DETERMINATION OF THE EFFECTIVENESS OF TRIANUM-P® (*Trichoderma harzianum*) AND TRICHOTECH® (*Trichoderma asperellum*) IN THE MANAGEMENT OF LATE BLIGHT DISEASE OF TOMATOES

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A thesis submitted to the Graduate School in partial fulfilment for the requirements of Master of Science Degree in Plant Pathology of Egerton University

EGERTON UNIVERSITY

NOVEMBER, 2016

DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work and has not been shared, presented or submitted wholly or in part for any award in any Institution.

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RECOMMENDATION

We confirm that this thesis was prepared under our supervision and has our approval to be submitted for examination as per Egerton University regulations.

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DEDICATION

This thesis is dedicated to my parents, my children (Ashley, Blessed and Shantel), my sisters and brothers for their love, support and patience.

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I shall remain eternally grateful to God, my parents, children, siblings, relatives and friends for their support during the entire period of study. My sincere gratitude goes to my supervisors: Prof. Nancy Mungai, Prof. Daniel Otaye and IITA supervisors Dr. Cargele Masso and Dr. Moses Thuita for their valuable time, patience and constant guidance throughout the study period. Their contribution in developing research ideas, encouragement, in depth discussions of the subject and mentorship is also gratefully acknowledged. I would also like to thank staff members of the Departments of Crops, Horticulture and Soils and Department of Biological Sciences of Egerton University for their support. My appreciation goes to the entire staff of COMPRO II Project under the International Institute of Tropical Agriculture (IITA) for their support especially the laboratory section for the technical skills. I thank COMPRO II project for generously funding the study.

ABSTRACT

The general objective was to evaluate the efficacy of *Trichoderma* species to promote the growth of tomato and to manage late blight disease under *in vitro* and *in vivo* conditions. A collection of 18 *Trichoderma* spp. were isolated from two commercial products (Trichotech® and Trianum-P®) that should have the antagonistic species *Trichoderma asperellum* and *Trichoderma harzianum* respectively. Cultural and molecular approaches were used to characterize the products. Differences on the growth rate and colony characterization including time of first appearance of green conidia and colony appearance were recorded on Potato dextrose agar and Rose Bengal media. Microscopy was done where the shape of the phialides, mass of conidia and branching of the conidiophores were observed. Displayed intra-specific variability related to *Trichoderma* spp. remains within the limit of each species and the growth curve of *Trichoderma* spp. isolates was distinctive of species on PDA medium. Molecular characterization of the genomic DNA was done using ITS1 and ITS4 universal primers. Isolates that had a single PCR band pattern at 600bp were selected for sequencing. The sequences were aligned using the MEGA 6.0 software and the BLAST program NCBI. Multiple sequence alignment (MSA) was done using cluster W and phylogenetic tree was generated using Sea-view. A greenhouse 3×5 factorial experiment, arranged in a randomized complete block design (RCBD) with three replicates was also set to determine the efficacy of Trianum-P®, Trichotech® and their combination in controlling *P. infestans* and production enhancement of tomato plants. Tomato biomass, number of leaves and height were used to assess growth for 12 weeks at 3 week intervals. The data was subjected to ANOVA and the means tested by LSD. Cluster analysis of the data demonstrated two major clusters that is, A and B, Cluster A, consisted of *Meyerozyma caribbica* (GK9) while cluster B was further subdivided into three clusters. Sub clusters B_1, B_2 and B_3 were *T. asperellum*, *T. longibrachiatum* and *T. harzianum* respectively. These clusters were supported at a bootstrap stability of 100% on the basal position. An inhibiting action was observed on the *Phytophthora infestans* mycelial growth of the isolates by the effect *T. asperellum* (82.9%), *T. harzianum* (71.5%) and the control was 0.0%. The greenhouse results showed that *Trichoderma* spp. strains reduced late blight of tomato plant by 6.6% (Trichotech® +Trianum-P®),12.7% Trichotech® and 17.2% Trianum-P®. Trichotech® resulted to an increase in tomato plant biomass thus significantly different compared to the other treatments.

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LIST OF ABBREVIATIONS AND ACRONYMS

AI	Active ingredient
BCAs	Bio control agents
BLAST	Basic Local Alignment Search Tool
CFUs	Colony forming units
CIP	International potato centre
CRD	Complete randomized design
GLM	General linear model
GUS	Glucuronidase gene
IPM	Integrated pest management
LSD	Least significant difference
MEGA	Molecular Evolutionary Genetics Analysis
MSA	Multiple sequence alignment
NCBI	National centre for biotechnology information
NPK	Nitrogen, phosphorus and potassium
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
RBA	Rose Bengal agar

RCBD	Randomized complete block design
SAHN	Sequential agglomerative hierarchical nested
TAE	Tris acetic acid
UPGMA	Unweighted pair group method with arithmetic average

CHAPTER ONE
INTRODUCTION

1.1 Background information

Tomato (*Lycopersicon esculentum* L) belongs to the Solanaceae family of plants, which contains many important food crops, including potatoes and eggplants /Aubergine (*Solanum melongena).* Tomato is an important crop throughout the world and is among the source of employment in a country's agricultural related economic activities (Fontenelle *et al.*, 2011). *Phytophthora infestans* is the most notorious species of genus *Phytophthora* which causes pre- and post-emergence damping-off and late blight of Solanaceae plants (Loliam *et al*., 2012). Among the diseases that affect tomatoes, late blight is the most important disease in Kenya which attacks all the above ground parts of the plant (Tumwine *et al*., 2002). The disease caused by *P. infestans* is difficult to control by chemical means, because of the high virulence of the pathogen and the increasing resistance to chemical fungicide. There is also an increasing urge to produce organic foods and therefore such chemical product-use is on the decline (Kim *et al.,* 2000).

There are toxicological concerns of the environment and the appearance of new resistant strains of pathogens due to the use of synthetic fungicides. Attention is increasingly being given to development of alternative control agents that are safer; more biodegradable hence eco-friendly and less costly for the control of plant pathogens (Parizi *et al*., 2012). Biological control of soil borne plant pathogens is a potential alternative strategy to substitute the use of environment-degrading inorganic fungicides (Perveen and Bokhari, 2012). Biological control is based on mycoparasitism and hyper-parasitism between microorganisms such as endophytic bacterial and fungal antagonists (Parizi *et al*., 2012). Fungal species belonging to the genus T*richoderma* are ubiquitous organisms or they exist in all environments and are found often on decomposing organic material as well as in plant rhizospheres. These opportunistic and non-pathogenic plant symbionts can very easily be isolated and grown readily on a variety of substrates. Certain *Trichoderma* species have the ability to induce plant resistance against some plant pathogens, promote plant growth and improve photosynthetic activity of plants (Mbarga, 2012).

Fungal species of the genus *Trichoderma* (teleomorph Hypocrea-Ascomycetes: Hypocreales), are considered as potential biological control agents (BCAs), and the modes of action include mycoparasitism, antibiosis, competition, enzyme activity and induced plant defence (Carvajal *et al*., 2009). *Trichoderma* has considerable activity against many pathogenic fungi, for example, *Fusarium, Pythium, Sclerotinia, Botrytis* and *Rhizoctonia* in a wide range of environmental conditions. The biological control has been obtained with *Trichoderma* isolates applied singly and in combination with other antagonists (Rosa and Herrera, 2009). Growth stimulation is manifested by increases in biomass, productivity, disease stress resistance and increased nutrient absorption. Increased crop productivity associated with the presence of *Trichoderma* has been observed in a broad range of plant species, such as carnation, *Chrysanthemums, Tagetes, Petunias*, cucumbers, eggplants, peas, and peppers (Carvajal *et al*., 2009).

During past decades, several potential bio-control organisms such as, *Trichoderma, Penicillum, Bacillus, Streptomycetes* and *Actinomyetes* have been isolated, characterized and commercialized, and thus, bio-control of plant diseases has received more consideration in plant disease control than using chemical controls (Rojan *et al*., 2010). *Trichoderma* as well as other fungal bio-control agents are being considered as alternatives to chemical fungicides. Unfortunately, some bio-products are contaminated and have a low count of microorganisms thus resulting in poor quality and less performance. This is linked to inappropriate strains and inefficient production technology (Alam, 2000). Molecular characterization provides a detailed source of data that can assist scientists in the study of identity, relatedness, diversity and selection of proper candidates for biological control (Gajera and Vakharia, 2010).

There are several BCA products of *Trichoderma* spp. in the agro-chemical stores containing conidia of *Trichoderma* spp. as active ingredients. These include Trianum-P® which is a wettable powder that contains 1.0×109 colony forming units (CFUs) per gram of *Trichoderma harzianum*, strain T22 and Trichotech® which is also a wettable powder that contains 4.0×109 colony forming units (CFUs) per gram of *Trichoderma asperellum*, isolate H22. According to the manufacturer, these *Trichoderma* spp. increase the resistance of plants to stress caused by diseases, sub-optimal feeding and watering regimes or climatic conditions, and increases nutrient uptake. They can also enhance the growth and development of roots and above-ground parts thus increase in yield. (www.koppert.com, 2008; www.finlays.net, 2011).

1.2 Statement of the problem

In Kenya, the management of late blight affecting tomatoes is a major production challenge because of the ineffective fungicide application strategies that have resulted in huge yield losses. Late blight, caused by *Phytophthora infestans* is one of the most economically important disease of tomato causing up to 90% losses in cool and wet weather conditions in the country. The disease affects leaves, stems and fruits of tomato and can lead up to 100 % losses if no control measures are taken.

Prevention and control of late blight disease has been based on different strategies, mainly on the use of synthetic fungicides which has not been successful to eradicate the pathogen due to pathogen resistance to these kinds of pesticides. On the other hand, excessive utilization of synthetic pesticides results in high production costs hence financial constrain and pollution of the environment as well as presence of pesticide residues in agricultural products which is a health hazard to the consumers.

Biological control of plant pathogens has received increasing attention as a promising supplement or alternative to chemical control. There are several BCA products in the market containing conidia of *Trichoderma* spp. as active ingredients, unfortunately, their poor performance is a major factor in their limited use by farmers which might be as a result of poor quality. This is primarily linked to inappropriate strains and inefficient production technology. Success of biological control in agriculture is primarily dependent on the quality of the bio-control products from the producers to the farmers. The poor quality of such products results in low yields, losses and general food insecurity. This study contributes to improvement in quality of biocontrols and an increase in agricultural products.

1.3 Objectives

1.3.1 General objective

Determination of the efficacy of Trianum-P® (Trichoderma harzianum) and Trichotech® (*Trichoderma asperellum*) as commercial products in the management of late blight of tomatoes to enhance production among farmers.

1.3.2 Specific objectives

1. To isolate and screen *Trichoderma* spp. in different production lots of Trianum-P® and Trichotech® using cultural and molecular approach.
2. To determine the antagonistic effect of the isolated *Trichoderma harzianum* and *Trichoderma asperellum* strains from Trianum-P®and Trichotech® respectively on *Phytophthora infestans.*
3. To determine the efficacy of Trianum-P®, Trichotech® and their combination on growth of tomato plants and late blight management of tomatoes in the greenhouse using soil from different agro-ecological zones in Kenya.

1.4 Hypotheses

1. *Trichoderma harzianum* in Trianum-P® and *Trichoderma asperellum* in Trichotech® were present as specified by the manufacturer.
2. Isolated *Trichoderma harzianum* and *Trichoderma asperellum* strains from Trianum-P®and Trichotech® respectively had the ability to antagonize *Phytophthora infestans.*
3. Trianum-P®, Trichotech® and their combination had a positive effect on growth of tomato plants and late blight treatment of tomatoes in different agro-ecological zones in Kenya.

1.5 Justification

A concern due to risks of non-target impacts of synthetic fungicides is increasing in Kenya as shown by increasingly more stringent standards on fungicide residue levels. Chemical fungicides can cause acute and chronic human health effects, contamination of atmosphere, ground and water surfaces. Other non-target organisms such as earthworms, termites and ant colonies have been affected negatively. Hence incorporation of biological control into an integrated pest management (IPM) programme reduces the environmental and safety challenges posed by insecticides, fungicides and other chemical control agents. They also reduce the cost of pest and pathogens management in the long-term. It is important that bio-control products meet the stipulated standards for effective pathogens management. The market share for bio-control products has steadily increased and this has seen an increase of such products in the market some of which are not of consistent quality. It is therefore important to assess the quality and efficacy of bio-control agents available in the market.

Phytophthora infestans is difficult to control by chemical means, because of the high virulence of the pathogen and increasing resistance to fungicides. Biological control is an alternative to the use of chemical pesticides. Among fungi used for control of soil borne pathogens are various species of *Trichoderma* spp which have received the most attention. *Trichoderma* spp. are fungal bio-control agents that attack a range of phytopathogenic fungi. *Trichoderma* spp. alone or in combination with another antagonistic species can be used in biological control of several plant diseases.

CHAPTER TWO

LITERATURE REVIEW

2.1 Constraints in tomatoes production

Tomato (*Lycopersicon esculentum* L.) is one of the most widely grown vegetable food crops not only in Kenya but in other parts of East Africa and the whole world at large, which is second only to potato. In the horticultural industry tomato is among the key crops and one of the leading processed vegetable crops in Kenya. Kenyan tomatoes satisfy the interior demand and have a strong export and processing demand. Despite this, there are seasonal scarcities. In addition, traditionally the tomatoes fruits have been marketed fresh picked from the field and are the best-selling fresh market vegetable crop in Kenya. The crop is an important dietary component, relatively nutritious and contains quantities of vitamin C and therefore used in salads, cooked as a vegetable or made into tomato paste and tomato sauce. It therefore contributes to improved nutrition and well living for both rural and urban inhabitants (Boriss and Bronke, 2005; Passam *et al*., 2007).

Low tomato productivity is caused by a combination of several factors such as nutritional disorders especially nitrogen deficiency which can cause restriction in growth rate and chlorosis on the oldest leaves, while excess nitrogen may cause poorly coloured, puffy fruits (Kirimi *et al*., 2011). The major soil pests that attack tomatoes are cut worms (*Agrotis* spp.), which suck plant sap and result in stunted growth of the plant (Waiganjo *et al*., 2006). Late blight (*Phytophthora infestans*), bacterial wilt (*Pseudomonas solanacearum*), early blight (*Alternaria solani*), root knot nematodes (*Meloidogyne* spp.) and some viral diseases are major constraints to tomatoes cultivation. Late blight is the most important disease in Kenya which attacks all above ground parts of tomatoes (Tumwine *et al*., 2002). The major tomato varieties grown in Kenya are Cal-J, Rio grande, Nema 1200, Nema 1400, Onyx, Eden, Roma and Monyalla F1 (Waiganjo *et al*., 2006). According to Musyoki *et al* (2006), tomato varieties with reduced susceptibility to late blight are Eden, Onyx and Rio Grande while Monyalla F1 can easily be affected by late blight especially at early stage if early spraying is not done. Cal J is also not resistant to the disease and is one of the tomato varieties commonly grown in Kenya because of its high demand which is due to its long shelf-life.

2.2 Disease cycle and symptoms

The fungal-like organism that causes late blight, *Phytophthora infestans,* affects both tomatoes and potatoes. *P. infestans* spores can be easily dispersed by wind from one area to another hence introduced on plant material (such as potato seed pieces), or on tomato transplants. Late blight of tomato or potato thrives under cool, wet conditions. Upon germinating, the spores may produce swimming structures that are ideal for spreading the disease under wet conditions (Eduardo *et al*., 2007). The ideal climatic conditions for infection, development and spread of late blight disease are night temperature of 10-16°C and day temperature of 16-21°C accompanied by rain, fog, or heavy dew and a relative humidity of 91-100% (Nelson, 2008).

Under moist conditions, the green to brown lesions on leaves may be ringed with the white fungus that causes late blight. Fruit also may be affected. Fruit quality may be affected after foliage dies, or the fungus may directly penetrate the fruit. The disease can spread and kill plants rapidly; so much so that it is common for affected tomatoes or potatoes to appear as if they had experienced a frost although none has occurred (Eduardo *et al*., 2007). Under these favourable conditions, symptoms may appear on plant tissues three to five days after infection and spore production begins on infected tissue one to two days later (Nelson, 2008).

Disease development is stopped at 30°C and above, but the pathogen can remain dormant in infected tissue and re-emerge if temperature changes to the disease favourable ranges. If the climatic conditions remain favourable to late blight, the crop can be destroyed within a week of the first appearance of symptoms (Seebold, 2011). Late blight is mainly controlled by repeated fungicide applications, which is time-consuming, costly and harmful to the environment. The control of this disease is also conducted via integrated management practices including sanitation, crop rotation, utilization of certified seeds and breeding programs for resistant cultivars. In the recent years, natural products such as plant extracts and microorganisms have been tested in the context of organic food production (Douglas, 2010).

2.3 Biological control of plant diseases

According to Haggag and Mohammed (2007), biological control of plant diseases is defined as the use of beneficial and natural microorganisms such as specialized fungi, bacteria or yeast to

attack and reduce the effects of undesirable organisms such as, fungi, nematodes, bacteria and parasitic plants. This strategy of control is eco-friendly, inexpensive and compatible with different models of agriculture such as organic, biological, and integrated pest and pathogen management (IPM) programs. Biological control is an alternative to the use of chemical pesticides. Biological fungicides may act to suppress the population of the pathogenic organisms through competition with the pathogens, stimulated plant growth, which may allow plants to quickly outgrow any effects from the pathogen or damage the pathogen by means of toxins produced. Bio-control agents are derived from natural materials such as animals, plants, bacteria, and fungi (Gomathinayagam *et al*., 2010).

Trichoderma harzianum has been reported to be effective against pathogenic fungi *Phytophthora megasperma* f. sp. glycinea root rot on soybean, *Gaeumannomyces gramminis* of wheat, *Sclerotium rolfsii* southern stem blight of tomatoes and *Cylindrocladium scoparium* damping-off of red pines (Chaverri *et al*., 2003). Past study indicate the ability of *T. asperellum* in inhibiting the mycelial growth of pathogen and production of cell wall degrading enzymes thus inhibiting growth of *Sclerotium rolfsii* (Rasu *et al*., 2012). According to Tondje *et al* (2007) *T. asperellum* has the ability to mycoparasitize on *Phytophthora* spp. that causes black pod disease of cocoa (*Theobroma cacao*). This includes *Phytophthora capcisi*, *Phytophthora palmivora* and *Phytophthora megakarya*. There is little information on the effect of *Trichoderma harzianum* and *Trichoderma asperellum* against *Phytophthora infestans*. However, among the *Trichoderma* spp. observed to have mycoparasitic action against *P. infestans* is *Trichoderma viride* (Zegeye *et al*., 2011).

2.4 Biopesticides used against *Phytophthora infestans*

There are potential bio-control agents that have been used to manage late blight these includes *Penicillum aurantiogriseum* and *Stachybotrys atra* to leaflets of potato plants with *P.infestans* has been carried out (Binyam, 2014). The most consistent results of biological control of late blight have been achieved with the *Xenorhabdus* spp. (Mizubuti *et al*., 2007). Bio control agents such as *Pseudomonas* species, fungal antagonists and *Arbuscular mycorrhizal* (AM) fungi have been used against diseases caused by *Phytophthora* (Gallou *et al*., 2011).

2.5 Role of *Trichoderma* spp. as bio-control agents

For many years, species of the filamentous fungus *Trichoderma* have been known to be able to attack and metabolize plant disease causing fungi, and therefore they are used as bio-control agents. *Trichoderma* spp. has the ability to survive under unfavourable conditions predominating in ecological niches like salt marshes (Omann and Zeilinger, 2010). These species belong to the genus *Trichoderma* (Hypocrea, Ascomycota, Hypocreales, and Hypocreaceae) (Druzhinina *et al*., 2010). According to Chaverri *et al* (2003), these fungi are easily recognized by their brightly coloured fructifications which are due to their green ascospores. Species within this genus are some of the most widely-utilized fungal biological control agents (BCAs) in agriculture.

2.6 Mode of action of *Trichoderma* spp.

These fungi act as mycoparasites by secreting hydrolytic enzymes such as chitinase and glucanase, which break down cell walls. *Trichoderma* spp. also produces antibiotic compounds which influence bio control capacity. Their rapid growth allows these species to directly compete for space and nutrients with phytopathogens while also indirectly fighting infection through stimulating plant growth and inducing acquired resistance mechanisms in the plant (Beaulieu *et al*., 2010; Druzhinina *et al*., 2010). Plant growth enhancement by *Trichoderma* isolates are as a result of different mechanisms such as exudation of plant growth regulators and their similarity with the fungi solubilization of phosphates, micronutrient and minerals such as Fe, Mn and Mg that have important role in plant growth, secretion of exogenous enzymes, siderophores and vitamins. The effect of *Trichoderma* isolates on plant growth and development is important, especially in nursery, because improvement of plant vigour to overcome biotic and abiotic stresses results in the production of stronger plants and increase in plant productivity and yields (Azarmi *et al*., 2011).

2.7 Effectiveness of bio controls in soil from different ecological zones

The widespread but limited ability of soils to suppress the growth or activity of soil borne pathogens have been referred to as ‘general suppression’, or ‘nonspecific antagonism’, or ‘biological buffering’. General suppression is related to the total microbial biomass in soil, which competes with the pathogen for resources or causes inhibition through more direct forms of antagonism. General suppression often is enhanced by the addition of organic matter, certain

agronomic practices, or the build-up of soil fertility, all of which can increase soil microbial activity (Weller *et al*., 2002).

The effect of soil type that has different nutrient status on the stimulatory efficiency of microbial inoculants may be important for successful root inoculation and plant growth stimulation (Egamberdieva, 2011). The chemical composition of soil controls the survival and biological activity of microorganisms (Kang and Mills, 2006). Hence the effectiveness of bio-control agents depends on several parameters, which include soil texture, water content, and pH and crop history. The genus *Trichoderma* includes the most common saprophytic fungi in the rhizosphere and is found in almost any soil (Hassanein, 2012).

Trichoderma spp. application therefore, is determined by environmental stress that could affect not only their survival in the soil, but also their ability to maintain their bio-control capacity. The effect of low temperatures, water activity and pH, the presence of heavy metals, pesticides or antagonistic bacteria have been tested as stress factors that may affect wild or mutant bio-control *Trichoderma* strains (Montealegre *et al*., 2009). Improvements in nutritional and other soil properties through the addition of compost promote the establishment, colonization and survival of *Trichoderma* spp. (Kibaki *et al*., 2012). The organically managed soils have a higher population of *Trichoderma* spp. than the inorganic one which involves application of synthetic fertilizers, fungicides and insecticides which inhibit the activity of these beneficial microorganisms (Liu *et al*., 2008).

2.8 Quality of bio-control products

The safety, quality and efficacy of a biological product are primarily the responsibility of the manufacturer. The Government usually establishes procedures for assuring that Biological products intended for use in the country are of adequate quality, safety and efficacy. In Kenya, the Kenya Plant Inspectorate Service (KEPHIS) is a state body that was established in 1996 for quality assurance. The corporation's activities and services involve offering inspectorate services on all matters related to plant health and quality control of agricultural inputs and produce. This responsibility should have a firm statutory basis backed by legislation (WHO, 2005). Despite these requirements, some bio products are contaminated and have a low count of microorganisms thus resulting to poor quality and performance. This is linked to inappropriate strains and

inefficient production technology (Alam, 2000). To ensure that the products of microbial BCAs do not affect the environment, human beings and other living organisms, quality control parameters should be set (Ramanujam *et al*., 2010). Quality is highly variable and a number of inoculants presently on the market, in countries where quality controls are not systematically practiced, are of poor quality (Catroux *et al*., 2001).

2.9 Assessment of biological control agents

Biological assays carried out in the laboratory are effective and rapid methods for identifying strains with bio control activity. Previous methods employed to identify strains of *Trichoderma* spp. are the use of dilution plates and staining techniques [based on reporter genes such as β-glucuronidase gene (GUS)]. These methods do not distinguish different strains (Rubio *et al*., 2005). Hence genetic variation of the population must be examined to assess the impact of the release of a single isolate on the natural population (Hintz *et al*., 2001). Molecular markers provide an immense source of data that can assist in the study of identity, relatedness, diversity, and selection of proper antagonists for biological control. Furthermore, molecular markers offer a means of constructing quality control tests that are essential throughout the developmental processes of these bio-control agents (BCAs) (Amal *et al*., 2005). Identification of variability within *Trichoderma* spp. isolates by morphological characters is very difficult, hence the adoption of the molecular techniques (Joerg *et al*., 2015).

From the literature, there is little information on the quality and performance of *Trichoderma* spp. as biocontrol agents in farming systems. The findings from this study about the effectiveness of the *Trichoderma* spp. on *P. infestans* will add more information on the mechanisms of action and uses of these fungi. In addition, the direct effects of these fungi on plant growth and development will be important for agricultural uses and for understanding the roles of *Trichoderma* spp. in natural and managed ecosystems. The quality and efficacy of Trichotech® and Trianum-P® was also verified for the purpose of quality assurance.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Objective 1: Isolation and screening of *Trichoderma* spp in different production lots of Trianum-p® and Trichotech® using cultural and molecular approach

3.1.1 Study area

Isolation of the microorganisms and testing for antagonism was carried out in the soil microbiology laboratory in Crop, Horticulture and Soil department Egerton University. Molecular work was carried out at IITA laboratory in Nairobi and gene sequencing was done at the International Livestock Research Institute (ILRI). Greenhouse trial will be conducted at the Biological Sciences glasshouse Egerton University, Njoro campus.

3.1.2 Sample Collection

The commercial bioproducts were requested for by Commercial Products II (COMPRO II) from private companies that are involved in manufacturing and supplying them. A total of 18 samples were collected, nine for Trianum-P® and nine for Trichotech®.

Table 1: Details of *Trichoderma* strains

Strain No.	Name of Bioagent	Strain code/Batch no	Source
GK1	*T. harzianum*	87LV3536	Trianum-P®
GK 2	*T. harzianum*	87LV3536	Trianum-P®
GK 3	*T. harzianum*	87LV3536	Trianum-P®
GK 4	*T. harzianum*	87TP2433	Trianum-P®
GK 5	*T. harzianum*	87TP2433	Trianum-P®
GK 6	*T. harzianum*	87TP2433	Trianum-P®
GK 7	*T. harzianum*	87TPP2433	Trianum-P®
GK 8	*T. harzianum*	87TPP2433	Trianum-P®
GK 9	*T. harzianum*	87TPP2433	Trianum-P®
GK 10	*T. asperellum*	51/013	Trichotech®
GK11	*T.asperellum*	51/013	Trichotech®
GK12	*T.asperellum*	51/013	Trichotech®
GK13	*T.asperellum*	51/013	Trichotech®
GK14	*T.asperellum*	51/013	Trichotech®
GK15	*T.asperellum*	51/013	Trichotech®
GK16	*T.asperellum*	51/013	Trichotech®
GK17	*T.asperellum*	51/013	Trichotech®
GK18	*T.asperellum*	51/013	Trichotech®

3.1.3 Isolation of *Trichoderma harzianum* from Trianum-P® and *Trichoderma asperellum* from Trichotech®

To isolate *Trichoderma* strains, a standard procedure as described by Carter, (2011), a serial dilution technique was followed for each sample (Trianum-P® and Trichotech®). Trianum-P® is a wettable powder that contains 1.0×109 colony forming units (CFUs) per gram of Trianum-P® strain T22. Trichotech® is also a wettable powder that contains 4.0×109 colony forming units (CFUs) per gram of Trichotech®, isolate H22. To determine the number of colony forming units (CFUs), one gram of each sample was suspended in 9ml of physiological water to make a full strength stock solution of 10ml in a test-tube. A ten-fold dilution series was made, 0.1 ml from selected dilutions ($10^{-}4$, $10^{-}5$, $10^{-}6$ and $10^{-}7$) was spread plated on Rose Bengal agar supplemented with 1g/L chloramphenicol and incubated at 28°C in the dark.

Distinct morphological characteristics were observed for identification, and the plates stored at 4°C (Rahman *et al*., 2009). Macroscopic examination was done by growing the *Trichoderma* spp. on PDA agar for five days. The mode of mycelia growth, colour, and changes of medium pigmentation for each isolate was examined every day. For microscopic study, examination of the shape, size, arrangement and development of conidiophores or phialides was done to identify *Trichoderma* spp (Chakraborty, 2010). Following the classification of Rifai (1969), these species were identified. The isolated *Trichoderma* spp.was stored at -20°C in a 10% glycerol solution until use (Hayfa *et al*., 2009).

3.1.4 Extraction of *Trichoderma* spp. DNA

Genomic DNA of the isolates was extracted by method described by Lee and Taylor with minor modifications (Yao *et al*., 2008). The four days old mycelia from cultures was scraped using a sterile microscope slide and transferred to sterile Eppendorf tubes. This was incubated in 500 µL lysis CTAB buffer (2.0 g CTAB, 100 mM Tris HCL pH 8.0, 1.4 M NaCl, 20 mM EDTA and 1 g PVP 40) at 65 °C for one hour in water bath followed by centrifugation at 12,000 rpm at 25°C for15 minutes. Supernatant was extracted and an equal volume of water saturated phenol was added and inverted thrice followed by centrifugation at 12,000 rpm at 25°C for 15 minutes.The supernatant was treated by adding equal volume of phenol: Chloroform: Isoamyl

alcohol (25:24:1) and inverted thrice followed by further centrifugation at 12,000 rpm at 25°C for 15 minutes.

The supernatant was extracted, an equal volume of chloroform: isoamyl alcohol (24:1) was added and inverted thrice followed by further centrifugation at 12,000 rpm at 25°C for15 minutes. Isopropanol 500 µL was added to the supernatant in order to precipitate the DNA and the content inverted thrice to mix before keeping the tubes overnight at -20°C. DNA was pelleted by centrifugation at 12,000 rpm at 4°C for 15 minutes. Pellets were washed with 70% ethanol followed by centrifugation at 12,000 rpm at 25°C for15 minutes. Pellets were air dried and re-suspended in 50 µL sterile double distilled water (Chakraborty *et al*., 2010).

3.1.5 DNA amplification

Internal transcribed spacer rDNA gene (ITS1-5.8S- ITS4) from the isolates was amplified using the polymerase chain reaction (PCR) with universal primers ITS1 (5' TCC-GTA-GGT-GAA-CCT-GCG-G 3') and ITS4 (5' TCC-TCC-GCT-TAT-TGA-TAT-GC 3') developed by White *et al*., 1990) by the PCR conditions described by Sim *et al*., 2010 with some modifications. The PCR amplification reactions were performed with a total 25 µl of the reaction that comprises of 12.5 µl commercial master mix, 8.5 µl of sterile distilled water, forward and reverse primers each 1 µl and 2 µl of template.

PCR was programmed with an initial denaturing at 94°C for 5 minutes followed by 36 cycles of denaturation at 94°C for 1 minute, annealing at a gradient of 60°C-55°C for 1 minute and extension at 70°C for 3 minutes and the final extension at 72°C for 10 minutes in a Thermocycler. A good amplification was checked by loading 3 µl of PCR product mixed with loading buffer (5 µ1) containing 0.25% bromophenol blue, 40% w/v sucrose in water and then loaded in 2% Agarose gel with 3.4g ethidium bromide for examination with horizontal electrophoresis. This was done at a 150volts for one hour. The gel was viewed under UV trans-illumination and photographed using gel documentation system.

3.1.6 Sequencing of PCR DNA products

Isolates that shared similar PCR banding patterns at 600bp were closely grouped for sequence analysis. Two master mixes were used; one containing 2 µl of the template and the

other contains 4 µl of the template for the isolates that formed weak bands. Universal primers ITS1 (5' TCC-GTA-GGT-GAA-CCT-GCG-G 3') and ITS4 (5' TCC-TCC-GCT-TAT-TGA-TAT-GC 3') developed by White *et al*. (1990) and PCR conditions modified by Sim *et al*., 2010. The PCR amplification reactions was performed with a total 25 µl of reaction that comprised of 12.5 µl commercial master mix, 8.5 µl of sterile distilled water, forward and reverse primers each 1 µl and 2 µl and 4 µl of template for the two master mixes.

PCR was programmed with an initial denaturing at 94°C for 5 minutes followed by 36 cycles of denaturation at 94°C for 1 minute, annealing at a gradient of 60°C-55°C for 1 minute and extension at 70°C for 3 minutes and the final extension at 72°C for 10 minutes in a Thermocycler. PCR product (3 µl) was mixed with loading buffer (5 µ1) containing 0.25% bromophenol blue, 40% w/v sucrose in water and then loaded in 2% Agarose gel with 3.4g ethidium bromide for examination with horizontal electrophoresis. This was done at a 150 volts for one hour. The gel was viewed under UV trans-illumination and photographed using gel documentation system.

3.1.7 PCR DNA product purification

Thermo Scientific Gene Jet PCR purification kit K0702 was used. A ratio of 1.1 volume of binding buffer was added to complete PCR mixture. This comprised of 20 µ1 of PCR product: 20 µ1 of binding buffer and vortexed to mix. The mix was transferred to the gene jet purification column and centrifuged for 1minute. The flow through was discarded, 700 µ1 of wash buffer (diluted with ethanol) to dissolve salts and centrifuged for 1minute. The flow through was discarded. The empty gene jet purification column was centrifuged for 1minute to completely remove any residual wash buffer. Fifty µ1 of elution buffer was added to the centre of the gene jet purification column membrane and centrifuged for 1minute. Gene jet purification column was discarded and the purified DNA was stored at -20°C.

3.2 Objective 2: Determination of the antagonistic effect of the isolated *Trichoderma harzianum* and *Trichoderma asperellum* strains from Trianum-P®and Trichotech® respectively on *Phytophthora infestans*

3.2.1 Isolation and identification of *Phytophthora infestans*

Infected tomato plant leaves showing the characteristic symptoms of late blight were collected from the diseased plants in Egerton University, Njoro and its environs. The infected leaves were used for the isolation of the pathogen. The samples were surface sterilized by immersion in 70% ethanol for 1 min and then washed in sterile water three times to remove the surface sterilization agents. Potato tubers was washed with tap water and peeled – off with a knife and surface sterilized with 70% alcohol. The tuber was sliced to about 0.5 cm thickness and two slices were placed in a petri dish having a filter paper.

The surface-sterilized samples of the infected parts were cut into small pieces having some dead and some living tissues. The pieces were placed in between the potato slices using forceps. The potato slices were incubated at 19°C for seven days. After the incubation period the mycelia had grown through the tuber slices (Chandrakala *et al*., 2012). A plug of the mycelia were placed on a V8 based medium [V-8 juice (100ml), 900ml distilled water, $CaCO_3$ (2g) and agar (15g/L)]. All ingredients for the media were mixed and autoclaved for 30 min at 104 kPa and allowed to cool and the media was poured into petri dishes (Ristaino *et al*., 2010; Nourulaini *et al*., 2012).

The 150ml of clarified V8 medium was first centrifuged for 5 min at maximum speed (6000 rpm) and then 100ml of the supernatant was used and 900ml distilled water, CaCO3 (2g) and agar (15g/L) was added following the protocol described in CIP manual (2007). Sub-culturing was done on malt extract (ME) agar supplemented with 5g/ml chloramphenicol and incubated at 25°C for three weeks (Kim *et al*., 2000). *Phytophthora infestans* was identified according to the following characteristics: The morphology of sporangium, sporangiophore, antheridia, oogonia, morphology of hyphae and presence or absence of clamydospores. This was based on the taxonomic key of Abad (2008); Mannon and Chuanxue (2008). The pure cultures of *P. infestans* were stored in 10% glycerol at -80°C (Bertier *et al*., 2013).

3.2.2 Determination of antagonism of *T. harzianum* and *T. asperellum* against *P. infestans*

The antagonistic test was carried out in the soil microbiology laboratory in Crop, Horticulture and Soil department Egerton University. The dual culture technique as described by Joshi *et al* (2010) was conducted to assess the inhibitory effect of the mycelial disc of *Trichoderma harzianum* and *Trichoderma asperellum* against *P. infestans*. The petri dishes containing PDA (Potato Dextrose Agar) were inoculated with mycelial discs (5mm in diameter) of seven days old culture of the pathogen and antagonistic strains. They were placed at equal distance from the periphery. The inoculated plates were incubated for five days at 25°C and the diameter growth of the pathogen was measured. The mycelial mats were gently picked with a needle from the zone of interaction between the antagonist strains and the pathogen. They were microscopically examined for hyphal interaction. The zone of inhibition in the diameter colony growth was also calculated. [Modified from Perveen and Bokhari (2012); Jagessar *et al*., 2008; Cornea *et al*., 2008]. Inhibition percentage was calculated using the following equation:

Inhibition Percentage $IP = \frac{(C - T)}{C} \times 100$

where:

IP is the inhibition percentage.

C is the mean colony diameter (mm) of the growth in the control treatment.

T is the mean colony diameter (mm) of the growth in the treatment tested (Amal *et al*., 2005).

The antagonistic experiment was designed as complete randomized design (CRD) with three replications (El-Fiky *et al*., 2006).

3.3 Objective 3: Determination of the effect of Trianum-p®, Trichotech® and their combination on growth and late blight treatment of tomatoes in different soils

3.3.1 Production of the test plants

Tomato seeds (Cal-J variety) were sown in a nursery bed in the glasshouse. According to Ezin *et al* (2010), a pot experiment was designed under greenhouse conditions using plastic cylinders (15.5cm diameter and 35.4cm height) containing 5 kg of solar sterilized soil. The experiment was established as a 3×5 factorial, arranged in a randomized complete block experimental design (RCBD) with three replications. This was used to assess the effect of two factors; A (soil) with three levels that is S1 (Egerton), S2 (Chuka) and S3 (Bungoma) soils. Agro-ecosystems of these three regions were selected on the bases of having a great variability in relation to soil chemical characteristics which have an influence in colonization of *Trichoderma* spp. Factor B (fungicide) had five levels that is (a) Trianum-P® -T1 (b) Trichotech® - T2(c) mixture of Trianum-P® and Trichotech® -T3 (d)Ridomil® - T4 which is a chemical fungicide that was used as a standard check and (e) negative control (distilled water) – T0.

Transplanting of 14 days old seedlings was done in pots containing sterile soil. The treatments were sprayed to the plants and for the negative control sterile distilled water was sprayed. Three days following the application of the control agents, 300 ml suspension (1×106cfu/ml) of *P. infestans* was sprayed to all the plants of the different treatments (Poornima, 2011). The counting of the spores was done using a haemocytometer and staining the spores using cotton blue.

3.3.2 Soil chemical analysis

Soils sampled in Egerton, Chuka and Bungoma sites in Kenya were air-dried and sieved through a 2 mm sieve for the analysis of soil pH, organic carbon, total nitrogen and available phosphorus. Soil pH was measured in water suspensions at a solid to-liquid ratio of 1:2.5. Organic C was determined by chromic acid digestion and spectrophotometric analysis (Heanes, 1984). Quantitative determination of organic nitrogen was done using a protocol by Kjeldahl 1883 whilst available P was extracted based on the method developed by Olsen *et al*., (1954).

3.3.3 Treatment application

Spraying of the treatments was done after a two weeks interval. Disease severity of late blight and percentage of healthy survival plants was assessed after 30 days from sowing.

Table 2: Schedule of treatments by soil drenching

Treatments	Dose (g/pot)	Dose (g/pot) 2WAP
T_1 =Trianum-P®	0.5g/pot	0.25g/pot
T_2 = Trichotech®	0.5g/pot	0.25g/pot
T_3=Trianum-P®and Trichotech®	0.5g/pot (each 0.25/pot)	0.25g/pot
T_4 =Ridomil®	25g/10L	25g/10L
T_0 =Untreated control	0g/10L	0g/10L

The recommended dosage by the manufacturer for Trianum- P® and Trichotech® is 3.0g/10L immediately after planting. The treatment should be repeated with half the dose that is (1.5g/10L), after every two weeks (www.koppert.com. 2008; www.finlays.net. 2011). The recommended dosage for Ridomil® is 25g/10L. In the field, it is recommended that a maximum of three treatments at an interval of 10 days during the rainy season be used (www.zfc co.zw. 2010).

Table 3: Chemistry of the three soil types used the greenhouse efficacy trial

Sample description	Soil textural class	pH	C ($g\ kg^{-1}$)	N ($g\ kg^{-1}$)	P ($mg\ kg^{-1}$)
Bungoma	Ferralsols	6.0	18.2	10.3	760
Chuka	Rhodic nitisols	5.8	26.6	8.5	690
Egerton	Vitric andosols	6.3	43.3	4.8	710

3.3.4 Assessment of tomato growth and late blight severity

Four weeks after sowing, one stem was randomly selected in every pot and tagged for assessments that were done once in a week over a period of 10 weeks from first observation of disease. The variables that were recorded to determine growth were plant height, number of leaves, fresh and dry weight of shoots and roots. Variables to determine disease management were number of healthy and diseased leaves. All variables were expressed as mean values for the assessment period. Disease severity (proportion of leaf area diseased) or the percentage leaf area

infected by late blight was estimated from five leaves between the 3rd and 7th leaf (from the top) on each of the sampling stem. The disease scoring (qualitative data) was done on scale rating of 0 to 3 where 0 = no symptoms on plants 1= $<$ 20% necrosis on leaves, 2 = $>$20 to 45% necrosis on leaves and 3 = severe symptoms with $>$45% necrosis (Bock *et al.*, 2010). The rating of disease severity was used in calculating percentage disease index (PDI) as follows;

$$\text{PDI} = \frac{\text{Sum of all individual ratings}}{\text{Total number of stems assessed} \times \text{Maximum disease category}} \times 100$$

Equation is modified from Sudha and Lakshmanan, (2009).

3.3.5 Data Analysis

The quantitative data on growth and disease severity and the statistical significance of antagonistic effect of *Trichoderma* spp. was analyzed using the general linear model procedure (GLM) of the SAS program version 9.3. Data was analysed using analysis of variance (ANOVA). Treatment means were separated using DMRT at $p<0.05$. The 3x5 factorial RCBD statistical models were fitted for the data collected during greenhouse study.

CHAPTER FOUR

RESULTS

4.1 Objective 1: Isolation and screening of *Trichoderma* spp in different production lots of Trianum-p® and Trichotech® using cultural and molecular approach.

4.1.1 Morphology of *T. asperellum*

The formation of concentric green and white rings was observed when these cultures were incubated at 28°C. The colony diameter was 8-9 cm which indicated rapid growth rate and had smooth edges. The mycelia colour was white and the green conidia produced after 4 days gave the colony a green colour. The reverse colony colour was pale yellow (Rex *et al*., 2001).

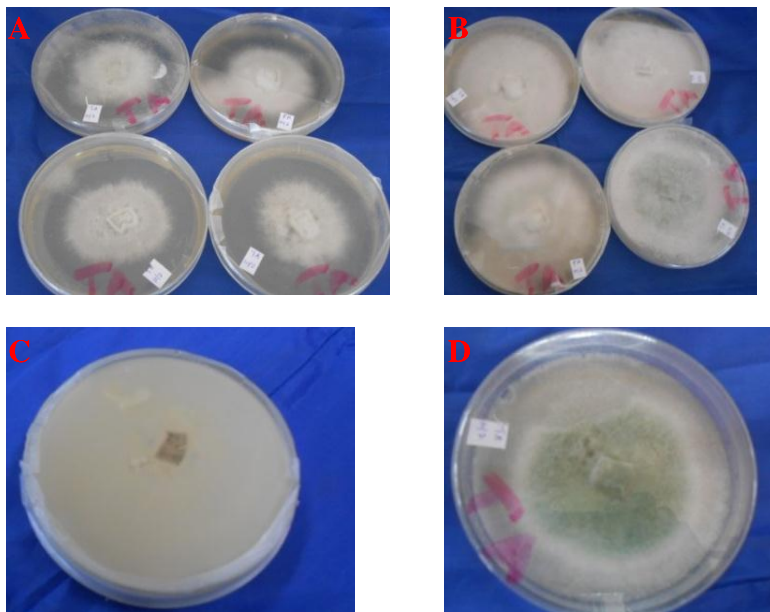

Plate 1*:* Colony appearance of *T. asperellum*. (A) 2days old colonies (B) 3days old colonies which indicated rapid growth (C) reverse colony colour after 4days was very pale yellow (D). The formations of concentric green and white rings were observed after 4 days.

Microscopic features indicated conidiophores showing their paired primary branches which were usually formed in nearly 90° to the main axis. Their phialides may be solitary or held in whorls of two to three. Those phialides held in whorls normally are flask-shaped, while

solitary phialides tend to be cylindrical and sharply constricted at the tips. Chlamydospores were produced after 7 days, appeared globose and were formed on the hyphal tips.

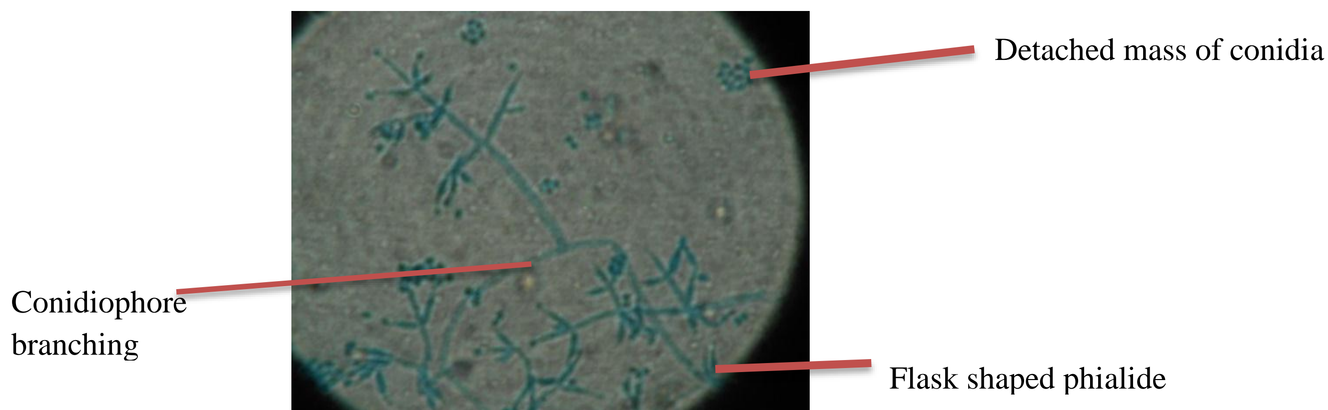

Figure 1: Microscopic features of 4 days old *T. asperellum* observed under a compound microscope at 400 ×

4.1.2 Morphology of *T. harzianum*

The formations of white concentric rings were observed when these cultures were incubated at 28°C. The colony diameter was 8-9 cm which indicated rapid growth rate and had wavy edges. The mycelia colour was white and no conidia produced after 4 days but were produced when grown on Rose Bengal. The reverse colony colour was pale yellow. Plate 2 shows (A) 2 days old colonies (B) 3 days old colonies which indicated rapid growth (C) reverse colony colour after 4days was pale yellow (D) The formation of white concentric rings were clearly observed after 4 days on PDA (E) green conidial production on Rose Bengal.

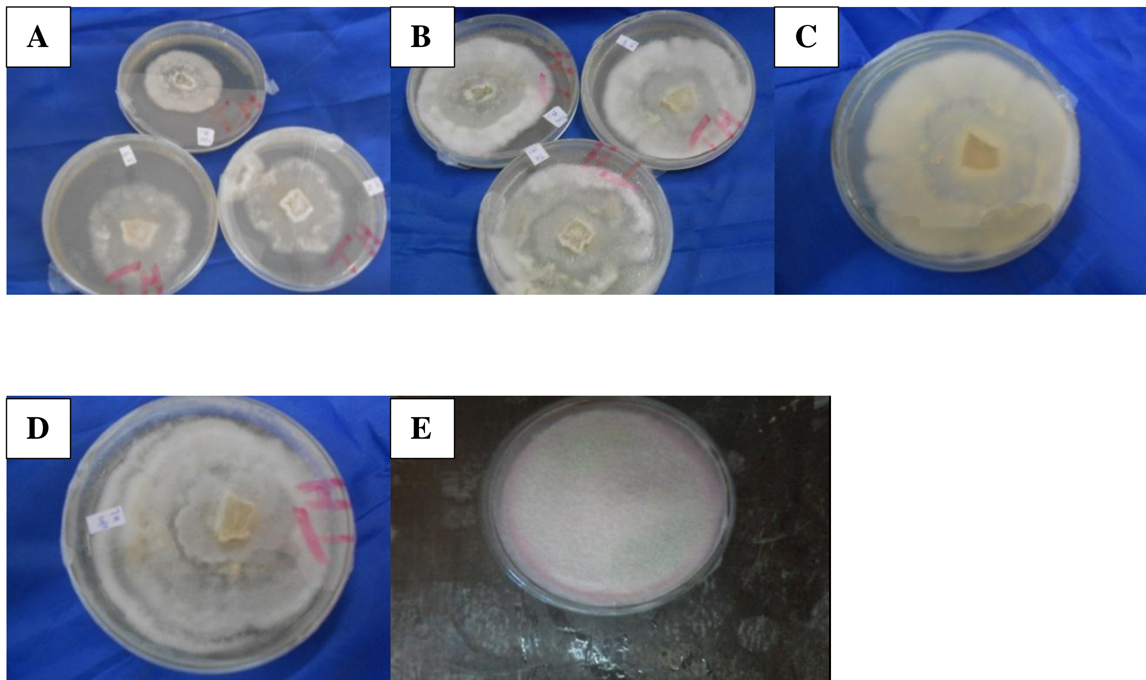

Plate 2: Colony morphology of *T. harzianum* on PDA media (plate A, B, C and D). Plate E colonies on Rose Bengal media.

Microscopic features shows paired primary branches, phialides held in whorls of two to three at ×400 under a compound microscope. *T. harzianum* had conidiophore branching patterns with short side branches, short inflated phialides, and smooth and small conidia. The conidiophore, with paired branches assumes a pyramidal aspect. Phialides were held in whorls, at an angle of 90° with respect to other members of the whorl.

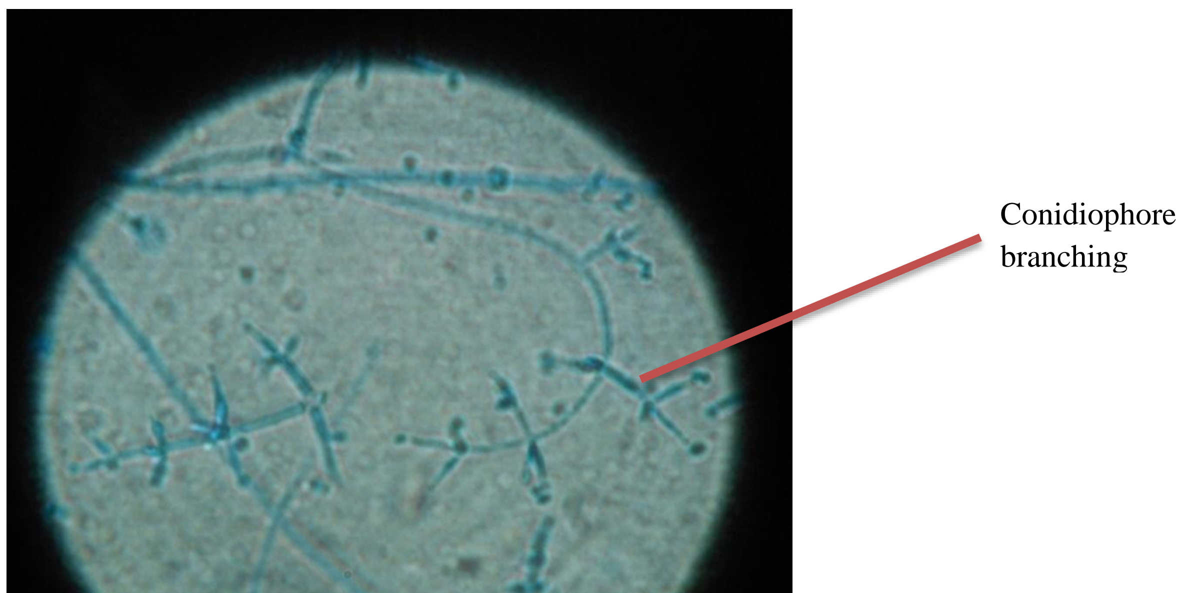

Figure 2: Microscopic features of 4 days old *T. harzianum* as observed under a compound microscope at 400 ×

4.1.3 Basic Local Alignment Search Tool (BLAST)

Nine isolates from different lots of Trichotech® and nine isolates of Trianum-P® were obtained using Rose Bengal media which was supplemented with chloramphenicol. Among the nine, three isolates were identified as shown in the figure below. Isolates 1-9 of Trichotech® was formation of bands in isolates 2(51/13B), 3(51/13C), 6(51/13F) when 2 µl of template was used. Isolates 10-18 a repetition of Trichotech® isolates but the template was increased to 4 µl and there was formation of bands in 1(51/13A), 5(51/13E) and 6(51/13F). Isolates 19-24 were from Trianum- P® whose template was also increased to 4 µl and only 6(87TP2433) showed positive result. The PCR products were prepared for sequencing.

Analysis of ITS1-5.8S-ITS4 region of the DNA showed that approximate 600 bp and size variation was observed. The results are in accordance with Mukherjee *et al*. (2002) who studied the identification and genetic variability of the *Trichoderma* isolates. These results are also in accordance with several workers who observed the amplified rDNA fragment of approximately 500 to 600 bp by ITS-PCR in *Trichoderma* (Venkateswarlu *et al*., 2008).

Restriction analysis of this region showed inter and intra -specific polymorphism (Latha *et al*., 2002).The ITS 1 and ITS4 DNA sequences of *Trichoderma* spp. were aligned using the

MEGA 6.0 software and the BLAST program at the US National Centre for Biotechnology Information (NCBI), (http://www.ncbi.nlm.gov/) was employed for nucleotide sequence analysis and database searches to identify the putative species of the fungi. The nucleotide sequences of the fungi strains were aligned against their respective test strains to further confirm their match and thus validating the identification.

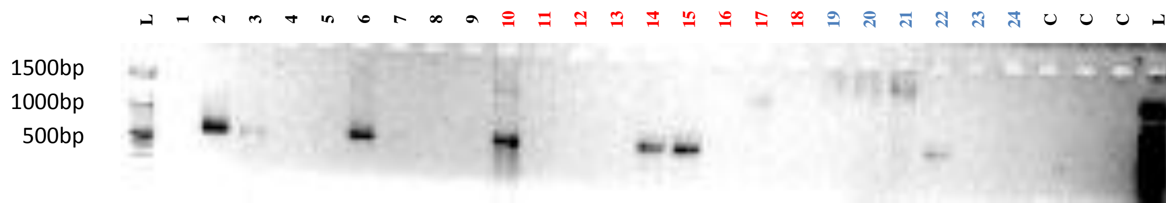

Figure 3: Gel image for PCR products of *Trichoderma* isolates after DNA extraction

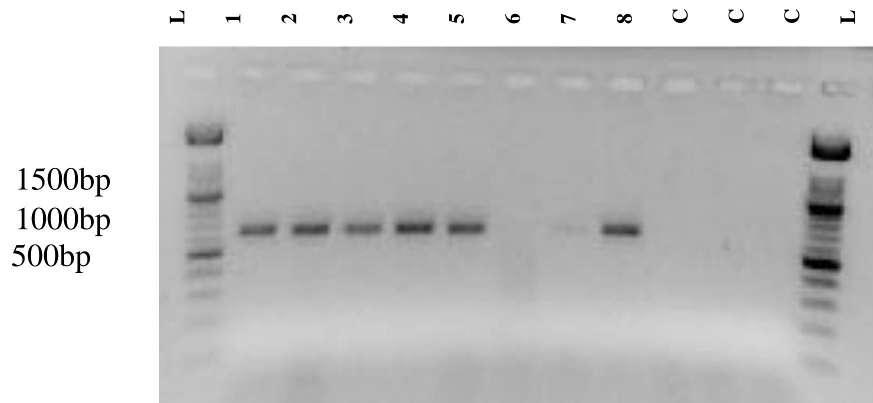

Figure 4: Gel image for PCR products of *Trichoderma* isolates for sequencing

Table 4: Cultural and molecular characterisation of *T. harzianum* and *T. asperellum* isolated from Trianum-P® and Trichotech® respectively

Lab ID	Expected strain	Description	Query Length	E Value	Query cover	Identity	Accession
Gk2F	*Trichoderma harzianum*	Not Related	-	-	-	-	-
Gk2 R	*Trichoderma harzianum*	*Trichoderma Harzianum*	797	0.0	79%	92%	KC582837.1
GK6F	*Trichoderma harzianum*	Not Related	-	-	-	-	-
Gk6R	*Trichoderma harzianum*	Not Related	-	-	-	-	-
GK7F	*Trichoderma harzianum*	Not Related	-	-	-	-	-
GK7R	*Trichoderma harzianum*	*Meyerozyma caribbica*	913	0.0	71%	89%	JQ425349.1
GK8F	*Trichoderma harzianum*	Not Related	-	-	-	-	-
GK8R	*Trichoderma harzianum*	*Trichoderma harzianum*	1043	3e-157	39%	91%	JX518925.1
Gk10F	*Trichoderma asperellum*	Not Related	-	-	-	-	-
GK10R	*Trichoderma asperellum*	Not Related	-	-	-	-	-
GK11F	*Trichoderma asperellum*	Not Related	-	-	-	-	-
GK11R	*Trichoderma asperellum*	Not Related	-	-	-	-	-

Table 5: Described samples after forming a contig alignment

Lab ID	Description	Query length	E value	Query cover	Identity	Accession no
GK9	*Meyerozima carribica*	1261	0.0	48%	88%	JQ425349.1
GK15	*Trichoderma asperellum*	698	0.0	81%	90%	LN846676.1

The samples whose E value was 0.0, cover query and identity was more than 50% verified that the sequences were of the described organism. Sample GK2 and GK8R showed that

the organisms were *Trichoderma harzianum*. Although GK8R E value was more than 0.0 and the query cover was less than 50%. GK15 as indicated in the table3 after forming a contig alignment assembly, that is, without separating the strands indicated that the organism was *Trichoderma asperellum*. The strands that had no significance did not match with any other sequences after blasting. However, GK7R and GK9 (in the table below after forming a contig alignment assembly, although the query cover was 48%) were expected to be *T. harzianum* but were described to be *Meyerozyma caribbica. Meyerozyma caribbica* is used as a biocontrol (Pedro *et al*., 2013). The yeast *Meyerozyma caribbica* was evaluated for their effectiveness against *Colletotrichum gloeosporioides* in the mango (*Mangifera indica* L.) cv. "Ataulfo". Electron microscopy showed that the yeast produced a biofilm adhering to the fruit and to *C. gloeosporioides* hyphae. *M. caribbica* showed competition for space and parasitism to the phytopathogen, furthermore it produced hydrolytic enzymes such as chitinase, N-acetyl-β-d-glucosaminidase and β-1, 3-glucanase. Thus, these results showed that one of the batches of Trianum-P® was contained *M. caribbica* which is also a biocontrol agent.

4.1.4 *Trichoderma* isolates sequence Alignment

For direct sequencing of the PCR products, a total of 8 isolates of *Trichoderma* PCR products produced sequences that could be aligned and showed satisfactory homology with ex-type strain (GK15) of *T. asperellum* sequences from the NCBI Genbank data base. The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponding to the actual ITS 1 region. A multiple sequence alignment was carried out that included the ITS 1 region, including gaps and the complete sequences align. There were quite a number of gaps that were introduced in the multiple sequence alignment within the IT S-1 region that were closely related and similar sequence indicated. From the sequence alignment, variations were observed between *T. harzianum*, *T. asperellum* and *Meyerozyma caribbica* (GK9) isolates and others species of *Trichoderma* isolates.

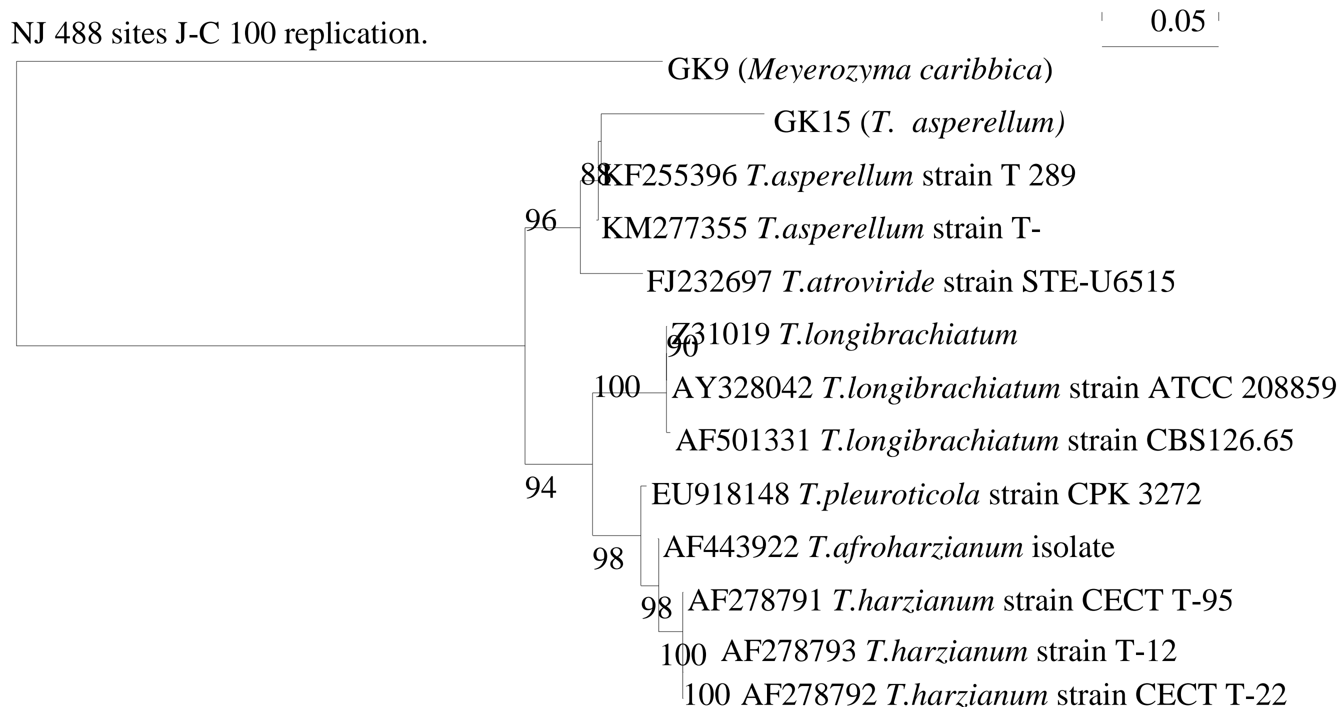

Figure 5: Neighbour-joining phylogenetic tree of *Trichoderma harzianum* and *Trichoderma asperellum* based on the ITS-rDNA gene sequences

4.1.5 Phylogenetic Analysis

Multiple sequence alignment (MSA) was done using cluster W and phylogenetic tree was generated using sea-view software. The ITS 1 region sequence was used in these analysis because it had showed to be more informative and closest phylogenetic relative in genus of *Trichoderma*. These strains were identified at the species level by morphological character using the existing taxonomic criteria analysis and by analysis of their ITS 1 region gene sequences. The strains of *T. atroviride* were used as out-groups because it had shown to be more closely related species of *T. asperellum* while *T. longibrachiatum*, *T. pleuroticola* and *T. afroharzianum* were used as out-groups because they had shown to be more closely related species of *T. harzianum*. The strains of *Meyerozyma caribbica* used as out-group for totally different gene sequence and it was isolated from Trianum-P® product.

The evolutionary history was inferred using the UPGMA (unweighted pair group method with arithmetic mean) method, bootstrap tree analysis with 1,000 bootstrap replications demonstrated two major clusters that is, A and B (Fig. 5). Cluster B was further subdivided into

three clusters, B1, B2 and B3. Cluster A, consisted of *Meyerozyma caribbica* (GK9) which was not closely related to *Trichoderma* spp in terms of genus although it is also a biocontrol agent. For isolate GK15, the ex-type strain (T 289, T-1 and STE-U6515) of *T. asperellum* indicated that the isolate fitted well into cluster B1 as established by UPGMA analysis. Hence GK15 strains could be referred to *T. asperellum*. Sub clusters B2 and B3 were *T. longibrachiatum* and *T. harzianum* respectively. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Fig. 5). (Michael and Martin, 2000).

4.2 Objective 2: Determination of the antagonistic effect of the isolated *Trichoderma harzianum* and *Trichoderma asperellum* strains from Trianum-P®and Trichotech® respectively on *Phytophthora infestans*

4.2.1 Microscopic confirmation of the pathogen

P. infestans was microscopically identified based on morphology in the form of a lemon shaped sporangia which is a semipapillae type that produces zoospores. It also had profusely branching mycelium that was aseptate (Fig. 6).

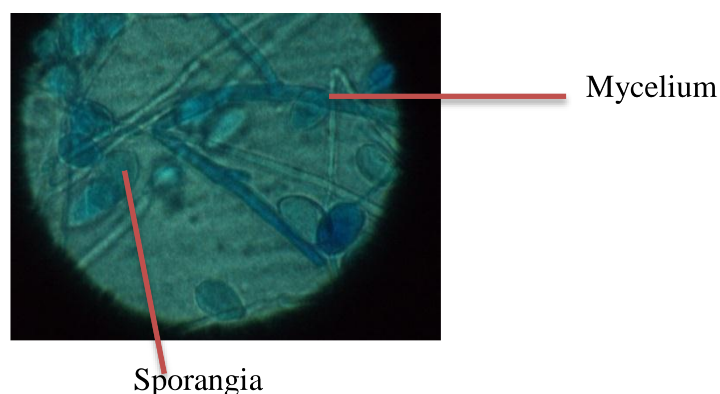

Figure 6: Morphology of *P. infestans* as observed under a compound microscope at 400×

T. harzianum and *T.asperellum* significantly reduced *P.infestans* growth under the in vitro conditions (Plate 3 and Table 6). Both *Trichoderma* spp. grew faster and occupied more space than *P. infestans*. In Table 6, use of the antagonists compared to the control significantly reduced the radial growth of the pathogen. *T. harzianum* and *T.asperellum* also inhibited the growth of

the pathogen by 72 % and 83 % respectively. The interactions between the antagonists and the pathogen were further observed (Plate 4). Once the antagonist came into contact with the pathogen, it attached itself, coiled and strangulated the host hyphae (pathogen) on the surface in a *Trichoderma-Phytophthora* interrelationship.

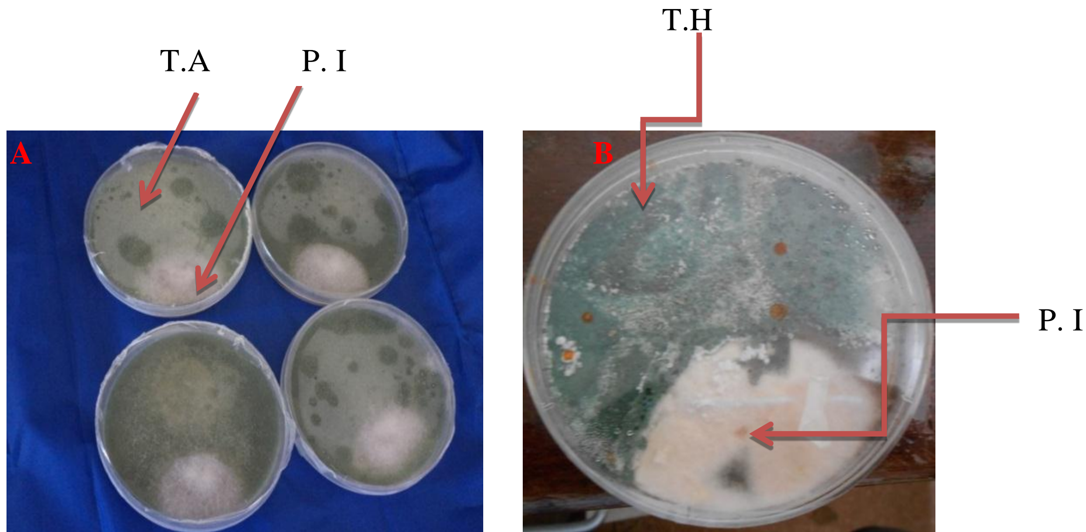

Plate 3: Effects of the antagonists on *P.infestans*. A = inhibition of growth of *P. infestans* (P. I) by *T. asperellum* (T. A); B = inhibition of growth of *P. infestans* (P. I) by *T. harzianum* (T. H).

Table 6: Mean comparison of radial growth and percentage inhibition of *P. infestans* in presence of *Trichoderma* spp. grown on PDA media

Isolate	Radial growth (cm)	Growth inhibition (%)
Trichoderma harzianum	2.57b	71.5
Trichoderma asperellum	1.54c	82.9
Control	8.67a	0.0
LSD	0.67	0.77
Significance level	***	***

Significance level: ***P < 0.001.

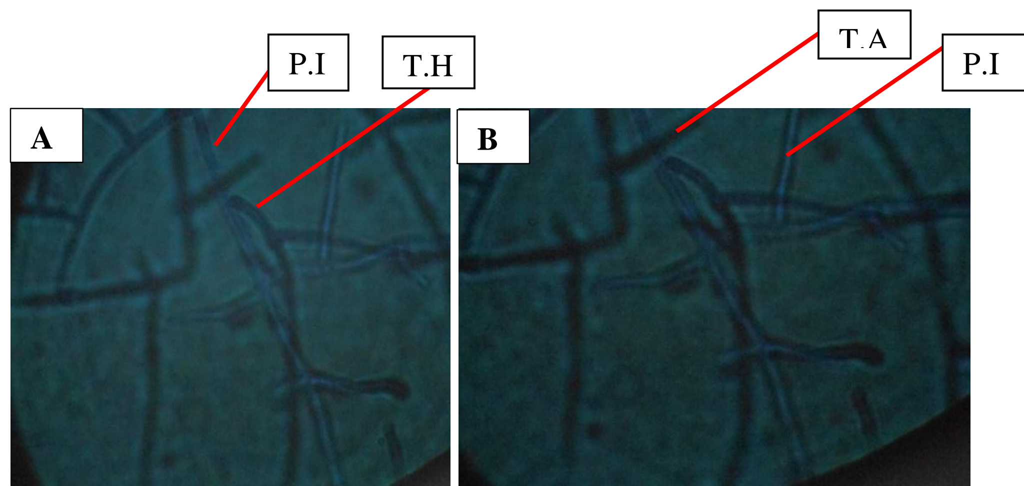

Plate 4: Hyphal interaction of *T. harzianum* and *T. asperellum* respectively with *P. infestans.*

Fungi of the genus *Trichoderma* have long been recognized for their ability to act as biocontrol agents against plant pathogens. During this time, research has described their mechanisms of action and how they might be used for various purposes (Harman, 2006). Antibiosis, mycoparasitism and food competition are the main mechanisms in biological control (Ranasingh *et al*., 2006; Ghildyal and Pandey, 2008; Umamaheswari *et al*., 2009 and Mohammad et al., 2014). Ghildyal and Pandey (2008) estimated that *Trichoderma* spp. produced diffusible and volatile metabolites. The production of volatile and non-volatile antibiotics by the species of *Trichoderma* also was reported by Ubalua and Oti (2007). Investigations into the biological activity of *Trichoderma* spp. have shown that they develop more rapidly than *P.infestans* in single as well as in double cultures. The intensive development of *Trichoderma* gives it a significant advantage in competition with pathogens for nutrient elements and space, even as it develops the system of mycotoxins (Barbosa *et al*., 2001). The contact between two fungi begins as early as at second day, with pathogen stagnation coming after, when *Trichoderma* is developing and spreading.

In plate 3, the petri plates are almost filled with *Trichoderma* spp., thus, it develops even over the culture of *P. infestans*. *In-vitro* antagonistic activity of *Trichoderma* sp. is expressed by suppressing test-fungi growth and its rapid growth, which is multiplying over the pathogen's colony (Faruk and Rahman, 2015). When the fragments are placed in near-contact, *Trichoderma* has a space advantage and bigger opportunities to stop pathogen development, and to develop its

mechanisms of antagonistic action. In a short time, it significantly reduces *P. infestans*. *T. harzianum* and *T. asperellum* inhibited the growth of the *P. infestans* through its ability to grow much faster than the pathogenic fungi thus competing efficiently for space and nutrients. Starvation was the most common cause of death for *P. infestans*, thus competition for limiting nutrients resulted in biological control of fungal phyto-pathogens (Sempere and Santamarina, 2007).

They were microscopically examined for direct hyphal interaction (plate 4) between *Trichoderma* spp. and the causal pathogen of late blight (*P. infestans*) in dual culture technique. Once the fungi came into contact, *Trichoderma* spp. attached itself to the host, coiled and strangulated the host hyphae and form appressoria and haustoria on the host surface in a *Trichoderma-Phytophthora* interrelationship. According to Almeida *et al*. (2007), inhibition of pathogen in double cultures begins soon after contact with the antagonist. *Trichoderma* spp. develops exactly on other fungi's hyphae, coils around them and degrades the cell's walls. This action of parasitism restricts the development and activity of pathogenic fungi.

Additionally, or together with mycoparasitism, some *Trichoderma* species release antibiotics (Laila *et al*., 2014). The growth of *P. infestans* was inhibited when exposed to the trapped headspace of compounds produced in the presence of *T. harzianum* isolates. The *Trichoderma* strains have been reported to produce different antimicrobial compounds like 6-pentyl-α-pyrone (Aline *et al*., 2008). The inhibition started after 24 hours and increased until end of the experiment (eight days) (Abdullah *et al*., 2007). However, Brunner *et al*. (2005) reported that their isolate of *T. atroviride* took five days to show parasitism on the pathogens such as *Pythium* and *Rhizoctonia*. This also suggests that there may be variations in pattern and time duration of parasitism depending upon the pathogen encountered.

4.3 Objective 3: Determination of the effect of Trianum-p®, Trichotech® and their combination on growth and late blight treatment of tomatoes in different soils

4.3.1 Symptoms of late blight infection

Lesions began as indefinite, water-soaked spots that enlarged rapidly into pale green to brownish-black lesions and can cover large areas of the leaf (Fig. 7). Lesions on the abaxial

surface of the leaf were covered with a grey to white mouldy growth. On the undersides of larger lesions, a ring of mouldy growth of the pathogen was visible.

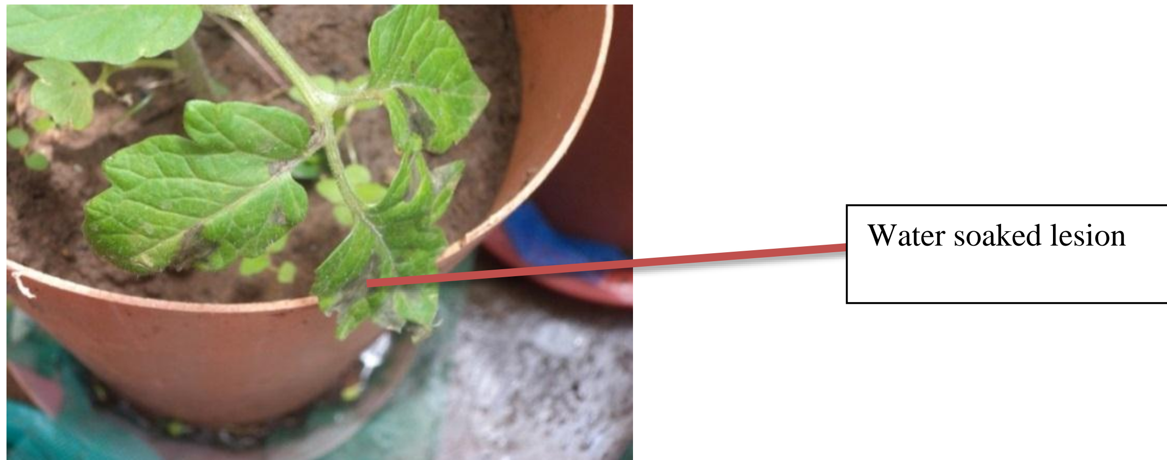

Figure 7: Lesions on the leaves of tomato plant was observed due to *P. infestans* as shown above at the third week.

4.3.2 Effect of different treatments on disease severity of tomato late blight

Disease severity was significantly low where a combination of Trianum-P®(*T. harzianum*) and Trichotech® (*T.asperellum*) was used followed by Trichotech®(*T. asperellum*) alone and Trianum-P® (*T. harzianum*) alone respectively, compared with chemical control and untreated control. In relation to previous study by Sharma *et al*. (2012) and Jan *et al*. (2013) reported that *Trichoderma* spp., have developed numerous mechanisms that are involved in attacking other fungi and reduce the plant diseases. These mechanisms include competition for space and nutrient, mycoparasitism and production of inhibitory compounds, inactivation of the pathogen enzymes (Roco and Perez, 2001) and induced resistance to crops (Kapulnik and Chet, 2000).

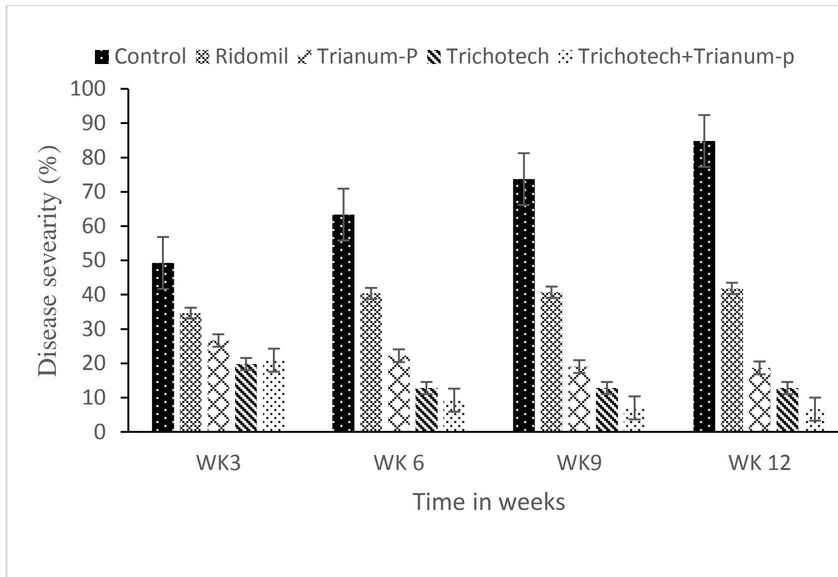

Figure 8: Effect of different treatments on severity of tomato late blight. The error bars are Standard Errors of the Difference.

4.3.3 Effect of different treatments on number of leaves and height of tomato plants

The biocontrol agent strains and Ridomil® had no significant difference on number of leaves and height of tomato plants; however, there was a significant difference compared with the control. Minimal number of leaves was observed where there was a combination of the two biocontrols but when applied singly there was increase in height of tomato plants and number of leaves (Fig. 9 and 10). Tomato plants treated with Trichotech® indicated a high growth rate compared to Trianum-P® and the combination of Trichotech® and Trianum-P®.

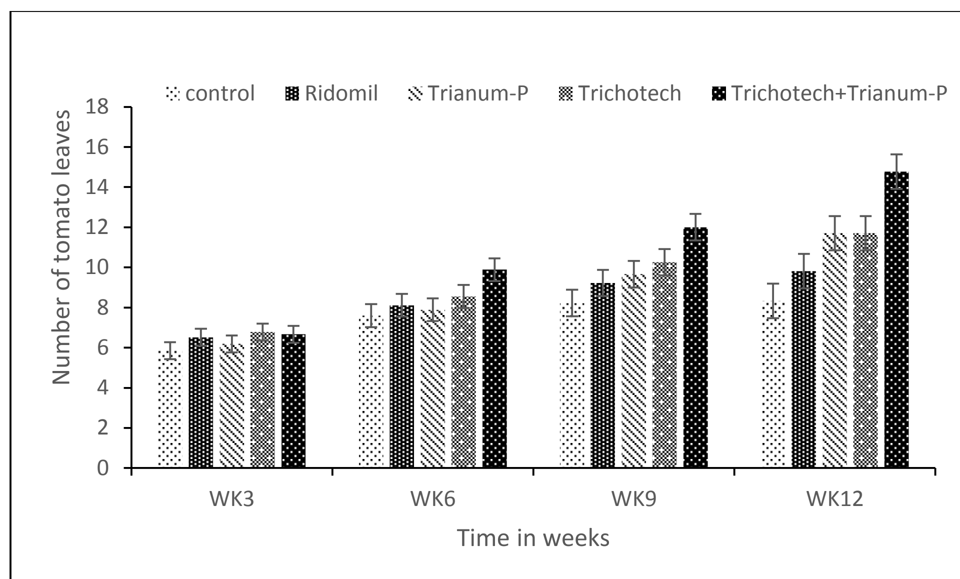

Figure 9: Effect of different treatments on tomato number of leaves. The error bars are Standard Errors of the Difference.

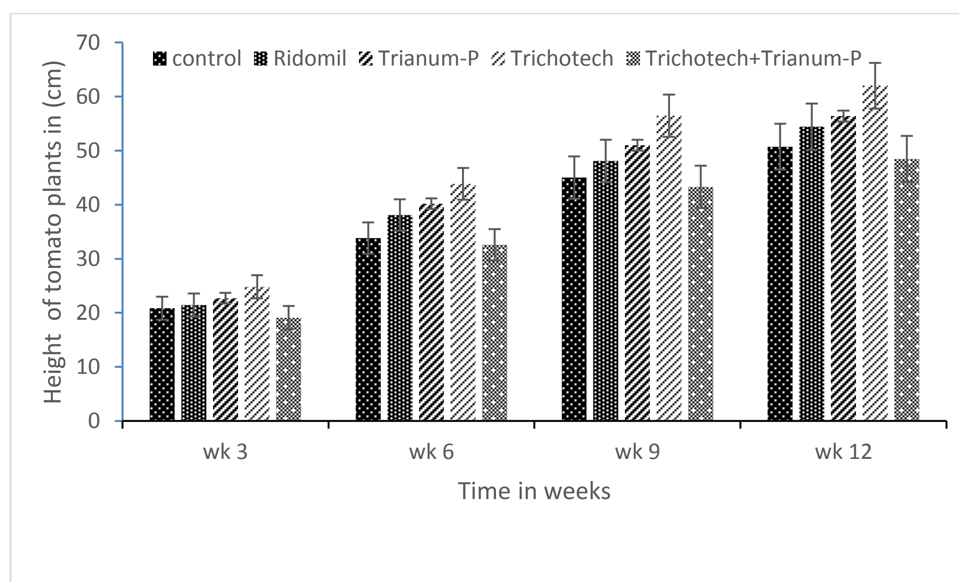

Figure 10: Effect of different treatments on tomato height. The error bars are Standard Errors of the Difference.

Effect of various treatments on tomato plants biomass

The analysis of variance of the data showed significant differences in treatments at P≤0.05 level. Tomato plants grown in Bungoma soil showed that the combined treatment of the *Trichoderma* spp. was significantly higher compared to the single application; chemical control

and the negative control on both shoot and root dry weight (Fig 11and12). While in Chuka soil single application of Trichotech® was significantly different compared to the other treatments on shoot and root dry weight. Single application of Trianum-p® and Trichotech® in Egerton soil was not significantly different from each other but was significantly different compared to the other treatments. This indicates that soil type is one of the abiotic factors that may have an influence on the biological control efficacy of *Trichoderma* isolates as well as promoting growth of plants.

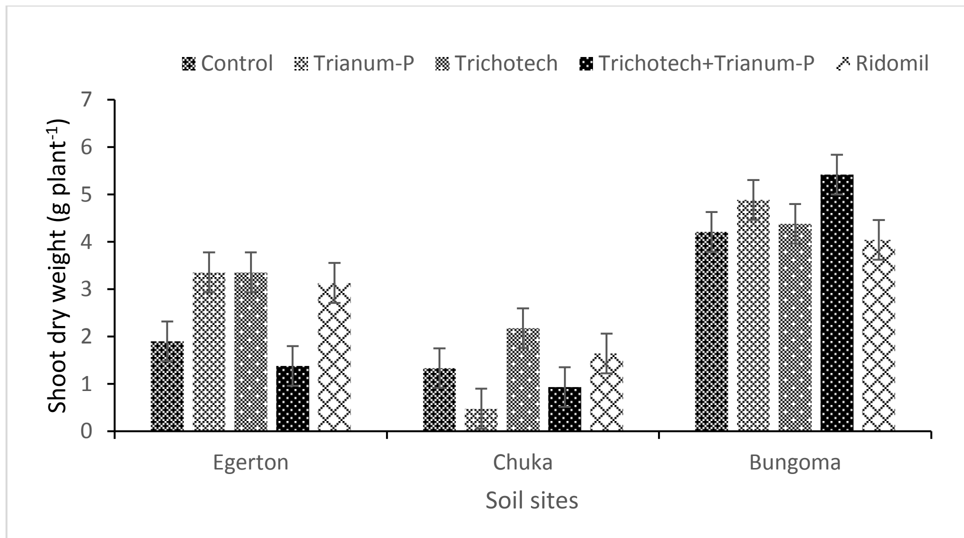

Figure 11: Effect of different treatments on tomato shoot dry weight in soils from different agro-ecological zones. The error bars are Standard Errors of the Difference.

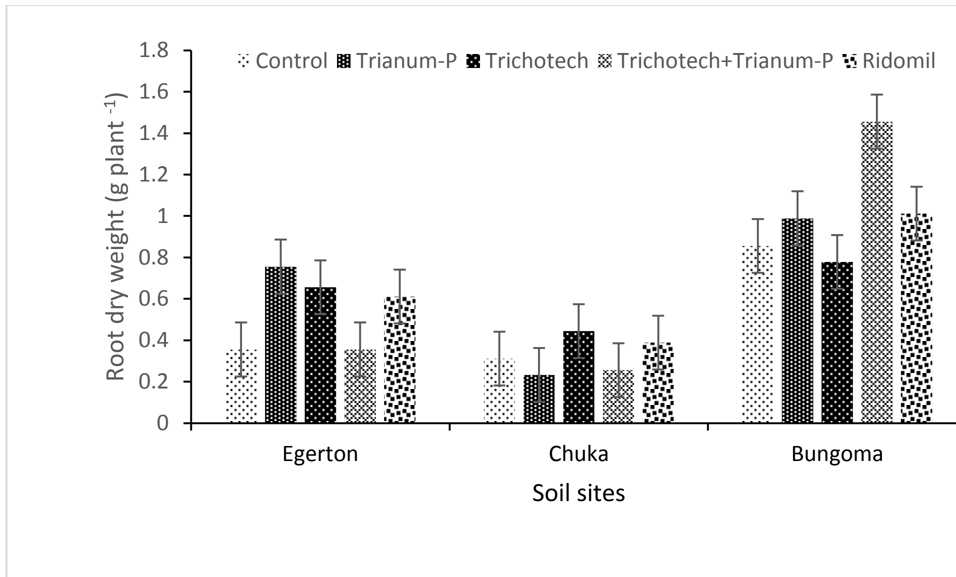

Figure 12: Effect of different treatments on tomato root dry weight in soils from different agro-ecological zones. The error bars are Standard Errors of the Difference.

Radjacommare (*et al*., 2004), El-Katatny *et al*., 2000) and Yedidia *et al*., 2000) reported that during interaction of biocontrol and plant-pathogens, an array of defense related proteins such as β -1, 3 glucanase, chitinase, peroxidase, polyphenol oxidase etc., are induced in plants resulting in reduction of disease incidence. From the present study it may be concluded that seedlings treatment with a group of these bio-agents (*Trichoderma* spp.) results in plant growth promotion and concurrently reduce the disease severity in contrast to application of single bioagent. *Trichoderma* spp. attack, parasitize and otherwise gain nutrition from other fungi.

CHAPTER FIVE

DISCUSSION

Molecular and morphological characterization of green mold, *Trichoderma* spp., isolated from commercial products showed some phylogenetic relationships. The phylogenetic relationships among *Trichoderma* spp., studies in which phenotype and DNA analyses were combined have tended to confirm species monophyly, as distinguished by Francesco *et al*. (2004). Thus, the results strongly indicated "*Trichoderma* aggregate species" with similar "DNA-based sequence", to accommodate with similar forms as referred to the species identifying concept. Most of the *Trichoderma* species are morphologically very similar and were considered as a single species for many years. DNA methods brought additional valuable criteria to the taxonomy of *Trichoderma* which are being used today for studies that include identification and phylogenetic classification. The study has also clarified that one lot of Trianum-P® contained *Meyerozyma caribbica* as the biocontrol agent rather than *Trichoderma harzianum* as indicated by the manufacturers. However, all the other lots have proved to contain the particular strains as expected.

The bottom line of this work is that among several criteria defined for an ideal biocontrol agent, one important criterion is a fast acting agent. An isolate of *Trichoderma* which can overcome the inhibition posed by the pathogen and parasitize the pathogen in a short span of time will be an efficient biocontrol agent. In this research *Trichoderma* isolate *T. asperellum* showed quicker action than *T. harzianum*. After reaching the verge of inhibition zone posed by *Phytophthora*, this agent took about two days to parasitize the pathogen. More isolates should be screened to find a better agent in terms of rapid activity and the present isolates shall be subjected to genetic modifications.

Trichoderma spp. isolates are very important to explore the antagonistic effects of their mechanisms expressing based on in-vitro dual culture technique. *Trichoderma* strains used as biocontrol agents can act in the following ways as: a) occupying a physical space and avoiding the multiplication of the pathogens; b) producing cell wall degrading enzymes against the pathogens, c) producing antibiotics that can kill the pathogens. It may be concluded from the present study that the two *Trichoderma* isolates could prevent or inhibit the mycelial growth of

P. infestans. Therefore, they have a large potential effect as biocontrol agent against *P. infestans* causing late blight of tomato.

From the results of present study it is concluded that, although all bio control agents applied individually reduced disease severity, the combination of *T. harzianum* and *T.asperellum* showed more protective effect on tomato in the greenhouse that were exposed to *P. infestans*. Minimal amounts of disease were observed on plants inoculated with a combination of the two products at $p \leq 0.05$ (at 6.6%) whereas the individual product had moderate (12.7% Trichotech® and 17.2% Trianum-P®) control. Trichotech® resulted to an increase in biomass thus significantly different compared to the other treatments on shoot and root dry weight.

It is widely known that environmental parameters such as abiotic (soil type, soil temperature, soil pH and water potential) and biotic (plant species and variety, and microbial activity of the soil) factors as well as other factors such as method and timing of applications may have influence on the biological control efficacy of *Trichoderma* isolates. Therefore, it is important that *Trichoderma* bio control potential in the greenhouse and field should be further evaluated. Although several biocontrol agents have been tried against late blight disease, still this lethal disease could not be controlled completely. In addition, mixture of bioagents of different genera or mixture of fungal and bacterial bioagents along with botanicals has to be tried to improve the level and extent of disease control under different environmental and soil conditions.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

Trichoderma harzianum in Trianum-P® and *Trichoderma asperellum* in Trichotech® are present as specified and the microbial content of the bio-control products is as identified by the manufacturer although one of the batches was contaminated with another biocontrol. Isolated *Trichoderma harzianum* and *Trichoderma asperellum* strain from Trianum-P® and Trichotech® respectively have the ability to antagonize *Phytophthora infestans*. Although the laboratory assay suggests that there may be variations in pattern and time duration of parasitism depending upon the pathogen encountered.

Trianum-P®, Trichotech® and their combination have a positive effect on growth, biomass and late blight treatment of tomatoes in different soils. Thus *T. harzanium* and *T. asperellum* are potential biological control agents. It can also be concluded that there are significant differences between *T. harzanium* and *T. aspellerum* in controlling late blight disease of tomato, where *T. aspellerum* was more effective.

6.2 Recommendations

1. There is a need for putting up proper quality control systems and regulations to ensure production of quality and efficient inoculants on non-disclosure. These systems can ensure production of good quality inoculants as well as sieving out the underperforming products.

2. Manufacturers should consider multi-strain products especially for strains that are synergistic.

3. More research to better understand the role of *Trichoderma* spp. as effective inoculants, both under controlled and field conditions.

4. Farmers should be aware that soil chemistry and PH of the soil has to be done before using *Trichoderma* strains. The reason being that these are the factors that determine their colonisation ability in different soils.

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