study had no much dissimilarity among them genetically

### 5.1.4. Conclusions

The present study clearly demonstrated that the evaluated isolates of *Trichoderma Reesei* and *Trichoderma koningii* have potential for use as biological control agents to protect bean plants from *Fusarium oxysporum* f. sp. *phaseoli*,. However, local isolates of *Trichoderma Reesei* demonstrated higher virulence. *Trichoderma* isolates can also increase the rate of seed germination, shoot and root growth of maize and bean seedlings under field conditions. The addition of fertilizers further enhanced *Trichoderma* activities. Result of molecular ITS marker demonstrated that rDNA sequences is a reliable clustering strategy for identification of isolates of *Trichoderma* based on similar rDNA sequence from available database. ITS markers could be useful for distinguishing the most efficient isolate for its use in biocontrol.

### 5.1.5. Recommendations

Efficacy studies are only an initial step in the process of developing *Trichoderma* isolates as a biocontrol agent for productivity and sustainability. However, accurate species identification based on virulence efficacy only is difficult due to paucity and similarity of useful molecular characters that can be distinguished through their DNA characters. For effective utilization of local isolates as biocontrol agents, further studies and evaluation under variable soil ecosystems is

recommended. The development and use of molecular identification tools for local *Trichoderma* species can help distinguish the isolates from each other.

## 6.0. REFERENCE

**Abeysinghe, S. (2007).** Biological control of *Fusarium* f. sp. *phaseoli.,* the causal agent of root rot of bean using *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 91: 82-88

**Adams P, De-Leij FA, Lynch JM. (2007).** *Trichoderma harzianum* Rifai 1295-22 mediates growth promotion of Crack willow (*Salix fragilis*) saplings in both clean and metal contaminated soil. Microbial Ecology 54:306-313

**Anasco, N., Uno, S., Koyama, J., Matsuoka, T., Kuwahara, N. (2010):** Assessment of pesticide residues in freshwater areas affected by rice paddy effluents in Southern Japan. *Environmental Monitoring and Assessment* 160: 371-383

**Bais, H. P. Weir, T. L., Perry, L. Gilroy, S. Vivanco. J. M., (2006).** The role of root exudates in rhizosphere interactions with plants and other organisms: Plant Biology 57: 233-266

**Barakat Radwan M., Barakat, Fadel Al-Mahareeq, Mohammed S. Ali – Shtayeh, M. A. (2007).** Biological Control of *Rhizoctonia solani* by Indigenous *Trichoderma spp*. Isolates from Palestine bean insect pests, and soil characteristics 57: 61–71

**Beddington.J\ (2009).** Food security: contributions from science to a new and greener revolution 13: 26–29

**Bell, D.K., Wells, H.D., Markham, C.R. (1982).** *In vitro* antagonism of *Trichoderma* spp against six fungal plant pathogens: Phytopathology, 72: 379–382

**Beneah, Edward Muya and Henry Roimen (2011).** Farmer's knowledge, attitudes and practices (KAP) in Embu and Taita benchmark sites before and after below-ground biodiversity project interventions 22(1): 13–36

**Benitez, T., Rincón, M.A., Limón, M.C. and Codón, C.A., (2004).** Biocontrol mechanisms of *Trichoderma* strains: International microbiology, 7: 249-260

**Brisa, R., Fernando, M. A. Arturo, P.E., Jose M. D. (2007).** The gene activity for new transcription (Eds) transcription of Fusarium oxysporunm is only expressed during infection of common beans 57-65

**Brunner K. Zeilinger S, Ciliento R. Woo SL., Lorito M, Kubicek CP, Mach RL. (2005).** Improvement of the fungal Biocontrol agent *Trichoderma atriviride* to enhance both antagonism and induction of plant systemic disease resistance. Environmental Microbiology 71(7) 3959-3965

**Chakraborty B.N., U. Chakraborty, A. Saha and P.L. Dey and K.Sunar (2010).** Molecular Characterization of *Trichoderma viride* and *Trichoderma harzianum* Isolated from Soils of North Bengal Based on rDNA Markers and Analysis of Their PCR-RAPD Profiles

**Chaudhary S. A. R., Anderson, S .J., Yu, K. (2006).** Comparison of screening methods for Resistance to *Fusarium* Root Rot in common Beans *Phaseolus vulgaris*

**Cooper, J., Dobson, H., (2007).** The benefits of pesticides to mankind and the environment Natural Resources Institute, University of Greenwich, Chatham Maritime

**Dennis, C., Webster, J., (1971).** Antagonistic properties of species groups of *Trichoderma* II. Production of volatile antibiotics: Trans. Br. Mycol. Soc., 57: 41–48. Department of Agriculture, PNG University of Technology

**Dubey, S. C., Suresh, M., Singh, B., (2007).** Evaluation of *Trichoderma* species against *Fusarium oxysporum* fsp. *Ciceris* for integrated management of chickpea wilt. Biol. Contr. 40: 118-127

**Edgar, R.C. (2004)**, MUSCLE: Multiple sequence alignment with high accuracy and high throughput, *Nucleic Acids Research* 32(5), 1792-97

**Eziashi, E. I., Omamo, I.B., Odigie, E. E., (2007).** Antagonism of *Trichoderma viridae* and effects of extracted water soluble compounds from *Trichoderma* species and benlate solution on *Ceratocystis paradoxa*. Afr. J. Biotechnology. 6(4):388-392

**Fatemeh Mastouri, Thomas Björkman, Gary E. Harman,(2010).** Treatment with *Trichoderma harzianum* Alleviates Biotic, Abiotic, and Physiological Stressesin Germinating Seeds and Seedlings 13(1), 26–29

**Filion, M., St-Arnaud, M., Jabaji-Hare, S. H. (2003).** Quantification of *Fusarium solani* f. sp. *phaseoli,* in mycorrhizal bean plants and surrounding mycorrhizosphere soil using realtime: 93:229-235

**Fox, J.E., Gulledge, J., Engelhaupt, E., Burow, M.E., McLachlan, J.A. (2007):** Pesticides reduce symbiotic efficiency of nitrogen-fixing rhizobia and host plants. *Proceedings of the National Academy of Sciences* 104(24), 10282-10287

**Geiger, F., Bengtsson, J., Berendse, F., Weisser, W.W., (2010):** Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. *Basic and Applied Ecology* 11(2):97-105

**Govinda Bhandari (2014).** An Overview of Agrochemicals and Their Effects on Environment in Nepal 39(3), 371–80

**Gwendolyn Ban, Shamsul Akanda and MacquinMaino (2013)** Study on the effectiveness of *Trichoderma* spp. on the growth of bean and tomato plants under greenhouse condition 641–648

**Harman G E, Howell C. R, Vitrebo A, Chet I, Lorito M. (2004a).** *Trichoderma* species opportunistic, arivulent plant symbionts. Nat Rev Microbiol 2:43-56

**Harman G. E, (2006).** Overview of mechanisms and uses of *Trichoderma* spp. Phytopathology 96: 190-194

**Harman, G.E., Howell, C.R, Viterbo A, Chet I, Lorito M (2004a).** *Trichoderma* species: opportunistic, avirulent plant symbionts. Nature Microbiology 2: 43–56

**Hexon, A. C., Lourdes, M. R., Carlos, C. P., Jose, L.B , (2009).** *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. Plant Physiology 149: 1579-159

**Howell, R.C., (2003).** Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. Plant Disease 87: 4-10

**Inbar, J., Hadar, I., (1997**). Fungal antagonists and mycoparasites. In: Wicklow D.T, Soderstrom B (eds) The Mycota IV: Environmental and microbial relationships 281(8): 5197–208

**Irum Mukhtar (2008).** Influence of *Trichoderma* species on seed germination in okra*J. Exp. Bot.* 52: 487–511

**Kamal Sharma *et al*., 2008.** Morphological, Biochemical and Molecular Characterization of *Trichoderma harzianum* Isolates for their Efficacy as Biocontrol Agents 22(1): 13–36

**Kimenju, J.W., Karanja, N. K., Okoth, S.A., Wachira, P. M., P. Jefwa, J.M. Nyamasyo, G., (2010),** Effect of land use in intensification and soil fertility management practices on soil Biodiversity in Embu and Taita, Kenya 51–60

**Kimura M. (1980).** A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16:111-120

**Lorito, M., (2004).** Application of *Trichoderma harzianum* (T22) and *Trichoderma atroviride* (P1) as plant growth promoters, and their compatibility with copper oxychloride: Journal of Zhejiang. University Science 30: 2–8

**M. R. Hermosa, I. Grondona, E. A. Iturriaga, J. M. Diaz-Minguez,C. Castro, E. Monte,* And I. Garcia-Acha (2010)** Molecular Characterization and Identification of Biocontrol Isolates of *Trichoderma* spp 9: 3151–3154

**Mausam, V., Satinde, K. Brar, R. Tyagi, D., Surampalli, R.Y., Valero, J. R. (2007).** Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control 24(2), 74–84

**Medvecky, B. A., Ketterings, Q. A., Nelson, E. B., Muchwezi-Mukiibi., (2001).** Relationships among soil borne bean seedling diseases, *Lablab purpureus* L. and maize Stover residue management, bean insect pests, and soil characteristics in Trans Nzoia District, Kenya 229–237

**Menale, K., Precious, Z. (2009).** The contribution of sustainable agriculture and land management to sustainable development 642–56

**Muriungi J.S, Mutitu E.W,Siboe M.G, (2013).**Biocontrol of *Fusarium* root rot in beans by antagonistic *Trichoderma* fungi.International journal of Agriscience 3(7):550-557

**Mutitu, E. W., Muthomi J. W. (2003).** Occurrences on mycotoxin producing Fusarium species and other fungi on wheat kernels harvested in selected districts in Kenya 11: 184

**Mutsotso, B., Muya, E., Roimen, H., (2011).** Farmers' Knowledge, Attitudes and Practices (KAP) in Embu and Taita Benchmark sites before and after Below Ground Tropical and Subtropical Agro ecosystems, 13: 51 – 58

**Mwangombe A.W., Thiongo, I.G., Olubayo M and Kiprop Z. E. K. (2007).** Occurrence of root rot disease of common bean (*Phaseolus vulgaris* L.) in association with bean stem maggot (*Ophyiomia* sp.) in Embu County, Kenya. Plant Pathology Journal 6 (2): 141 – 146

**Oerke E.C., (2005).**Crop losses to pests.The Journal of Agricultural Science.31-43

**Okoth, S. A., Roimen, H, Mutsotso B., Muya E., Okoth, P. (2007).** Land use systems and distribution of *Trichoderma* species in Embu region, Kenya. Journal of Tropical and Subtropical Agroecosystems, 7:105-12

**Okoth, S., Siameto E., (2010).** Suppression of *Fusarium* spp. in a maize and beans intercrop by soil fertility management 1(2), 035-043

**Pal, R., Chakrabarti, K., Chakraborty, A., Chowdhury, A. (2005):** Pencycuron application to soils: Degradation and effect on microbiological parameters. *Chemosphere* 60(11): 1513-1522

**Potera, C. (2007):** Agriculture: Pesticides Disrupt Nitrogen Fixation. *Environ Health Perspect* 115(12)

**Rojo F.G., Reynoso M.M., Ferez M., Chulze S.N., Torres A.M., (2007).** Biological control by *Trichoderma* species of *Fusarium solani* causing peanut brown root rot under field conditions. *Crop Protection* 26: 549-555

**Saba H.1, Vibhash D.1, Manisha M.1, Prashant K.S.1, Farhan H.1 and Tauseef A.1 (2014)** *Trichoderma* a promising plant growth stimulator and biocontrol agent. 39: 371–80

**Saitou (1987).** The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4:406-Samuels, G.J. (2006). *Trichoderma:* Systematics, the sexual state, and ecology. Phytopathology 96: 195-206

**Saremi(2011). J.** Epidemiological aspects of bean decline disease caused by *Fusarium* species and evaluation of the bean resistant cultivars to disease in Northwest Iran 13: 26–29

**Serpil Savci (2011),** an Agricultural Pollutant: Chemical Fertilizer, *International Journal of Environmental Science and Development, Vol. 3, No. 1, February 2012*

**Shalini S, Narayan KP, Lata, Kotasthane AS (2006).** Genetic relatedness among Trichoderma isolates inhibiting a pathogenic fungi Rhizoctonia solani. Afr. J. Biotechnol. 6: 580-584.

**Sharma. P, Mata. G, (2011),** Complexity of Trichoderma – Fusarium interation and manifestation of Biological control, Pachpendi, Jabalpur- 482001, M.P., India

**Tahia B. Rincón., A. M., Limon, M. C., Codón, A. C. (2004 ).** Biocontrol mechanisms of *Trichoderma* strains 22:13–36

**Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S., (2011).** MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* **(In press)***

**Vadakattu V.S.R. Gupta (2012).** Beneficial microorganisms for sustainable .Agriculture. Microbiology Australia 113-115

**Vinale F., Krishnapillai S., Emilio L., Ghisalberti R. M., Sheridan L., Woo, M, L., (2008).** *Trichoderma*- plant- pathogen interactions. Soil Biology & Biochemistry 40: 1–10

**Whipps, J.M. (2001).** Microbial interactions and Biocontrol in the rhizosphere. Journal of Experimental Botany 52: 487-511

**World health Organization (WHO), Geneva, (1990).** Public Health impact of Pesticide use in Agriculture

**Yedidia (2001).** Effect of *Trichoderma harzianum* on microelement concentration and increased growth of cucumber plants. Plant Soil 235-242

## 7.0. APPENDICES

### APPENDIX I: CALCULATION OF PERCENTAGE INHIBITION OF AVERAGE RADIAL GROWTH BETWEEN *TRICHODERMA REESEI AND FUSARIUM*

Radial growth of pathogens was measured and the percentage inhibition of average radial growth was calculated to the control according to Edington *et al*., 1971 formula (L = [(C-T)/C x100) where; L is the percentage inhibition of radial mycelia growth, C is the radial growth of the pathogen in the control in cm; while T is radial growth of the pathogen in the presence of *Trichoderma* species .

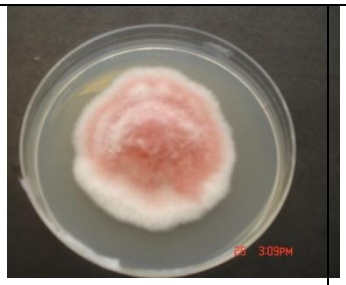
| **Inhibition calculation of mycelia growth at Day 6** | | | | | | |
|---|---|---|---|---|---|---|
| ***Trichoderma koningii*** | ***Fusarium*** | **Control *Fusaruim*** | **Control *Trichoderma*** | **C** | **T** | **L= [(C-T/C] x 100** |
| 4.3 | 1.4 | 1.7 | 5.0 | | | |
| 4.1 | 1.4 | 1.7 | 4.7 | | | |
| 4.1 | 1.4 | 1.5 | 5.2 | | | |
| **4.17** | **1.40** | **1.63** | **4.97** | **1.38** | **1.2** | **55.20** |
| ***Trichoderma reesei*** | ***Fusarium*** | **Control *Fusarium*** | **Control *Trichoderma*** | | | |
| 4.3 | 1.4 | 1.7 | 5.7 | | | |
| 4.5 | 1.4 | 1.7 | 5.7 | | | |
| 4.4 | 1.2 | 1.5 | 5.3 | | | |
| **4.40** | **1.33** | **1.63** | **5.57** | **1.38** | **1.1** | **60.02** |
| ***Trichoderma asperellum*** | ***Fusarium*** | **Control *Fusarium*** | **Control *Trichoderma*** | | | |
| 4.1 | 1.2 | 1.7 | 5.1 | | | |
| 4.2 | 1.7 | 1.7 | 4.9 | | | |
| 4.1 | 1.5 | 1.5 | 4.8 | | | |
| **4.13** | **1.47** | **1.63** | **4.93** | **1.38** | **1.2** | **50.38** |
| ***Trichoderma atroviride*** | ***Fusarium*** | ***Control Fusarium*** | **Control *Trichoderma*** | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4.1 | 1.4 | 1.7 | 5.2 | | | |
| 4.3 | 1.5 | 1.7 | 5.1 | | | |
| 4.3 | 1.4 | 1.5 | 5.2 | | | |
| **4.23** | **1.43** | **1.63** | **5.17** | **1.38** | **1.2** | **52.79** |
| ***Trichoderma harzianum*** | ***Fusarium*** | **Control *Fusarium*** | **Control *Trichoderma*** | | | |
| 3.6 | 1.4 | 1.7 | 4.4 | | | |
| 3.8 | 1.5 | 1.7 | 4.7 | | | |
| 3.8 | 1.6 | 1.5 | 4.5 | | | |
| **3.73** | **1.50** | **1.63** | **4.53** | **1.38** | **1.3** | **47.97** |

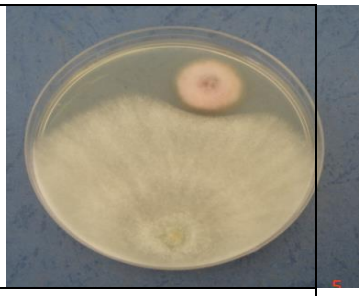
## APPENDIX II: ANTAGONISM TEST ON CULTURE PLATES

Plates were observed daily and growth antagonism ratings classified as follows; (Classes 1 to 5) as follows; Class 1 = the antagonist completely overgrown the pathogen (100 % overgrowth). Class 2 = the antagonist overgrown at least 3/4th of pathogen surface (75% overgrowth). Class 3 = the antagonist colonized on half of the growth of the pathogen (50% overgrowth). Class 4 = the pathogen and the antagonist locked at the point of contact. Class 5 = the pathogen overgrown the mycoparasite.

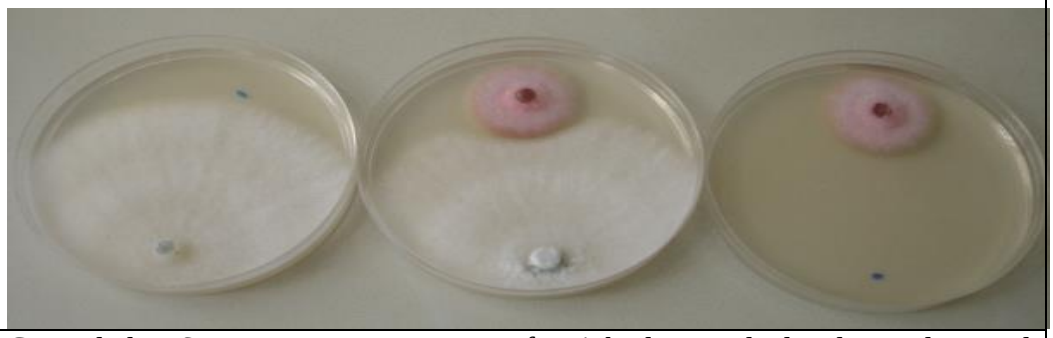
Mean radial growth (mm) between *Trichoderma reesei and Fusarium* at day 6



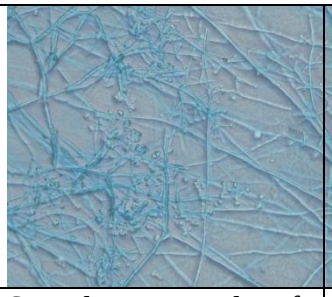
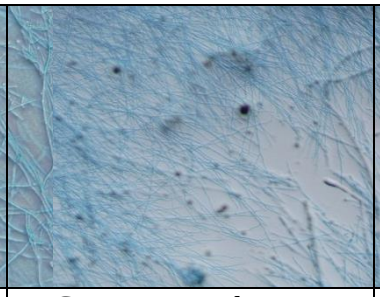
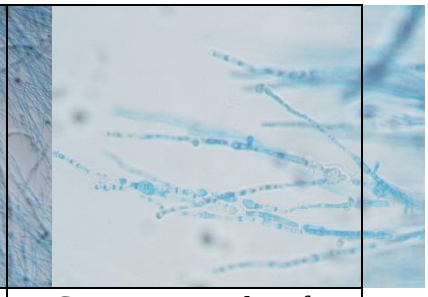
*Fusarium* mycelial growth



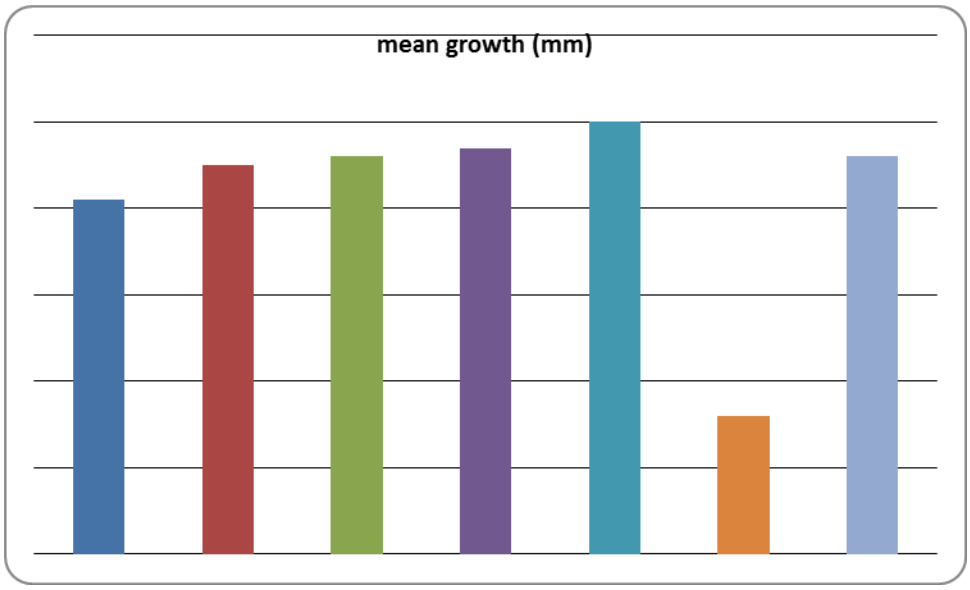
Mean radial growth (mm) between *Trichoderma reesei and Fusarium* at day 5



Control day 6 in repeat experiments of *Trichoderma*, dual culture plate and *Fusarium* mycelia

| | | |
|---|---|---|
|  |  |  |
| Sporulating mycelia of *Trichoderma reesei* | Contact area showing inhibition Mean radial growth (mm) between *Trichoderma reesei and Fusarium* at day 6 | Growing mycelia of *Trichoderma reesei* |

**APPENDIX III: MEAN RADIAL GROWTH OF TRICHODERMA SPECIES AGAINST *FUSARIUM OXYSPORUM* F. SP. *PHASEOLI*,.**



**APPENDIX IV: GREEN HOUSE ANALYSIS**

| **1. control - sterile soil** | | | | | | |
|---|---|---|---|---|---|---|
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight root** | **Fresh weight shoot** | **Disease severity** | **% Disease severity** |
| 1 | 2 | 21.10 | 1.20 | 3.50 | - | |
| 1 | 2 | 54.75 | 0.80 | 5.65 | - | |
| 1 | 0 | 19.00 | 0.40 | 4.10 | 2.00 | |
| 1 | 2 | 32.00 | 1.35 | 4.25 | - | |
| 1 | 1 | 23.00 | 0.50 | 4.70 | 1.00 | |
| 1 | 2 | 24.80 | 1.20 | 4.00 | - | |
| 1 | 1 | 35.00 | 1.90 | 5.90 | 1.00 | |
| 1 | 0 | - | - | - | 2.00 | |
| | **10** | **209.65** | **7.35** | **32.10** | **6.00** | |
| 8 | 16 | 8 | 8 | 8 | | |
| | **63%** | **26.21** | **0.92** | **4.01** | | **45%** |
| **T1** | **1.25** | **26.20625** | **0.91875** | **4.0125** | | |
| **2, *Fusarium* pathogen** | | | | | | |
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight root** | **Fresh weight shoot** | | |
| 2 | 1 | 38.00 | 1.80 | 5.40 | 1.00 | |
| 2 | 1 | D | - | - | 1.00 | |
| 2 | 1 | 21.50 | 0.80 | 3.70 | 1.00 | |
| 2 | 2 | 30.50 | 1.00 | 3.65 | - | |
| 2 | 2 | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 29.00 | 0.95 | 4.60 | - | |
| 2 | 2 | 35.20 | 1.70 | 4.40 | - | |
| 2 | 0 | - | - | - | 2.00 | |
| 2 | 1 | 24.50 | 2.20 | 7.50 | | |
| | **10** | **178.7** | **8.45** | **29.25** | 1.00 | |
| 8 | 16 | 8 | 8 | 8 | **6.00** | |
| | **63%** | **22.34** | **1.06** | **3.66** | | 60% |
| T2 | **1.428571** | **25.52857** | **1.207143** | **4.178571** | | |
| **3. *Fusarium* pathogen and *Trichoderma koningii*** | | | | | | |
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight root** | **Fresh weight shoot** | | |
| 3 | 3 | 29.63 | 1.43 | 3.37 | - | |
| 3 | 2 | 25.70 | 1.25 | 4.20 | - | |
| 3 | 1 | 22.50 | 0.60 | 4.00 | 1.00 | |
| 3 | 1 | 33.90 | 1.60 | 5.10 | 1.00 | |
| 3 | 2 | 29.70 | 0.65 | 2.80 | - | |
| 3 | 0 | - | - | - | 2.00 | |
| 3 | 2 | 43.50 | 1.60 | 4.90 | 0 | |
| 3 | 2 | 30.80 | 1.90 | 4.10 | 0 | |
| | **13** | **215.73** | **9.033333** | **28.46667** | **4.00** | |
| 8 | 16 | 8 | 8 | 8 | | |
| | 0.8125 | 26.96625 | 1.129167 | 3.558333 | | 30.80% |
| T3 | **1.857143** | **30.81857** | **1.290476** | **4.06667** | | |
| **4. Manure** | | | | | | |
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight** | **Fresh weight** | | |

| | | | root | shoot | | |
|---|---|---|---|---|---|---|
| 4 | 2 | 35.75 | 1.25 | 4.50 | - | |
| 4 | 0 | - | - | - | 2.00 | |
| 4 | 2 | 37.50 | 1.45 | 4.80 | - | |
| 4 | 2 | 34.00 | 0.90 | 3.85 | - | |
| 4 | 2 | 21.70 | 1.10 | 3.20 | - | |
| 4 | 1 | 37.00 | 1.00 | 3.90 | 1.00 | |
| 4 | 2 | 52.30 | 1.60 | 3.10 | - | |
| 4 | 2 | 36.30 | 1.60 | 3.20 | - | |
| | **13.00** | **254.55** | **8.90** | **26.55** | **3.00** | |
| 8 | 16 | 8 | 8 | 8 | | |
| | 0.81 | 31.82 | 1.11 | 3.32 | | 23.10% |
| T3 | 1.857143 | 36.36429 | 1.271429 | 3.792857 | | |
| **5. Mavuno** | | | | | | |
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight root** | **Fresh weight shoot** | | |
| 5 | 2 | 11.00 | 0.20 | 1.50 | - | |
| 5 | 0 | - | - | - | 2.00 | |
| 5 | 2 | 1.20 | 0.10 | 1.50 | - | |
| 5 | 0 | - | - | - | 2.00 | |
| 5 | 2 | 16.75 | 0.45 | 2.70 | - | |
| 5 | 2 | 10.80 | 0.10 | 1.50 | - | |
| 5 | 1 | 11.35 | 0.70 | 4.70 | 1.00 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 5 | 0 | - | - | - | - | |
| | 9 | 51.1 | 1.55 | 11.9 | **3.00** | |
| 8 | **16** | **8** | **8** | **8** | | 33.30% |
| | 0.5625 | 6.3875 | 0.19375 | 1.4875 | | |
| T4 | 3 | 17.03333 | 0.516667 | 3.966667 | | |
| ***6. Trichoderma koningii* and Mavuno** | | | | | | |
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight root** | **Fresh weight shoot** | | |
| 6 | 0 | 7.80 | 0.20 | 1.40 | 2.00 | |
| 6 | 2 | 9.25 | 1.70 | 3.40 | - | |
| 6 | 1 | 17.00 | 0.40 | 3.30 | 1.00 | |
| 6 | 2 | 16.75 | 0.45 | 2.70 | - | |
| 6 | 2 | 12.00 | 0.20 | 2.30 | - | |
| 6 | 2 | 19.00 | 0.40 | 2.60 | - | |
| 6 | 0 | - | - | - | 2.00 | |
| 6 | 2 | 10.50 | 0.60 | 3.40 | - | |
| | **11** | **92.3** | **3.95** | **19.1** | **5.00** | |
| 8 | 16 | 8 | 8 | 8 | | 45.40% |
| | 0.6875 | 11.5375 | 0.49375 | 2.3875 | | |
| | 1.571429 | 13.18571 | 0.564286 | 2.728571 | | |
| ***7. Trichoderma koningii* and Manure** | | | | | | |
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight root** | **Fresh weight shoot** | | |
| 7 | 2 | 35.75 | 0.70 | 4.05 | - | |
| 7 | 0 | - | - | - | 2.00 | |
| 7 | 1 | 10.20 | 1.60 | 6.70 | 1.00 | |

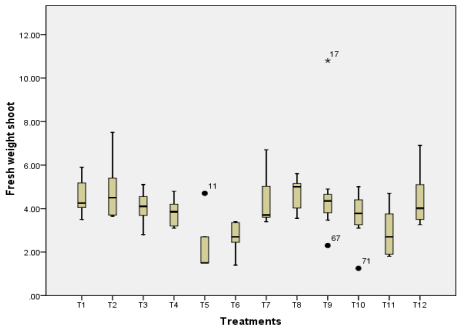
| | | | | | | |
|---|---|---|---|---|---|---|
| 7 | 1 | 55.00 | 0.70 | 6.00 | 1.00 | |
| 7 | 2 | 32.20 | 0.80 | 3.50 | - | |
| 7 | 2 | 34.30 | 1.10 | 3.40 | - | |
| 7 | 2 | 49.00 | 1.20 | 3.70 | - | |
| 7 | 2 | 30.00 | 1.60 | 3.70 | - | |
| | **12** | **246.45** | **7.7** | **31.05** | **3.00** | |
| 8 | 16 | 8 | 8 | 8 | | |
| | 0.75 | 30.80625 | 0.9625 | 3.88125 | | 25.00% |
| | 1.714286 | 35.20714 | 1.1 | 4.435714 | | |
| ***8. Trichoderma koningii*** | | | | | | |
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight root** | **Fresh weight shoot** | | |
| 8 | 2 | 39.75 | 0.95 | 5.30 | - | |
| 8 | 2 | 26.00 | 1.10 | 3.55 | - | |
| 8 | 2 | 32.75 | 1.65 | 5.00 | - | |
| 8 | 2 | 29.25 | 1.05 | 4.00 | - | |
| 8 | 2 | 36.50 | 1.05 | 4.05 | - | |
| 8 | 1 | 41.00 | 1.70 | 5.60 | 1.00 | |
| 8 | 0 | - | - | - | 2.00 | |
| 8 | **1** | **37.80** | **2.20** | **5.00** | **3.00** | |
| | 12 | 243.05 | 9.7 | 32.5 | | |
| 8 | 16 | 8 | 8 | 8 | | |
| | 0.75 | 30.38125 | 1.2125 | 4.0625 | | 25% |
| | 1.714286 | 34.72143 | 1.385714 | 4.642857 | | |
| ***9. Trichoderma reesei*** | | | | | | |

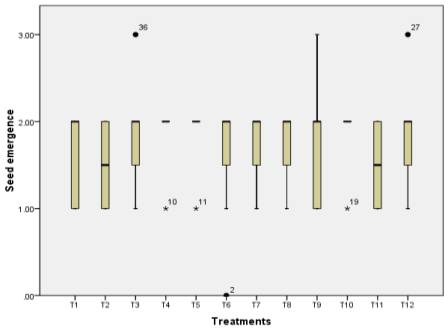
| Treatment no. | Seed emergence | Shoot Height | Fresh weight root | Fresh weight shoot | | |
|---|---|---|---|---|---|---|
| 9 | 1 | 30.50 | 1.90 | 4.35 | 1.00 | |
| 9 | 1 | 34.65 | 3.50 | 10.80 | 1.00 | |
| 9 | 2 | 32.00 | 1.25 | 4.15 | - | |
| 9 | 3 | 30.33 | 1.00 | 3.47 | - | |
| 9 | 1 | 47.00 | 1.50 | 4.90 | 1.00 | |
| 9 | 2 | 37.00 | 1.10 | 4.40 | - | |
| 9 | 2 | 26.40 | 0.80 | 2.30 | - | |
| 9 | 0 | - | - | - | 2.00 | |
| | **12** | **237.8833** | **11.05** | **34.36667** | **5.00** | |
| 8 | 16 | 8 | 8 | 8 | | 41% |
| | 0.75 | 29.73542 | 1.38125 | 4.295833 | | |
| | 1.714286 | 33.98333 | 1.578571 | 4.909524 | | |
| ***10. Trichoderma reesei and Fusarium*** | | | | | | |
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight root** | **Fresh weight shoot** | | |
| 10 | 1 | 35.00 | 1.15 | 4.00 | 1.00 | |
| 10 | 2 | 39.50 | 1.75 | 4.70 | - | |
| 10 | 2 | 29.50 | 1.40 | 3.55 | - | |
| 10 | 2 | 35.75 | 1.00 | 5.00 | - | |
| 10 | 2 | 24.00 | 1.10 | 1.25 | - | |
| 10 | 2 | 35.00 | 1.00 | 3.10 | - | |
| 10 | 2 | 38.30 | 1.00 | 4.10 | - | |
| 10 | 2 | | | | | |

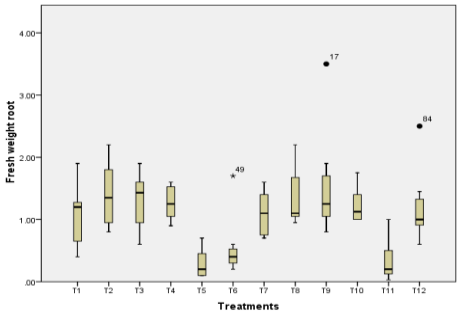
| | | | | | | |
|---|---|---|---|---|---|---|
| | | 31.80 | 1.40 | 3.40 | - | |
| | 15 | 268.85 | 9.8 | 29.1 | - | |
| 8 | 16 | 8 | 8 | 8 | | |
| | 0.9375 | 33.60625 | 1.225 | 3.6375 | | 6.70% |
| | 1.875 | 33.60625 | 1.225 | 3.6375 | | |
| ***11. Trichoderma reesei and Mavuno*** | | | | | | |
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight root** | **Fresh weight shoot** | | |
| 11 | 2 | 19.00 | 1.00 | 4.50 | - | |
| 11 | 2 | 11.35 | 0.70 | 4.70 | - | |
| 11 | 1 | 7.50 | 0.05 | 1.80 | 1.00 | |
| 11 | 1 | 11.50 | 0.03 | 1.80 | 1.00 | |
| 11 | 2 | 12.00 | 0.20 | 2.40 | - | |
| 11 | 1 | 9.50 | 0.30 | 3.00 | 1.00 | |
| 11 | 2 | 11.50 | 0.20 | 2.00 | - | |
| 11 | 1 | 12.00 | 0.20 | 3.00 | 1.00 | |
| | **12** | **94.35** | **2.68** | **23.2** | **4.00** | |
| 8 | 16 | 8 | 8 | 8 | | 33.30% |
| | 0.75 | 11.79375 | 0.335 | 2.9 | | |
| | 1.5 | 11.79375 | 0.335 | 2.9 | | |
| ***12. Trichoderma reesei and Manure*** | | | | | | |
| **Treatment no.** | **Seed emergence** | **Shoot Height** | **Fresh weight root** | **Fresh weight shoot** | | |
| 12 | 3 | 35.27 | 0.97 | 3.83 | 0 | |
| 12 | 1 | 20.00 | 1.00 | 5.20 | 1.00 | |
| 12 | 2 | 20.00 | 1.45 | 3.25 | - | |
| 12 | 2 | | | | | |

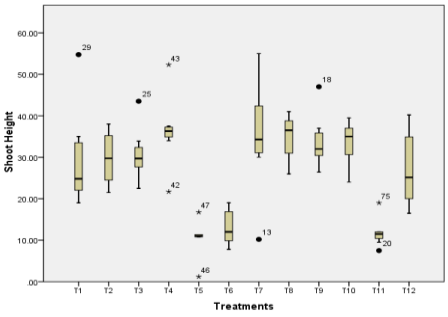
| | | | | | | |
|---|---|---|---|---|---|---|
| | | 28.00 | 1.20 | 4.20 | - | |
| 12 | 2 | 40.20 | 0.85 | 5.00 | - | |
| 12 | 2 | 22.30 | 1.00 | 3.50 | - | |
| 12 | 2 | 34.50 | 0.60 | 3.50 | - | |
| 12 | 1 | 16.50 | 2.50 | 6.90 | - | |
| | **15** | **216.77** | **9.566667** | **35.38333** | **1.00** | |
| 8 | 16 | 8 | 8 | 8 | | 6.70% |
| | **0.9375** | **27.09625** | **1.195833** | **4.422917** | | |
| | 1.875 | 27.09625 | 1.195833 | 4.422917 | | |
| | Percentage disease incidence= Number of infected plant X 100 | | | | | |

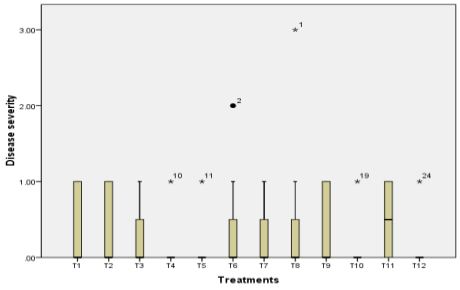
## APPENDIX V: STIMULATIVE EFFECT OF *TRICHODERMA* SPP. ON BEAN SEEDLING GROWTH AND DEVELOPMENT



## APPENDIX VI: PCR AMPLIFICATION OF ITS OF *TRICHODERMA* ISOLATES

Fragments of the ribosomal DNA (rDNA) containing the internal transcribed spacer (ITS) regions, ITS1 (5'- TCT GTA GGT GAA CCT GCG G) and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC) were amplified and sequenced as described by Zhang *et al*., 2005.

Primers ITS1 and ITS4 were synthesized by Microsynth AG Switzerland. The PCR reagents were supplied by Bioneer AccuPower® HotStart PCR PreMix. Genomic DNA was amplified by mixing the template DNA (30 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase.

Polymerase Chain Reaction amplifications was performed on each sample in a total volume of 20µl reaction mix containing 1x PCR Buffer (200 mM Tris-HCl, (pH 8.0); 500 mM KCl), 1 unit of HotStart DNA Polymerase, 200 µM of each dNTP, 0.5 µM reverse and forward primers and 1 µl of 30 ng template DNA.

## APPENDIX VII: SEQUENCE DATA ANALYSIS

The sequenced PCR product was aligned with ex-type strain sequences from NCBI GenBank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm. Multiple alignment parameters used were gap penalty= 10 and gap length penalty =10. Both of these values are aimed to prevent lengthy or excessive number of gaps. The fault parameters were used for the pair wise alignment. The use of Clustal W determines that once the gap is inserted it can only be removed by editing. Therefore, the final alignment adjustments were made manually in order to remove artificial gaps.

The phylogenetics analysis was completed using MEGA package (version 4.01). No gaps generated through insertion or deletions, including equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic inference was performed by UPGMA method. Bootsrap tests with 1000 replications were conducted to examine the reliability of the interior branches and the validity of the tress obtained. There were a total of 22 positions in the dataset. The phylogenetics analysis was conducted in MEGA 4 as described by Tamura *et al*., 2011.