BIOCONTROL POTENTIAL OF *Trichoderma harzianum* Rifai, (1969) AND *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) AGAINST *Phytopthora infestans* AND *Alternaria solani* CAUSING BLIGHT IN TOMATO

EMMY JEPTOO CHERUIYOT

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DECLARATION

Declaration by the student

This thesis is my original work and has not been presented for a degree in any other University. No part of this thesis may be reproduced without the prior written permission of the author and/or University of Eldoret

Emmy Jeptoo Cheruiyot

_____ Date**_____**

SC/PGB/001/15

Declaration by the Supervisors

This thesis has been submitted with our approval as the University supervisors.

_____ Date**_____**

Prof. Lizzy A. Mwamburi

University of Eldoret, Eldoret, Kenya

_____ Date**_____**

Dr. Billy A. Makumba

Moi University, Eldoret, Kenya

DEDICATION

To my children Shanice, Ryan and Shayne. The sky is the limit. May God lift you to greater heights of Education.

ABSTRACT

The most devastating fungal pathogens in tomato (*Solanum lycopersicum* L.) production are *Alternaria solani* and *Phytophthora infestans* which cause early blight and late blight diseases respectively. The pathogens thrive well in humid and wet conditions that make it difficult for fungicide application. Tomato is a popular vegetable that is grown mainly in small holder farms for home consumption and local market with many benefits ranging from nutrition to source of income. It is grown in 95% open fields and 5% greenhouse. This study sought to find alternative control to these pathogens that employs the use of *Trichoderma harzianum* and *Beauveria bassiana. T. harzianum* has been widely used against plant pathogens whereas *B. bassiana* has been rarely used. *T. harzianum* was isolated selectively from soil using Potato Dextrose Agar whilst *B. bassiana* was isolated from a dead beetle by use of corn meal agar. Both pathogens were isolated from diseased tomato plant tissues showing the symptoms of early and late blight. Both the potential biocontrol agents and the pathogens were identified using cultural, morphological and microscopic characteristics. *In vitro* tests of antagonism by dual culture technique, volatiles and non-volatiles production of each biocontrol agent (BCA) were performed. *In vivo* tests were performed to compare the efficacy of the two BCAs. Daily growth recording of the pathogen colonies under different inhibitory treatments were taken for seven days. Dual culture of *T. harzianum* and *A. solani* resulted in a significant reduction in the colony growth of the pathogens. Subjecting the seventh day recordings to t-test resulted in a statistically different result at $P < 0.001$. There was a growth reduction of about 65% in the case of *P. infestans*. Treatment with volatile metabolites produced by *T. harzianum* also resulted in inhibition of *A. solani* colonies by 48.25% (P =0.00034), While it also significantly slowed *P. infestans* colonies growth by 38.7%. *Trichoderma harzianum* culture filtrate (non-volatile metabolites) however was not effective against *A. solani* colonies. The colonies' diameter did not significantly differ from that of the controls ($p > 0.05$). As for *P. infestans* however, the highest concentration of the culture filtrate (15% v/v), showed stronger inhibition albeit weak (control; 70.33mm, 5%; 70mm, 10%; 69.66mm and 15%; 62mm). *Beauveria bassiana* on the other hand was not effective in dual culture against *A. solani* but there was a very clear zone of inhibition against *P. infestans*. Its volatiles reduced the growth of *A. solani* by 22.5% while that of *P. infestans* was reduced by 45.9%. The culture filtrate proved effective against *A. solani* (10%v/v; 33%, 15%v/v; 38.88%) while for *P. infestans,* none of the concentrations effectively inhibited the colonies. Greenhouse experiments utilized spore suspensions of 3×10^8 conidia ml^{-1}. It was revealed that *B. bassiana* was more effective in the reduction of disease severity. *T. harzianum* reduced disease severity by 25% and 28.75% against *A. solani* and *P. infestans* respectively. *Beauveria bassiana* reduced the disease score against *A. solani* by 50% while against *P. infestans* by 46.25%. The biocontrol agents used in the current study were therefore effective against the test pathogens. More experiments ought to be carried out to establish the extent of potency of *B. bassiana* against phytopathogens.

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LIST OF ABBREVIATIONS

˚C	Degree Celcius
AMF	Arbuscular Mycorhizal Fungi
ANOVA	Analysis of Variance
BCA	Biocontrol Agent
cm	Centimetre
F.A.O	Food and Agriculture Organisation of the United Nations
g	Grams
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
ml	Mililitre
PDA	Potato Dextrose Agar
PGA	Potato Glucose Agar
PGPR	Plant Growth Promoting Rhizobacteria
SD	Standard Deviation
SDA	Saboraud Dextrose Agar
spp	species
THSM	*Trichoderma harzianum* Selective Medium
TWOWS	Third World Organization of Women in Science
VOCs	Volatile Organic Compounds

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CHAPTER ONE

INTRODUCTION

1.1. Background information

Tomatoes *(Solanum lycopersicum* L.*)* are the most popular and widely grown vegetables in the world (Kaoud, 2014). The crop's popularity is attributed to its high nutritive value especially many important minerals such as phosphorus and potassium and vitamins B and C (Sabbour, 2014). It is also very important in diet since it's phytochemical content including carotenoids and lycopenes work against breast and prostate cancer that are quite common (Kaoud., 2014). Tomato products have gained popularity in fast food industry in Kenya where tomatoes are used in form of pastes, ketchups, salads or in cooked form (Geofrey *et al.,* 2014).

Soil borne pathogens inflict a lot of diseases and economic yield loss to tomato plants (Babalola & Glick., 2012). Such diseases include bacterial wilt, root knot nematode diseases, early blight, late blight, buck eye rot, and Fusarium wilt. These are destructive diseases of tomato worldwide (Strayer *et al.,* 2016). Various disease management practices that can be implemented to manage these diseases include; crop rotation, mulching, spacing, fungicide applications, and use of resistant and tolerant varieties (Geofrey *et al.,* 2014). One such variety is that which is resistant to *F. oxysporum* f. sp. *lycopersici* races 1 and 2 (Arie *et al.,* 2007). These methods of disease control are either not very efficient or are difficult to apply. Additionally, some of them, such as development of disease resistant varieties, take too long. It is for these reasons that alternative methods of controlling diseases have or are being explored.

The term "biological control of plant pathogens" refers to the application of microorganisms and/or their metabolites to lower plant pathogen activity or survival. Biological control has become more important than chemical control of disease causing pathogens and perhaps replacing chemical control of disease methods in plants like *Solanum lycopersicum* (Herforth & Van, 2017). Numerous biological defenses against plant diseases have been discovered (Jaber & Ownley, 2018). These mechanisms may act alone or in combination. For example, the biocontrol organism plays a key role in antibiosis, competition, lysis, and mycoparasitism, whereas others, such as induced systemic resistance and increased growth, involve the organism's endophytic colonization of the plant, which eventually causes responses in the plant that lessen or eliminate plant disease (Keswani *et al.*, 2019).

To reduce competition in an ecosystem, some microorganisms have evolved mechanisms of producing metabolites that impede the growth of their competitors (Barrat *et al*., 2018). This antagonism is therefore exploited by humans to develop biological control agents of phytopathogens. The Mechanisms of antagonisms occur differently. Direct antagonism exists where there is physical contact between the biological control agent and the pathogen and/or a high degree of selectivity for the pathogen by the biological control agent (Kaushal & Wani., 2017). When used properly, biological control of plant pests and diseases increases the ecological and financial sustainability of farming systems by lowering the risk of crop losses and health risks to humans. (Fahad *et al.*, 2015).

One of the promising bio-control agents, the genus Trichoderma, has been reported as having potential for biological control of soil-borne plant diseases (Moosa *et al.*, 2017;

Alani, 2019). *Trichoderma* uses different mechanisms for the control of phytopathogens which include mycoparasitism, competition for space and nutrients, secretion of antibiotics and fungal cell wall degrading enzymes (Harman *et al*. 2004; Vinale *et al*. 2008). In addition, *Trichoderma* could have a stimulatory effect on plant growth (Naseby *et al*, 2000) as a result of modification of soil condition. Successful control of blight disease in many crops by application of different species of *Trichoderma* has been reported (Sharma & Gothalwal, 2017). However, all isolates of *Trichoderma* spp. are not equally effective in controlling pathogens both in *in vitro* and *in vivo* conditions (Islam *et al.,* 2017).

Beauveria sp. is an entomopathogenic hyphomycete that naturally occurs in the soil. Fungal entomopathogens like *Beauveria bassiana* have been identified as biological plant pathogen controllers (Jordan *et* al., 2021). *B. bassiana* has a lot of potential as a biological control because it is affordable to cultivate in large quantities (Qazzaz *et al.,* 2015). *B. bassiana* has been tested as a biocontrol agent for pest management and has been successfully formulated for use in many countries. The ability and efficacy of *B. bassiana* to prevent crops damage is extremely rare. However, recommendations have encouraged studies to use it in biological control of crop pathogens (Qayyum *et al.,* 2015). Perhaps this is because *B. bassiana* is capable of endophytically colonizing most plant species (Doolotkeldieva *et al.*, 2019).

1.2. Statement of the Problem

Tomato is a popular vegetable in Kenya, both in the fresh market and processing industry (Kirimi *et al.,* 2011). It is the highest vegetable income earner and ranks third after kales

and cabbages in metric tons annually in Kenya (Kirimi *et al.,* 2011; Karuku *et al.,* 2016). This production makes Kenya among Africa's leading producers of tomato and is ranked sixth in Africa. Tomato makes up 6.72% of all horticulture crops and 14% of all vegetable production (Karuku *et al.,* 2016). The area under tomatoes in Kenya is estimated at 20,111 hectares where 95% is done in open field while 5% is done in controlled environments. These are mainly small holder farms whose produce is for home consumption and local market (Short *et al.,* 2014). The crop plays a critical role in meeting domestic and nutritional food requirement, generation of income, foreign exchange earnings and creation of employment to a majority of the Kenyan citizens (Geofrey *et al.,* 2014), where 60% of the labour force come from women. It is therefore among the promising commodities of horticultural expansion and development in Kenya.

Phytopthora infestans, the causal pathogen of late blight and *Alternaria solani* that causes early blight are the most ubiquitous and devastating pathogens of tomato and thrives well in humid and wet conditions (Roy *et* al., 2019). Production losses as a result of the two diseases have been estimated to be above 60% with complete crop failures in cool and wet conditions. These diseases pose serious challenges to tomato farming hence there is a need to be addressed.

Fungicide application is normally employed by farmers to control the blight diseases. However, because of serious health hazards that are posed by chemical fungicides, efforts are being made to find alternative control methods. Fungicides have also been reported to kill various beneficial organisms alongside the target organism (Fromme *et al*., 2017). In

addition, their toxic forms persist in soil and contaminate the whole environment (Nakhungu *et al.,* 2021). There is therefore an urgent need to find alternative ways of managing the diseases effectively without affecting the environment which can best be done through biocontrol.

1.3. Justification of the Study

The most important factors responsible for the low productivity of tomato are diseases and insect pests. Early and late blight of solanaceous vegetables are two of the diseases, and they are the most pervasive and destructive in tropical, subtropical, and temperate regions of the world (Amin,*et al*., 2013). The most pervasive and destructive diseases that infect tomatoes in the North rift region of Kenya are said to be *Phytophthora infestans*, which causes late blight, and *Alternaria solani*, which causes early blight. (Annastacia *et al.,* 2011). The predicted range of production losses due to late blight is 65-70%. Complete crop failures have been reported in cool and wet weather conditions. These diseases are responsible for serious loss in yield, quality as well as reduction in its marketability value (Roy *et al*., 2019).

Numerous crops have been effectively treated with several types of *Trichoderma* to prevent Fusarium wilt. (Sundaramoorthy & Balabaskar., 2013) (a). On the other hand, *B. bassiana* has been reported to reduce diseases caused by soil borne plant pathogens for example *Pythium, Rhizoctonia* and *Fusarium* (Canfora *et* al., 2017). It also has endophytic colonization effect on plant tissues of both monocots and dicots (Mwamburi *et al*., 2016).

The introduction of biological control systems using biocontrol agents produced by the genus *Trichoderma* and *Beauveria* in the control of tomato blight diseases will bring

about a new crop protection method that has not been used in Kenya. It has also been reported that these microorganisms release antibiotics and other chemicals that are harmful to pathogens hence inhibit their growth (Brandt, 2015).

1.4. Objectives of the Study

1.4.1. General Objective

To determine the antifungal activity of *Trichoderma harzianum* and *Beauveria bassiana* in controlling early and late blight disease of tomato caused by *Alternaria solani* and *phytophthora infestans* respectively.

1.4.2. Specific Objectives

1. To evaluate the antagonistic ability of isolates of *Trichoderma harzianum* and *Beauveria bassiana* against *Alternaria solani* and *phytophthora infestans* by the dual culture technique *in vitro.*
2. To determine the efficacy of volatile and non-volatile substances produced by *Trichoderma harzianum* and *Beauveria bassiana* against *Alternaria solani* and *phytophthora infestans in vitro.*
3. To compare the efficacy of *Trichoderma harzianum* and *Beauveria bassiana* against early and late blight diseases *in vivo*.

1.5 Research Questions

1. Do *Trichoderma harzianum* and *Beauveria bassiana* isolates exhibit any antagonism towards *Alternaria solani* and *Phytophthora infestans in vitro*?

2. How effective are volatile and non-volatile substances produced by *Trichoderma harzianum* and *Beauveria bassiana* against *Alternaria solani* and *Phytophthora infestans in vitro?*
3. How do the efficacy of *Trichoderma harzianum* and *Beauveria bassiana* on early and late blight diseases *in vivo* compare?

CHAPTER TWO

LITERATURE REVIEW

2.1. The history of Tomatoes and its importance

The cradle of tomatoes is thought to be in the tropical Americas, around the present day Ecuador and the wilderness of Peru (Ochida *et al*., 2019). Even this still remains unclear, it is generally speculated that the Incas and Aztecs were the earliest cultivators of tomatoes (ChitraMani & Kumar, 2020). At around the 16th century the Spanish introduced it in Europe from where it spread to other parts of the world. Tomatoes form part of an essential human diet and is ranked as the most significant vegetable crop globally (FAO, 2018). Its designation as a vegetable is derived from the mode through which it is consumed, botanically however it is a fruit (Alam & Goyal, 2007). Tomatoes are used both fresh and cooked and are liked due to their extensive range of colours, shapes, sizes, and flavours (Zörb *et al*., 2020). The carotenoid lycopene gives the tomatoes their red colour and is believed to have health promotion aspects such prevention and control of prostrate, breast and lung cancer (Rao & Agarwal, 2000). Other tomato colours albeit rare are yellow, pink, black, brown, orange and green (Okwori *et al*., 2010). Phenols, flavonoids, and ascorbic acid (vitamin C) are other elements that promote health. .

2.2. Tomato (*Solanum lycopersicum* L) varieties

Determinate and indeterminate cultivars of tomatoes are both available. Determinate types are required for open field production, including Onyx, Eden, Tanzanite and Monyalla as they produce a high yield while Cal. J (Kamongo) despite being prone to

disease, it is well-liked due to its high market value and lengthy shelf life. The greenhouse production requires indeterminate tomato varieties like Kenom, Marglobe, Monset, Nemonneta and Anna F1 (Geofrey *et al.,* 2014: Annastacia *et al.*, 2011).

2.3. Cultivation of Tomatoes

Globally, tomatoes represent approximately a total of 4.8 million ha of land area producing an estimate of 162 million tonnes (Ochida *et al*., 2019). China is the leading producer of tomatoes (50 million tonnes) followed by United States of America and India (Guan *et al*., 2018). Nigeria, Egypt and Morocco are the largest producers in Africa (Nadia *et al*., 2014).

Tomatoes respond well to high temperatures. The range of temperature for seed germination is 10^{o}C to 35^{o}C and an optimum range of 17 to 20^{o}C. An optimum growth rate is obtained at 22^{o}C with reductions occurring above 30^{o}C and below 12^{o}C. Fruit setting is inhibited above 30^{o}C and below 16^{o}C (Chepkoech *et al*., 2019). Rough fruit results from growing temperatures below 16^{o}C. Root growth does not occur below 16^{o}C. Tomatoes require adequate, even moisture particularly at flowering and during fruit set (Blancard , 2012).

Tomatoes grow best in well-drained soils that are well supplied with organic matter. Sandy soils are suited for early production, loam and clay loam soils are suited for later production (Chepkoech *et al*., 2019). Shelter from wind is important especially for early production. Plastics are used to assist this crop as well, such as ground mulch, row coverings, and tunnel homes. Weather has a significant impact on the marketable yields of ripe tomatoes which is about 10,000 to 30,000 kg per hectare (Kirimi *et al.,* 2011).

2.4. Diseases of the Tomato plant

Diseases of tomato plant lead to yield losses that are attributed to both premature death of foliage, diseased stems and also diseased fruits. These diseases are more severe in humid and high rainfall areas than in dry areas. Diseases significantly contribute to the yield gap hence there is need for management of tomato crops against the pathogens to maximize the crop yield (Mulugeta & Selvara, 2013). A wide range of tomato diseases are a serious problem in Kenya and other tomato growing countries. They include bacterial diseases like bacterial wilt, bacterial spot, etc.; fungal diseases, such as early blight, late blight, and Fusarium wilt; and viral diseases like leaf curl and spotted wilt (Annastacia *et al.*, 2011).

2.4.1. Early blight

Early blight, caused by *Alternaria solani*, is economically the most important disease of tomatoes internationally. In Greece, the pathogen was first discovered in tomato plants in 1936. In 1995 to 1998 there was an increase in the incidence and severity of the disease in many tomato growing areas in mainland Greece. A. solani has been documented to infect tomato and potato plants in both dry and wet conditions and can grow in a wide range of temperatures between 4°C and 36°C (Vloutoglou & Kalogerakis, 2000). Control is done by removal and destruction of the affected plant parts, practicing crop rotation and spraying the crop with fungicides (Fry & Goodwin., 1997).

2.4.1.1. The pathogen classification and morphology

Alternaria solani belongs to the kingdom Fungi, the domain Eukarya, the class Hyphomycetes, the order Hyphales, the series, and the Porosporae (Neergaard 1945; Jones *et al*., 2016). The form of colonies can vary greatly, but they are often effusive, greyish, brown to black, and have a cotton felt or velvet-like texture (El-Nagar *et al*., 2020). Growth is rapid on many growth media, but special conditions are required for sporulation. Orange to dark red pigments are produced which colour the medium (Govan., 2014).

Conidiophores can grow up to 110 mm in length and 6–10 mm in diameter, are thick-walled, straight to flexuous, septate, and can appear alone or in small groups. Conidia are typically 0-8 longitudinal or oblique, 6-19 transverse, obclavate to elongate, double walled, pale to olivaceous-brown, formed individually or in short chains, straight or somewhat flexuous, 75-350 m in length, and 20-30 m in diameter in the more broad part. Beaks are 5 to 9 mm in diameter, filiform, straight or flexuous, septate, hyaline to pale brown, and approximately half to one-third the length of the conidium (Kumar *et al*., 2017).

There is variability in spore dimensions hence there is overlap of spores with dimensions of other large spored *Alternaria* species. Leaf symptoms, host range, and cultural traits all help with identification. Biochemical or molecular techniques best verify the identity of the pathogen. The special conditions are required for sporulation (Rodrigues *et al.,* 2010).

2.4.1.2. Signs and symptoms of the disease

Tomato early blight affects the foliage at any stage of growth (Roy *et al*., 2019). As spots grow, a bull's-eye pattern of concentric rings can be observed in the center of the infected area by the time they are a quarter inch in diameter or greater. Spots enlarge, and by the time they are a quarter inch in diameter or larger, concentric rings in a bull's eye pattern can be seen in the center of the diseased area. The tissue around the patches may become yellow. Much of the foliage gets lost at this time if the temperature and humidity are extreme. Similar to leaf lesions, stem lesions can occasionally girdle the plant if they develop close to the soil line. The calyx or stem attachment is typically where the fungus enters the fruit to cause infection. Concentric rings are also found on the fruit, and lesions can grow to be quite large, typically covering nearly the whole fruit (Adhikari *et al*., 2017).

2.4.1.3. Host susceptibility

At all growth stages, tomato plants are vulnerable to *Alternaria solani*, but as plants mature, their vulnerability rises (Moghaddam *et al*., 2019), Plants with scant foliage and strong yield or early maturity are more susceptible than those with extensive foliage and poor yield or late maturity. (Alizadeh-Moghaddam *et al*., 2020). Susceptibility between tomato cultivars and hybrids are similar (Annastacia *et al.,* 2011). The age of the host affects how susceptible tomato plants are to *A. solani* infection. Favorable weather conditions may not induce epidemics in young, resistant tomato plants. Host susceptibility is determined biologically by factors such as primary inoculum level, and physiological age of the host (Vloutoglou & Kalogerakis., 2000).

2.4.1.4. The Pathogens and their Life Cycles

Early blight is widespread in most areas where tomatoes or potatoes are grown, but is particularly prevalent in tropical and temperate zones (Jones, 1991). Due to the possibility of several cycles of infections over a season, *A. solani* is a polycyclic pathogen. It is an imperfect fungi as the sexual mode of reproduction is currently unkown (Adhikari *et al*., 2017). Primary infections on new plantings of tomatoes or potatoes are caused by overwintering inoculums (Fooland *et al*., 2008). The pathogen overwinters in plant detritus as mycelium or conidia. Additionally, chlamydospores have been identified as a source of early blight's overwintering inoculum, enabling the disease to endure chilly conditions in or on the soil (Dhaval *et al*., 2021). For five to eight months, inoculum can survive in detritus in uncultivated soil. The hyphae's resistance to lysis is increased by their dark color. Spores survive most frequently in infected debris and seed (Moghaddam *et al*., 2019). Primarily meteorological, edaphic and biotic factors determine survival in debris and seed. The fungus survives best in dry, fallow fields and between crops in plant debris and on seed (Roy *et al*., 2019). It can also survive on volunteer tomato plants (warm climates) and on other cultivated and wild solanaceous plants for example potato, eggplant, horse nettle and black nightshade (Mc Govan, 2014). Warm and humid conditions favour the germination of spores with optimum temperature ranging from 8-32˚C (Adhikari *et al*., 2017). Germination involves the growth of germ tubes penetrating the host directly through either the stomata or lesions on the plant.

The primary inoculum produces conidia in the spring, which are then splashed or wind dispersed to the lower leaves of the plant where they germinate and infect (Mc Govan., 2014). Wind, rain and insects are the principal methods of dissemination of the spores. A

rapid rate of dispersal does not set in until infection has reached the stage at which whole leaves dry up and plants begin to die (Vloutoglou & Kalogerakis., 2000). The curve of wind velocity resembles that of spore dispersal (Kumar, 2013).

Free moisture helps spore germination during an infection, but relative humidity close to saturation can also cause it (Fooland *et al*., 2008). At 20°C, spores could germinate after just two hours of wetness. Elongation of the germ-tube requires a longer wetting period. Germ-tubes form appressoria, and penetrate the epidermis directly or through wounds or stomata. Depending on the age and susceptibility of the plants, incubation varies significantly (Adhikari *et al*., 2017). The initial infections became necrotic and develop chlorotic haloes. Conidia produced by mycelium from necrotic areas infect healthy plants and start secondary infections (Mc Govan, 2014). Sporulation takes place between 5 to 30 degrees Celsius, with 20 degrees being ideal. The heaviest sporulation occurs after heavy rain or dew. Large numbers of spores are produced during alternating wet and dry period. Spore production is initiated by daylight, but spores accumulate over a 7–14-day period and are then dispersed during the day (Adhikari *et al*., 2017).

2.4.1.5. Epidemics

Moisture plays a major role in the development of early blight (Dhaval *et al*., 2021). Free water is essential for disease development, and the length of leaf wetness can explain up to 90% of the variation in disease onset and severity, according to studies. Early blight development is also aided by increased leaf maturity, heavy fruit loads, crowded plants, above average rainfall or dew, and shade (Alizadeh-Moghaddam *et al*., 2020). *A. solani* reacts differently to weather conditions, depending on the circumstance. In some circumstances, weather factors may have an indirect effect by affecting the host's

sensitivity (Annastacia *et al.,* 2011). Short photoperiods are linked to a drop in the sugar content of leaves, while cooler temperatures may slow the growth of the plant (Gao *et al*., 2017). Epidemics do not generally occur until late in the season, when the plants are most susceptible (Blancard, 2012).

2.4.2. Late blight

2.4.2.1. The causal agent

The *Phytophthora infestans* fungus is the cause of late blight. It is thought to be a fungus-like organism rather than a truel fungus (Birch & Whisson, 2001). Currently, this pathogen is categorized as an oomycete, which belongs to the Chromista kingdom. (Stramenopiles or Straminopiles) (Judelson & Ah-Fong, 2010). Saprolegniales and Peronosporales are the two orders to which oomycetes belong. Phytophthora species and a number of other crucial plant pathogenic taxa, such as the genus Pythium, are found in the order Personosporales (Kazan & Gardiner, 2017).

Phytophthora infestans is found throughout the world, although the most serious epidemics happen in regions with regular cool, moist weather (Jaime *et al.,* 2000). However, chilly, damp weather frequently precedes epidemics. P. infestans' primary host range is restricted to solanaceous plants including tomato, nightshade (*Solanum nigrum*), and potato (Leesutthiphonchai *et al*., 2018). *P. infestans* has been reported in numerous strains both domestically and abroad (Kazan & Gardiner, 2017). They differ genetically in terms of virulence, are capable of defeating previously resistant plants cultivars, and also carry the risk of evolving resistance to particular classes of fungicides. At any stage

of plant development, *P. infestans* infects all aboveground sections of susceptible plants (Leesutthiphonchai *et al*., 2018).

The pathogen is heterothallic, with the A1 and A2 compatibility mating types. Oospores are formed whenever these two mating kinds are present in the same plant tissue; otherwise, only asexual sporangia are created (Wang *et al*., 2019). Up until the early 1980s, A1 was the most common mating type found worldwide, with the exception of central Mexico. According to reports, mating type A1 is likely to blame for tomato late blight epidemics because there are no oospores in samples of tomatoes with late blight (Zhang *et al.,* 2003).

2.4.2.2. The disease cycle

When sporangia or mycelia fragments are spread by the wind from infected plant parts, dissemination takes place. Either sporangia produce motile zoospores that encyst on host organs and penetrate the tissues via a penetration peg, or sporangia directly germinate by penetrating a plant organ via germ tubes (Jaime *et al.,* 2000).

Inoculation occurs when sporangia or mycelia fragments land on the plant producing characteristic necrotic late blight symptoms (Narouei-Khandan *et al*., 2020). In nature, asexual reproduction is more common than sexual reproduction (Wang *et al*., 2019). With an optimal temperature range between 18 and 22°C, sporangiophores harboring asexually generated zoosporangia form on sick tissues at relative humidity levels of 91% to 100% and temperatures ranging from 4°C to 26°C (Cai *et al*., 2019). When both mating kinds are present, *P. infestans* can live in plant waste, on stray tomato plants, and on perennial weeds like black nightshade. The pathogen produces the thick-walled oospores (A1 and A2), which are long-term survival propagules (Zheng *et al.,* 2003).

The infection is dispersed by wind, rain, or with human assistance when infested or contaminated things, including seeds or tools (Majeed *et al.,* 2017). The optimal conditions for the infection and spread of the late blight disease include relative humidity near 100%, daytime temperatures between 16 and 21°C, and nighttime temperatures between 10 and 16°C (Demissie, 2019). Large, densely planted crops, chilly, wet weather, and high relative humidity are all risk factors (Cohen *et al.,* 1994). It also occurs when humid conditions coincide with mild temperatures for prolonged periods. If conditions are ideal for disease development, the disease will be rapid causing severe economic losses (Fry & Goodwin, 1997).

2.4.2.3. Symptoms and disease development

As a result of the pathogen's mycelium immediately penetrating cell walls and ramifying intercellularly throughout host tissues, the disease quickly takes hold and the tissues are destroyed (Cohen *et al.,* 1994). On the leaves, lesions are first big, irregular, greenish-black, and wet. These spots coalesce turn brown, and, in humid environments, produce a white moldy growth close to the edges of the sick area on the underside of leaves or on stems. Under humid conditions, the disease spreads quickly destroying significant sections of fruit and leaf tissue. Lesions that form on the leaves are initially asymmetrical, big, greenish-black, and wet (Demissie, 2019). These spots quickly grow in size, turn brown, and, in humid environments, produce a white moldy growth close to the edges of the sick area on the underside of leaves or on stems. Fruit lesions are big, green to dark brown lesions that typically appear on the fruit's upper half, however they can also appear on other areas. White moldy growth may also appear on fruits under humid conditions (Pliego et al 2011).

2.4.2.4 Control of Late Blight

Control practices include rotating fields so as not to replant potatoes or tomatoes in the same land, avoiding planting tomatoes near potatoes, using disease-free seeds and transplants. Adopting certain measures can also control the disease. First, the seed material should be obtained from a disease-free area. The seeds should be treated before planting. The plants must be sprayed with fungicides at 15 days interval, starting from 30 days after transplanting (Fry & Goodwin, 1997).

2.4.2.5. Isolation and identification of *Phytopthora infestans*

Morphological characteristics are the basis for species identification and taxonomy. The classification of Phytopthora species is based on factors such as sporangia's ease of detachment, the type of antheridial attachment, the mating system, the morphology of the sporangiophore, the presence of chlamydospores and hyphal swellings, and whether or not sexual reproduction is heterothallic or homothallic. Therefore, the genus is divided into six major groups which are intended to aid in identification (Drenth & Sendal, *2*011)

2.4.2.6. Media and antibiotics for isolation of *Phytophthora* from diseased plant tissue

Special techniques are required for isolation of Oomycetes because they are not true fungi. *In vitro*, the majority of *Phytophthora* species grow slowly. Bacterial populations need to be kept low because they may suppress the growth of *Phytophthora* by direct competition, by antagonism caused by antibiotic production, or by direct parasitism (Demissie, 2019) To overcomes these problems, there is need to use selective media. Antibiotics are added to the isolation media in order to suppress the growth of bacteria. Suitable antibiotics that are effective against bacteria include ampicillin, penicillin,

rifampicin, and vancomycin, used alone or in combination (Pliego *et al* 2011). *Phytophthora* sp. are out-competed by many fungi hence the media used should be low in nutritional components and have suitable antibiotics for antifungal activity (Majeed *et al*., 2017). The media commonly used is synthetic cornmeal agar. Other desirable basal media include water agar or V8 juice agar (Leesutthiphonchai *et al*., 2018). The antibiotics comprise pimaricin and nystatin. Nystatin is typically more affordable and accessible than pimaricin. This reduces the growth rate of fungal contaminants, allowing colonies of *Phytophthora* to become established (Selim., 2015).

2.4.2.7. Pathogenicity of *P. infestans*

Phytophthora spp. are the most effective plant pathogens because of the ability to produce different types of spores including sporangia and zoospores for short-term survival and spread, chlamydospores and oospores for more long-term survival (Fooland *et al*., 2008). Sporulation occurs rapidly on host tissue within 3-5 days after infection therefore resulting in a rapid building up of inoculum. This leads to epidemics when the environmental conditions are favorable (Drenth & Sendall., 2011)

Zoospores of *P. infestans* have the ability to be attracted to root tips through positive chemotaxis as a result of their mobility by swimming to the actively growing root tips where they encyst, and infect young susceptible root tissue (Leesutthiphonchai *et al*., 2018). They also have the ability to survive in or outside the host tissue as oospores or chlamydospores for long periods of time (Wang *et al*., 2020). Oospores are also known to survive passage of the digestive systems of animals. *P. infestans* produces sporangia that are airborne and sporangiospores that, in humid and cool environments, can differentiate

into 4 to 32 zoospores, resulting in many infections from a single sporangium. (Pragya *et al.*, 2017)

Oomycetes have different biochemical pathways compared to the true fungi. Therefore, many fungicides are not very effective against *P. infestans* pathogens (Fry *et al*., 2015). They thrive under humid and wet conditions, which make them difficult to control because protectant fungicides are difficult to apply and least effective in humid conditions. *Phytophthora* spp. pathogens can cause many different diseases and disease symptoms on a wide range of plant species (Drenth & Sendall., 2011*)*

2.5. Biological Control of Diseases

Biological control is now increasingly being considered as an alternative to chemical control of plant disease in sustainable agriculture (Moghaddam *et al*., 2019). Its measures rely on the use of organisms that are antagonistic to the target pathogens (Padmavathi, 2011). Mycoparasitism, which can be caused by physical interhyphal interference or by the generation of volatile and nonvolatile metabolites, is one way that antagonistic organisms might interact (Pusztahelyi *et al*., 2015). Several microorganisms, including the obligate mycoparasite fungus *Verticillium biguttatum*, have been reported as effective biological control agents (BCAs) against *R. solani* (Demirci *et al*., 2009). The genus *Trichoderma* remains an economically efficient BCA that is commercially produced at a large scale and is applied against several fungal pathogens (Lahlali & Hijri, 2010). Increased research efforts on biological control of soil plant pathogens have been made worldwide as a result of the lack of resistant crop varieties, the unfavorable effects of

applying large quantities of fungicides to soil, and the emergence of resistance in soil borne plant pathogens. (Padmavathi, 2011).

Use of biological controls products for soil borne pathogen has gained popularity in recent years due to environmental concerns raised on the use of chemical products in disease control (Demirci *et al*., 2009). Biological control methods have been widely accepted and advocated for as key practice in sustainable agriculture with the biggest potential of the biological control being microorganisms, arbuscular mycorrhizal fungi (AMF) and some naturally occurring antagonistic rhizobacteria such as *Bacillus* sp., *Pseudomonas* sp. (Dorjey *et al*., 2017) Use of AMF in agricultural crops can provide protection against soil-borne pathogens by reducing the root diseases caused by a number of soil pathogens. A number of mechanisms are involved in controlling and suppression of the pathogen by mycorrhizal fungi, among them exclusion of pathogen, changed nutrition, lignifications of cell wall, and exudation of low molecular weight compounds (Dorjey *et al*., 2017).

Other biological agents that have been used for the disease management include; Fluorescent pseudomonads such as *Pseudomonas fluorescens,* which are antagonistic to soil-borne pathogens by production of antimicrobial substances, competition for space, nutrients and indirectly through induction of systemic resistance (Shen *et al*., 2013). Bacteriophages, which are capable of attacking the *Ralstonia solanacearum*, have been used as a biocontrol agent (Tan *et al*., 2010). Plant growth promoting bacteria (PGPR) strains are reported to be promising biocontrol agents to control *R. solanacearum.* A fungus, *Pythium oligandrum* has been reported to suppress bacterial wilt caused by *R.*

solanacearum but it is yet to be produced and formulated for use on a commercial scale (Onduso, 2014).

2.5.1. *Beauveria* sp. as a biological control agent

Agostino Bassi de Lodi identified the fungus entomopathogen *Beauveria bassiana* in 1835 (Shapiro-Allan *et al*., 2002). It was discovered to be effective in dwindling the number of silkworms. The potential of *B. bassiana* wasn't realized until 1991. Bing & Lewis (1991) identified *B.* bassiana as a potential endophytic biocontrol agent and found that the foliar application of conidial suspension to the whorl-stage of corn plants (*Zea mays L.)* reduced *Ostrinia nubilalis* populations and persisted to provide season-long suppression of the insect indicating the successful establishment of *B. bassiana* as an endophyte. Around the world, *Beauveria bassiana* are known to spread a variety of plant diseases and insect pests (Shapiro-Allan *et al*., 2002). These entomopathogenic fungal genera are recognized to be able to disturb and kill insects, making them effective biocontrol agents. Other research examined the effects of inoculating tissue grown banana (*Musa* sp.) plants with endophytic *B. bassiana* strains on larval development and the reduction of banana weevil (*Cosmopolites sordidus)* damage (Akello *et al.,* 2009). The coffee berry borer (*Hypothenemus hampei* Ferrari) damage could be lessened by using endophytic *B. bassiana* strains (Vega *et al.,* 2008), millet stem borer, *Chilo partellus* Swinhoe (Reddy *et al.,* 2009), and cotton bollworm, *Helicoverpa zea* (Lopez & Sword, 2015) on their respective host plants.

Beauveria bassiana also has a potential for biologically controlling plant pathogens (Wang & Zheng, 2012). There is now a lot of proof that *B. bassiana* can shield plants from some soil-borne plant diseases (Akello *et al.,* 2009). It generates a variety of

bioactive metabolites, some of which have been shown to inhibit *in vitro* the growth of fungal plant pathogens (Tall & Meyling, 2018) such as *Pythium, Rhizoctonia*, and *Fusarium* (Wang & Zheng, 2012). Additionally, it might be advantageous to fungi and plants. It is well known that plant species significantly influence the microbial communities that are linked with plants (Tall & Meyling, 2018). *B. bassiana* colonizes tomato (*Solanum lycopersicum* L.), various dicots, and monocots in addition to growing as an endophyte. (Bonnie *et al.*, 2009).

It's possible that *B. bassiana* suppresses plant diseases through more than one mechanism (Saragih, 2019). Numerous secondary metabolites produced by the fungus are known to have antifungal, antibacterial, insecticidal, and cytotoxic, properties. Examples include beauverolides, bassianolides, beauvericin, oosporein, cyclosporine A, and oxalic acid (Bonnie., 2009). Recently, the antibacterial substance bassianolone from a fermentation culture of *B. bassiana* under low nitrogen conditions was described (Ssaragih, 2019). Bassianolone is active against Gram-positive cocci and fungi. *In vitro* antibiosis tests using *B. bassiana* against a variety of plant pathogens have been described, but the antimicrobial substances used have not been identified. (Canassa *et al*., 2019). *Rhizoctonia solani* and *Pythium myriotylum*, two soil-borne plant pathogens, are suppressed by the *Beauveria bassiana* strain 11-98. (Zhang & Li., 2011). It is unknown whether the beauvericin and oosporein produced by this isolate contribute to the control of plant disease. Given that *B. bassiana* 11-98 is a plant colonizing fungus, resource rivalry is likely to be a factor in the biological control of plant diseases with this strain. When *B. bassiana* 11-98 was applied to tomato seed, seedlings became endophytic and epiphytic colonized, and they were then protected from damping-off (Canassa *et al*.,

2019). A surface-sterilized tomato seedling's root, stem, and leaf portions all contained the strain. *B. bassiana* 11-98 has been found in the foliage, stem, and root tissues of 18-week-old tomato plants grown from treated seed in addition to seedlings. Numerous plants, both those that are grown naturally and those that have been infected using a variety of techniques, have had *B. bassiana* isolated as a fungal endophyte (Bonnie *et al.*, 2009).

In vitro antagonism of *Beauveria bassiana* an entomopathogenic fungus has been also tested against *Fusarium oxysporium* f. sp. *vasinfectum*, pathogen of cotton wilt and *F verticillioides*, pathogen of cotton red rot. On dual culture, *B. bassiana* showed obvious inhibition on the growth of the two pathogens. Different doses of conidia had different inhibitory effects, with inhibition rate linearly correlated to inoculum, indicating that the antagonism was associated with competition for nutrition and space. The inhibition on the two pathogens was both over 90% at an inoculum of 6×10^{7} spores ml^{-1}, slightly higher on *F. verticillioide*. The antagonism was also associated with antibiotic substances, antibiosis was considered to contribute more than competition. The main inhibitory substances were polysaccharide and protein with high molecular weight (Zhang & Li., 2011).

2.5.2. *Trichoderma* sp. as a biological control agent

Person originally identified the genus Trichoderma in 1794, and more than 100 species have since been defined at the genetic level. Many of these species lack information on their sexual life cycle and are therefore regarded as fungi imperfecti (Mukherjee *et al.*, 2013). Trichoderma spp. are widespread, free-living soil borne fungus that can be isolated and grown in a variety of soil types, from temperate to tropical (Mukherjee *et al.*,

2013). Trichoderma species were formerly thought of as common soil saprophytes, but research conducted over the past three decades has revealed that they are capable of creating opportunistic, avirulent symbiotic partnerships with plant root systems (Druzhinina *et al.,* 2011). More than 80 identified species of *Trichoderma spp*., which includes a large number of fungal strains, operate as biological control agents against numerous soil-borne phytopathogens (Bailey *et al*., 2008; Howell, 2003), and foliar pathogens including *Sclerotinia sclerotiorum* (Castillo *et al.,* 2011).

In contrast to other fungi, Trichoderma species grow rapidly on a variety of substrates, create secondary metabolites, and may be mycoparasitic against a variety of diseases (Mukherjee *et al.,* 2013). *Trichoderma* spp.'s antagonistic traits are based on the activation of a number of processes, including mycoparasitism, antibiosis, plant growth promotion, competition, and induced systemic resistance (Bailey *et al.*, 2008; Howell, 2003). *Trichoderma* has some antibiotic producing properties. As a result, it serves as a biocontrol agent (Ghisalberti & Sivasithamparam, 1991). Isolates of *Trichoderma* including isolates from the same species produce a range of volatile antibiotics that have different effects on different fungi (Harman, 2004). It is possible that the isolates produce either the same blend of metabolites in different proportions or completely different chemical compounds (Howell, 2003).

Nutrient competition, mechanical barriers, or pH changes are some of the antagonistic effects (Ghisalberti & Sivasithamparam, 1991). Both fungi and bacteria are able to synthesize a wide range of metabolites with fungicidal and bactericidal capabilities. These antibiotics are an alternative biological protection to conventional fungicides. Additionally, screening the isolates individually or in combination is necessary to choose

effective isolates that will enhance plant growth and biomass (Padmavathi & Madhumathi, 2011).

Several *Trichoderma* species have been extensively studied for their biocontrol potential. *Trichoderma harzianum* isolate T-22 is marketed for the control of many different diseases in many different crops (Akladious & Abbas, 2012). T-22 is applied as a seed treatment or soil amendment but has also been used for foliar applications to control diseases of apple, grape, and other fruit crops. Numerous studies have been conducted on *T. harzianum* isolate T39 to control gray mold on grapes using foliar sprays. Witches' broom is managed in the field using *T. stromaticum*, a mycoparasite of *C. perniciosa*. Numerous plant species have proved to be resistant to disease when exposed to isolates of *Trichoderma* species including *T. harzianum*, *T. hamatum*, *T. asperellum*, and others (Anees *et al.,* 2012).

Although *Trichoderma* has been extensively studied for its biocontrol potential, the endophytic ability of *Trichoderma* has only recently received attention. Even little consideration has been given to the idea of Trichoderma as an endophyte of tissues from above-ground plants. Typically, Trichoderma species are regarded as soil and root endophytes. Several of the *Trichoderma* isolates efficient as endophytic colonizers of the above ground portions of the cacao seedling has been studied by inoculating away from the soil surface between the cotyledons (Bailey *et al.,* 2008).

The crucial element of this relationship is Trichoderma's ability to enter the plant's root system and the fungus's continued existence within living plant tissues (Cripps-Guazzone, 2014; Cripps-Guazzone *et al.,* 2016; Hohmann *et al.,* 2011, 2012). Recent

research has shown that *Trichoderma harzianum* can invade *Theobroma cacao* leaves in addition to the roots (Bailey *et al.,* 2009) and cabbage (Zhang, 2014). It was discovered that *T. atroviride* LU132 greatly increased the root and shoot biomass of *B. napus* (Maag *et al.,* 2013) this implied that the plant had been endophytically colonized by the infected fungi. Additionally, it has been demonstrated that adding T. virens LU556 and *T. hamatum* LU593 as maizemeal-perlite (MP) soil incorporations reduces *S. sclerotiorum* infection of cabbage. (Jones *et al.,* 2014) and suggested that it was rhizosphere competence and therefore induced resistance. In another study, pre-inoculation of *Trichoderma* spp. on cotton against *Aphis gossypii* resulted in all leaves being colonized by the fungus (Gurulingappa *et al.,* 2010) and enhanced protection. Geraldine *et al.* (2013) described that inoculating common beans with *Trichoderma* spp. against *S. sclerotiorum* resulted in reduction of apothecia density and disease severity, subsequently increasing the number of pods per plant and yields up to 40% compared to controls. Apart from other mechanisms of biocontrol by the fungus, the ability of *Trichoderma* spp. to effectively colonize the plant rhizospheres can result in protection of the host from both biotic and abiotic stresses including protection against phytopathogens ((Padmavathi & Madhumathi., 2011).

2.6. Use of resistant cultivars and its limitations

Host resistance is an efficient and effective component in integrated management of plant diseases and some tomato cultivars provide moderate resistance against diseases for example bacterial wilt disease (Juan *et al*., 2005). This is having been made possible by genetic improvement on varieties to increase tolerance against pathogens for example *R. solanacearum.* The use of resistant varieties has been reported to be the most effective

and practical method to control diseases. Although the species occur worldwide, distribution of individual strains is not uniform leading to resistance breakage of a highly tolerant variety from a different geographical area (Onduso, 2014).

2.7. Volatile organic compounds

Volatile organic compounds (VOCs) are carbon-based solids and liquids that readily enter the gas phase by vaporizing at 0.01 kPa at a temperature of approximately 20^{o}C (Hung *et al*., 2015). Most are lipid soluble and thus have low water solubility (Bennet *et al.*, 2012). Despite some methodological and technological constraints, researchers have detected and characterized approximately 250 VOCs from fungi which have characteristic odors and are produced during primary and secondary metabolism (Hung *et al*., 2015). They occur as mixtures of simple hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives, including, among others, benzene derivatives, and cyclohexanes. Fungal VOCs may be important in the success of some bio-control species of *Trichoderma* and also play important signaling roles for fungi in their natural environment (Shannon *et al.*, 2012: Bennet, 2015).

Many VOCs have distinctive odors, and can diffuse through the atmosphere and soil. For example, the distinct bouquets of macro fungi such as mushrooms and truffles, highly valued in the culinary arts, include mixtures of different VOCs, of which alcohols, aldehydes, terpenes, aromatics and thiols dominate (Hung *et al*., 2015). Many ecological interactions are mediated by VOCs, including those between fungi and plants, arthropods, bacteria, and other fungi. The diverse functions of fungal VOCs can be developed for use in biotechnological applications for biofuel, biocontrol, and myco-fumigation (Shannon *et al.*, 2012).

Volatiles represent a new frontier in bioprospecting, and the study of these gas-phase compounds promises the discovery of new products for human exploitation and will generate new hypotheses in fundamental biology (Bennet *et al.,* 2012).

2.7.1. Antifungal activity of volatile compounds

Trichoderma is widely used in agricultural biotechnology and have been already used as biocontrol agents against numerous plant pathogens and quite a few have been developed for commercial use (Shannon *et al.,* 2012). The research demonstrated that *Trichoderma harzianum* strain SQR-T037 produced volatile compounds that can inhibit the growth of *F. oxysporum* up to 40%, while the non-volatile isolates extracted from the liquid culture significantly inhibited the growth of *F. oxysporum.* In the dual culture assay, the strain SQR-T037 overgrew the strain *F. oxysporum.* The incubation time of six days with a temperature of 30^{o}C and pH of 6 were found optimum for the maximum production of compounds by strain SQR-T037. This research showed the great potential of strain SQR-T037 as an antagonist to control the *Fusarium* wilt disease under greenhouse and field conditions (Yanar. 2011: Morath & Bennet., 2012).

Hundreds of volatile compounds are produced by *Trichoderma* spp (Shannon *et al.,* 2012). Many biocontrol antagonistic strains have been reported to produce volatile compounds that inhibit the growth of pathogenic fungi significantly (Yanar. 2011). Volatile compounds produced by *T. harzianum* decreases the growth of *Colletotrichum capsici* which causes leaf blight on basil, chickpea and pepper as well as dieback in pigeon pea (Hung *et al*., 2015). In addition, these volatile compounds are also helpful for producing strains of *Trichoderma* as conidiation is induced by its own volatile (Kramer & Abraham., 2012).

2.7.2. Antifungal activity of non-volatile compounds

The research of antifungal activity of non-volatile or diffusible compounds produced by *Trichoderma* strain SQR-T037 showed that the extracted isolates significantly inhibited the growth of *F. oxysporum* in agar diffusion assay (Morath & Bennet, 2012). The *Trichoderma* strains have been reported to produce different antimicrobial compounds like 6-pentyl-α-pyrone, with a strong, coconut-like aroma produced by *T. harzianum* in liquid culture (Yanar, 2011). This compound showed broad-spectrum antimicrobial characteristics against species such as *Rhizoctonia solani*, *F. oxysporum* f. sp. *lycopersici*, *Botrytis cinerea* and *F. moniliforme.* The compoinds' production is most important mechanism of action for the effective biocontrol of soil borne diseases under green house or field condition (Waseem *et al.*, 2013).

2.8. Mechanisms of plant disease suppression by biocontrol fungi

2.8.1. Antibiosis

The mechanism of antibiosis includes production of antibiotics, bioactive volatile organic compounds (VOCs), and enzymes (Suprapta, 2012). Volatile bioactive compounds include acids, alcohols, alkyl pyrones, ammonia, esters, hydrogen cyanide, ketones, and lipids. The fungal endophyte *Muscodor albus* produces a mixture of VOCs that are lethal to a variety of microorganisms (Suprapta., 2012).

In the first report of VOCs released by a fungal entomopathogen *B. bassiana* carbon source played a major role in VOC production. When cultured on glucose-based media, the VOCs identified were di-isopropyl naphthalenes (50%), ethanol (≈ 10%) and sesquiterpenes (6%), but in media with n-octacosane (an insect-like alkane), the primary VOCs were n-decane (84%) and sesquiterpenes (15%) (Hung *et al*., 2015).

Enzymes involved in antibiosis are distinctly different from those involved in mycoparasitism of plant pathogens (Bailey *et al.,* 2008).

2.8.2. Competition

Fungal biocontrol organisms actively compete against plant pathogens for niche or infection site, carbon, nitrogen, and various microelements such as iron (Blagodatskaya *et al*., 2014). The site of competition is often the rhizosphere, phyllosphere or intercellularly within the plant. Successful competition is often as a result of proper timing as resources are likely to go to the initial colonizer (Lagos *et al*., 2015).

2.8.3. Mycoparasitism

This is the parasitism of one fungus by another (Viterbo & Horwitz, 2010). Varying degrees of host specificity are displayed by mycoparasites. Within a given species of a mycoparasite, some isolates may infect a large number of taxonomically diverse fungi, while others demonstrate a high level of specificity (Poveda, 2021). Parasitism by the biocontrol fungus Trichoderma begins with detection of the fungal host before contact is made. The fungus produces low levels of an extracellular exochitinase, which diffuse and catalyze the release of cell-wall oligomers from the target host fungus. This activity induces the fungus to release fungitoxic endochitinases, which also degrade the fungal host cell wall (Haran *et al*., 1996).

Attachment of the mycoparasite to the host fungus is mediated by binding of carbohydrates in the Trichoderma cell wall to lectins in the cell wall of the fungal host. Upon contact, hyphae of Trichoderma coil around the host fungus and form appressoria. Several lytic enzymes are involved in degradation of the cell walls of fungal and

oomycetous plant pathogens, including chitinases, ß-1, 3 gluconases, proteases, and lipases (Bailey *et al.,* 2008).

2.8.4. Induced systemic resistance (ISR)

Plants are sessile organisms that must develop a complex chemical mechanism in order to withstand biotic and abiotic attack (Pieterse *et al*., 2014). Colonization of plants with non-pathogenic fungi and bacteria can lead to ISR in the host plant. Induced resistance is plant-mediated biocontrol mechanisms whereby the biocontrol agent and the phytopathogen do not make physical contact with one another. Plants react to the presence of a pathogen with a rapid expression of defense-related genes. The signaling mechanisms for this induced resistance are based on Jasmonic acid (JA) and ethylene. Induction of systemic resistance via the JA/ ethylene signaling pathway has been reported primarily for plant growth-promoting bacteria, however, it is also operative for many mycorrhizal fungi and biocontrol fungi (Owney *et al.,* 2010).

In conclusion, the mechanisms of biocontrol in many cases are not mutually exclusive, i.e. multiple mechanisms may be operating against a specific plant pathogen, or a given biocontrol fungus may employ different mechanisms against different phytopathogens. For example, control of *Botrytis cinerea* on grapes with Trichoderma involves competition for nutrients and mycoparasitism of sclerotia, (the overwintering, long-term survival structure of *Botrytis*). Both mechanisms contribute to suppression of the pathogen's capability to cause and perpetuate disease (Bailey *et al*., 2008).

CHAPTER THREE

METHODOLOGY

3.1 Study Area

This study was carried out in Uasin Gishu county, Soy constituency, Soy Sub County, soy division, Barsombe location and Barsombe sub location. The coordinates of the area under study are 0^{o} 33’ 00’’ N and 35^{o} 44’ 00’’ E. Farmers in this area engage in all year round tomato production supplemented with irrigation water from the nearby River Moiben. It is for this reason that the area was selected for this study.

3.2 Collection of materials

Diseased tomato leaves and fruits showing symptoms of early and late blight were obtained from the sited above. The samples were separately placed in biodegradable paper bags and transported to the University of Eldoret Biotechnology laboratory. A scanning light microscope was used to observe the specimens in order to study the symptoms of pathogens causing early blight and late blight (Swapan & Chakra, 2012).

3.3 Isolation and characterization of biocontrol agents

3.3.1 *Trichoderma harzianum*

Trichoderma harzianum was selectively isolated from soils obtained from root rhizosphere of tomatoes planted by farmers in the study area. One gram of soil was added to 0.1% Tween 80 in a bottle, shaken and allowed to rest for 10 minutes. Serial dilutions were performed up to the tenth dilution, from which, 100 microliters were pipetted and placed on a plate containing *Trichoderma harzianum* selective medium (THSM) while streptomycin was used as an antibiotic. This was then incubated at 28^{o}C for 4 days. Sub culturing of colonies of interest was done on Sabouraud Dextrose Agar (SDA) and later

on Potato Dextrose Agar (PDA) media in preparation for subsequent use (Ephrem *et al.*, 2011).

Characterization was done by colony observation and spore was observed under the microscope. Greenish white colony color was a preliminary identification of the fungus. Observation of spores held on highly branched conidiophore and flask shaped phialide confirmed the identity of the isolates as *Trichoderma harzianum*.

3.3.2. *Beauveria bassiana*

Beauveria bassiana was isolated from a dead beetle showing signs of infection by the entomopathogenic fungi. The beetle was obtained from the study area and was brought to the University of Eldoret Biotechnology laboratory. It was surface sterilized by use of 1% Sodium hypochlorite (NaOCl) solution then rinsed with sterile distilled water. The beetle was then put on a culture plate containing selective Peptone Glucose Agar (PGA) medium (2.5 g/l peptone, 5g/l glucose, 15g/l agar) with the addition of Streptomycin and Tetracycline after autoclaving at a temperature range of 55-60^{o}C. The plate was then incubated at 28^{o}C for 7 days. After which, colonies of interest were sub-cultured onto PDA to obtain a pure colony for subsequent use (Owney *et al.*, 2010).

Characterization was done morphologically and microscope observation of the spores. The typical white cottony *B. bassiana* colonies were sought for in the isolates. Microscope observation of rachis and the typical conidiogenous cells confirmed the identity of the isolates as *B. bassiana*.

3.3.3 Isolation and purification of test pathogens

The infected tissues from tomato leaves and fruits obtained from the study area were surface sterilized with 1% sodium hypochlorite for 5 minutes, then sterile distilled water was used to wash them three times subsequently. The infected tissue was inoculated on PDA and incubated for 5 days separately at the University of Eldoret laboratory at temperatures of 20^{o} and 25^{o}C for *Phytophthora infestans* and *Alternaria solani* respectively. The resulting culture of *A. solani* was sub-cultured on PDA containing streptomycin whilst *P. infestans* was sub-cultured on Corn Meal Agar for 7 days to obtain a pure cultures. The antibiotics used were streptomycin, Nystatin, Rifampicin and vancomicin. Both pathogens were preserved by transferring the tip of the mycelia into the PDA slants which were refrigerated and maintained as stock cultures (Swapan & Chakra, 2012).

3.3.3.1 Identification and characterization of the test pathogens

The identification of the pathogens was done using cultural and morphological characteristics with the help of published identification fungal Keys (Swapan & Chakra, 2012; Meya et al., 2014).

3.4 *In vitro* tests for antagonism by *T. harzianum* and *B. bassiana* against *P. infestans* and *Alternaria solani*

The antagonistic effect of *T. harzianum* and *B. bassiana* against *A. solani* and *P. infestans* under *in vitro* was evaluated using the dual culture technique. The *A. solani* and *P. infestans* isolate was cultured, separately, on PDA medium for 7 days at room temperature. *T. harzianum* and *B. bassiana* isolates was also sub cultured separately on PDA medium for 7 days incubated at 25^{o}C and 20^{o}C respectively. Discs of 5 mm-

diameter from *T. harzianum* and *B. bassiana* mycelial colonies that were actively growing were separately inoculated on the surface of PDA medium 1 cm from one side of Petri dish and at the centre of the petridish for *T. harzianum* and *B. bassiana* respectively. Similarly, 5 mm diameter discs of of *A. solani* and *P. infestans* were also inoculated separately at equal distance on the opposite side of each antagonist on the same Petri dishes. The treatments included: *T. harzianum* vs *A. solani*, *T. harzianum* vs *P. infestans*, *B. bassiana* vs *A. solani* and *B. bassiana* vs *P. infestans*. Petri dishes inoculated with the two pathogens without the bioagents served as controls. Treatments were replicated thrice. The inoculated Petri was incubated simultaneously at 25^{o}C for 7 days. The plates were examined and radial growth of the pathogen towards the antagonist was measured for 7 days so as to compare with those of the controls (Suprapta, 2012). The radial growth (r) towards the antagonist and radial growth in the absence of the antagonist (R) in control plates were taken for 7 days. The radius of inhibition was then obtained by the formula R-r (Makumba, 2016).

3.5 Determination of the efficacy of volatile compounds of *T. harzianum* and *B. bassiana* against *A. solani* and *P. infestans*

The effect of volatile compounds from the antagonists on the growth of *A. solani* and *P. infestans* was determined by the following procedure:

The bottom portion of Petri plate containing PDA was inoculated at the centre with a 5mm disc of actively growing pathogen (*A. solani*). Another bottom plate was inoculated at the centre with 5 mm disc of T. *harzianum.* The Inoculated bottom plates were placed facing each other and sealed with parafilm. They were then incubated at 25^{o}C for 7 days. The same procedure was repeated for T. *harzianum* with *P. infestans*, *B. bassiana* with *A.*

solani, and *B. bassiana* with *P. infestans*. Petri dishes containing PDA with 5 mm discs of pathogens inoculated at the centre of the plate and without antagonist disc on the other plate served as controls. The colony diameter of each of the test pathogen in the treatmenst in comparison with that of controls were taken for 7 days consecutive (Ajith & Lakshmidevi, 2010).

3.6 Determination of the efficacy of non-volatile compounds of *T. harzianum* and *B. bassiana* against *A. solani* and *P. infestans*

The effect of non-volatile (culture filtrate) compounds from *T. harzianum* and *B. bassiana* on the growth of *A. solani* and *P. infestans* was also determined as follows:

The antagonists were grown in potato dextrose broth at 25ºC with intermittent shaking at 150 rpm. The culture filtrate for each were collected after 10 days and filtered first by use of sterile filter papers and later by nitrocellulose filter during amendment. The sterilized filtrate was amended in PDA to make 5, 10 and 20% concentration in petriplates. The solidified agar plates in triplicates were inoculated at the centre with 5 mm diameter mycelial disc of the pathogens (*A. solani* and *P. infestans*) and incubated at 25^{o}C for 7 days. Three plates each without filtrate for each set up served as controls. The colony diameter for each treatment was recorded and compared with the controls to obtain the diameter of inhibition (Ajith & Lakshmidevi, 2010).

3.7 Determination of the efficacy of the *T. harziunum* and *B. bassiana* against early and late blight diseases *in vivo*.

3.7.1 Preparation of microbial suspensions for foliar spraying

3.7.1.1. Preparation of biocontrol fungi conidia

A 250 ml suspension of *T. harzianum and B. bassiana* conidia were prepared from 9 days old culture plates which were grown on PDA at 25^{o}C and 20^{o}C respectively (Ajith & Lakshmidevi, 2010). The plates were rinsed with sterile distilled water and the mycelia carefully scraped off the agar with a glass rod. The suspension was then filtered through a sterile white cotton strainer and filter paper to separate the spores from the mycelia. The concentration was adjusted to 3.0×10^{8} conidia/ml with the help of haemocytometer (Ephrem *et al.,* 2011)

3.7.1.2 Preparation of Pathogen Spores

A 250 ml suspension of conidia of *A. solani* and *P. infestans* were prepared from 9-day old cultures which were grown on PDA at 25^{o}C and 20^{o}C for *A. solani* and *P. infestans* respectively. Mycelia were harvested from the plates by rinsing the mycelia mat with sterile distilled water then scrapped the mat using glass rod (Ajith & Lakshmidevi., 2010. The suspensions were filtered through four folds of sterile cotton cloth to separate the conidia/sporangia from the mycelia. The concentration of the conidia/sporangia was adjusted to 1.0×10^{3} conidia/sporangia per ml with the help of haemocytometer (Ephrem *et al.,* 2011).

3.7.2 Growing of seedlings

Seedlings of Rio Grande variety of tomatoes (Kenya Seed) were raised on seedbeds containing strilized soil measuring 1m long, 1m wide and 15cm high. The seeds were sown at a depth of 0.5 cm in rows of 15cm spacing between rows (Ephrem *et al.,* 2011).

Seedlings were transplanted after 25 days onto sterile plastic pots of 2 litre capacity with sterile soils. Soil sterilization was done by autoclaving at 121^{o}C for one hour repeated twice and thereafter aired and dried for 3 days (Gatachew., 2017). Di ammonium phosphate (0.6 g) were added to each pot (Ephrem *et al.*, 2011). Three replicates were used for each treatment and the pots were arranged in a completely randomized design (Gatachew, 2017).

3.7.3 Foliar spraying of antagonists and the pathogen

Tomato seedlings were planted in pots containing the sterilized soil. The seedlings were transplanted after 25 days. Three days later, all seedlings were sprayed with sterile distilled water, and covered with transparent polythene bags for 3 consecutive days to provide high humidity hence moisten their leaves. After an additional 3 days, the seedlings were treated by spraying with the biocontrol agents (*T. harziunum* and *B. bassiana*) at a concentration of 3.0×10^{8} conidia/ml and the pathogens (*A. solani* and *P. infestans*) at a concentration of 1×10^{3}conidia/sporangia/ml. Triplicate positive control set ups were only sprayed with the pathogens without the biocontrol agents. Negative control set ups were sprayed with sterile distilled water. Individual plants were covered with polythene bags to prevent cross infection among adjacent plants (Ephrem *et al.*, 2011).

3.8 Disease assessment

The disease progress was observed from the onset of signs and symptoms until death of the tomato plant. The lower leaf level of the individual plants on each of the three replicates were chosen and for each leaf level, one deseased leaf was picked for the study and rated on weekly intervals for eight weeks for percentage of the area blighted on the

leaves showing symptoms of early and late blight (Ephrem *et al.*, 2011). To calculate the leaf area, one millimeter graph paper method was used (Pandey & Hema, 2011) to get the percentage that was to be used for rating in the scale of 0-9 (Honeburg & Becker, 2011). Disease grades obtained were analyzed.

3.9 Data analysis

Data analysis was done for each objective independently. Cleaned, coded and managed data were analysed with statistical package (SPSS version 23). Data obtained from dual culture experiments, volatile compounds produced by *Trichoderma harzianum* and *Beauveria bassiana* against *Alternaria solani* and *Phytophthora infestans in vitro* were analysed using student's T-test. Efficacy of non-volatile substances produced by *Trichoderma harzianum* and *Beauveria bassiana* against *Alternaria solani* and *phytophthora infestans in vitro* was tested by Analysis of Variance (ANOVA). Comparison of efficacy of *Trichoderma harzianum* and *Beauveria bassiana* against early and late blight diseases *in vivo* was tested using ANOVA.

CHAPTER FOUR

RESULTS

4.1 Collection of Materials

Three adjacent farms were visited and all isolates showed similar morphological and cultural characteristics. *The f*ruit lesions were large, brown and mostly occured on the upper half of the fruit. The upper half may be moldy with white growth under humid conditions.

Alternaria solani was isolated from fruits obtained from the three adjacent farms (Plate 4.1a) and colonies showed similar morphological characteristics. The lesions appeared dark pigmented and showed concentric growth rings in a bull's eye pattern.

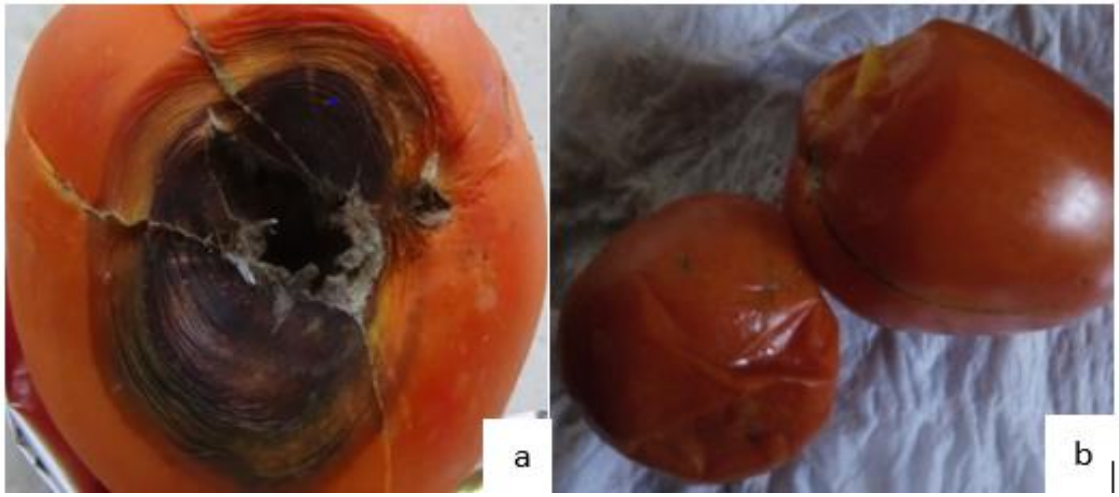

Plate 4.1: Diseased tomato samples. a) Tomato fruit showing symptoms of early blight caused by *Alternaria solani*. b) Tomato fruit showing symptoms of late blight *P. infestans*.

4.2 Isolation and identification of biocontrol agents

4.2.1 *Trichoderma harzianum*

The fungus was fast growing hence occupied the 9 cm diameter plate in 4 days. The colony morphology was whitish green in colour forming concentric rings of green conidial production (Plate 4.2a). The green colour was denser in the center and towards the margin (Plate 4.2). Under a light microscope at magnification of 400×, the following features were seen: soft fluffy mycelia, green ring like conidia and highly branched conidiophore and flask shaped phialide (Plate 4.2b).

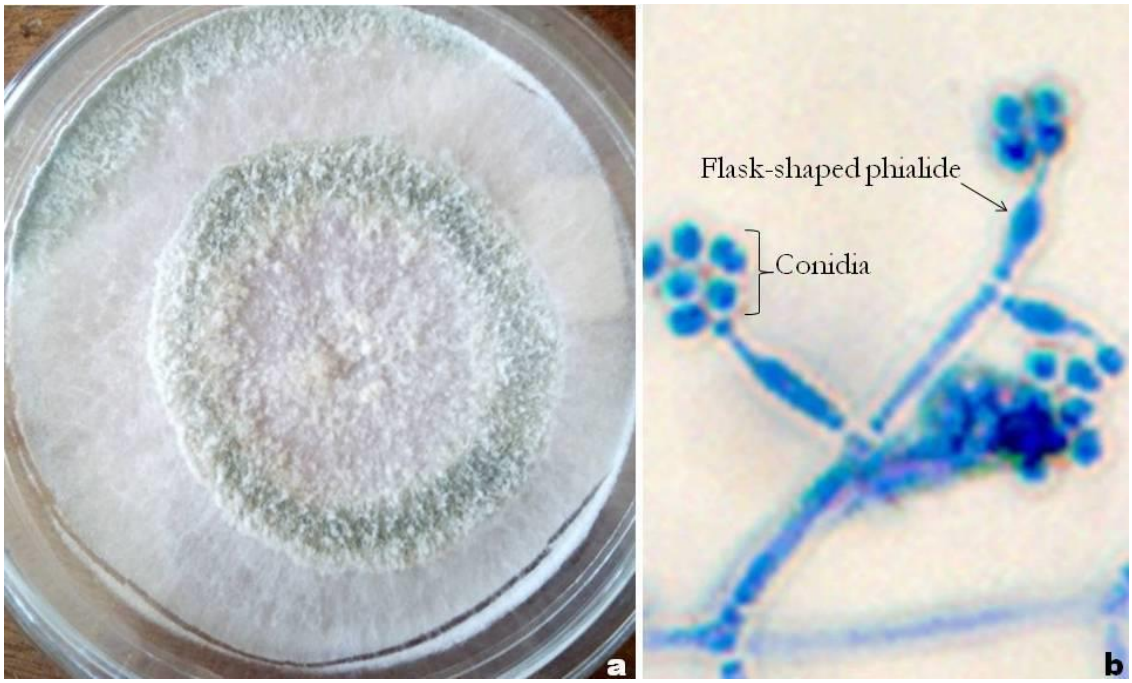

Plate 4.2: a) *Trichoderma harzianum* colony (7 days old) on PDA. Note the concentric rings with green colouration (conidia) and the white fluffy mycelium. b) A micrograph of *T. harzianum* under the light microscope at 400× showing a clump of conidia at the tip of a flask-shaped phialide.

4.2.2 *Beauveria bassiana*

Beauveria bassiana was isolated on PDA. Its growth was slow. The colony is white in colour, densely wooly and had raised aerial hyphae (Plate 4.3a). Cells that were short and ovoid were seen when the slide was viewed under a microscope at 400×. The cells were narrow at the apex forming an extension called rachis. There was a large number of conidiogenous cells (Plate 4.3b).

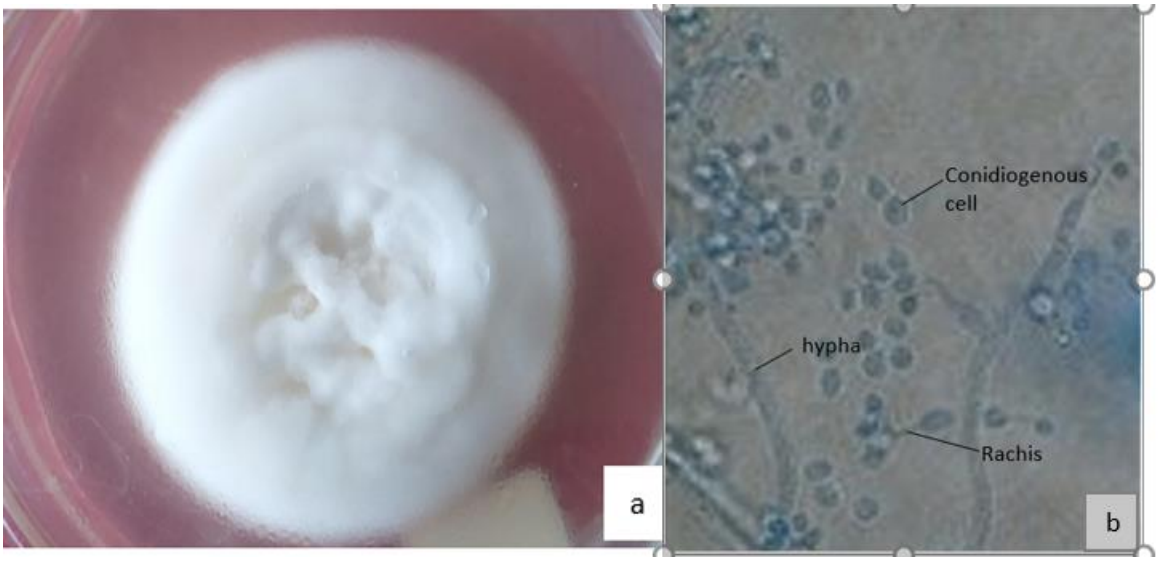

Plate 4.3: a) *Beauveria bassiana* colony (10 days old) on PDA. Note the densely cottony mycelium with raised aerial hyphae. b) A micrograph of B. *bassiana* under the light microscope at 400× showing ovoid conidiogenous cells and the rachis

4.3 Isolation and identification of test pathogens

4.3.1 *Phytophthora infestans*

The colony character was pale white in colour, round in shape with flat elevation, smooth surface, entire margin and thin mycelial mat as shown in Plate 4.4a. The isolate was relatively fast growing and covered the 9 cm plate in ten days. When viewed under a microscope at 400×, the hyphae appeared aseptate and thin walled. Sporangia were

terminal, ovoid or lemon shaped. The tip of the sporangium appeared to be semi papillate (Plate 4.4b).

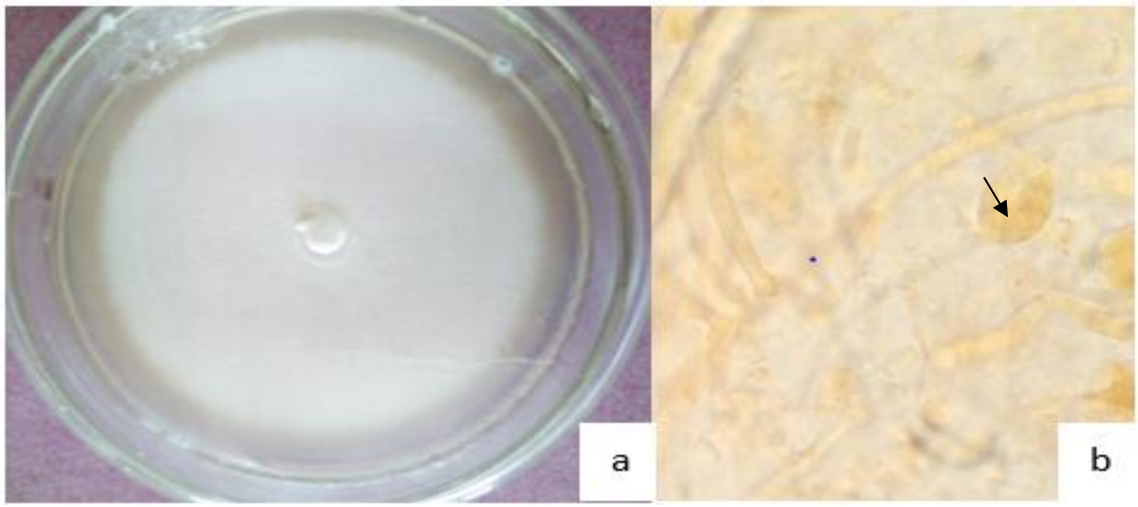

Plate 4.4: a) *Phytophthora infestans* colony (7 days old) on PDA. Note the pale white colony with a thin mycelial mat. b) A micrograph of *P. infestans* under the light microscope at 400× showing aseptate hyphae (shown by the arrow) and an ovoid sporangium.

4.3.2 *Alternaria solani*

The colony morphology was effuse grey in colour, had curved margin, umbonate elevation, cotton felt surface and produced dark red pigmentation that colored the medium (Plate 4.5a). When viewed under a microscope at 400X, conidiophores were brown, thick walled, septate and straight (Plate 4.5b). Conidia were club-shaped and occurred singly and some in groups.

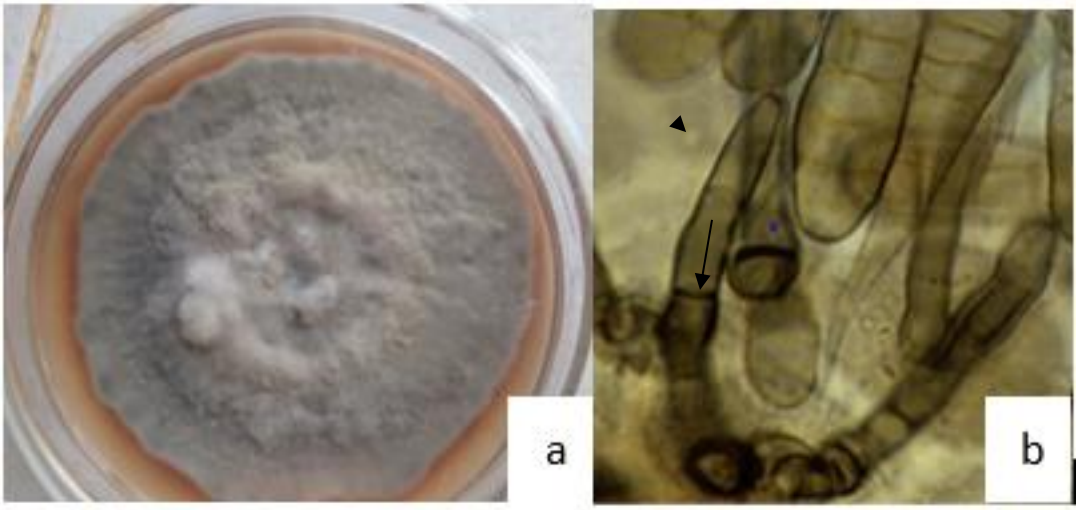

Plate 4.5: a) *A. solani* colony (7 days old) on PDA. Note the dark red pigmentation in the medium and the effuse grey mycelium. b) A micrograph of *A. solani* under the light microscope at 400× showing brown, thick walled and septate (shown by the arrow) conidiophores with club shaped conidia.

4.4 *In vitro* tests for antagonism by *T. harzianum* and *B. bassiana* against test pathogens

In vitro antifungal activities was determined in three ways; dual culture, emission of volatiles and use of diffusible metabolites.

4.4.1 Growth inhibition of *A. solani* by *T. harzianum*

4.4.1.1 Dual culture

Antagonism by *T. harzianum* against *A. solani* was tested *in vitro* by use of dual culture technique. The radius of the colonies from the treatment plates in triplicates and those of controls in triplicate were recorded for 7 days (Table 4.1, Fig 4.1 and Plate 4.6 Fig 4.1). From the graph in fig 4.1 below, the colony diameter becomes apparent from the third day after inoculation.

Table 4.1. *A. solani* colony diameter variations due to inhibition by *T. harzianum*.

Time (Days)	d (mm)	D (mm)
1	4.67±0.6	4.67±0.6
2	8.67±0.6	9.67±0.6
3	13.67±1.5	15.00±1.0
4	16.00±1.7	20.00±1.0
5	17.33±2.1	26.67±0.5
6	19.00±1.7	32.67±1.5
7	20.67±1.5	39.67±2.5

Initially on inoculation there was equal distance between the bioagent and the pathogens. As the time progress the inhibition showed an increased diameter of pathogen away from the bioagent as opposed to towards the bioagent.

The values represent means of three replicates±SD. ‘d’ represent the colony diameter growing towards the *T. harzianum*, while ‘D’ represent measurements of colony diameter growing away from *T. harzianum.*

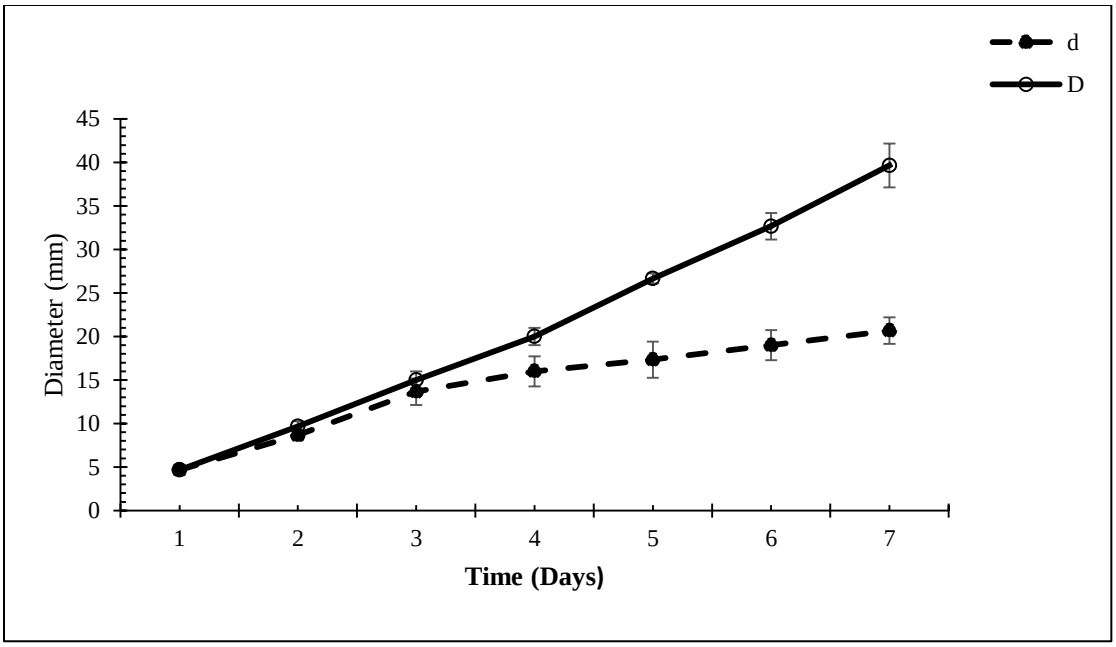

Fig 4.1: A line graph showing the progress of *A. solani* mycelial growth for 7 days in a petri plate. The dotted line (d) presents the fungal colonies growing in the presence of *T. harzianum* while the continuous line (D) represents the control.

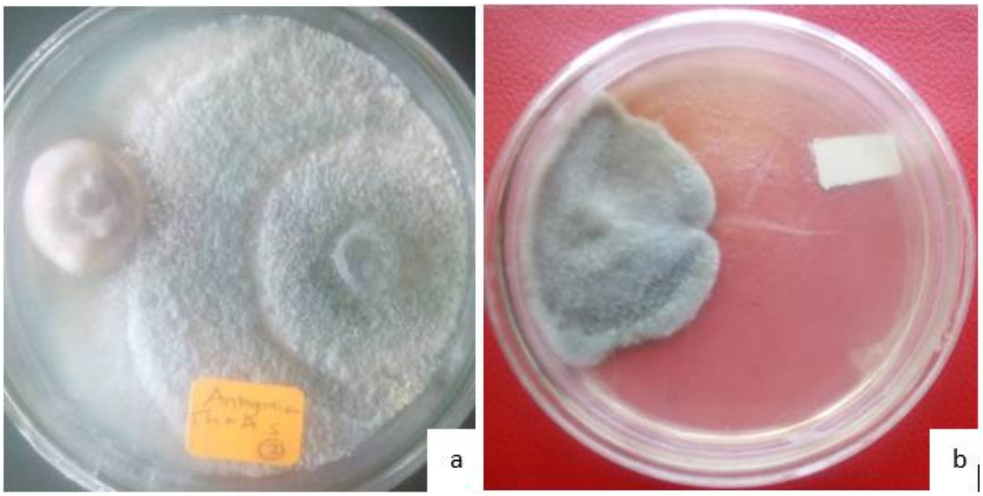

Plate 4.6: a) Dual culture of *A. solani* and *T. harzianum* colonies (7 days old) on PDA. Note the radius of *A. solani* towards the antagonist is *(T. harzianum)* smaller compared to the radius away from antagonist. b) A colony of *A. solani* (control).

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From the first to the third day, the two antagonistic colonies were not close for the effect of *T. harzianum* to be effected on *A. solani*. Subjecting the data collected on the seventh day to t-test reveals a statistical significant difference (P = 0.00018) in the colony diameters of the two treatments (Table 4.2).

Table 4.2: T-test output of *A. solani* diameter recordings 7 days after incubation

t-Test: Two-Sample Assuming Equal Variances			
		d	***D***
Mean		20.66667	39.66667
Variance		2.333333	6.333333
Observations		3	3
Pooled Variance		4.333333	
Hypothesized	Mean		
Difference		0	
df		4	
t Stat		-11.1786	
P(T<=t) one-tail		**0.00018**	
t Critical one-tail		2.131847	
P(T<=t) two-tail		0.000365	
t Critical two-tail		2.776445	

Fungal colonies growing together with *T. harzianum* attained diameters of approximately 21 mm while the control reached an approximate diameter of 40 mm in 7 days. Plate 4.6 shows the respective colonies as they appeared after 7 days. *T. harzianum* colony grew very fast covering the whole plate by surrounding the colony of *A. solani* hence suppressing its growth.

4.4.1.2 Antifungal activity of volatile metabolites

Effectiveness of antifungal activity of volatile compounds produced by *T. harzianum* against *A. solani* was tested *in vitro* (Plate 7). Daily measurement of diameter reveals that

the effect of the volatiles impacted the growth *of A. solani* from the start and the potency increased with time (Table 4.4 & Fig 4.2). Subjecting the seventh day recordings to t-test reveals a statistical significant difference (P = 0.00038) in the colony diameters of the two treatments (Table 4.3).

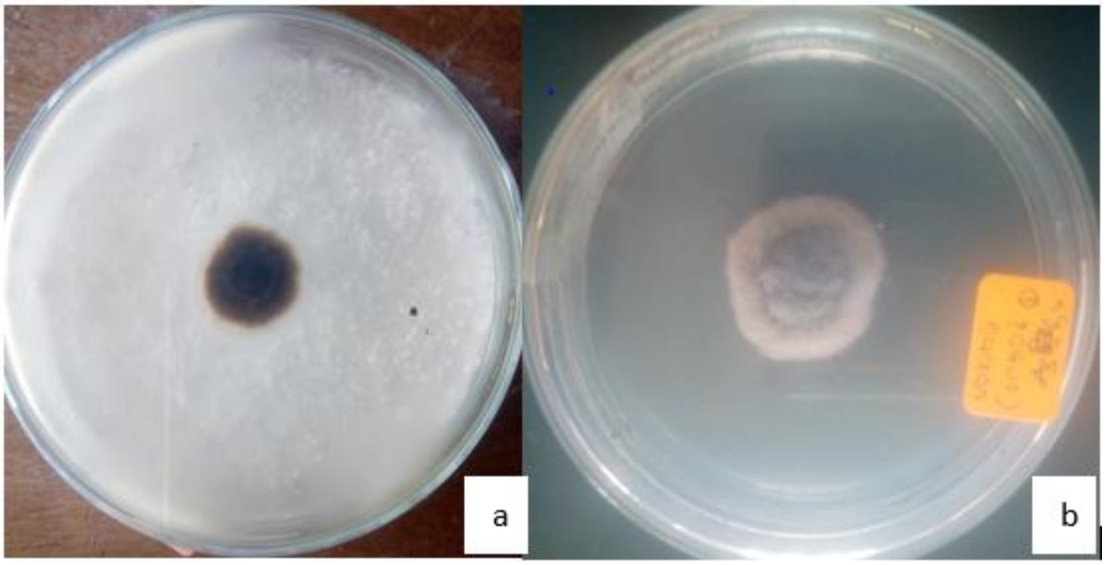

Plate 4.7: a) Petri plate with *A. solani* colony inverted over a plate with *T. harzianum* colony (7 days old). b) *A. solani* colony (control). Differences in colony sizes between the two colonies with different treatments can be noted. The colony receiving *T. harzianum* volatiles is smaller than the control.

Table 4.3: T-test output of *A. solani* colony diameters 7 days after subjecting to *T. harzianum* volatiles

t-Test: Two-Sample Assuming Equal Variances			
		Treated	***Control***
Mean		20.66667	40
Variance		8.333333	4
Observations		3	3
Pooled Variance		6.166667	
Hypothesized Mean Difference		0	
df		4	
t Stat		-9.53514	
P(T<=t) one-tail		**0.000338**	
t Critical one-tail		2.131847	
P(T<=t) two-tail		0.000676	
t Critical two-tail		2.776445	

Table 4.4. *A. solani* colony diameter variations due to inhibition by *T. harzianum* volatiles.

Days	Treated	Control
D1	13.67±0.6	16.00±1.0
D2	16.33±0.6	19.33±0.6
D3	16.67±1.2	25.67±1.5
D4	18.00±1.7	29.00±2.0
D5	19.67±2.9	33.33±1.2
D6	20.67±2.9	36.67±1.6
D7	20.67±2.9	40.00±2.0

'Treated' represent the diameter measurements of colonies exposed to *T. harzianum* volatiles while 'Control' represent measurements of colonies not exposed to any volatiles.

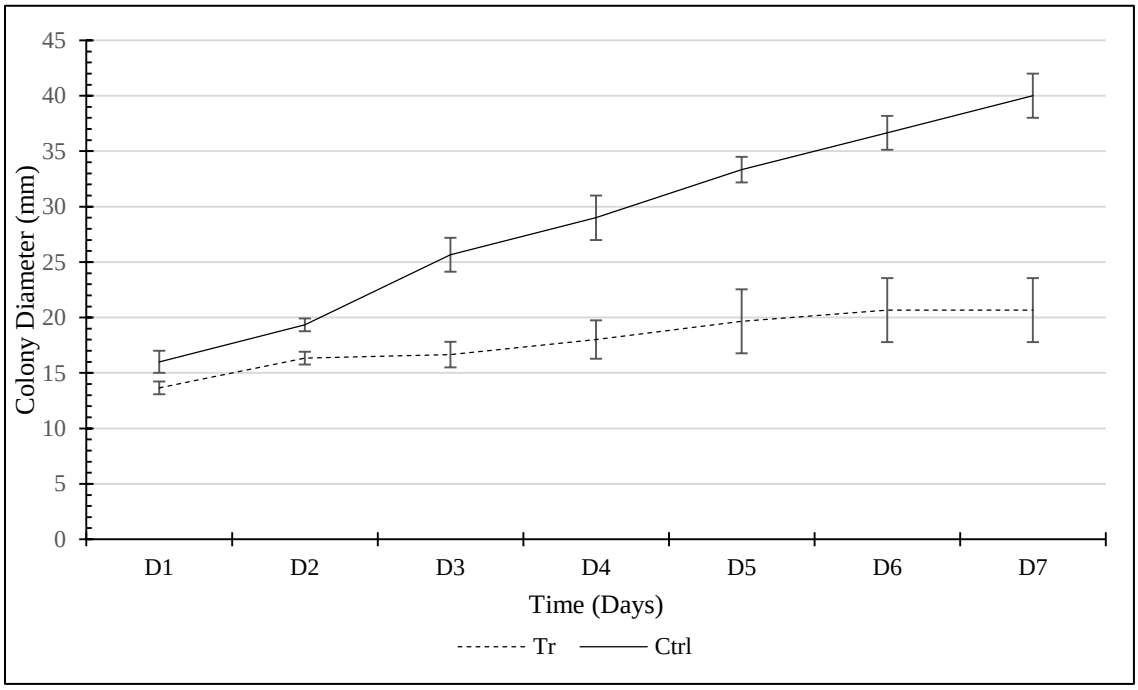

Fig 4.2: Line graph showing the progess of growth of the *A. solani* colonies exposed to *T. harzianum* volatile metabolites. Inhibition was effected from the 24 hours as shown in the graph. Inhibition only resulted in impeded growth.

4.4.2 Growth inhibition of *P. infestans* by *T. harzianum*

4.4.2.1 Growth inhibition Dual culture

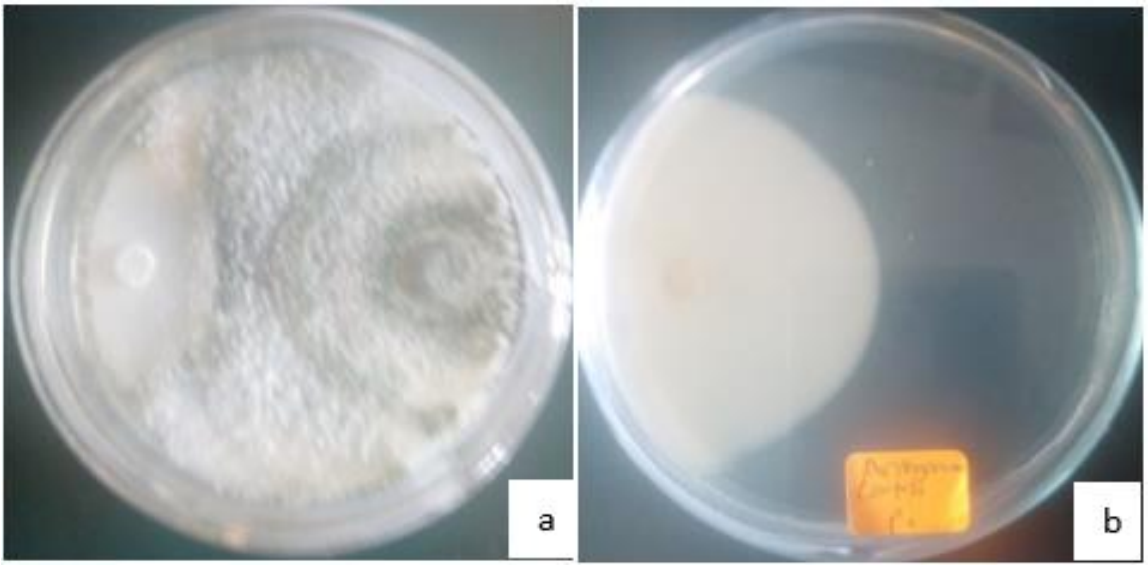

Plate 4.8: Growth inhibition of *P. infestans* by T. *harzianum*. a) Dual culture of *P. infestans* colony (left) and *T. harzianum* on the right, on PDA b) *P. infestans* colony (control on PDA). Note that all colonies are 7 days old. (Source: Author 2020).

The colony growth of *P. infestans* co-cultured with *T. harzianum* was subdued within a short time. The relatively fast growing colony of *T. harzianum* quickly outgrew and prevented further growth of the *P. infestans* (plate 4.9). this happened on the fifth day since the colonies were co-cultured (Table 4.7).

Table 4.5: *P. infestans* colony diameter variations due to inhibition by *T. harzianum*..

Control	Antagonized
15.67±2.1	15.33±1.5
23.67±2.1	21.33±0.6
39.00±2.0	26.67±0.6
44.67±2.5	21.67±0.6
52.33±2.1	22.00±1.0
65.67±1.5	27.00±1.0
72.00±2.0	27.02±1.2

‘Antagonized’ represent measurements of colony diameter growing towards *T. harzianum*, while ‘Control’ represent measurements of colony diameter growing away from *T. harzianum.* Values represent means±SD.

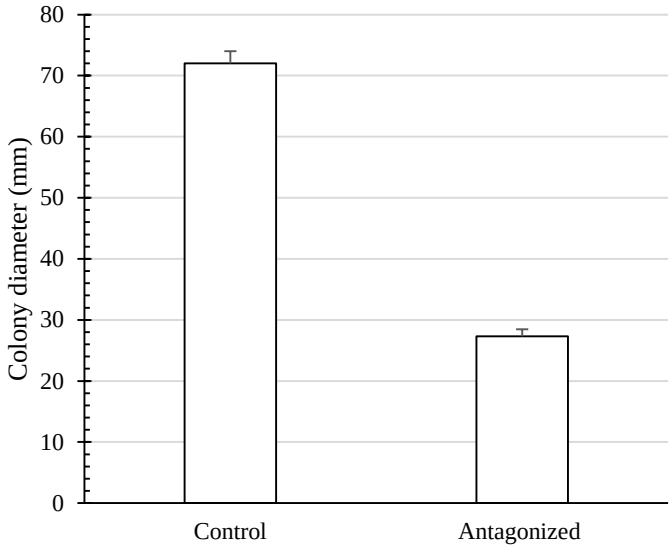

Fig 4.4: A bar graph depicting the difference in colony diameter between *P. infestans* co-cultured with *Trichoderma harzianum* (antagonized) and the control 7 days after incubation.

As shown in fig 4.4 above, the diameter of the controls reached about 72 mm which was significantly different from the antagonized colonies. After the 7th day, the diameters of the antagonized colonies was not even half of what the controls.

4.4.2.2 Growth inhibition of *P. infestans* by *T. harzianum* volatiles

T. harzianum volatile metabolites were also inhibitory to the growth of *P. infestans* colonies. Slight evidence of inhibition was visible from the third day of incubation and henceforth, the rate of growth of *P. infestans* colonies were severely reduced (fig 4.5). A point of significant departure between the colonies exposed to *Trichoderma* volatiles and control colonies occurred on the 4th day (Table 4.8). All these culminated in the control plate colonies attaining a diameter of 62.33mm while the colonies exposed to volatiles were at 38.33mm at day 7. This is an approximate of 39% growth inhibition. Plate 10 clearly depicts the discrepancy in size between the two colonies.

Table 4.6: *P. infestans* colony diameter variations due to inhibition by *T. harzianum* volatiles.

Days	Treated	Control
D1	11.33±0.6	11.33±0.6
D2	22.33±2.1	21.67±1.5
D3	27.67±2.5	31.00±2.6
D4	32.67±3.2	39.33±3.8
D5	36.00±2.6	45.00±3.0
D6	37.33±2.1	50.33±2.9
D7	38.33±1.5	62.33±3.0

'Treated' represent the diameter measurements of colonies exposed to *T. harzianum* volatiles while 'Control' represent measurements of colonies not exposed to any volatiles. Values present means±SD.

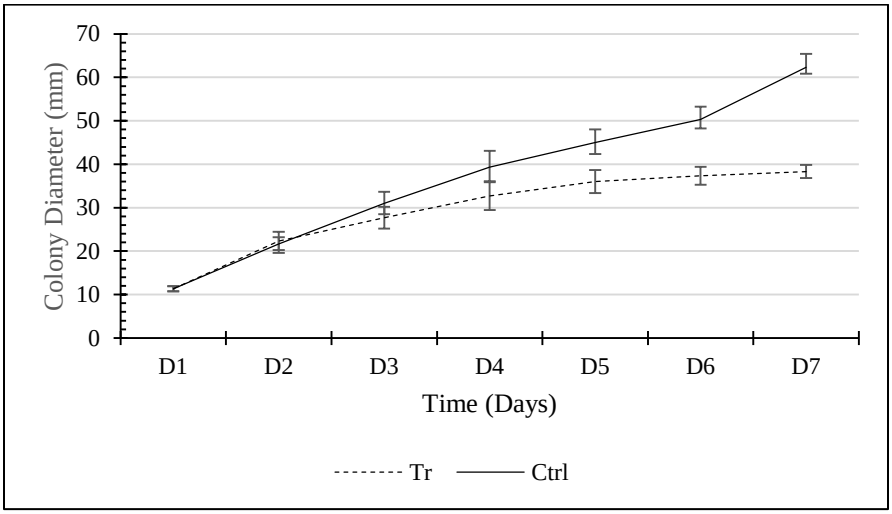

Fig 4.5. Line graphs showing different growth rates of *P. infestans* colonies resulting from the effect of *Trichoderma* volatile metabolites different treatments.

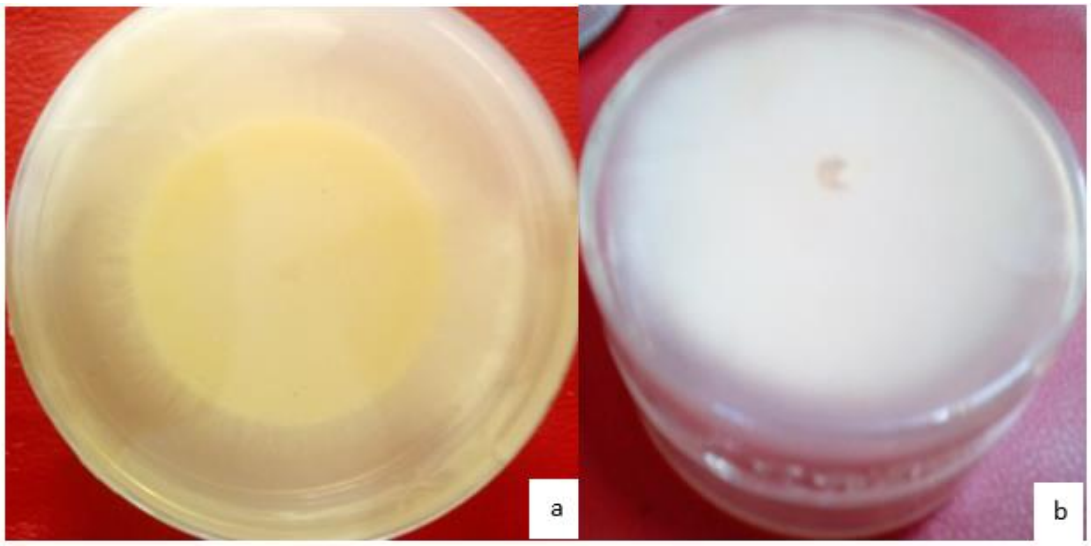

Plate 4.9. Seven days old P. infestans colonies. In plate a, the colony is exposed to T. harzianum volatiles and as evident, the growth is clearly impeded when compared to the control plate b.

4.4.2.3 Growth inhibition of *P. infestans* by different concentrations of *T. harzianum* non-volatile metabolites

Despite being very strongly antifungal in the dual culture, *T. harzianum* diffusible metabolites did not prove to be equally effective when acting alone (Table 4.9). As depicted in the line graphs in fig 4.5, no real difference in the rate of growth can be seen among the colonies receiving different concentrations of the volatile metabolites. The bar graph which only displays the sizes of the colonies in the last four days, shows some mild inhibition by the highest concentration of the diffusible compounds obtained from *T. harzianum*. On the 8th day the plates amended with 15% v/v diffusible metabolites, had smaller diameter (62 mm) compared to the rest (fig 4.6).

Table 4.7. Diameters of different colonies of *P. infestans* exposed to varying concentrations of *T. harzianum* diffusible compounds.

Time (Days)	5%	10%	15%	Ctrl
1	15.00±0.0	15.33±0.6	15.00±1.0	14.67±0.6
2	23.00±1.0	21.33±0.6	20.00±1.7	23.67±0.6
3	29.67±0.6	29.00±0.0	27.00±1.0	31.67±0.6
4	38.33±1.5	36.33±1.2	35.00±1.0	32.67±2.3
5	44.67±1.2	43.67±1.2	43.33±1.5	38.67±2.3
6	53.67±1.5	50.00±1.0	49.33±0.6	51.00±4.6
7	62.33±1.1	60.33±1.5	52.00±1.0	61.67±2.1
8	70.00±1.0	69.67±2.1	62.00±2.6	70.33±2.1

Values represent Means±SD.

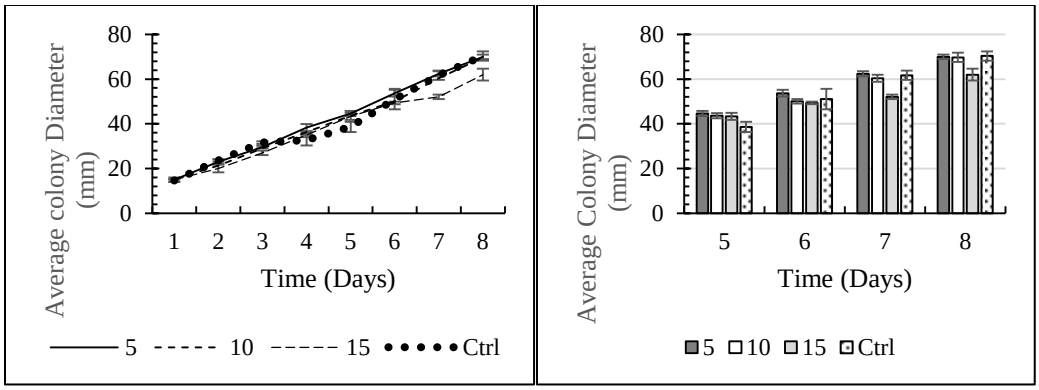

Fig 4.6. Line and bar graphs showing the growth behavior of *P. infestans* colonies under different *T. harzianum* metabolite concentrations.

Line graphs display the growth behavior in all the 8 days while the bar graph shows the growth behavior in the final 4 days.

This is corroborated by the colony pictures in plate 4.11. It is evident that the sizes of the colonies in a (5%v/v), b (10%v/v) and d (negative control) are of similar sizes. The smaller colony is growing on a medium amended with the highest concentration of the *T. harzianum* culture filtrate. Control plates had the largest diameters showing an average of 70.33mm. Highest concentration of the culture filtrate therefore impeded the growth of the colonies by 11.8%. This hints to dose-dependent antifungal activity of the *T. harzianum* culture filtrates.

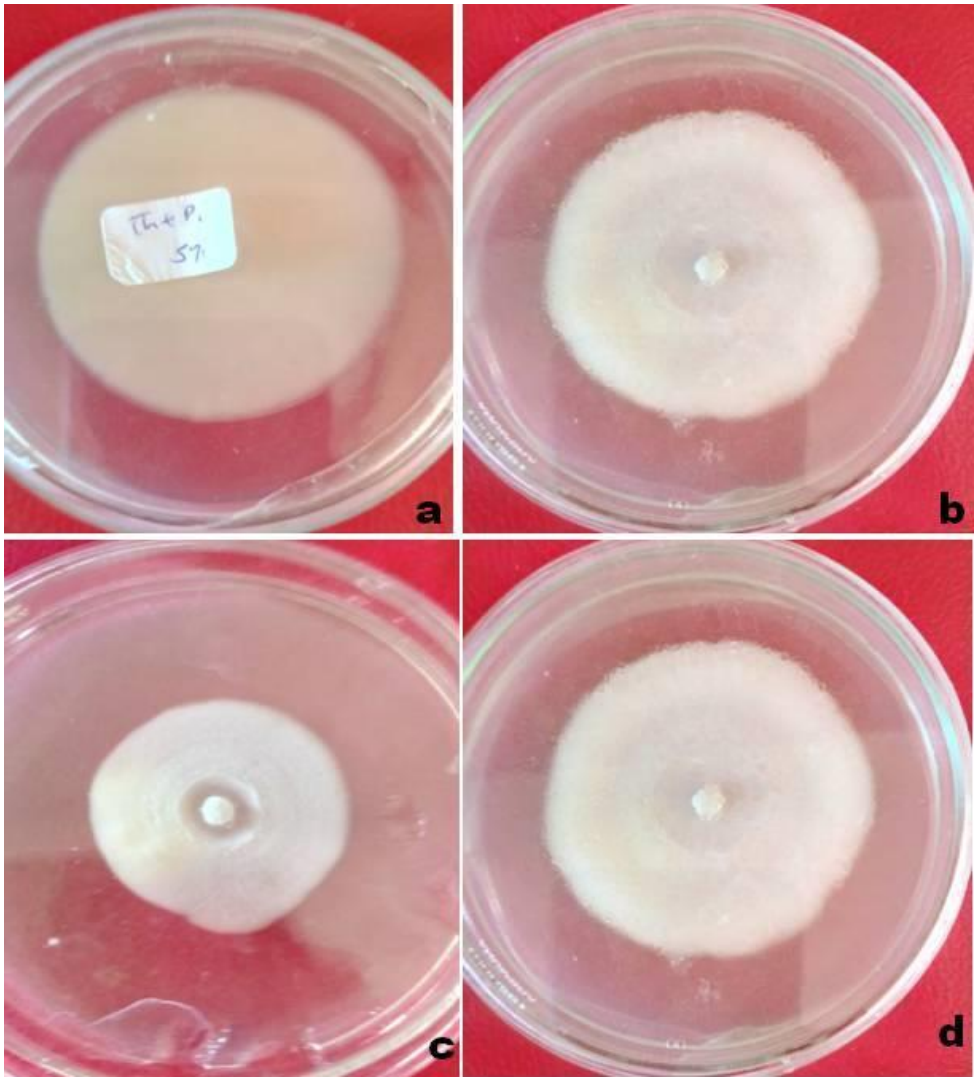

Plate 4.10: 7-day-old colonies of *P. infestans* on PDA amended with different concentrations of *T. harzianum* non-volatile compounds. a) *P. infestans* colony on PDA amended with 5% of *T. harzianum culture* filtrate. b) *P. infestans* on PDA amended with 10% of *T. harzianum culture* filtrate. c) *P. infestans* colony on PDA amended with 15% of *T. harzianum culture* filtrate. d) *P. infestans* colony on un-amended PDA.

4.4.1.3 Antifungal activity of diffusible compounds

Effectiveness of antifungal activity of diffusible compounds produced by *T. harzianum* were tested *in vitro* against *Alternaria solani*. Experimentation involved the use of plates with PDA amended with 5%, 10%, and 15% *T. harzianum* culture filtrate. The antifungal action of the diffusible metabolites has been demonstrated in plate 4.8 and figure 4.3. Colony diameter differed slighty in size with the colony growth in 15% amendment being relatively smaller.

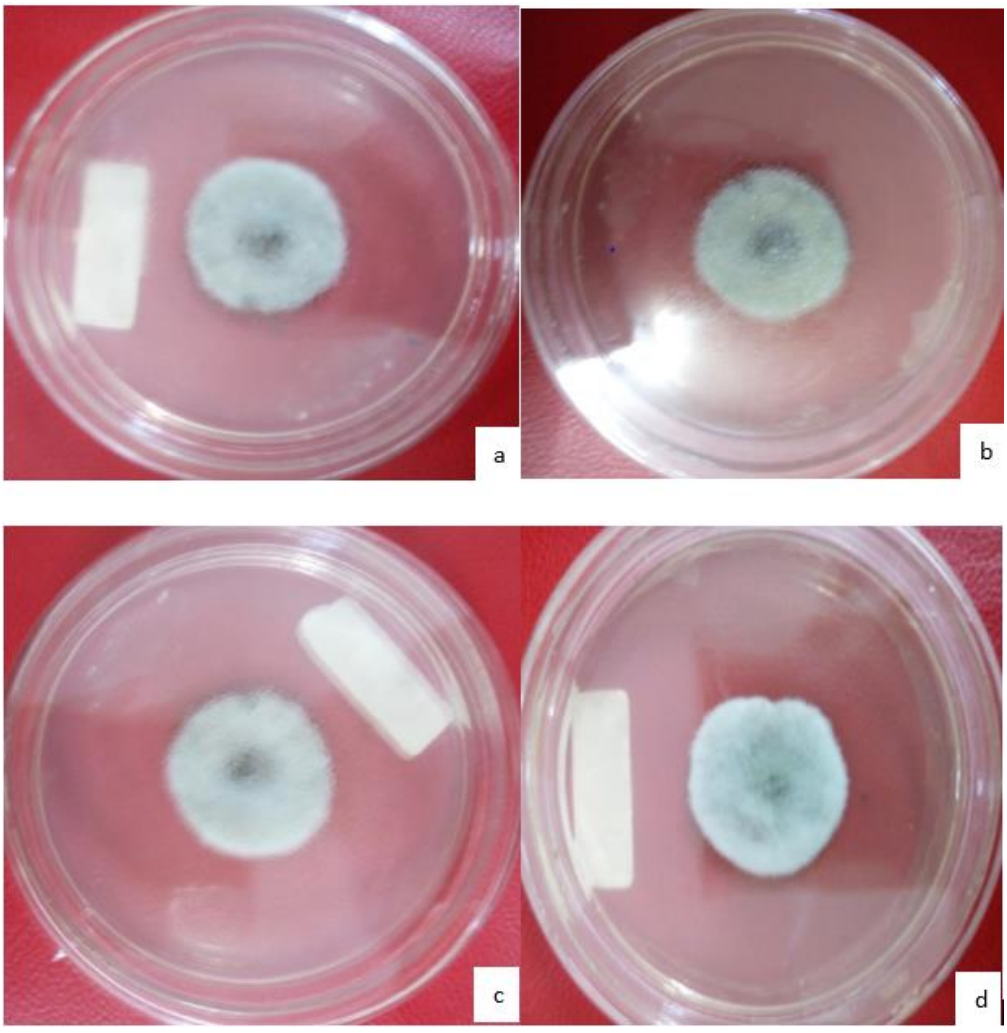

Plate 4.11: a). 7-days-old *A. solani* colony on 5% *T. harzianum* diffusible compounds amended PDA, b) on 10% amended PDA, c) on 15 % amended PDA and d) control.

The colony diameters of *A. solani*, as depicted from plate 8 above do not show much difference among all the treatments (Table 4.5). This is more clearly shown in fig 4.2 where all the lines representing different treatments cross each other.

Table 4.8. Diameters of different colonies of *A. solani* exposed to varying concentrations of *T. harzianum* diffusible compounds.

Time (Days)	5%	10%	15%	Ctrl
1	11.33±0.6	9.33±0.6	10.33±0.6	10.67±1.5
2	16.00±1.0	13.33±0.6	13.33±0.6	14.67±0.6
3	18.33±0.6	16.67±2.5	17.33±0.6	18.33±0.6
4	21.67±2.1	19.67±2.5	20.67±1.5	21.00±1.7
5	25.67±1.5	25.00±2.0	26.33±1.5	26.33±1.5
6	33.00±3.0	30.00±2.0	30.33±1.5	30.67±1.2

Values represent Means±SD. Ctrl stands for the control

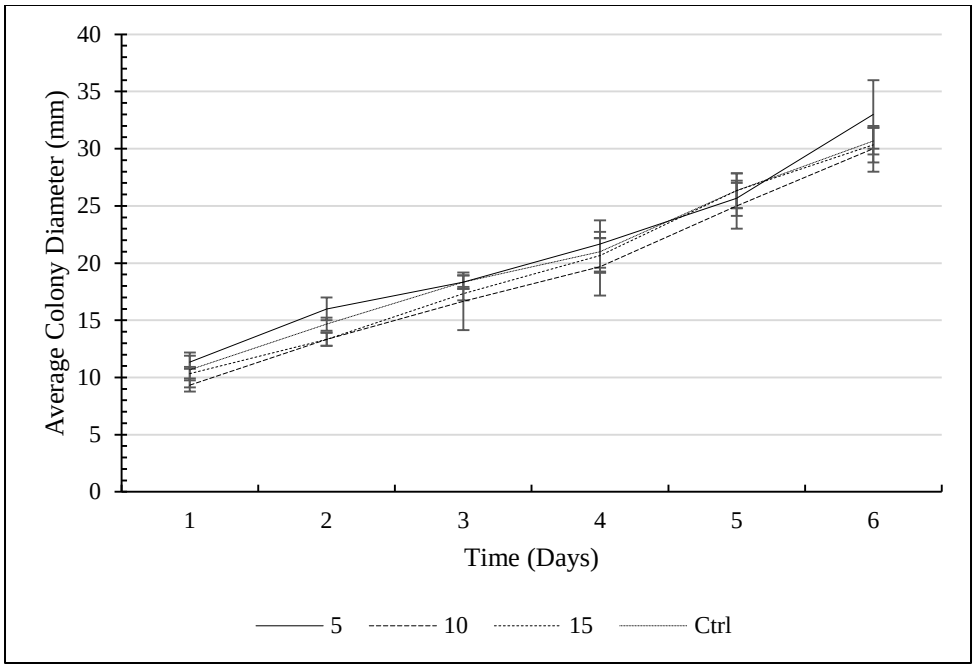

Fig 4.3. A graph depicting the rate of growth of *A. solani* under different treatments of diffusible compounds of *T. harzianum* at 6 days. Note that the graph lines are packed close together and crisscross one another.

Analysis of variance further shows that the differences in the colony diameter of the colonies in the sixth day (P = 0.3299) were not significantly different (Table 4.6). Therefore, the diffusible metabolites obtained from the *T. harzianum* culture filtrate does not have any effect on the growth of *A. solani* colonies. The inhibition of *A. solani* is therefore not contributed by the volatile compounds of *T. harzianum*..

Table 4.9: ANOVA table of the effect of *T. harzianum* diffusible metabolites on *A. solani.*

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	16.66667	3	5.555556	1.333333	**0.329989**	4.066181
Within Groups	33.33333	8	4.166667			
Total	50	11				

4.4.3 Growth inhibition of *B. bassiana* against *A. solani*

4.4.3.1 Growth inhibition by Dual culture

The biocontrol fungus *B. bassiana* did not have any apparent impact on the growth of *A. solani*. The colonies grew until they touched one another and growth continued beyond their colony edges (Plate 4.12).

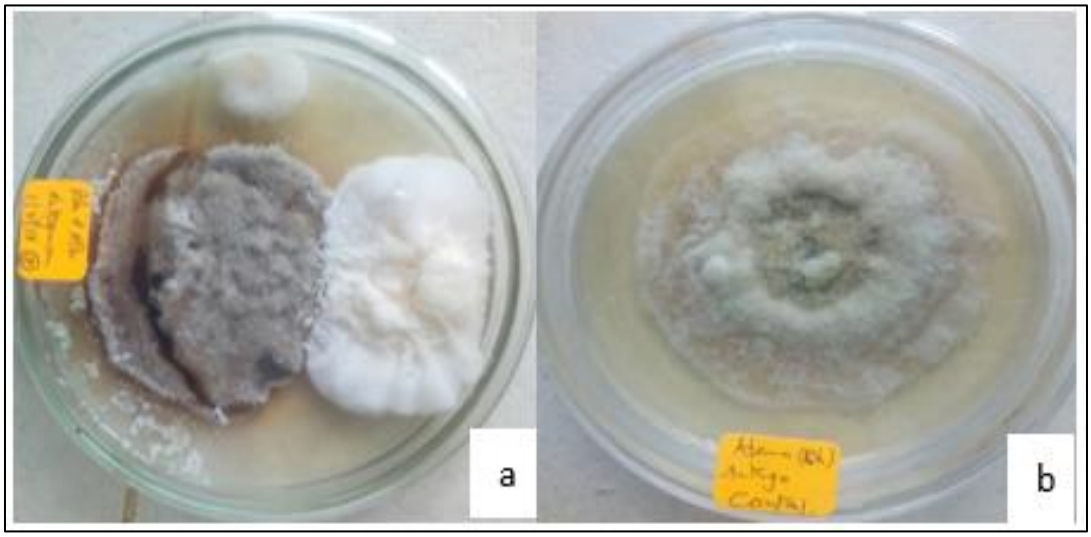

Plate 4.12: a) 7-day-old dual culture of *A. solani* and *B. bassiana* colonies on PDA. b) Control plate bearing *A solani* alone.

4.4.3.2 Growth inhibition of *A. solani* by *B. bassiana* volatile compounds

The colony diameters of the controls and the colonies exposed to *B. bassiana* colonies were measured daily for 7 days (Table 4.10). For the first three days, the difference was not apparent (fig 4.7, Plate 4.13). On the third day the diameter of the colonies exposed to the volatiles averaged at 24.33mm while the controls were 24.67mm. Some form of inhibition was observed from the 4th day to the final seventh day where a plateau in growth was witnessed. The difference in colonies sizes between these days was significant.

Trend lines shown in the bar graphs in fig 4.7 clearly show the difference in the growth rates. In the treated colonies, there was not much increase from the fourth and the final day. Between the fourth and the final day the unexposed colonies increased their diameter from 31.33mm to 40.33mm.

Table 4.10 *A. solani* colony diameter variations due to inhibition by *B. bassiana* volatiles.

	D1	D2	D3	D4	D5	D6	D7
Tr	14.67±1.2	20.33±3.2	24.33±2.1	28.00±1.7	28.33±1.5	28.67±1.5	28.67±1.5
Ctrl	15.67±1.2	21.67±1.5	24.67±3.1	31.33±3.5	34.00±2.6	37.33±2.1	40.33±1.5

'Tr' represents the measurement values from the colony diameter that were exposed to the volatiles while 'Ctrl' are controls. Values represent means±SD

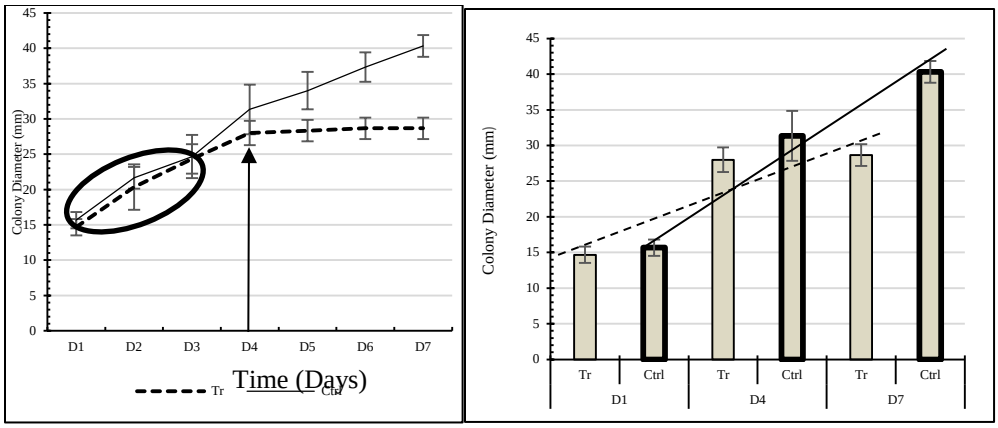

Fig 4.7. Line and Bar graphs showing the gradual growth of the colonies of *A. solani* as affected by the *B. bassiana* volatiles.

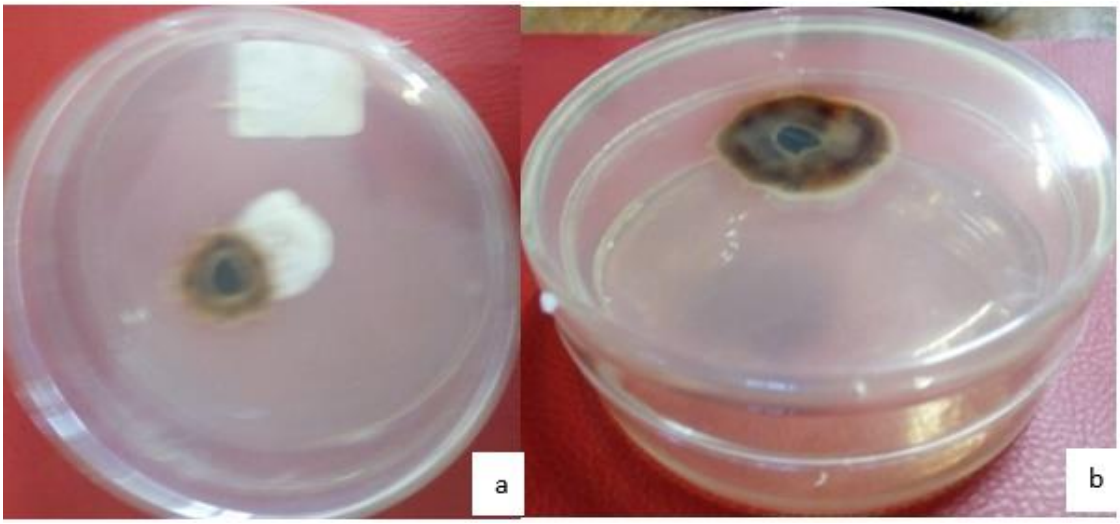

Plate 4.13. a) Superimposed plates of *A. solani* and *B. bassiana* on PDA b) Control plate of *A. solani* superimposed against a non-inoculated PDA plate.

4.4.3.3. Antifungal activity of non-volatile compounds produced by *B. bassiana* against *A. solani*.

Seven-day old colonies of *A. solani* inoculated on PDA amended with 5%, 10% and 15% culture filtrate of *B. bassiana* were observed and recorded. They were as shown in plate 4.14 a, b, c respectively. Plate 4.14 d is the control. As depicted in figure 4.8, the

treatments split into two groups. One treatment group is the control and the 5% concentration and the other is the 10% and 15% concentration treatments. The same pattern can be observed in the colonies shown in plate 4.14. Looking at the bar graphs, largest diameter is by the colonies in the control plate with 36mm diameter followed by the 5% v/v (33.33mm) treated plate. This difference was statistically significant ($P < 0.01$) (Appendix II). The 10% concentration on the other hand, recorded a diameter of 24.67mm and the 15% concentration resulted in the smallest diameter of 22mm. The *B. bassiana* extracts are therefore showing dose dependent antifungal activity.

Table 4.11. Diameters of different colonies of *A. solani* exposed to varying concentrations of *B. bassiana* diffusible compounds.

Time (Days)	5%	10%	15%	Ctrl
1	11.00±0.0	10.33±1.2	10.00±0.0	13.67±0.6
2	13.67±1.2	11.67±0.6	11.67±0.6	17.67±1.2
3	21.33±2.5	16.67±0.6	13.33±0.6	22.00±1.0
4	27.33±1.2	20.33±1.2	18.33±1.5	28.67±0.6
5	30.33±2.5	22.67±0.6	20.00±2.0	30.33±1.5
6	31.67±2.1	23.33±0.6	20.33±2.1	32.67±1.5
7	33.33±1.5	24.67±0.6	22.00±2.6	36.00±1.0

Values represent means±SD. Inhibition is dose-dependent.

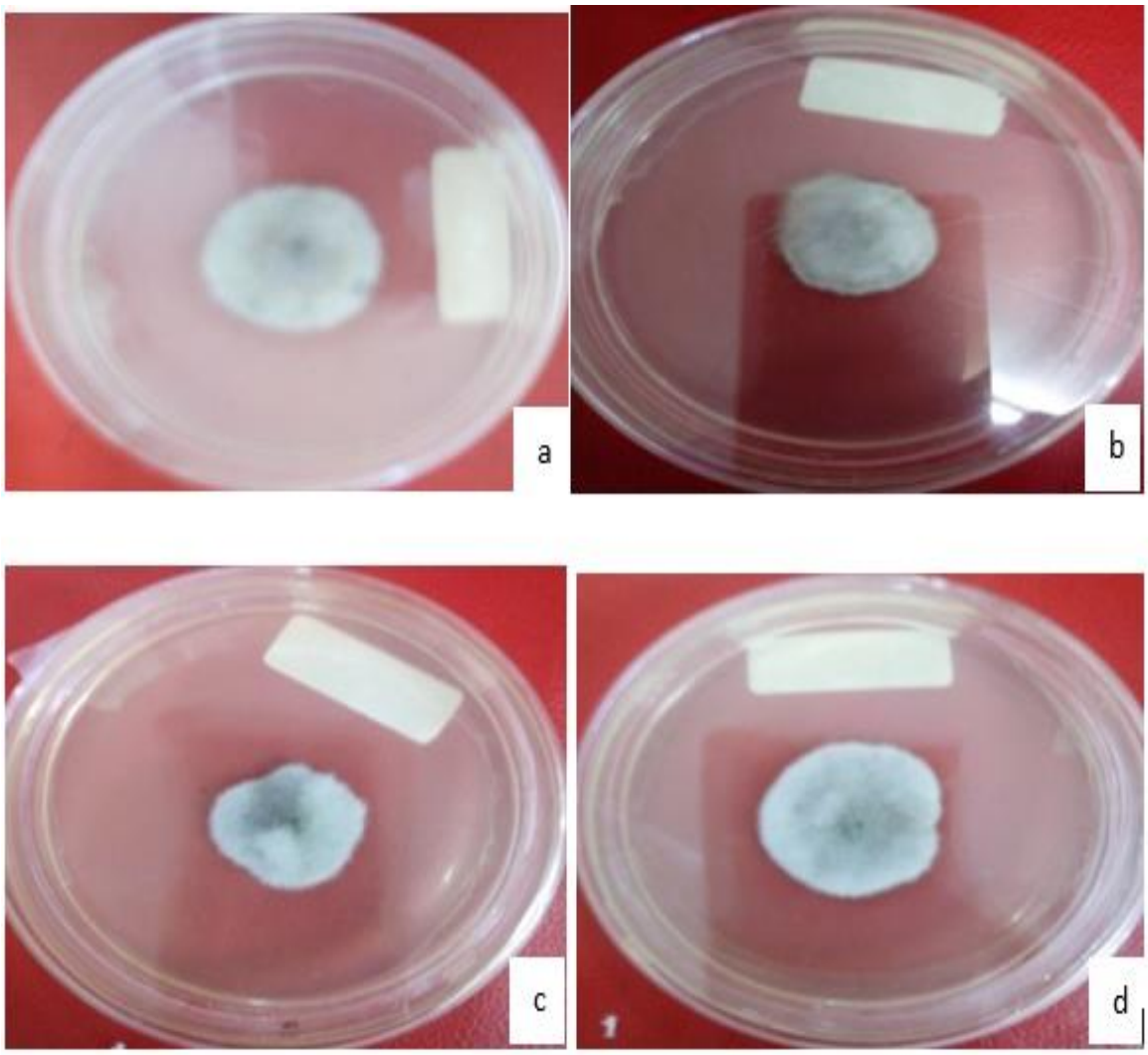

Plate 4.14: a) 7-day-old colony on PDA amended with 5% *B. bassiana* culture filtrate .b) 10%mmended PDA c) 15% amended PDA d) control.

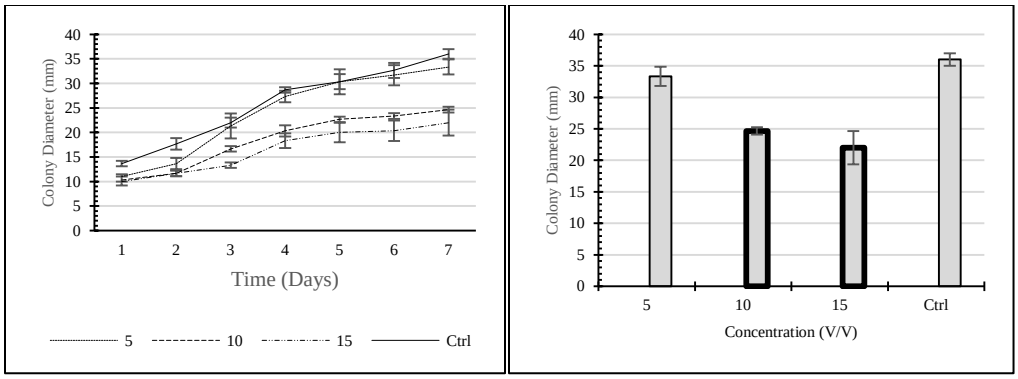

Figure 4.8: Lines and Bar graphs of growth inhibition of *A. solani* by *B. bassiana* non-volatile metabolites.

4.4.4 Growth inhibition of *P. infestans* by *B. bassiana*

4.4.4.1 Antagonism of *B. bassiana* against *P. infestans* by the dual culture method

Antagonism of *B. bassiana* against *P. infestans* was tested *in vitro* by dual culture technique. The radius of the *P. infestans* colony towards the antagonist (r) was smaller than radius away from antagonists (R).after 7 days of incubation. It was also noted that the overall colony diameter of the control was relatively larger than that of the treatment. After 7 days, the colony of *B. bassiana* grew into the *P. infestans* showing greater inhibition. The results are as shown in plate 4.15.

The black arrow in plate 13 shows the clear zone of inhibition as a result of antifungal activity displayed by *B. bassiana* towards the *P. infestans* colony. Though it is clearly evident that *P. infestans* a superior growth rate, *B. bassiana's* diffusible metabolites are strongly antifungal and impedes the growth of the colony towards it..

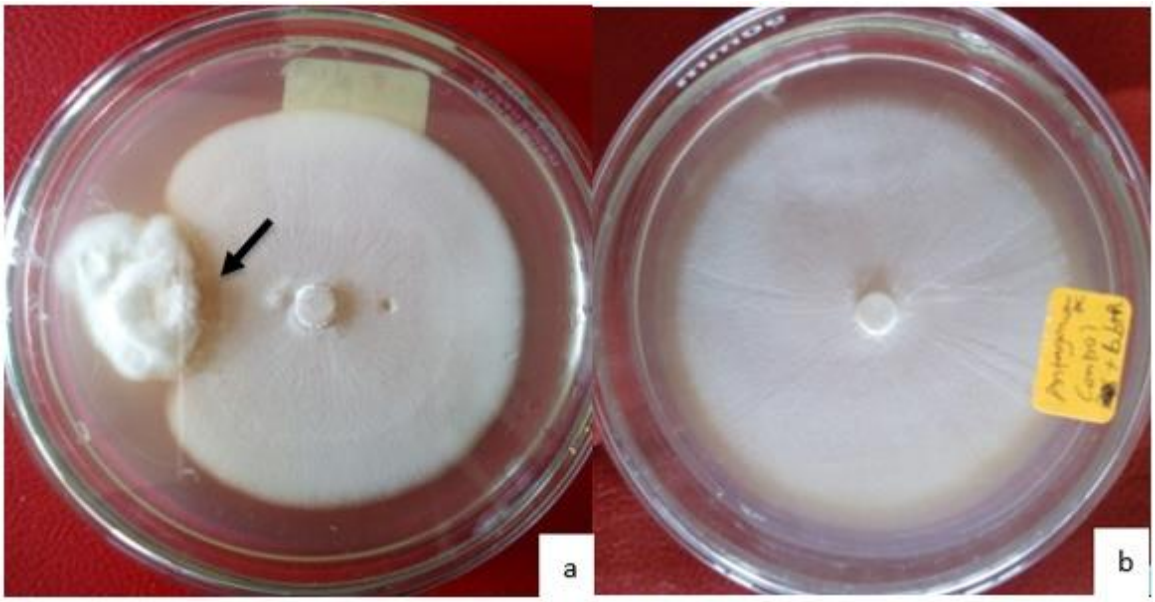

Plate 4.15: a) 7-day-old dual culture of *P. infestans* and *B. bassiana* colonies on PDA. b) Control (7 days old) showing uniform radius in all directions.

4.4.4.2 Antagonism of *B. bassiana* volatiles against *P. infestans*

Effectiveness of antifungal activity of volatile compounds produced by *B. bassiana* was tested *in vitro* against *P. infestans*. Colony diameters in the treatment and the control plates were taken after 7 days (Table 4.12). From the third day, the inhibitory effect was evident on some exposed colonies (fig 4.9). The effect was clear from the fourth day onwards as the difference in the diameters between the fungi receiving the different treatments widened henceforth. On the final day of measurement (7^{th} day), the colony diameters of the control colonies averaged at 61 mm while those which were exposed to volatiles were at 33 mm. These measurements were statistically different at $p < 0.05$ as shown by the different colony sizes (plate 4.16).

Table 4.12 *P. infestans* colony diameter variations due to inhibition by *B. bassiana* volatiles.

	D1	D2	D3	D4	D5	D6	D7
Tr	11.33±0.6	22.33±0.6	28.67±3.8	30.00±4.6	30.33±4.6	31.33±4.0	33.00±4.0
Ctrl	11.33±0.6	23.67±0.6	32.33±2.1	39.67±3.2	46.67±3.2	50.33±2.9	61.00±5.3

Signs of inhibition can be observed from the second day after inoculation. Values represent means±SD.

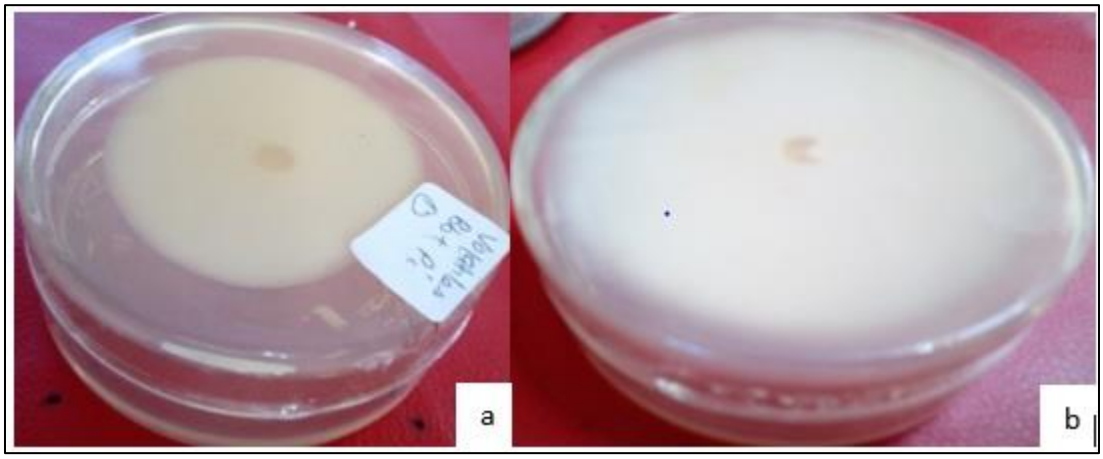

Plate 4.16: Effect of *B. bassiana* volatile compounds against *P. infestans*. a) Dual culture of *P. infestans* colony inverted over *B. bassiana* on PDA b) *P. infestans* colony (control) inverted over a petri plate with PDA only. All colonies are 7 days old

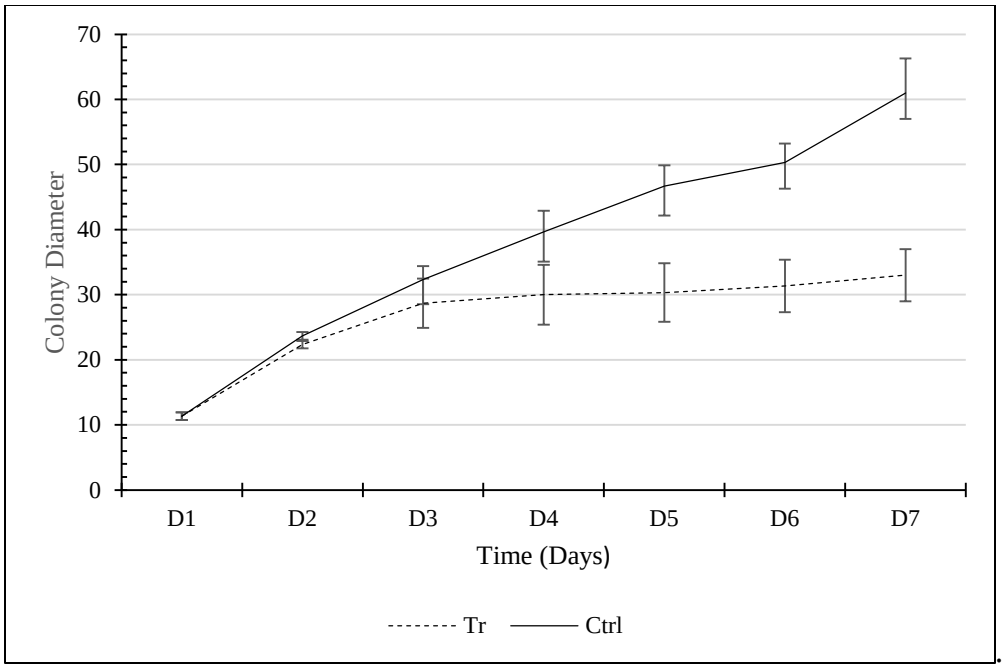

Fig 4.9. Line graph showing the rate of growth of *P. infestans* under two different treatments. The dotted line shows the rate of growth of the colonies exposed to *B. bassiana* volatile metabolites.

4.4.4.3 Antagonism of *B. bassiana* non-volatiles against *P. infestans*

Effectiveness of antifungal activity of non-volatile compounds produced by *B. Bassiana* was tested *in vitro* against *P. infestans*. The colony diameters for the treatments and controls were measured for 7 days. The results were as shown in plate 4.17. The diameters of the treatments compared to those of the controls reduced with a very small margin (plate 16) with ANOVA revealing non-significant difference (Appendix II).

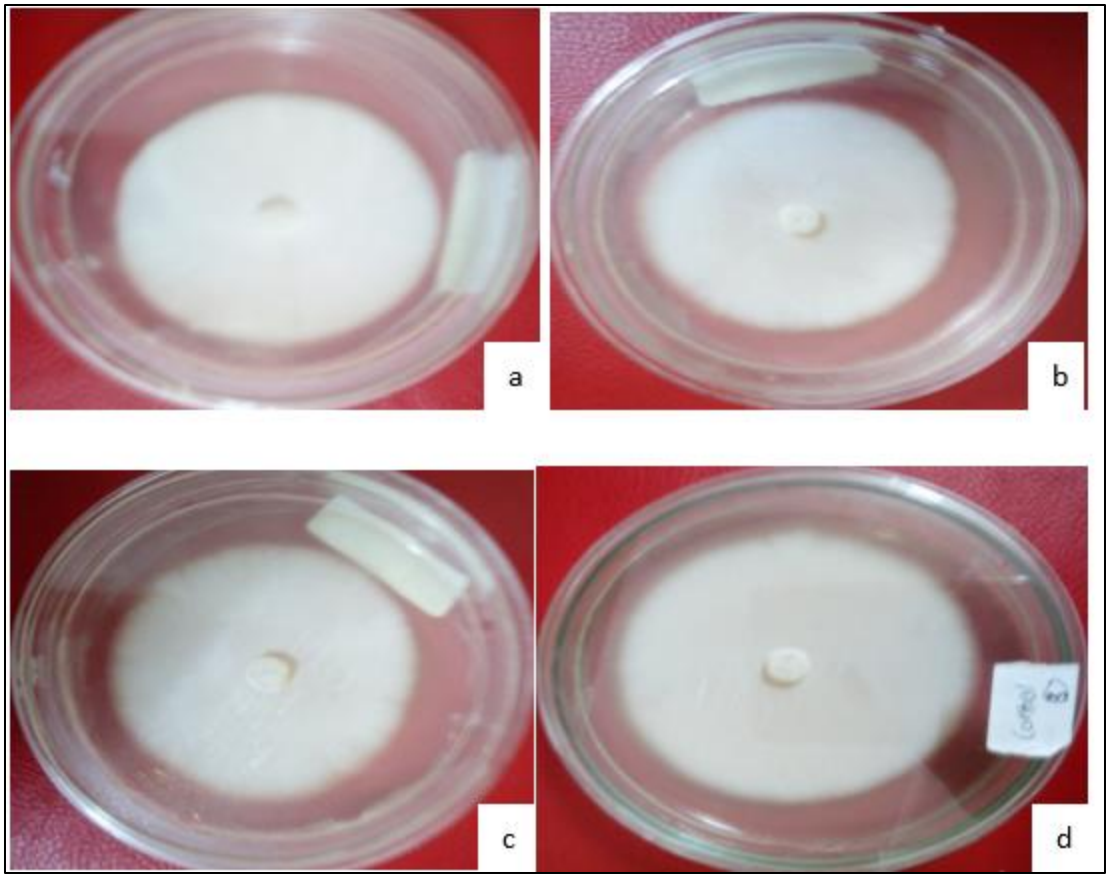

Plate 4.17: Effect of *B. bassiana* non-volatile compounds against *P. infestans*. a) *P. infestans* colony on PDA amended with 5% of culture filtrate. b) *P. infestans* on PDA amended with 10% culture filtrate. c) Colony on medium amended with 15% of *B. bassiana culture* filtrate. d) *P. infestans* colony on plain PDA.

Table 4.13. Diameters (mm) of different colonies of *P. infestans* exposed to varying concentrations of *B. bassiana* non-volatile compounds.

5%	10%	15%	Control
17.67±2.1	16.33±2.1	16.00±2.6	16.00±0.0
25.00±2.6	22.33±1.2	23.33±2.3	26.00±2.6
31.33±0.6	30.00±1.0	31.33±1.5	34.00±3.6
45.00±1.7	41.67±3.5	38.33±2.1	42.33±3.2
49.33±1.2	48.00±3.0	43.67±1.2	53.00±1.7
57.33±1.5	56.67±2.5	53.33±2.5	60.33±1.5
65.00±2.0	64.33±2.5	62.33±2.5	66.00±1.0

The values means±SD. Measurements do not differ significantly.

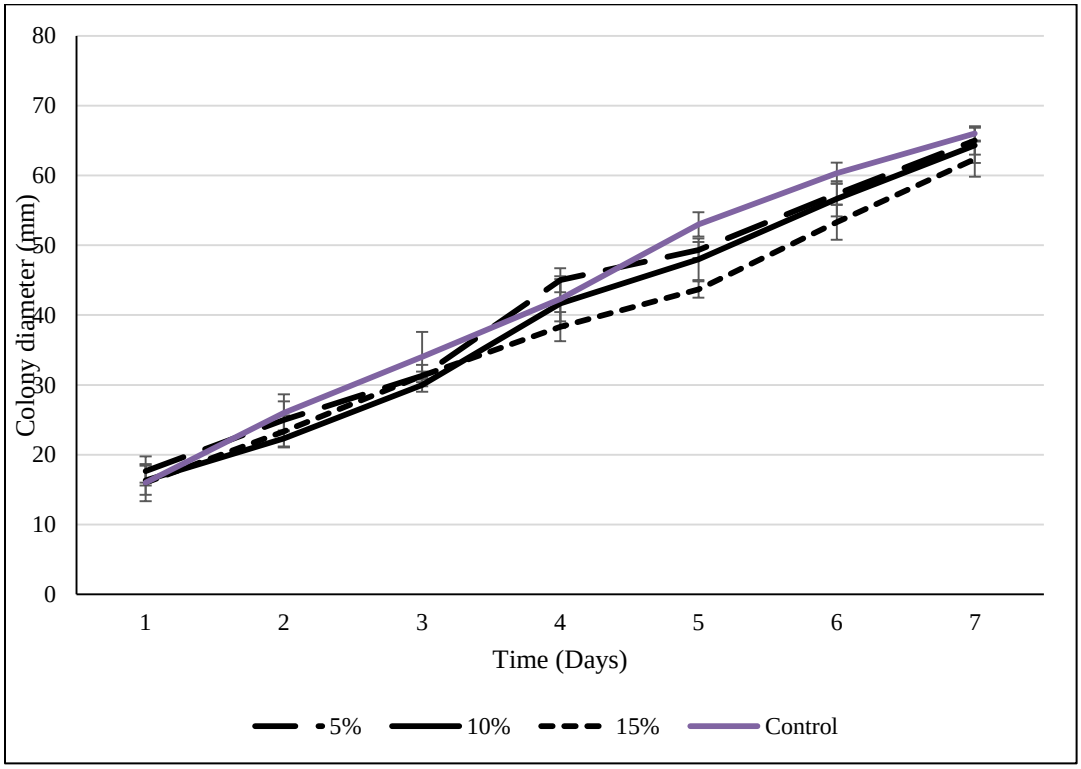

Figure 4.10. Line graphs showing the growth of *P. infestans* colonies under different treatments.

The line graphs (fig 4.10) shows a steady growth rate for all the *P. infestans* colonies regardless of the treatment. There is some evidence of dose-dependent antifungal activity as the colonies that received the highest concentration of the culture filtrate appears to have had the lowest growth rate. However, on the final day as depicted by the error bar, there was no significant difference in the sizes of the colonies. This is depicted both by fig 4.10 and plate 4.17 and Appendix III where $P > 0.05$.

4.5 *In vivo* determination of the efficacy of the *T. harzianum* and *B. bassiana* against early and late blight diseases

Images of the leaves under study were taken to show the extent of reduction of disease severity when the two biocontrol agents were used. They are given in plates 4.18 and 4.19.

Plate 4.18: Greenhouse images of different treatments on tomato plants. a) Plant inoculated with both *A. solani* and *B. bassiana*. b) Plant inoculated with *A. solani* alone (+ve control of (a)). c) Plant inoculated with both *A. solani* and *T. harzianum*. d) Plant inoculated with *A. solani* alone (+ve control of (c)).

Plate 4.19: Greenhouse images of different treatments on tomato plants. e) Plant inoculated with both *P. infestans* and *T. harzianum*. f) Plant inoculated with *P. infestans* alone (+ve control of e). g) Plant inoculated with both *P. infestans* and *B. bassiana*. h) Plant inoculated with *P. infestans* alone (+ve control of g).

Greenhouse studies revealed that in the first 21 days after infection, there were no apparent differences in the degree of disease grade among all the treatments. A sharp difference began to emerge between the negative controls and the infected tomatoes treated with the fungal antagonists. This is evidenced in fig 4.11 as disease grades shows sudden departure from the rest of the treatments. From the fourth week onwards, the negative controls were highly affected by the diseases with further increase between the fourth and sixth week. The severity graph of *A. solani* induced blight in the negative controls took a plateau (8.33) at the sixth week, while that of *P. infestans* maximum disease grade was in the seventh week. Between the two biocontrol agents tested, *B. bassiana* is more superior in the control of both the pathogens. However marked differences could only be obtained after the seventh week of disease development. Maximum disease grade achieved in *A. solani*-inoculated tomatoes treated with *T. harzianum* was 6.00, while that of *P. infestans*-inoculated-tomatoes was 5.67. Even though *P. infestans* appeared to have been suppressed by a greater degree, this difference was not statistically significant. In the plants treated with *B. bassiana* maximum disease score was 4.00 (*A. solani*) and 4.33 (*P. infestans*).

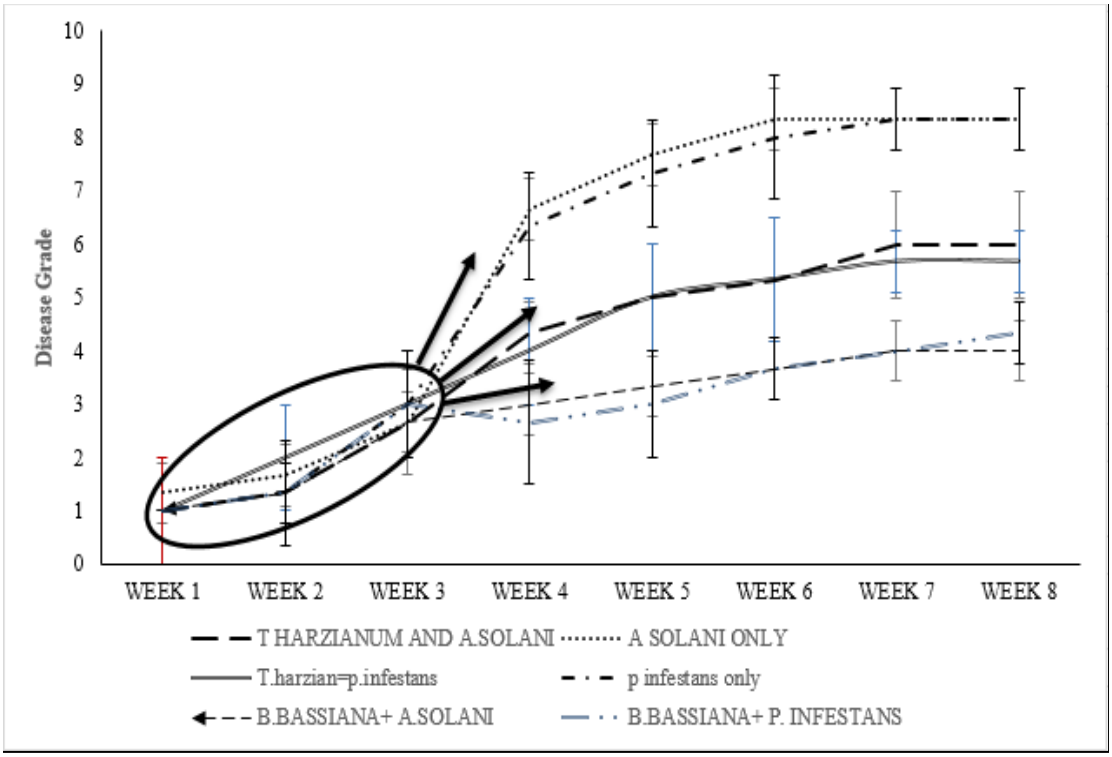

Fig 4.11: Graph depicting the progress of the diseases under different treatments. Uppermost pair represents the negative controls; the middle pair represent *T. harzianum* treatments while the lowermost pair shows the *B. bassiana* treatments. Error bars represent standard deviation.

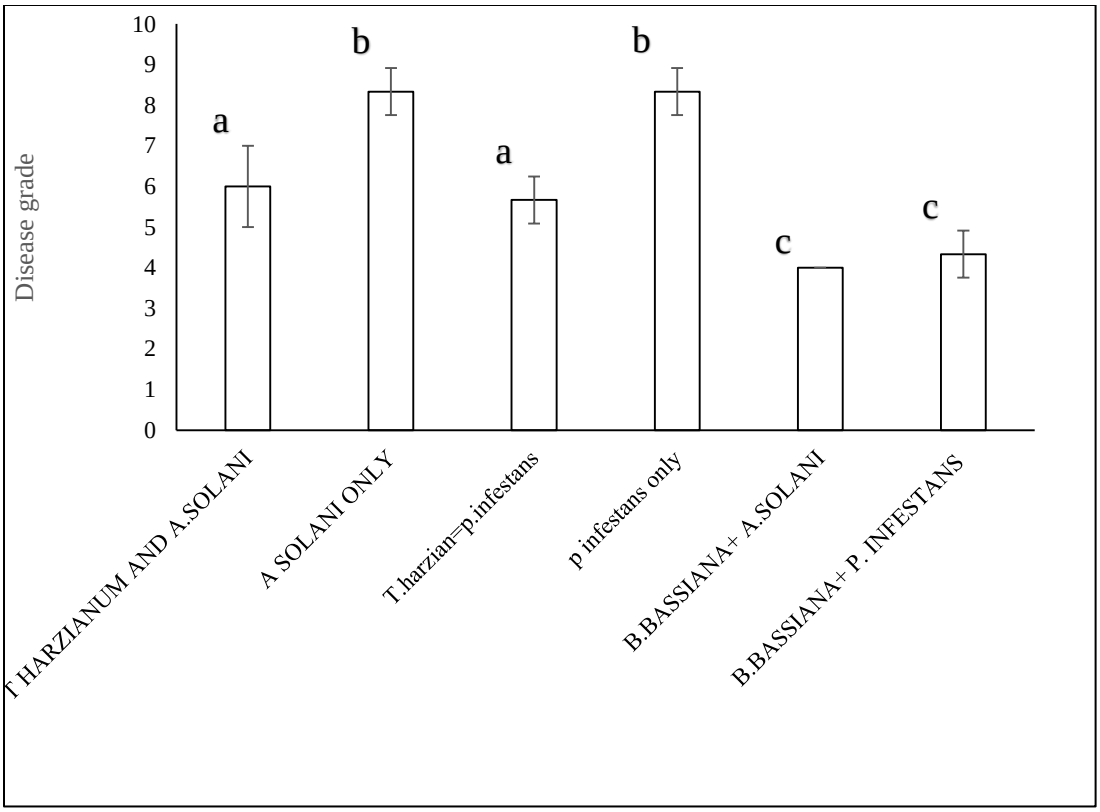

Fig 4.12: Bar graph showing the extent of disease grade on plants on the final week. Bars having the same letters depict means of disease grade that are not statistically significant (*P* = 0.000). There are three sets of similar means based on treatment.

Taking the data of the final day, the superiority of *B. bassiana* is more apparent (fig 4.12). Disease thrived more in the negative controls, followed by those plants treated with *T. harzianum*. Plant treated with *B. bassiana* were least affected by the inoculated pathogens. Differences in the means of disease grade taken on the last day were significant ($P < 0.001$) (Appendix I).

CHAPTER FIVE

DISCUSSION

5.1 Isolation and identification the of biocontrol fungi and the pathogens

Trichoderma harzianum was isolated from soils collected from tomato rhizosphere while *B. bassiana* was isolated from a dead beetle that was found within the locality under study as hinted by (Ephrem *et al*. 2011). These habitats were just as appropriate given that *T. harzianum* is free living and a ubiquitous soil borne fungus that thrives well in all types of soils whereas *B. bassiana* on the other hand grows naturally in soils throughout the world and parasitize on various arthropods (Culebro *et al.,* 2017). Isolation of the biocontrol agents was done on selective media followed by their identification using cultural, morphological characteristics. They were then sub-cultured on PDA for subsequent use

Phytophthora infestans was isolated from infected tomato fruits as was performed by Zheng *et al*. (2003). The pathogen affects all above ground plant parts at any stage of development (Ephrem *et al*. 2011). Corn meal agar medium was used for isolation of *P. infestans* because it was the most available selective media with low nutritional value (Nowicki *et al*., 2013).This property, coupled with suitable antibiotics like Nystatin encourages the growth of *P.infestans* (Suprapta, 2012).This is because other fungi that would have out-competed the pathogen were prevented from growing in low nutrient content. (Ephrem *et al*. 2011) also put forward that bacteria population must be kept low so as not to suppress the growth of *P. infestans* by antagonism, antibiotic production and direct parasitism. This was achieved by adding suitable antibiotics such as vancomycin, ampicillin, rifampicin, streptomycin and penicillin.

Alternaria solani was isolated from infected fruits and leaves. The pathogen occurs on foliage at any stage of growth causing spots and blight in fruit and leaves (Adhikari., 2017). Rodrigues *et al*. (2010) pointed out that isolation of *A. solani* is done on many growth media but special conditions are required for sporulation. In the current study, isolation was done on PDA with special antibiotics including streptomycin. Identification of *A. solani* was made simple by the use of leaf and fruit symptoms as well as cultural characteristics.

5.2 *In vitro* antifungal activities of *T. harzianum*

The *in vitro* antifungal activity was tested via dual culture, production of volatiles and the use of diffusible/non-volatile metabolites (culture filtrate). *T. harzianum* had a relatively fast growing rate compared to both *A. solani* and *P. infestans.* For this reason, the phytopathogens were naturally subdued. Indeed, fast growth rate endows a microorganism possessing it colonizing advantage in any ecosystem (Jeyaseelan, 2014). However, the main mode of action for *T. harzianum* is hyperparasitism. Majorly, mycoparasitism is involved when there is physical interhyphal contact between two fungi (Mukherjee *et al.,* 2013). This was clearly evident in both cases in the current study (Plates 4.6 & 4.9). A fungus lacking hyperparasitism capability cannot restrict the growth of another fungus by just interaction of the hyphae. In the experiments carried out in this study, *T. harzianum* colony not only impeded the growth of the test pathogens but also spread over the colonies of both *A. solani* and *P. infestans* indicating parasitism. In this way, *T. harzianum* uses the test pathogens used in the experiments here as substrates for its growth.

The test for antagonism via volatiles and diffusible metabolites eliminates the possibility of colony contact. These approaches assess the ability *of T. harzianum* to produce inhibitory substance (s). *T. harzianum* strongly inhibited the growth of both colonies via volatiles. However, for the non-volatiles (diffusible metabolites) antifungal activity only appeared in the highest concentration (15%v/v) used albeit mild. The other concentrations of diffusible metabolites never showed any signs of antagonism. *P. infestans* was more susceptible to the culture filtrate (Plate 4.11). This result corroborates the observation made by Zhang *et al*. (2018) where a different species of *Trichoderma* (*T. longibrachiatum*), was stronger on *P. infestans* compared to other test fungi. They however further characterized the culture filtrate and found a number of antifungal compounds with varying antifungal potential. But as also reflected here, the antifungal activity was dose-dependent. *P. infestans* is classified as fungi like organism unlike *A. solani* which which is a true fungi (Alaux *et al*., 2018). This fact must play a big role in determining level of susceptibility to antifungal agents.

Previously, antagonism by dual culture also reflect and possibly explains the dynamics portrayed by use of volatiles and diffusible metabolites. The slow growth of the test pathogens must have been as a result of volatile metabolites produced by the *T. harzianum*. Further, the fact that the *T. harzianum* colony came into contact with both pathogens attests to the proven apparent inability to produce inhibitory substances. This is however not totally expected; a fungus that feeds on another fungus wouldn't produce substances to repulse its 'prey' but would rather restrict its growth. The experiments by Zhang *et al.,* (2018) however did not involve dual culture. As argued elsewhere, *T. harzianum* first employs other arsenals like fast growth rate before producing toxins

(Kucuk & Kivanc, 2004). Mycoparasitism by *Trichoderma* spp therefore also involves production of substances to further subdue the parasitized fungus. Further, it has been shown that the environment where the *Trichoderma* spp has been isolated influences its biological activity (Mayo-Prieto *et al*., 2020). Antifungal activity will work better if *Trichoderma* spp is isolated in the soil infested with the pathogen it is intended to control. In the case of this study however, the pathogens and the *Trichoderma* isolate were obtained from different environments.

5.3 In vitro antifungal activities of *B. bassiana*

This fungus, *B. bassiana,* is more reputable for its entomopathogenic capabilities than antimicrobial activity. Unsurprisingly therefore, as discussed here, its antifungal activity was generally mild but dose dependent. But to have an edge against competing organisms, it must produce some antimicrobial substances such as oosporein (Sahab, 2012). The antifungal activity of *B. bassiana* against the two fungal pathogens varied greatly. First, in the dual culture with *A. solani*, there was no effect imparted on the *A. solani* colony (Plate 4.12). Just like it was the case with *Trichoderma harzianum*, the colonies came into contact. However, in the current case of *B. bassiana*, the *A. solani* colony was not parasitized. In the dual culture with *P. infestans* however, there was a very clear zone of inhibition produced. *B. bassiana* strongly inhibited the growth of *P. infestans* even though the growth rate was considerably slow compared to that of *P. infestans* (Plate 4.15).

Volatile organic compounds were mildly effective against *A. solani.* Its antifungal activity was only evident from the fourth day after inoculation (fig 4.7). It was however

again very strongly inhibitory against *P. infestans*. Even though the culture filtrate was mild against *A, solani*, it had no effect on *P. infestans.* As observed, the *P. infestans* colonies appeared different when cultured with *B. bassiana* (plate 4.17). The hyphae were not as thick as in the controls. This was also observed on the colonies (both pathogens) when grown on media amended with *B. bassiana* culture filtrates. This may be due to the action of *B. bassiana* which inhibits pathogen growth by collapsing the pathogen mycelium (Zhang & Li., 2011; Culebro *et al*., 2017). The metabolites interact with hyphae of fungal pathogens and collapse the mycelia of pathogenic fungus before direct contact occurs (Bucarei, 2019). Cell wall degrading enzymes are produced efficiently at 5 to 7 days after inoculation leading to maximum growth inhibition after 7 days (Lamsal *et al*., 2013)

5.4 *In vivo* determination of the efficacy of the *T. harziunum* and *B. bassiana* against early and late blight diseases

The current study showed that *T. harzianum* reduced the mean disease score of early blight caused by *A. solani* by a value of 1.825 and *that* of late blight caused by *P.infestans* by a value of 2.088. On the other hand, *B. bassiana* reduced the mean disease score of early blight by a value of 2.625, and that of late blight by 2.513.These reduction in mean disease score implies that the area of the leaf with blight spot was reduced hence disease severity reduced.

The study results revealed that both the antagonists are more effective in the control of the two pathogens under study. However, the use of *B. bassiana* in controlling *A. solani* and *P. infestans* is more effective than the use of *T. harzianum* on the same. This implies that there is a statistically significant difference in reduction of mean disease score when

tomato plants infected with *P. infestans* and *A. solani* are treated with *T. harzianum* and *B. bassiana.*

B. bassiana therefore is the most effective in reduction in the size of necrotic and chlorotic leaf area, hence high reduction in disease severity. Bonnie *et al*. (2001) acknowledged that colonization by *B. bassiana* is not restricted to growth as an endophyte but also as an epiphyte resulting in subsequent protection against diseases. More than one mode is operative in suppression of plant diseases ranging from antibiosis to production of bioactive metabolites that limit growth of plant pathogens *in vivo*. Bennet *et al.* (2012) and Aneeset *et al.* (2012) indicated that biocontrol agents induce resistance in the above ground plant parts.

Apart from other mechanisms of biocontrol by the fungi, *T. harzianum* can effectively colonize the plant resulting in protection of the host from biotic and abiotic stress and against phytopathogens (Mukherjee *et al*., 2013). The effective inhibition by the two biocontrol fungi could also be attributed to competition which is a result of proper timing because resources go to the initial colonizer. This is the reason why the biocontrol fungi spores were sprayed first onto tomato seedlings followed by the pathogen spores so as to be the first colonize the plant before the pathogen spores (Zhang & Li, 2011).

Other researchers established that *B. bassiana* when used *in vivo* decrease plant index disease by 1.4 and reduce growth of pathogen (Greenfield, 2016). Enzymatic activity and release of metabolites act directly on the pathogen by antagonism and or induced systemic resistance in plants resulting in a decrease in disease development **(**Chowdappa *et al*., 2013). *B. bassiana* also produces appressoria that penetrates the leaf cuticle, stem

cell walls or roots. On the other hand, stem application, foliar application and soil application has been shown to reduce disease development in tomato plants by lowering disease severity (Jeyaseelan & Thamila, 2014)

Disease symptoms were not suppressed completely indicating that the growth of pathogenic fungi was not inhibited completely (Lopez &Sword, 2015). This could be attributed to the fact that amount of fungal inoculum used may have affected degree of colonization in plants and infection rates (Akello, 2007). During foliar application the inoculum spend significant amount of time on the leaf surface and germination could be affected by radiation, temperature and humidity conditions (Buccarei, 2019). *B. bassiana* is able to colonize plant tissues above and below the soils. It exhibits poor competition in the soil hence encountering more competitive microbiota in the soil roots. Control of pathogens too is dependent on other factors too for example biotic and abiotic stresses. Furthermore, antagonist must be applied regularly to maintain antagonistic population dynamics because its survival can be lower than pathogens (Vos *et al*., 2015).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

1. Both *T. harzianum* and *B. bassiana* showed significant antagonistic ability against *A. solani* and *P. infestans in vitro*. Both *T. harzianum* and *B. bassiana* produced volatile organic compounds that were effective against *A. solani* and not effective against *P. infestans in vitro*. The culture filtrate (non-volatile) of both *T. harzianum* and *B. bassiana* were effective against *A. solani* and *P. infestans in vitro*.
2. Fungal growth medium amended by 15% culture filtrate of *B. bassiana* showed higher reduction in diameter of the colony of *A. solani* and *P. infestans* in vitro followed by 10% and lastly 5% for both *T. harzianum* and *B. bassiana.* This showed that the higher the concentration of the culture filtrate, the higher the inhibition of growth of the pathogen.
3. Both *T. harzianum* and *B. bassiana* reduced disease severity of *A. solani* and *P. infestans in vivo* however, *B. bassiana* showed a higher reduction in disease severity through significant reduction of blighted area of the leaf as compared to *T. harzianum.*

6.2 Recommendations

1. *T. harzianum* and *B. bassiana* can be harnessed and be used in the control of early and late blight in tomatoes.
2. More research to be done to establish the specific metabolites produced by *T. harzianum* and *B. bassiana*, that are responsible for the control of early and late blight disease of tomato.
3. There is need to investigate more on the biocontrol potential *B. bassiana* against plant pathogens both *in vitro* and *in vivo*. This is because this study has established that its potential is great as compared other researches that did more on its entomopathogenic potential

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APPENDICES

Appendix I: ANOVA table; in vivo experiments to test the efficacy of the bio-control agents

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	53.1111	5	10.6222	27.3143	**3.66E-06**	3.10588
Within Groups	4.66667	12	0.38889			
Total	57.7778	17				

Appendix II: ANOVA Table; comparison of colony diameters of A. solani exposed to different concentrations of B. bassiana culture filtrate.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2079.981	4	519.9952	12.4726	4.32E-06	2.689628
Within Groups	1250.73	30	41.69101			
Total	3330.711	34				

Appendix III: ANOVA table for the comparison of the antifungal activity of the various concentrations of B. bassiana culture filtrate against P. infestans

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	71.20635	3	23.73545	0.077414	0.971604	3.008787
Within Groups	7358.54	24	306.6058			
Total	7429.746	27				

Appendix IV: Similarity Report

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