Biological control of damping off disease caused by *Pythium aphanidermatum* using *Bacillus subtilis* and *Trichoderma asperellum*

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A thesis submitted in partial fulfilment for the degree of Master of Science in Agricultural and Environmental Biotechnology in the Jomo Kenyatta University Of Agriculture And Technology

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DECLARATION

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

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DEDICATION

To my father Simion Kimunai Ledama, my mother, Elizabeth C. Chelegoi; and my siblings for the love, care and encouragement. Above all I offer my sincere gratitude to the Almighty God for making this journey possible.

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LIST OF ABBREVIATIONS

BCA…………………………………………………….……... Biological Control Agent

CFU…………………………………………………………..Colony Forming Units

FAO……………………………….................. Food and Agriculture Organization

GRAS…………………………………………………...Generally Regarded as Safe

IRS……………………………………………………Induced Systemic Resistance

JKUAT…………………….Jomo Kenyatta University of Agriculture and Technology

NA…………………………………...…………………………...Nutrient Agar

NB………………………………………………………………...…..Nutrient Broth

PDA………………………………………………………...Potato Dextrose Agar

SAR…………………………………………………Systemic Acquired Resistance

SPSS………………………………..……Statistical Package for the Social Science

USFDA…………………………………………… US Food and Drug Administration

UV…………………………………………………………..Ultraviolet Radiation

ABSTRACT

Seedling damping off caused by *Pythium aphanidermatum* is an important disease in tomato production in Kenya. The disease causes seedling losses up to 100% in most tomato growing regions in Kenya. A study was conducted from 2012 to 2014 at Jomo Kenyatta University of Agriculture and Technology (JKUAT) to assess the efficacy of two biological control agents (BCAs), *Bacillus subtilis* and *Trichoderma asperellum,* against *P. aphanidermatum.* The antagonistic activity of the *B. subtilis* and *T. asperellum* isolates against *P. aphanidermatum* was assessed both *in vitro* and *in vivo*. The storability of the two BCAs when applied as a seed dress was also determined at 25 °C ±2. *Bacillus subtilis* and *T. asperellum* significantly (P≤0.05) inhibited the *in vitro* radial growth of *P. aphanidermatum* by 68% and 69% respectively. Similar results were observed in the greenhouse test with fewer post-emergence damping off cases for seedling coated with *B. subtilis* and *T. asperellum* (20.19% and 24.07% respectively) while 65.89% of the control (non-coated) developed damping off symptoms. The antagonistic ability of *B subtilis* significantly (P≤0.05) declined in the presence of Mefenoxam 1g/l to 60.09%, and to 32.4% in presence of Propineb/cymoxanil 15.6 g/l. *Trichoderma asperellum* declined in the presence of Mefenoxam 1g/l to 6.7%, and to 3.7% in presence of Propineb/cymoxanil. Coating of seed with *B. subtilis* and *T. asperellum* at concentrations of 10^{13} and 10^{9} CFU/ml resulted in drastic decline in the concentration compared with a coating concentration of 10^{6} CFU/ml. A combination of NPK fertilizer and biocontrols in seedling management resulted to a significantly higher dry mass compared to the use of either biocontrol agent or fertilizer alone (P<0.001). The study recommends that *B. subtilis* BS01 and *T. asperellum* T900 be considered among the strategies for controlling damping off in tomatoes. It also recommends that seed coating with BCAs at 10^{6} CFU/ml should be used for damping off control before the 7th week to prevent loss of efficacy due to decrease of BCA concentration below the effective concentration.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Tomato (*Solanum lycopersicum*) is a crop of economic importance worldwide. However, pest and diseases challenge the production of tomatoes. Damping off caused by *Pythium aphanidermatum* is a disease of economic importance in tomato production in the tropics (Owen-Going *et al*., 2003). The disease attacks seedlings causing pre- and post-emergence seedling mortality, which results in losses of up to 100% (Muriungi *et al*., 2014).

Damping off is controlled through prophylactic synthetic fungicides application (Leammlen, 2001). Fungicides commonly used to control *Pythium* spp. include propamocarb-hydrochloride, fosetyl-Al, metalaxyl and azoxystrobin. However, the use of these synthetic pesticides in vegetable production results in detrimental environmental effects (Bajpai and Giri, 2003; Muriungi *et al*., 2014). These include pesticide residues in foods, contamination of surface and ground water and the undesirable effects on non-target organisms (Torres and Ruberson, 2004; Stuart and Banks, 2003). The environmental and economic losses due to pesticides use in the USA translates to billions of dollars with estimated $1.1 billion in health sector, $1.5 billion in pesticides resistance, $2.2 billion due to bird losses from feeding on pesticide contaminated crop and $2 billion loss due to ground water contamination (Pimentel, 2005). These losses may be higher in developing nations where the use of pesticides often go unchecked. This therefore calls for alternative methods of disease management (Rosenzweig *et al.,* 2001).

Biological control agents may provide an alternative to synthetic pesticide use. They are eco–friendly and highly specific to target organism, (Gupta and Dikshit, 2010). Currently, there are various fungal and bacterial based BCA products approved for use on plant pathogens (O' Callaghan *et al*., 2006). These products are steadily gaining global market share (Thakore, 2006). Increased adoption of BCAs is based on

their efficacy against plant pathogens. However, the efficacy of the BCAs is influenced by the mode of application (McIntyre and Press, 1991). BCA has been mostly applied as soil drench (Kurze *et al*., 2001), or incorporated into the planting media (Abdel-Kader, 1997). Application of BCA through seed coating has not been used in a commercial set up (Shah-Smith and Burns, 2010). The method provides timely control of seedling and seed-borne plant pathogens (Martin and Loper, 1999; Jensen *et al*., 2004). Coating of onions seeds with *Pseudomonas fluorescens* F113 is reported to effectively control seedling damping off (O'Callaghan *et al*., 2006). *Trichoderma* spp. applied as a seed coat in vegetable seeds reduces the damping off disease caused by *Rhizoctonia solani* (Harman *et a*l., 1980b; Papavizas *et al*., 1982). The use of BCAs as seed coats is however influenced by various factors. The presence of residual synthetic pesticides in the soils can reduce the population of the BCAs inoculated into the soils, hence its efficacy (Torres and Ruberson, 2004). The use of broad-spectrum pesticides such as Mefenoxam, Propineb and cymoxanil in crop production may interfere with BCAs activity (Razaei *et al.,* 2007). The storability of the BCAs on coated seeds also influences the effectiveness of the organisms. Seed coating practices that utilises seed wetting can cause the pregermination of the BCAs spores (Larena, *et. al*., 2003). Pre-germinated spores are susceptible to physical and chemical changes that lead to desiccation and hence spore death (O'Callaghan *et al*., 2006). This may lead to low concentration of the BCAs on the seed surface during sowing, which results in reduced efficacy (Harman *et al*., 1980a; Hong *et al*., 2005; Shah-smith and Burns, 2010).

In this study the survival of *Bacillus subtilis* and *Trichoderma asperellum* coated seeds in high damping off disease pressure was assessed. The efficacy of *B. subtilis* and *T. asperellum* against *P. aphanidermatum* and the influence of pesticides residue on BCA effectiveness were also evaluated. The storability of both microorganisms at room temperature was also assessed.

1.2 Statement of the problem

Production of tomatoes in Kenya has been on a decline from 2010 to date due to disease and pest infestation (Babalola and Glick, 2012; FAOSTAT, 2014). The

restriction imposed on the use of synthetic pesticides, due to the associated undesirable environmental effects, is leading to high economic losses (Rosenzweig *et al*., 2001). The potential use of *B. subtilis* and *T. asperellum* as an alternative is impeded by the lack of information on their efficacy against *P. aphanidermatum,* which vary with the type of strain and method of application used (Akello *et al*., 2007). Control of *P. aphanidermatum* through coating of seeds with *B. subtilis* and *T. asperellum* is also affected by the shelf life of the BCAs (Adekunle *et al*., 2006). The compatibility of *B. subtilis* and *T. asperellum* with synthetic pesticides commonly used in tomato production also determines the efficacy of the BCAs against damping-off.

1.3 Justification of the study

The use of BCAs for the control of damping off disease reduces the environmental threats associated with chemical pesticides (Gravel *et al*., 2005; Bajpai and Giri, 2003). *Bacillus subtilis* and *T. asperellum* are the best candidates for use as seed coats in the control of damping off because of their availability in soils and their suggested efficacy against fungal pathogens. *Bacillus subtilis* and *Trichoderma* spp. are effective in control of crop fungal pathogens (Daghman *et al*., 2006, Asaka and Shoda, 1996). The use of seed coating for the delivery of *B. subtilis* and *T. asperellum* contains huge promise in the control of *P. aphanidermatum* damping off in tomatoes. To encourage the adoption of these BCAs it is necessary to establish efficacy and the shelf life of both *B. subtilis* and *T. asperellum*. This information will aid in the adoption of the stakeholders make decision on the effectiveness of *B. subtilis* and *T. asperellum* coated seeds in the control of damping off.

1.4 Research questions

I. Do *B. subtilis* and *T. asperellum* have *in vitro* ability to inhibit *P. aphanidermatum*?

II. What is the longevity of *B. subtilis* and *T. asperellum* on coated tomato seed?

III. Do *B. subtilis* and *T. asperellum* have ability to control damping off disease in greenhouse tomato seedlings?

IV. What is the effect of the interaction between fertilizers and biological control agents on the growth of tomato seedlings?

1.5 Null hypothesis of the study

Bacillus subtilis and *T. asperellum* have no efficacy against *P. aphanidermatum* in tomato.

1.6 Objectives of the study

1.6.1 Main objective

To assess the efficacy of *B. subtilis* and *T. asperellum* against *P. aphanidermatum* in tomato.

1.6.2 Specific objectives

I. To assess the *in vitro* sensitivity of *B. subtilis* and *T. asperellum* to synthetic pesticides and their efficacy against *P. aphanidermatum.*

II. To determine the longevity of *B. subtilis* and *T. asperellum* isolates on coated tomato seed.

III. To determine the greenhouse performance of tomato seeds coated with *B. subtilis* and *T. asperellum* against *P. aphanidermatum.*

IV. To assess the interaction of fertilizers and *B. subtilis* and *T. asperellum* in promoting growth.

CHAPTER TWO

LITERATURE REVIEW

2.1 Importance of tomato in Kenya

Tomato (*Lycopersicon esculentum mill*) is amongst the promising commodities in horticultural expansion and development in Kenya. It accounts for 14% of the total vegetable produce and 6.72% of the total horticultural crops (Gok, 2012). Tomato is grown either on open field or under greenhouse technology. Open field production account for 95% while greenhouse technology accounts for 5% of the total tomato production. Kenya is among the Africa's leading producer of tomato and is ranked 6th in Africa with a total production of 397,007 tones (FAO, 2012). The major tomato producing Counties in Kenya are Kirinyaga (14%), Kajiado (9%) and Taita Taveta (7%) as shown in Table 1. In 2011, area under production was 19,000 ha, from which 600,000 MT valued at KES 14.2 billion were produced (HCDA, 2011).

Table 1.1. Production of tomato in selected counties in Kenya

Counties	Area (Ha)	Quantity (tonnes)	Values (KSHs.) Milions
Kirinyanga	1978	54524	1070
Kajiado	1551	36460	990
TaitaTaveta	548	27400	959
Bungoma	1022	21720	887
Kiambu	930	20972	884
Total in kenya	18613	397007	12840

Source: HCDA (2013).

2.2 Challenges affecting tomatoes in Kenya

Despite its contribution in poverty alleviation, the tomato industry is faced with a myriad of constraints. These challenges facing tomatoes in Kenya have resulted in a steady decline in yield since 2009 as indicated in fig 2.1 below (FAOSTAT, 2014).

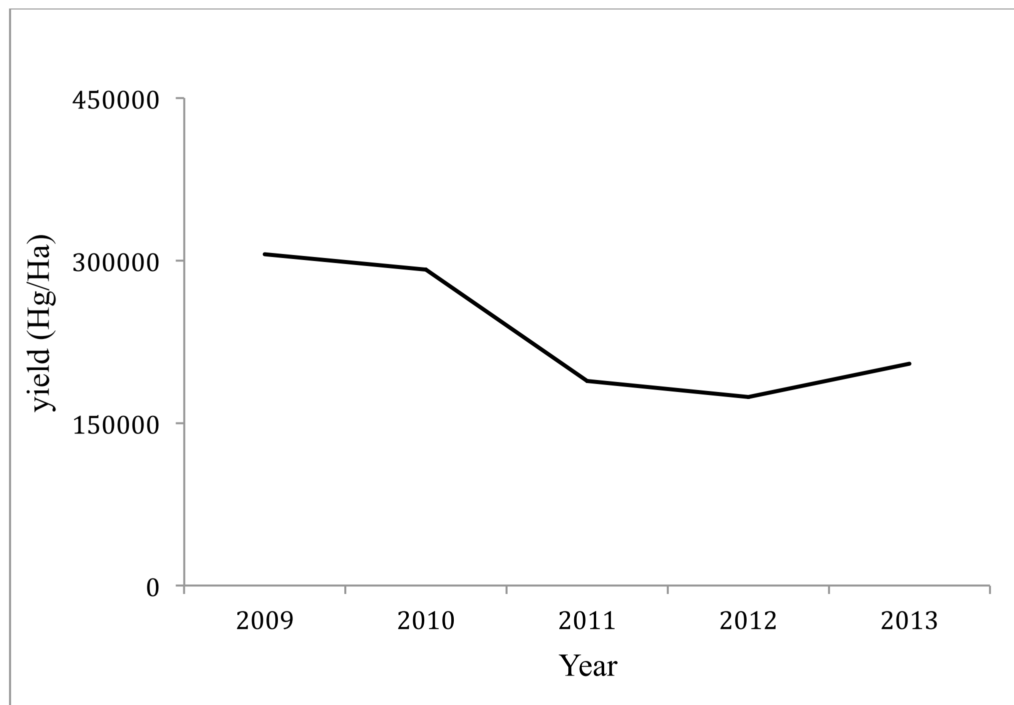

Figure 2.2: Tomato production (yield) trend between 2009 and 2013.

These include agronomic constraints like incidence of pest and diseases and physiological disorders (cracking, sunburn or scald); institutional constraints like poor post-harvest technologies that has ten perish ability and poorly organized rural and urban market infrastructures that permit unpredictable price fluctuation. Tomato late and early blight diseases that are caused by the fungi *Phytophthora infestans* (Mont.) and *Alternateria solani* respectively have been identified as the main constraint in its production (Tumwine et al., 2002).

The tomato late blight disease is caused by *P. infestans* while *A. solani* the fungus causing early blight disease, which are difficult diseases to manage and cause significant reduction in yield. These diseases, which are caused by seed borne fungi, influence the overall health germination and final crop stand (Bissdorf, 2005; Pandey et al., 2006). Fungal diseases such as damping off disease caused by *Pythium* spp. and

Rhizoctonia spp. also cause heavy losses in seedling numbers. These constraints adversely affect the production and marketing of quality tomatoes and should be prudently managed through periodic monitoring and improvement of every function (stakeholder), in order to develop a productive, sustainable and robust tomato value chain. An estimated 60% of the Africa's rural population lives in areas of good agricultural potentials, but face poor market access for their agriculture produce. Therefore, improving market infrastructure by providing better and affordable transportation is deemed necessary for enhancing commercialization in developing countries like Kenya (Shilpi & Umali-Deininger, 2008). The whole portion of tomato produced from Kenya is locally marketed within and around East African countries with nothing left for the international market. The key constraints that cause the dismally export market for Kenyan tomatoes; include poor quality, poor health standards and capricious constant supply of substantially high quantities of the commodity in western markets (Humphrey, 2009).

2.3 Damping off disease

Damping off disease of tomatoes seedling caused by *Pythium* species is widely spread (de Cock and Lévesque, 2004). The disease is of economic importance in all tomatogrowing regions of the world (van West *et al*., 2003). Among the *Pythium* species, *P. aphanidermatum* is the most widely distributed and an important crop parasitic pathogen in tropical regions (Waterhouse and Waterston, 1964). The disease is characterised by the affected parts becoming water soaked and mushy and results in eventual wilting and falling off of the affected seedling. Damping off can occur in different phases of crop development, the disease can attack the seed or the seedling before it emerges from the soil surface resulting in pre-emergence seed rotting. Pre-emergence damping off results in poor stand often mistaken for poor seed quality, soil fertility and other abiotic factors (Laemmlen, 2001). The disease can affect the seedling after germination or after transplanting resulting in a post-emergence damping-off.

The genus *Pythium* is one of the largest *Oomycete* genus consisting of more than 130 recognized species which are isolated from different regions of the world (Paul *et al*., 2006; Bala *et al*., 2010; Robideau *et al*., 2011). Some species of *Pythium* are

beneficial while most species are known to parasitize and cause infections in the roots of crop plants and ultimately damage them (Van der Plaats-Niterink, 1981). *Pythium* diseases of vegetables and field crops are considered an important limiting factor in successful cultivation of crop plants throughout the world. *Pythium* spp. survive as oospores in the soil and germinates attacking root hairs and root tips initiating progressive damage of the root system (Abdelzaher, 2004). It is estimated that diseases caused by *Pythium* species in different crops are responsible for multibillion-dollar losses worldwide (van West *et al*., 2003). *Pythium* species is mainly responsible for pre-emergence phase of the disease; however it can also attack the stem at or below the soil surface resulting in post-emergence death of the seedling (figure 2.2).

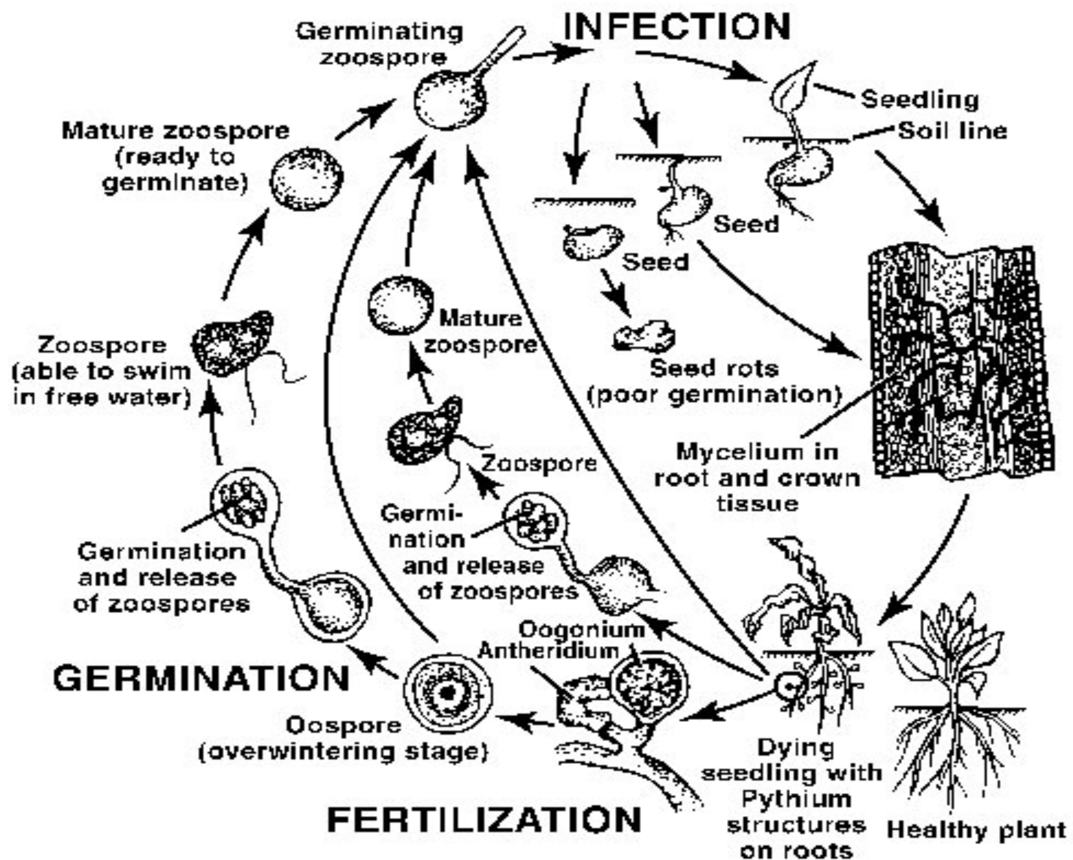

Figure 2.2: Disease cycle of damping off caused by *Pythium* species (Agrios, 1997)

Damping off disease has been documented to be fatal for tomato seedling because it is hard to control once the disease process is underway (Leammlen, 2001).

2.4 Control of damping off disease

Damping off diseases are most effectively controlled by eliminating the pathogen. Measures should be taken to treat the soil or the seeds prior to planting, (Sonoda, 1976). This is important since once the disease sets in, it causes destruction of seedlings in the first two weeks of germination (Agrios, 2005). Various cultural practices have been used in the past to limit the occurrence of the disease. Practices such as the use of clean planting material and healthy seeds and seedlings have greatly reduced the incidence of damping off (Menzies and Belanger, 1996). The use of soil-less germination mix or pasteurized soil has been practiced in the past

(Stephens and Stebbins, 1985). However the cultural methods are cumbersome to apply in large-scale production practices.

Chemical control of damping off offers a reliable control of the disease. Chemical fungicides such as Thiram are commonly used to control damping off diseases (Leammlen, 2001). However, the adverse environmental effects associated with chemical pesticides are causing concern (Bajpai and Giri, 2003). Chemical pesticides are known to be non-specific hence have the potential to affect the natural ecological equilibrium (Witzgall, 2001). The threat posed by pesticides is evident by the number of people recorded to die due to pesticide poison placed at 20,000 per year (Gravel *et al.*, 2004). Apart from threat to humans the target organisms have developed resistance to the pesticide (Howell, 2003).

Various alternative strategies to minimise pesticides use are being tested. Increased monitoring and integrated application of various controls have led to efficient pest control (Dubey *et al.*, 2007). This approach though requires proper understanding of the onset of pest infestation and infestation progress. Accessing this information in developing countries is a challenge to farmers partly due to the failure of extension workers to deliver agricultural information to farmers hence little or no access to such information.

Through biotechnology, genetically engineered crops capable of resisting pest attack have been developed. Genetic manipulation of *Bacillus thuringiensis* (Bt) genes encoding for proteins toxic to insects offers an opportunity to produce genetically modified strains with more potent and transgenic plant expressing Bt toxin which confers resistance to the transformed plants (Tipvadee, 2002). Despite the advantages associated with genetic engineering, public opinion is still divided in Kenya on the risks and benefits of the technology, and the competing political priorities are hindering full implementation of genetically engineered crops for disease prevention.

2.5 Biological control agents as a better alternative to controlling damping off

BCAs have a great potential in agriculture for the control of crop pests (Rodgers, 1993). They provide a safe, effective and environmental-friendly method for plant

disease management (Gravel, 2005). They decompose more quickly in the environment and are generally less toxic towards non-target species (Thakore, 2006). In addition to their potential to directly reduce the incidence of diseases, some microbial products promote plant nutrition and growth (biofertilizers and phytostimulators) and/or facilitate interaction between the host plant and other beneficial organisms (Antoun and Prevost, 2006). Some microbial agents produce enzymes that degrade a precursor of ethylene thus limiting the levels of this hormone in the plant thereby increasing plant growth especially under stress conditions (Lugtenberg and Kamilova, 2009). In addition, better nutrition of the plant often enhances its overall resistance against pathogens and other stress factors (Bent, 2006). The gradual appreciation of the BCAs has been reflected in the growth in their global market share (Thakore, 2006). This growth indicates the realization among the farmers and industry players of the potential the BCAs have in solving the challenge of plant diseases (fig 2.3).

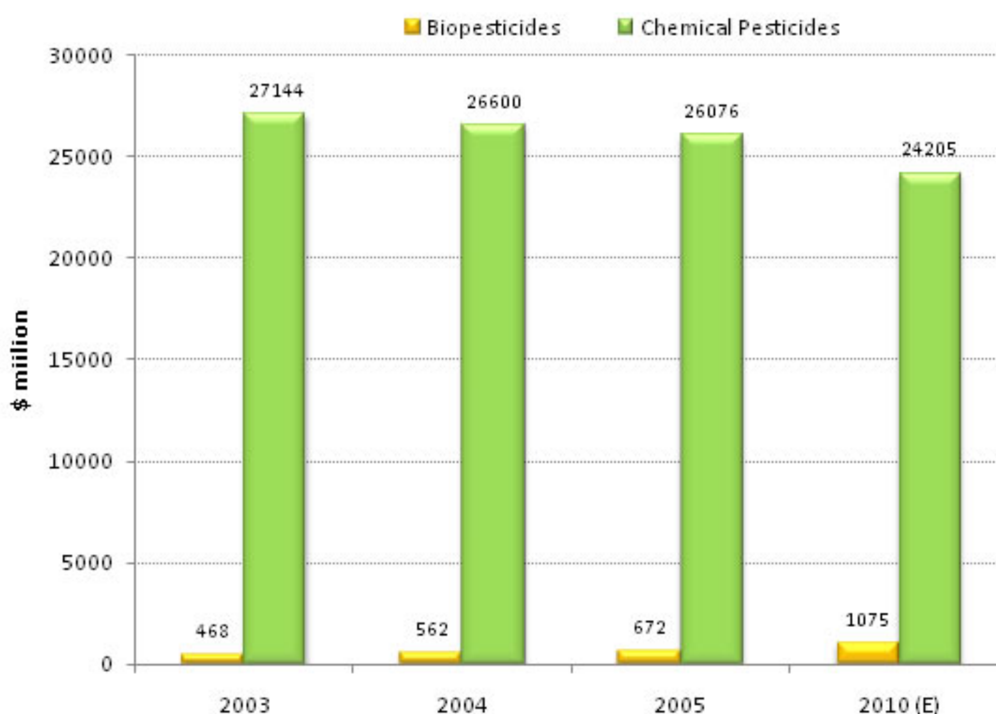

Figure 2.3: Trend of global pesticide vis-à-vis biopesticide market (Thakore, 2006).

Some of the promising biocontrols for commercial use include *Trichoderma harzianum* (UPM40), which has been shown to actively attack *Rhizoctonia solani in vitro* (Daghman, 2004). *Trichoderma harzianum* has also been used effectively in controlling damping off in *Brassica rapa* (Daghman *et al*., 2006). Inoculation of banana tissue culture seedlings with *Fusarium oxysporum* reduced nematode population densities by >45% and damage by >20% over one growth cycle. Coating tomato seeds with *T. harzianum* increased seedling germination rate by 82% (Okoth *et al*., 2011). Isolates of *T. harzianum* are reported to significantly reduce the severity of seedling wilt in tomatoes caused by *Fusarium oxysporum* (Mwangi *et al*., 2011). *Bacillus subtilis* have been shown to produce antibiotic activity that suppress damping off diseases in tomatoes caused by *R. solani* (Asaka and Shoda, 1996). However, information on their use as seed coats against tomato damping off is still lacking.

2.6 Characteristics of biological control agents

2.6.1 Characteristic and taxonomy of *Bacillus* spp.

Bacillus subtilis cells are rod-shaped, Gram-positive bacteria that are naturally found in soil and vegetation. *Bacillus subtilis* grow in the mesophilic temperature range. Originally named *Vibrio subtilis* in 1835, this organism was renamed *Bacillus subtilis* in 1872. Other names for this bacteria also include *Bacillus uniflagellatus*, *Bacillus globigii*, and *Bacillus natto*. *Bacillus subtilis* bacteria were one of the first bacteria to be studied. These bacteria are a good model for cellular development and differentiation (Entrez Genome Project). The taxonomic classification of B. subtilis is as follows: Bacteria, phylum: Firmicutes, class: Bacilli, order: Bacillales, family: Bacillaceae (Entrez Genome), the Genus is *B. subtilis.*

The optimal temperature is 25-35 degrees Celsius (Entrez Genome Project). Stress and starvation are common in this environment; therefore, *Bacillus subtilis* has evolved a set of strategies that allow survival under these harsh conditions. One strategy, for example, is the formation of stress-resistant endospores. Another strategy is the uptake of external DNA, which allows the bacteria to adapt by recombination. However, these strategies are time-consuming. *Bacillus subtilis* can also gain protection more quickly against many stress situations such as acidic, alkaline,

osmotic, or oxidative conditions, and heat or ethanol. The alternative sigma factor B is a global regulator of stress response. Heat, acid, or ethanol and glucose or phosphate starvations are all stimuli that activate B (Bandow 2002).

Bacterial biocontrol agents represent the majority of the microorganism-based biopesticides (Shoresh *et al.*, 2010). *Bacilli* are present in a wide range of environments. They have the capacity to produce spores capable of withstanding high temperatures, and unfavourable pH (Piggot and Hilbert, 2004). This is important since sporulation can be induced in industrial production (Monteiro *et al.*, 2005). This greatly facilitates post-culture conditioning as bacterial suspensions can be converted to powder formulations without bacterial mortality observed (Lolloo *et al.*, 2010). Beside its spore forming ability, *B. subtilis* possesses several characteristics that enhance its survival in the rhizosphere and thus its effectiveness as a BCA (Losick and Kolter, 2008; Rosas-Garcia, 2009).

Bacillus subtilis known to live in aerobic environments can also behave as facultative anaerobe surviving and evolving under low oxygen concentration (Nakano and Hulett, 1997). Additionally, *B. subtilis* is a motile bacterium that readily moves towards and on the root surface that facilitates colonization of new ecological niches. Another reason for the high interest in *Bacilli* is the diversity of their modes of action; one strain may often acts through several mechanisms. This enables these bacteria to be effective in many conditions (variety of pathogens, plants, environmental conditions).

2.6.2 Characteristic and taxonomy of *Trichoderma* spp.

Christiaan Hendrik Persoon described the genus in 1794, but the taxonomy has remained difficult to resolve. For a long time it was considered to consist of only one species,*Trichoderma viride*, named for producing green mold (Druzhinina & Kubicek, 2005). The genus was divided into five sections in 1991 by Bissett, partly based on the aggregate species as follows: *Pachybasium* (20 species); *Longibrachiatum* (10 species);*Trichoderma*; *Saturnisporum* (2 species); *Hypocreanum* (Bissett, 1991).

With the advent of molecular markers from 1995 onwards, Bissett's scheme was largely confirmed but *Saturnisporum* was merged with *Longibrachiatum*. While *Longibrachiatum* and *Hypocreanum* appeared monophyletic, *Pachybasium* was determined to be paraphyletic, many of its species clustering with *Trichoderma*. Druzhina and Kubicek (2005) confirmed the genus as circumscribed was holomorphic. They identified 88 species, which they demonstrated could be assigned to two major clades (Druzhinina & Kubicek, 2005). Consequently, the formal description of sections has been largely replaced by informal descriptions of clades, such as the Aureoviride clade or the Gelatinosum clade.

The belief that *Trichoderma* was monotypic persisted until the work of Rifai in 1969, who recognised nine species (Samuels, 2006). Currently there are 89 accepted species in the *Trichoderma* genus. *Hypocrea* are *teleomorphs* of *Trichoderma* which themselves have *Hypocrea* as anamorphs (Samuels, 2006)

Species of the filamentous ascomycete genus *Trichoderma* are among the most commonly isolated saprotrophic fungi. They are frequently found in soil and growing on wood, bark, other fungi and innumerable other substrates, demonstrating their high opportunistic potential and their adaptability to various ecological conditions (Brotman *et al.,* 2010; Jaklitsch, 2011). The potential of *Trichoderma* species as biocontrol agents in plant disease control was first recognized in the early 1930s (Weindling, 1932) and subsequently they were applied successfully as biocontrol agents against several plant diseases in commercial agriculture (Howell, 2003). Control may be achieved by competition, production of antibiotics or by mycoparasitism (Campbell, 1989).

Several superior strains have been identified and formulated into commercial biopesticides (Agrios, 1997). Otadoh *et al*. (2011) evaluated the antagonistic ability of *T. asperellum*, *T. atroviride*, *T. koningii*, *T. harzianum* and *T. reesi* against *Fusarium oxysporum* f. sp. *phaseoli* under laboratory and green house conditions and found *T. reesei* and *T. koningii* as the most effective isolates against the pathogen and for stimulation of plant growth. Akrami *et al*., (2011) evaluated *T. harzianum* (T1), *T. asperellum* (T2) and *T. virens* (T3) against *F. oxysporum* of lentil and found that all of

them could effectively inhibit growth of the fungus in laboratory tests. Although effective in reducing disease incidence, *Trichoderma* spp. often fails to establish in the rhizosphere (Papavizas *et al*., 1982) or may not be as effective as currently labelled fungicides when applied as seed treatments (Kommedahl *et al*., 1981).

Trichoderma asperellum possess swollenin a protein that carries a cellulose-binding module and can disrupt the crystalline cellulose structure of plant cell walls (Saloheimo, 2002). It contributes to root colonization in *T. asperellum* and induces local defence responses. (Brotman et al., 2008). Swollenin has sequence similarity to expansins, which are plant proteins that facilitate expansion of the plant cell wall in roots and root hairs (Guo, 2011), and *Trichoderma* spp. may take advantage of a swollenin-induced increase in root surface area when establishing in the plant rhizosphere. *T. asperellum* also exhibit systemic acquired resistance (SAR), normally associated with the second stage of the plant immune response, but it is in a concentration-dependent manner and may occur in the early stages of the fungal interactions with roots (Segarra, 2007).

2.7 Mode of action of biological control agents

Biological control agents inhibit the growth of the pathogen through different means. The modes of action of the different organism may vary depended on the strain of the organism. Below are some of the modes employed various BCAs against the target pathogen

2.7.1 Competition

Competition is defined as niche overlap, resulting from a situation where there is simul taneous demand for the same resource by two or more microbial populations (Droby & Chalutz, 1994). Competition for nutrients (e.g. carbohydrates, nitrogen, oxygen) and space is often suggested as a potential mechanism of action in biological control systems (Spadaro et al., 2010). For this, both the pathogen and the antagonist must have the same requirement for a specific nutrient or resource. Competition can be an effective biocontrol mechanism when the antagonist is present in sufficient quantities at the correct time and location and if it can utilize limited nutrient s or resources more efficiently than the pathogen (Larkin et al., 1998). In terms of

competition for space, certain microorganisms (yeasts and bacteria) have the added advantage of the formation of an extracellular polysaccharide capsule that can promote adhesion to the fruit surface (Spadaro & Gullino, 2003).

The effects of competition show that there are many different possibilities for biological control such as (i) reducing inoculum potential by nutrient competition, (ii) increasing saprotrophic competition for initial resources in substrate colonization and (iii) reducing the actual amount of the pathogen in either the dormant survival or pathogenic growth phases (Spurr, 1994). Competition for nutrients and space seem stop lay a major role; however, appropriate methods are lacking to separate the various action mechanisms Janisiewicz & Korsten, 2002). Competition is a mechanism of biocontrol that is likely to be used by many antagonists (yeasts and bacteria) (Vero et al., 2009; Spadaro et al., 2010). For the control of post-harvest diseases, the use of microorganisms that compete with pathogens for nutrients may be preferable to the use of antibiotic-producing microorganisms, because of potential issues related to human toxicity and build-up of antibiotic resistance within the pathogen population.

2.7.2 Antibiosis

Antibiosis is defined as the inhibition or destruction of a microorganism by substances such as specific or nonspecific metabolites, lytic agents, or enzymes that are produced by another microorganism (Melin et al., 2007). Antibiotics are volatile or non-volatile substances produced by microorganisms, which operate at low concentrations (less than 10 ppm). Certain microorganisms start producing antibiotics only when a substantial quantity of substrate mainly carbon is available, but other microorganisms start producing antibiotics when the substrate availability decreases. This strategy is thought to serve in extending the general activity of certain microorganisms by preventing other microorganisms from using the remaining quantity of substrate. Antibiosis refers to the inhibition or destruction of the pathogen by a metabolic product of the antagonist, such as the production of a specific toxin, antibiotics or enzymes (Heungens & Parke, 2001). To be effective, antibiotics must be produced in situ insufficient quantities at the precise time of interaction with the pathogen (El-Ghaouth et al., 2002). It was discovered that bacteriocins, which are

antibacterial proteins, produced by bacteria, kill or inhibit the growth of other bacteria (Cleveland et al., 2001). Bacteriocins function by forming pores in the membrane of target cells and depleting the trans-membrane potential. This results in the leakage of cellular materials (Cleveland et al., 2001). One well-known example is Pyrrolnitrin, a natural product produced by some *Pseudomonas* spp. This compound provided the chemical model for development of Fludioxonil, a broad-spectrum fungicide used as seed treatment, foliar sprays or soil drenches (Gardener & Fravel, 2002).

Pyrrolnitrin produced by *Pseudomonas cepacia* LT-4-12W (Janisiewicz & Roitman, 1988) reduced in vitro growth and conidia germination of the stone fruit pathogen *Monilia fructicola*, and pome fruit pathogens *Penicillium expansum* and *Botrytis cinerea*, respectively. Both strains controlled fruit decays caused by the respective pathogens (Pusey & Wilson, 1984; Janisiewicz & Roitman, 1988), and strain LT-4-12W also controlled various decays on citrus (Smila-nick & Denis-Arrue, 1992) and stone fruits (Smilanick et al., 1993). These fruit decays were also controlled by applications of the respective antibiotics alone (Pusey et al., 1986; Janisiewicz et al., 1991). However, the significance of the antibiotics in these biocontrol situations is not clear, because strain LT-4-12W still provided substantial control of blue mould decay on oranges inoculated with laboratory-derived mutants of *Penicillium itallicum* resistant to pyrrolnitrin (Janisiewicz & Korsten, 2002). The mechanism(s) of biocontrol of *P. syringae* strains ESC-10 and ESC-11 used in BioSave products has not been elucidated. Bullet al., (1998) showed that on some media both strains can produce syringomycin E, which is inhibitory to a variety of fungi and that the purified compound can control green mould (*Penicillium digitatum*) of lemons. However, the role of syringomycin E in biocontrol is in doubt because efforts to isolate this compound from fruit wounds treated with the antagonist have been unsuccessful (Bull et al., 1998), and rapid growth and colonization of the wounds was important for biocontrol. This suggests that com-petition for nutrients and space may have played a major role (Bull et al., 1997).

The production of antimicrobials substances by *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus subtilis* against several avocado post-harvest pathogens was demonstrated using the dual culture technique as well as using the indirect agar

plate method (Korsten, 2006). In a study conducted by Jiang et al. (2001), *B. subtilis* was found to be the most effective antagonist against the main pathogens of lychee fruit (*Pernophythora itchi*). In addition, *B. subtilis* produces iturin, which is a powerful antifungal peptide as well as gramicidin S (Cho et al., 2003). Another well-known example of bacteria that produce toxic compounds is *Bacillus thuringiensis*, which produces a BT toxin (Gerhardson, 2002). The main concern, related to the use of antibiotics in food products, is the development of human pathogens resistant to these compounds and the possible development of resistance in fruit pathogens (Melin et al., 2007).

2.7.3 Parasitism

Parasitism or predation occurs when the antagonist feeds on or within the pathogen, resulting in a direct destruction or lysis of propagules and structure (Bull et al., 1998). Direct parasitism by the antagonist on the pathogen propagules has been reported to play an important role in biological control systems, particularly in soil-borne and to a lesser extent foliar diseases (Bonaterra et al., 2003). Direct parasitism by the antagonist on the pathogen propagules has been reported to play a role in biological control of foliar plant diseases (Droby & Chalutz, 1994). Methods to prove parasitism include burying and retrieving propagules of the pathogen to isolate the antagonist (Gardener & Fravel, 2002). Mycoparasites utilize fungal cell-wall-degrading enzymes such as chitinases, glucanases and b-1, 3-glucanase to dissolve their fungal hosts' cell walls and penetrate the cells (Elad et al., 1983).Through ultrastructural and cytochemical studies, El-Ghaouth et al. (1998) found that *Candida saitoana* yeast cells, when cultivated together with *B. cinerea* mycelium, are associated with fungal hyphae showing cytological damage, such as papillae and other protuberances in the cell wall and degeneration of the cytoplasm.

Wisniewski et al. (1991) observed a strong in vitro adhesion of *Pichia guilliermondii* antagonist cells to *B. cinerea* mycelium, perhaps due to a lectin link. Moreover, *P. guilliermondii* shows a high activity of b-1, 3-glucanase enzymes that could result in the degradation of the fungal cell walls (Jijakli &Lepoivre, 1998). *Aureobasidium pullulans* in apple wounds produces extracellular exochitinase and b-1, 3-glucanase, which could play a role in the biocontrol activity (Castoria et al., 2001). Results

showed that the yeast *Pichia anomala* strain K, effective in the control of grey mould of apple, increased production of exo-b-1,3-gluconase three-fold in the presence of cell wall preparations of *B. cinerea* in apple wounds, reducing lesion size by more than half compared to the antagonist. Higher b-1, 3-glucanase and chitinase activity was also detected in apple wounds treated with strains of an antagonist, *A. pullulans*, effective in controlling various decays on apple, table grape and other fruits (Vero et al., 2009). Chitinolytic enzymes have been considered important in the biological control of the post-harvest pathogens because of their ability to degrade fungal cell walls, whose major component is chitin (Saravanakumar et al., 2008).

The yeast *Candida famata* reduced green mould decay (caused by *P. digitatum*) on oranges and increased the phytoalexins scoparone and scopoletin 12-fold in fruit wounds after 4 days when inoculated alone (Arras, 1996). However, the significance of phytoalexins in this biocontrol is not clear because of their relatively slow production. Electron microscopic observations indicate rapid colonization and partial lysis of the pathogen's hyphae by the antagonist.

2.7.4 Induced resistance in the host tissue

Plant defence mechanisms include the hypersensitive response, synthesis of phytoalexins, lignification of plant cell walls, synthesis of lytic enzymes, as well as expression of a wide range of pathogenesis-related proteins. Induced systemic resistance can be demonstrated by applying a biocontrol agent at a location separated from the plant organ that is challenged by a pathogen, whereas the suppression of disease by dead cells of the inducer may demonstrate a locally induced resistance (Zhu et al., 2010). Induced systemic resistance caused by various microorganism scan protect plants against soil or foliar pathogens (Castoria et al., 2001). Several Candida strains, applied to the fruit surface, are able to cause chemical and osmotic changes in apple tissues, favouring antagonist settlement (Spadaro & Gullino, 2003). Fun-gal pathogens must overcome several barriers before they are able to initiate disease in plants. The pathogen must locate and adhere to susceptible host tissue and initiate infection. Contact with underlying plant tissues present a different set of barriers, most notably, preformed antibiotic compound, morphological barriers and phytoalexins induced by the host (Zhu et al., 2010). Evidence suggests that preformed

antifungal compounds might be involved in the resistance on fruits to fungal development and many plants may have the ability to accumulate phenolic compounds, especially tannins, in response to stress (Kumar & McConchie, 2009). A phytoalexin accumulation (scoparon and scopoletin) was noted in citrus fruits treated with yeast cells (Spadaro & Gullino, 2003). A variety of pathogenic microorganisms can induce plant defence and may be useful as biocontrol agents. In a study by Qinet al., (2003) salicylic acid was used to enhance biocontrol efficacy in cherries. The salicylic acid treatment induced a significant increase in polyphenoloxidae, phenylalanine ammonia-lyase and b-1, 3-glucanase activities in cherry fruit thereby increasing the biochemical defense response. Wilson & Pusey (1985) suggested that part of the mechanism of action of yeasts against certain citrus fruit rots might involveinduced resistance in the fruit and this was confirmed later by Droby et al., (2009). Some antagonists can interact with the host tissue, particularly wounds, increasing the cicatrisation processes (Droby et al., 2009). Several antagonistic yeasts are as effective if applied before pathogen inoculation. This observation has suggested that yeast cell application-induced resistance processes in the fruit skin. Some *Candida* spp. strains are able to cause chemical and osmotic changes in apple tissues, favouring antagonist settlement (Mohamed & Saad, 2009). A *P. guilliermondii* strain has been shown to stimulate the production of ethylene, a hormone in loquat fruit able to activate the phenylalanine ammonium lyase (Liu et al., 2 010), an enzyme involved in the synthesis of phenols, phytoalexins and lignins. The increased amount of these enzymes was attributed to higher production by the antagonist and to the induction of the enzymes in the fruit itself. The source of these enzymes and their significance in biocontrol warrant further investigation. Further evidence for the significance of these enzymes in biological control could come from studies evaluating disease suppression by mutants with a disrupted b-1, 3-glucanase genes. Accumulation (scoparon and scopoletin) was noted in citrus fruits treated with yeast cells (Spadaro &Gullino, 2003). In addition to controlling decays, *A. pullulan* scan cause a transient increase in b-1, 3-glucanase, chitinase and peroxidase activities on apple fruit, starting 24 h after treatment. The increased quantities of these enzymes were attributed to higher production by the antagonist and to the induction of the enzymes in the fruit itself. The source of these enzymes and their significance in biocontrol warrant further

investigation. Further evidence for the significance of these enzymes in biological control could come from studies evaluating dis-ease suppression by mutants with a disrupted b-1, 3-glucanasegene.

2.8 Coating of seed with biological control agents: An ideal application method

Seed coating, in the broadest sense, includes any process for the addition of materials to the seed; in the simplest form, it is the direct application of a material to seeds. The term "seed coating" is used to denote the application of a useful material(s) to the seed without changing its general size or shape.

Biocontrol application method is crucial to their efficacy and compatibility with farming practice (Akello *et al*., 2007). Dubois *et al*., (2006) underlined the importance of an efficient application method in the utilisation of BCAs. To overcome this hurdle, coating of seeds with appropriate BCAs prior to planting has been proposed. The objective of dressing the seed with microorganisms is to provide timely protection of the seeds and seedlings against soil-borne pathogens. Seed treatment with BCAs is now practiced commercially in Europe, USA and Israel, with several products developed for seed and seedling treatment against nematodes, insects and diseases (Bennett and Whipps 2008). However, little use has been made of this technology in sub-Saharan Africa in spite of its great potential to have an immediate low-cost impact on crop establishment and plant growth enhancement. Existing use on high value crops and commercial trials clearly demonstrate the benefits of seed treatments. Seed dressing involves the wetting of seeds prior to coating. This has the potential of raising water activity, which in turn may affect shelf life of the spores (Hong *et al*., 2005).

Dry powders may be applied to seeds and are widely used as planter-box treatments. These materials do not adhere well to the seed surface and result in poor loading, lack of uniformity, and dust problems (Leaver & Roberts, 1984). Active agents may be dispersed or suspended in water to form slurry. Application of slurries improves uniformity and helps overcome other problems associated with dry powder application. Slurry treatments may include adhesives generically termed stickers, glues, or binders to improve retention of materials applied to seeds. Adhesives used

for this application include: methylcellulose, dextran, gum arabic, vegetable or paraffin oils (Scott & Archie, 1978). Conventional seed-coating equipment developed for laboratory and commercial applications has been reviewed (Leaver & Roberts, 1984) and is not discussed in this review.

A more recent development, "film coating", is being pursued primarily by the seed industry (Tonkin, 1979). Active materials are dispensed or dissolved in a liquid adhesive and applied to seeds either with a fluidized-bed treater or pharmaceutical coating drum. This technology permits the application of multiple coatings and the increase in seed weight ranges from 1-10%. Recovery rates have been reported as great as 90% and seed-to-seed variability is low.

Coating technologies have been investigated to ameliorate the environmental stresses on germination and seedling establishment. A hydrophilic polymer, hydrolyzed starch-graft-polyarylonitrile (H-SPAN) was evaluated to maintain a high water potential around germinating seeds (Silcock & Smith, 1982). The H-SPAN was applied to sweet corn seeds and seeds were sown in a soil of known matric potential. Polymeric coatings have been investigated to retard imbibition rates when seeds are sown in a wet soil (Baxter & Waters, 1986).

Seed coatings with peroxide compounds that provide oxygen to seeds have been studied under anoxic or near-anoxic soil conditions. Beneficial results have been reported on rice seeds coated with $Ca0_2$ and sown under flooded conditions (Jeffs & Tuppen, 1986).

Macro-and micronutrients have been applied to seed in seed coatings and reported to improve early plant growth (Halmer, 1988). However, there are limitations to the quantity of fertilizer that can be applied effectively without injury to the germinating seed. Beneficial microorganisms that may fix nitrogen or enhance nutrient uptake can also be applied to seeds. Results from seeds inoculated with Rhizobium or other beneficial microorganisms have been reviewed (Tonkin, 1984).

The term "Nanotechnology" was first defined in 1974 by Norio Taniguchi of the Tokyo Science University is the study of manipulating matter on an atomic and

molecular scale. By and large nanotechnology deals with structures in the size range between 1 to 100 nm and involves developing materials or devices within that size (Arivalagan et al., 2011). The properties and possibilities of nanotechnology, which have great interest in agricultural revolution, are high reactivity, enhanced bioavailability and bioactivity, adherence effects and surface effects of nanoparticles.

Nano-coating of seeds using elemental forms of Zn, Mn, Pa, Pt, Au, Ag will not only protect seeds but reduces the requirement of elements to far less quantities than done today. The use of quantum dots (QDs) technique, developed by Su et al. 2004, as a fluorescence marker coupled with immunomagnetic separation for E coli 0157:H7, proved useful to separate unviable and infected seeds. Seeds can also be imbibed with nanoencapsulations with specific bacterial strain termed as Smart Seed as it will thus reduce seed rate, ensure right field stand and improved crop performance. A Smart Seed can be dispersed over a mountain range for reforestation and programmed to germinate when adequate moisture is available. Nano-membranous coatings on seeds allow sensing the availability of water to seeds to imbibe only when time is right for germination, aerial broadcasting of seeds embedded with magnetic particle, detecting the moisture content during storage to take appropriate measure to reduce the damage and use of bio analytical nanosensors to estimate ageing of seeds are some upcoming thrust areas of research (Chinnamuthu & Boopathi, 2009).

2.9 Factors affecting the efficacy of biological control agents applied as seed coats

2.9.1 Timing of application

Filonow and Dole (1999) highlighted the importance of establishing the right time of BCA inoculation. Timing of inoculation is important because most of Pythium attack on plants occur at early stages of plant development, between the 2nd and 3rd week (Hoch and Abawi, 1979). Proper timing also ensures efficient control of the pathogen before the efficacy of the biocontrol is lost (Liu *et al*., 2007). Lumsden and Locke (1989) demonstrated that *Pythium ultimum* is effectively controlled by the BCAs without the need for long exposure time of the pathogen to *Gliocladium virens* prior to planting. However, Filonow and Dole (1999) reported *Pythium* control by allowing 5-7 days of pre incubation of *Pythium* infested medium with the BCAs prior to

planting. Hoch and Abawi (1979) observed that *Corticium* sp. retained its activity against *P. ultimum* beyond 21 days after incubation. Some of the bacteria strains however are reported to quickly diminish in their ability to control *Pythium* in less than 60 hours (Liu, *et al*., 2007). Since the literature points to variation in the time the BCAs remain active, it is important to establish the time the BCAs remain active before utilising it.

2.9.2 Biological control agents coating concentration

Liu *et al*., (2007) established that the rate at which the rhizospheric BCAs are applied affects the performance of the bacteria against *Pythium*. Working with two rates of the bacteria, 10^3 and 10^4CFU/ml Liu *et al*. (2007) observed that higher rates of 10^4 CFU/ml offered protection to the plants against both *P. aphanidermatum* and *P. dissotocum* by 11- 39% compared to a lower concentration of 10^3 CFU/ml.

2.9.3 Biological control agents shelf life on the Seed

Determination of the BCAs shelf life, efficient product delivery, and enhanced bioactivity are crucial to the commercialization of the BCAs (Schisler et al., 2004). Biological control agent should have stability during storage and a good shelf life (Andrews, 1992). For optimum performance these microorganism must have sufficient viable spores during use. The viable spores present will ensure high colonization of the plant rhizosphere. High colonization is vital for control of the pathogen. The quality and quantity of viable spores can be influenced by nutritional composition of the media (Jackson & Schisler, 1992).

The storage time and conditions, formulations and mode of application are among the major factors influencing the efficacy of BCA (Larena et al., 2003). The mode of application of the biological agent and its influence on the viability of the biological agent used has been a subject of intense debate. Several modes of application employed in the use of the biological agents exist today. One mode of application is seed coating; this involves the dressing of the seed with desired microorganism prior to planting. The objective of dressing the seed with microorganisms is to provide timely protection of the seeds and seedlings against soil-borne pathogens (Jensen et al., 2004). It is a simple and inexpensive mode of biological agent application. Seed

dressing involves the wetting of seeds prior to coating. This has the potential of raising water activity that in turn may affect shelf life of the spores (Hong et al., 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Isolates of *Trichoderma asperellum* TRC 900 and *Bacillus subtilis* BS 01 used in the study were obtained from REAL IPM (Kenya) Ltd. The isolates were stored in soil slants at 4°C. The pathogen, *P. aphanidermatum,* used in the study was obtained from ICIPE and stored in sterile water at 25°C. The laboratory media used in the study include the following: Potato Dextrose Agar (PDA), Nutrient broth (NB) and Nutrient agar (NA) obtained from HiMedia Laboratory, Pvt. Ltd., India.

Two synthetic formulations of fungicides used in the study included Mefenoxam (Mefenoxam Gold®) that was provided by Syngenta agro and a combination of Propineb and cymoxanil (Milraz 607-SL, 722 g/ L) that was obtained from Bayer Crop Science Kenya. The Tomato seeds (cultivar "Rio Grande") and the foliar fertilizer (NPK 17:17:17) were obtained from the Kenya Seed Company (Nairobi, Kenya). The Artificial media (Coco peat) used to grow the tomato seedling was obtained from Oshwal Chemical Company (Nairobi, Kenya).

3.2 *In vitro* efficacy of *Bacillus subtilis* and *Trichoderma asperellum* against *Pythium aphanidermatum*

Three *in vitro* experiments were performed and repeated four times. The first set of experiments was performed to determine the sensitivity BCAs and the pathogen to synthetic pesticides. The second and third sets of experiments were performed to assess the efficacy of BCAs against the pathogen in pesticide and non-pesticide amended media.

3.2.1 *In vitro* inhibition of *Pythium aphanidermatum* growth by *Bacillus subtilis* and *Trichoderma asperellum*

The *in vitro* inhibitory effect of *B. subtilis* and *T. asperellum* on *P. aphanidermatum* was evaluated by placing a mycelia plug of *P. aphanidermatum* taken from the periphery of actively growing culture using a sterile cork borer on both 24 PDA and

24 NA plates at 25 mm from the centre of the plates. Twelve PDA and 12 NA plates containing the *P. aphanidermatum* plugs were labelled as *B. subtilis* and *T. asperellum* treatment respectively. The remaining 12 PDA and NA plates containing *P. aphanidermatum* were used as the control treatments. To the *B. subtilis* treatment plates, a colony plug of *B. subtilis* taken from a 24 hour old swab cultures was placed approximately at 45 mm from the incubated *P. aphanidermatum* plug. *Trichoderma asperellum* plugs was also introduced in the same way as *B. subtilis* to the *T. asperellum* treatment plates. In this experiment, the *P. aphanidermatum* alone plates were used as the controls. The percentage radial growth inhibition was determined after 4 days of incubation at 25^{0}C ±2 using the formula of Skidmore (1976) as follows:

$\%\ \text{Radial growth in hibition} = \left(\frac{x-y}{x}\right) \times 100$..................................Equation 1

Where X represents the radius of the control *P. aphanidermatum* growth, and Y the radius of the *P. aphanidermatum* growth towards the BCAs

3.2.2 *In vitro* effect of synthetic pesticides on the growth of biological control agent and *Pythium aphanidermatum*

The following concentrations were used for the study: 0, 2, 2.5, 3.9, 7.81, 15.6, 20, 31.2 and 40 g/l of Propineb/cymoxanil and 0, 0.1, 0.3, 0.5, 1, 5, 10, 12 and 15 g/l of mefenoxam. The concentration levels were chosen based on the manufacturers label rates for each pesticide (Mefenoxam: 1.25-4.0 g/10 L propineb/ cymoxanil; 350-400 g/ 100 L water). The various concentrations were picked randomly above and below the manufacturers label rates. The media with no pesticides was used as the control. A set of stock solutions of each fungicide concentration was made using sterile distilled water. The molten (50°C) growth media was then amended with 1 ml of the individual fungicide concentration stock solution prior to pouring to the plates (Locher & Lorenz, 1991). Control plates were not amended with fungicides. Mycelial plugs of approximately 10 mm in diameter obtained from the margins of 3 days old culture of *T. asperellum* and *P. aphanidermatum* and 10mm diameter colony of *B. subtilis* obtained from three day old culture was placed upside down on the fungicide-

amended and fungicide-free PDA media in Petri dishes and incubated at 25°C in the dark. After 3 days, colony diameter of each isolate was measured in two directions excluding the diameter of inoculation plug and the Percent inhibition (PI) of each fungicide concentration was calculated using the formula below:

Percent inhibition = $\frac{(a-b)}{a} \times 100$................................equation 2

Where a = colony diameter of control plate and b = colony diameter of fungicide amended plate.

The IC_{50} values calculated from the data obtained were interpreted based on the guidelines provided of Edgington and Klew (1971). The sensitivity was therefore categorized as follows: IC_{50} > 50 g/L; insensitivity, 10 <IC_{50} ≤50 g /L; low sensitivity, 1< IC_{50} ≤10 g/L; moderately sensitive and highly sensitive, when IC_{50}<1 g/ L.

3.2.3 *In vitro* effect of synthetic fungicides on the efficacy of *Trichoderma asperellum* and *Bacillus subtilis* against *Pythium aphanidermatum*

The experiment to assess efficacy of *T. asperellum* and *B. subtilis* against *P. aphanidermatum* consisted of treatments in which biological control agents and *Pythium* were grown in media with various concentrations of different pesticides as shown in table 3.1.

Table 3. 1 Treatment of the *in vitro* effect of synthetic fungicide

P. aphanidermatum with	Pesticides	Concentration (g/ L)
B. subtilis	Mefenoxam	0.3; 0.5; 1
B. subtilis	Cymoxanil/Propineb	2; 3.9; 15.6
T. asperellum	Mefenoxam	0.3; 0.5; 1
T. asperellum	Cymoxanil/Propineb	2; 3.9; 15.6
None	Mefenoxam	0.3; 0.5; 1
None	Cymoxanil/Propineb	2; 3.9; 15.6

Media amended with a particular pesticide at specified concentration (as describe in the table above) was prepared as described in section 3.2.2. A mycelia plug of *P. aphanidermatum* taken from the periphery of 3 days old culture using a sterile cork borer was then introduced at 25 mm from the centre of all fungicide amended plates. A colony plug of *B. subtilis* taken from three day old swab cultures was then placed at approximately 45 mm away from *P. aphanidermatum* plug in fungicide amended NA plates (treatments 1-6). *Trichoderma asperellum* mycelial plugs were also introduced in the same way as *B. subtilis* to the fungicide amended PDA plates (treatments 7-12). The remaining treatments 13-18 with *P. aphanidermatum* were used as the control treatments. All the treatments were replicated four times and the plates arranged randomly in 4 rows, with each row containing 18 plates stacked on each other in groups of 3s (6 groups made of 3 plates stacked on each other). The plates were then incubated at 25^0C ±2 for 4 days. The radius of the *P. aphanidermatum* colony in all the treatments was then measured and the percentage radial growth inhibition was

determined using equation 2 above. The experiment was repeated four times.

3.3 Storability of *Bacillus subtilis* and *Trichoderma asperellum* isolates coated on tomato seeds

3.3.1 Seed coating

Maize seeds were bought from the Kenya seed company (Nairobi Kenya). The maize seeds were used because of their easy to handle. A lot of 2200 maize seeds of equal size were selected. The seeds were then divided into 6 groups of 720 seeds each. The individual seeds groups were then coated by thoroughly mixing with either *B. subtilis* or *T. asperellum* suspensions at a concentration of either 10^6, 10^9 or 10^{13} CFUs/ ml, respectively. The concentration was formulated as described in section 3.3.2. After coating, seeds were dried in open trays at room temperature away from direct sunlight. The dried coated seeds were then divided into 4 replicates of 90 seeds. For each replicate, 5 seeds were separately packaged in a sealable paper bag and stored at 25^0C ±2 until assessment.

3.3.2 Experimental design

The shelf life experiments were performed four times between the years 2013 and 2014. The experiments consisted of six treatments of seeds coated with either *B. subtilis* or *T. asperellum* at three different levels of concentrations (10^6 10^9 and 10^{13} CFU/ ml). The treatments were laid out in a complete randomised design and replicated four times

The shelf life of the bio-control was assessed at weekly intervals for 18 weeks. Three samples of coated seeds were selected from each treatment. The seeds were washed in 10 ml distilled water by vortexing in a test tube for 30s, allowed to standing for 2 minutes and vortexed again for 30s. The resulting suspension was serial diluted. 0.2 ml of the diluted concentration was cultured on PDA plates and incubated for 48 hrs at 25^0C±2 in the dark. The number of colony forming units (CFUs) was assessed per plate by counting at the 3rd day after inoculation. The CFU counted were used to calculate the concentration of viable BCA on the seed and the percentage concentration at a given time was calculated as follows:

$$\text{Percentage concentration} = \frac{\text{concentration at a given time}}{\text{initial concentration}} \times 100 \text{.....................Equation 3}$$

3.4 Control of *Pythium aphanidermatum* damping off of tomato using *Bacillus subtilis* and *Trichoderma asperellum* coated seeds

3.4.1 Site description

Three greenhouse trials were conducted yearly between 2013 and 2014 at JKUAT (1537 MASL; 01°05'25. 6"S, 037°00'45.5"E). The greenhouse used was an 8 X 15 m tunnel made of polycarbonate material whose light penetration is 80-84 per cent and has the ability to filter UV harmful rays.

3.4.2 Biological control agent and pathogen inoculum preparation

An isolate of *T. asperellum* stored in sterile soil slants was started on PDA and incubated for 4 days at 20 °C in the dark to induce sporulation. One ml of sterile water was then poured onto the 4 day old culture and fungal spores were scraped using sterile glass rods into 20 ml sterile bottles containing nutrient broth. The bottles with the spores were then shaken in a reciprocal shaker (Taiyo Recipro shaker SR-1, Tokyo Japan) for 3 days to dislodge the cells from the media. Spore concentration of 10^6 spores/ml was then formulated by serial dilution in distilled water and final concentration determined using a haemocytometer.

Growth of *Bacillus subtilis* isolate BS 01 was started in NA and transferred to NB after four days. The suspension was shaken in a reciprocal shaker for 72 hours at 25 °C and concentration adjusted to approximately 10^6 CFU/ml using a haemocytometer.

Isolates of *P. aphanidermatum* stored in distilled water were started in PDA at 20 °C and kept in the dark for 4 days. A spore concentration of 10^4 spores/ml was formulated as described above in the formulation of *T. asperellum* inoculum.

3.4.3 Seed coating

Six grams of tomato seeds were dipped in distilled water for 2 minutes and water drained off immediately. Three grams of the wet seeds were thoroughly mixed manually with the prepared concentration (10^6 CFU/ml) of either *B. subtilis* or *T.*

asperellum suspensions. The coated seeds were then spread on open plastic trays and stored at 25^0C ±2 away from direct sunlight to dry for 24 hrs.

3.4.4 Experimental design

The experiment consisted of four treatments two of which consisted of coated tomato seeds (one with *B. subtilis* the other *T. asperellum*) and 3rd consisted of non- coated seeds all were planted in growth media inoculated with *P. aphanidermatum*. The 4th was made up of non- coated seeds all were planted in *P. aphanidermatum* free growth media. All the treatments replicated three times and laid out in completely randomised design.

For each treatment, plastic seedling trays (plug size 3 by 3 by 4cm, 66 plugs/tray) were filled with cocopeat. *Bacillus subtilis*, *T. asperellum* and control treatment were then inoculated with *P. aphanidermatum* by drenching 1ml of pre-formulated 10^4 spores/ml *P. aphanidermatum* suspension into each plug cell. Two tomato seeds were sown per plug for each treatment. The pre-emergence seedling mortality was assessed daily up to 14th day after sowing. The percentage pre-emergence damping off was calculated based on the positive control as follows.

$$\text{Pre}-\text{emergence damping off}=\frac{\text{N}-\text{X}}{\text{N}}\times 100 \qquad \text{...Equation 4}$$

Where N maximum germination in +ve control and X maximum germination in the treatment

The post-emergence damping off was assessed from the day of germination to the 28th day based on characteristic symptoms of damping off infection (stem Girdling). The percentage post-emergence damping was the calculated using the following formula:

$$\text{Percentage damping off}=\frac{\text{X}-\text{n}}{\text{X}}\times 100 \qquad \text{...Equation 5}$$

Where X is the number of germinated seedlings in a given treatment and n number of seedling with damping off symptoms and/or succumbed to damping off.

3.5 Effect of the interaction between fertilizer and seedlings pre-coated with *Bacillus subtilis* and *Trichoderma asperellum* on growth

The experiment consisted of six treatments divided into two groups. The first group consisting of two treatments of coated seeds (one with *B. subtilis* the other *T. asperellum*) prepared by as described in 3.4.3 and 3rd consisted of non-coated seeds was grown with fertilizer application. While the second groups with similar treatment as the first was grown without fertilizer application. All the treatments were replicated three times and laid out in a completely randomised design.

Plastic seedling plug trays were filled with coco peat and two tomato seeds were sown per plug. After germination, the seedlings in the first treatment groups were drenched with 200 ml /tray of NPK fertilizer (400 ppm) for a period of three days interspersed by a day of watering with no fertilizers. The treatment in the second group was watered daily with no fertilizer application for the experimental period of 28 days. On the 28th day a sample of 30 seedlings was obtained from each treatment and washed in running tap water for 15 minutes to remove any coco peat residues. The seedlings were then wrapped in old newspapers and oven dried (EYELA windy oven, Tokyo) at 70^0 C for 48hrs. The weight of the dried seedlings was assessed using weighing balance (Traveller TA 302, d=0.001g, OHAUS CORPORATION, USA).

3.6 Data analysis

The sensitivity of the pathogen and the BCA to the two synthetic pesticides was determined by calculating the IC_{50} values. The equation of the curve of percentage inhibition against $\log_{10}$ of concentration was used to calculate the IC_{50} values. The % inhibition of the pathogen by the BCA was calculated based on the equation 2 and the means of the % growth inhibition for the different treatments compared by ANOVA test at p=0.05. Where significance difference was established between the means, Tukey test was used to identify which means differ. The statistical tool used was the SPSS version 20.0.

Storability data (number of colony forming units (CFU) g^{-1} of each treatment) were log transformed before subjecting to analysis of variance (ANOVA) then Tukey test

(P = 0.05) was used to compare the means. The log transformed means of colony forming units (CFU) g^{-1} of each treatment was then plotted against time.

The data on the efficacy of the BCA against *P. aphanidermatum* in the greenhouse were analysed by transforming the percentage seedling mortality data by arcsine square root transformation. Then Lervene test was performed to test for normality before subjecting to generalised linear models procedure of SAS. Factor interaction was assessed and whenever there was a significant factor interaction, analysis was performed for the interacting factor at each level of the other factor. Means were compared using the Tukey test. All tests were performed at 5% level of significance.

CHAPTER FOUR

RESULTS

4.1 *In vitro* efficacy studies

The results from the three sets of in vitro experiments are presented in sections below

4.1.1 *In vitro* inhibition of *Pythium aphanidermatum* by *Bacillus subtilis* and *Trichoderma asperellum* isolates in pesticides free media

Bacillus subtilis and *T. asperellum* significantly inhibited the growth of *P. aphanidermatum* compared to the controls (P≤0.05). The pathogen percentage inhibition was significant throughout the experimental period. *Bacillus subtilis* and *T. asperellum* inhibited the pathogen to the same extent (68% and 69% respectively at the 120th hour) (Table 4.1 and plate 4.3).

Table 4.1: Inhibition of Pythium aphanidermatum radial growth by Bacillus subtilis and Trichoderma asperellum.

Treatment	Growth radius (cm)			Percentage inhibition (P.I)		
	24 hrs	72hrs	120hrs	24 hrs	72hrs	120hrs
Control	2.94a	3.80a	4.43a	0	0	0
B. subtilis	1.03b	1.27b	1.38b	64.07a	67.64a	69.53a
T. asperellum	0.90b	1.23b	1.35b	69.39b	66.58b	68.85a

In table 4.1 Radius refer to the growth radius of *P. aphanidermatum* in the different treatments; P.I refer to the percentage inhibition of *P. aphanidermatum* in the different treatments. The values with different letters are significantly different at $p<0.05$

4.1 Effect of synthetic pesticides on the growth of *Trichoderma asperellum, Bacillus subtilis* and *Pythium aphanidermatum*

Propined/cymoxanil inhibited the growth of the three organisms to a varying extent. *Trichoderma asperellum* and *P. aphanidermatum* were inhibited to the same extent throughout all the fungicide concentration levels. Propined/cymoxanil however inhibited the growth *B. subtilis* to a smaller extent throughout all the concentration levels at $P \leq 0.05$. (Figure 4.1 and plate 4.1).

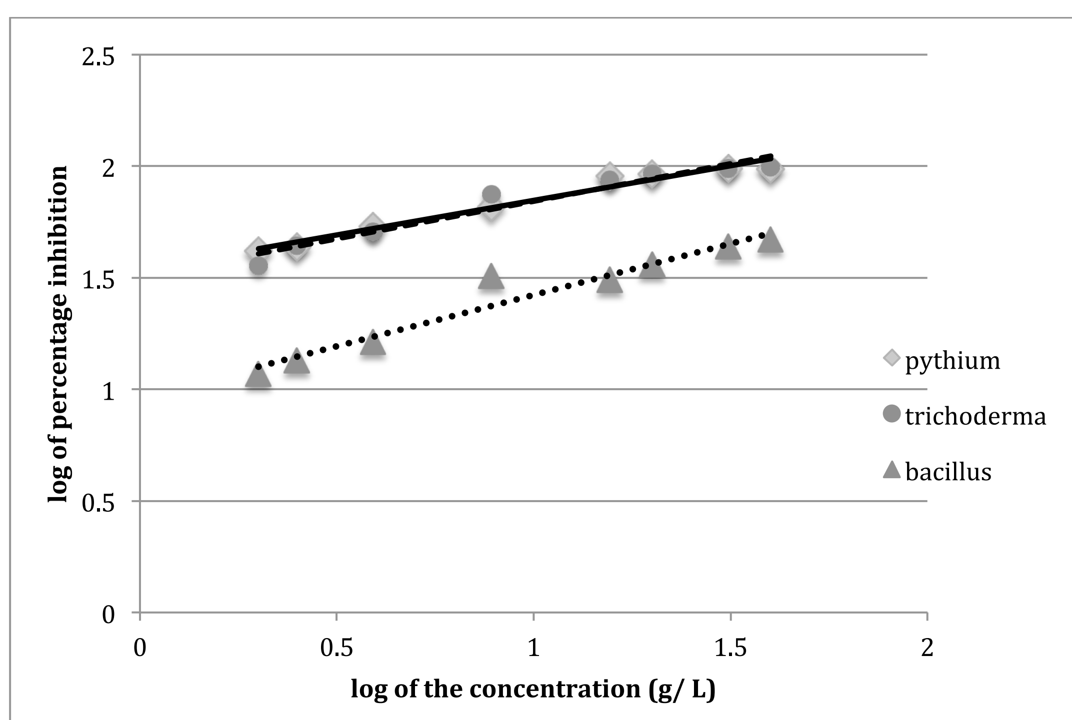

Figure 4.3: Inhibition of *Pythium aphanidermatum*, *Bacillus subtilis* and *Trichoderma asperellum* growth by cymoxanil/Propineb.

Increase in the concentration of the Mefenoxam in the growth media resulted in increased growth inhibition across all the treatments (figure 4.2 and plate 4.2). A significantly lower growth inhibition, at $p=0.05$, was observed with *B. subtilis* compared to *T. asperellum* and *P. aphanidermatum* across all the concentration.

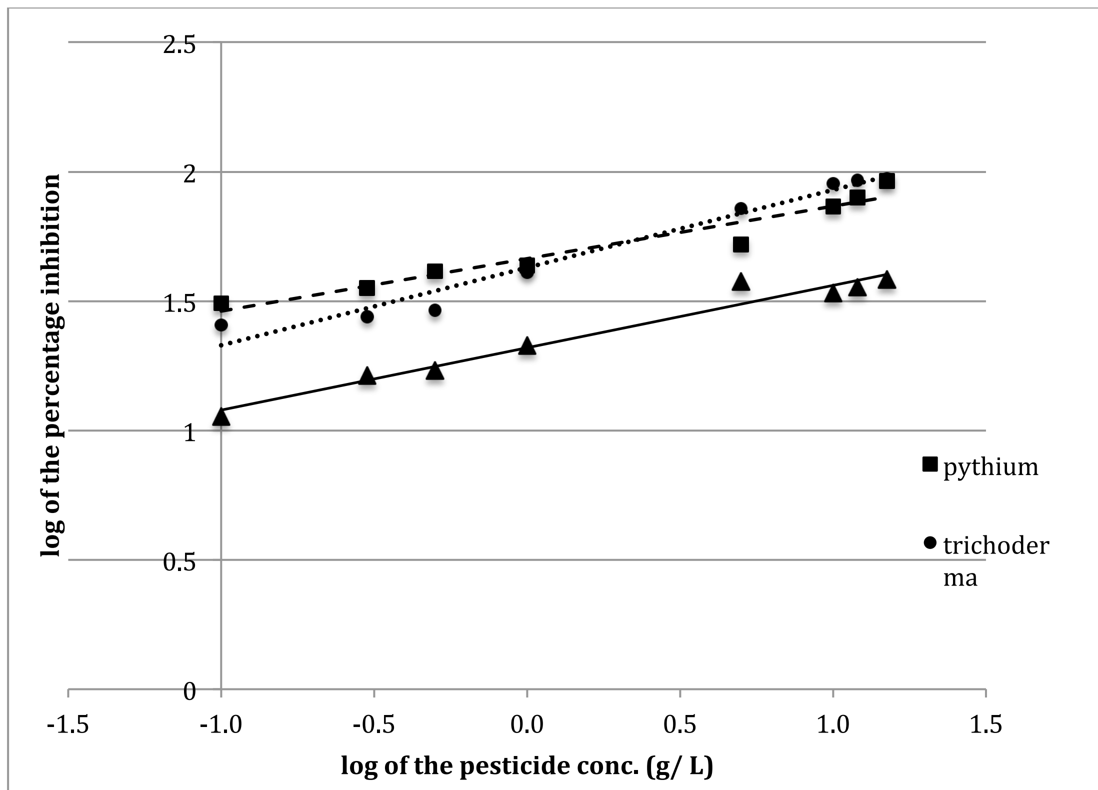

Figure 4.2: Inhibition of Pythium aphanidermatum, Bacillus subtilis and Trichoderma asperellum growth by Mefenoxam.

The inhibitory concentration (IC_{50}) of mefenoxam and cymoxanil/ propined on the BCAs and *P. aphanidermatum* growth varied greatly and was calculated from the generated equations (De Rossi *et al.*, 2015). The results of this study show that *B. subtilis* has low sensitivity to both mefenoxam and Cymoxanil/ Propined. *T. asperellum* and *P. aphanidermatum* has the same level of sensitivity to both fungicides with both organism being moderately sensitive to mefenoxam and Cymoxanil/ Propined fungicides.

Table 4.2: The IC_{50} for the growth inhibition of the biological control agents and *Pythium aphanidermatum* by the pesticides

Fungicide/conc. range (g/ L)	Biological control agent	Equation*	IC_{50} (g/ L)**
Mefenoxam Range: 0.1-15	*T. asperellum*	y =0.2027x +1.664	1.49
	B. subtilis	y =0.3001x +1.629	37.47
	P. aphanidermatum	y =0.2410x +1.320	1.71
Cymoxanil/Propineb Range: 2-40	*T. asperellum*	y =0.3365x +1.506	3.75
	B. subtilis	y =0.459x + 0.963	39.93
	P. aphanidermatum	y =0.31lx +1.5366	3.34

*Y= percentage inhibition, x= $\log_{10}$(pesticide conc.)

** Concentration calculated using the equation

4.1.3 Effect of synthetic pesticides on antagonistic ability of *Trichoderma asperellum* and *Bacillus subtilis* against *Pythium aphanidermatum*

The ability of *T. asperellum and B. subtilis* to inhibit the growth of *P. aphanidermatum* in the growth media was lowered by the presence of Propined/cymoxanil and mefenoxam pesticides (Tables 4.2 and 4.3). The inhibitory ability of *T. asperellum* and *B. subtilis* in non- amended growth media was higher at P= 0.05 (62.5% and 61.6% respectively) than in the pesticide-amended media. The reduction in the inhibitory ability of the assessed BCAs varied with the concentration of the pesticide used and the BCA. A significantly (P=0.05) higher percentage inhibition of *P. aphanidermatum* by *T. asperellum* was observed in the media amended with 3.9 gm/l of propineb/cymoxanil (11.3%) compared to the media amended with 2 gm/l and 15.6 gm/l of the same pesticide (Tables 4.3 and plate 4.5). The inhibition of *P. aphanidermatum* growth by *B. subtilis* however reduced

gradually with increase in propined/cymoxanil concentration in the media (Tables 4.4 and plate 4.4).

Table 4.3: Inhibition of Pythium aphanidermatum by Trichoderma asperellum and Bacillus subtilis in propined/cymoxanil-amended media

Treatment	% Inhibition in media amended with propineb/cymoxanil at conc. of			
	0 gm/ L	2 gm/ L	3.9 gm/ L	15.6 gm/ L
B. subtilis	61.6±2.01[a]	59.4±1.62[a]	43.6±2.69[b]	32.4±2.34[c]
T. asperellum	62.5±1.4[a]	4.4±1.05[c]	11.3±2.16[b]	3.7±0.43[c]

In table 4.3 the values with similar letter (a or b) within the rows indicate no significant difference at $p<0.05$.

Table 4.4: Inhibition of Pythium aphanidermatum by Trichoderma asperellum and Bacillus subtilis in mefenoxam-amended media

Treatment	% Inhibition in media amended with mefenoxam at conc. of			
	0 gm/ L	0.3 gm/ L	0.5 gm/ L	1 gm/ L
B. subtilis	61.6 ±2.01[a]	51±1.35[c]	57±1.69[b]	60.1 ±1.63[a]
T. asperellum	62.5±1.40[a]	9.7±1.58[b]	5.7b±2.84[c]	6.7b±2.42[bc]

In table 4.4 the values with similar letter (a or b) within the rows indicate no significant difference at $p<0.05$.

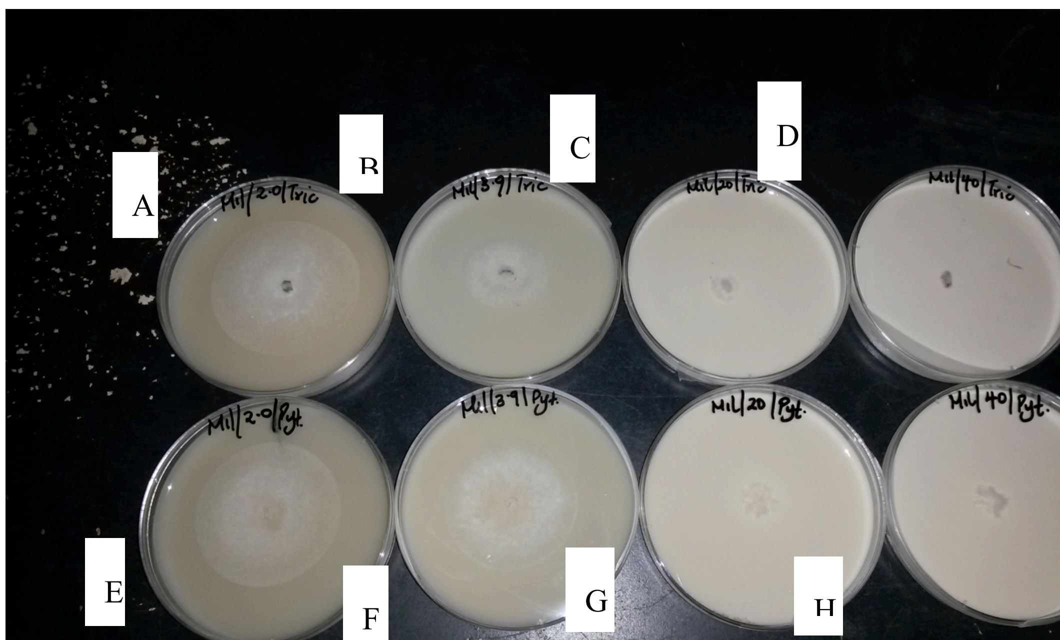

Plate 4.1: The growth *Pythium aphanidermatum* and *Trichoderma asperellum* in different conc. of propined/cymoxanil (2.0,3.9, 20 and 40 gm/l)

The key below explains the labels used in the image 4.1:

A: Growth *T. asperellum* in media amended with 2.0 g/l of cymoxanil/Propineb.

B: Growth *T. asperellum* in media amended with 3.9 g/l of cymoxanil/Propineb.

C: Growth *T. asperellum* in media amended with 20 g/l of cymoxanil/Propineb.

D: Growth *T. asperellum* in media amended with 40 g/l of cymoxanil/Propineb.

E: Growth *P. aphanidermatum* in media amended with 2.0 g/l of cymoxanil/Propineb.

F: Growth *P. aphanidermatum* in media amended with 3.9 g/l of cymoxanil/Propineb.

G: Growth *P. aphanidermatum* in media amended with 20 g/l of cymoxanil/Propineb.

H: Growth *P. aphanidermatum* in media amended with 40 g/l of cymoxanil/Propineb.

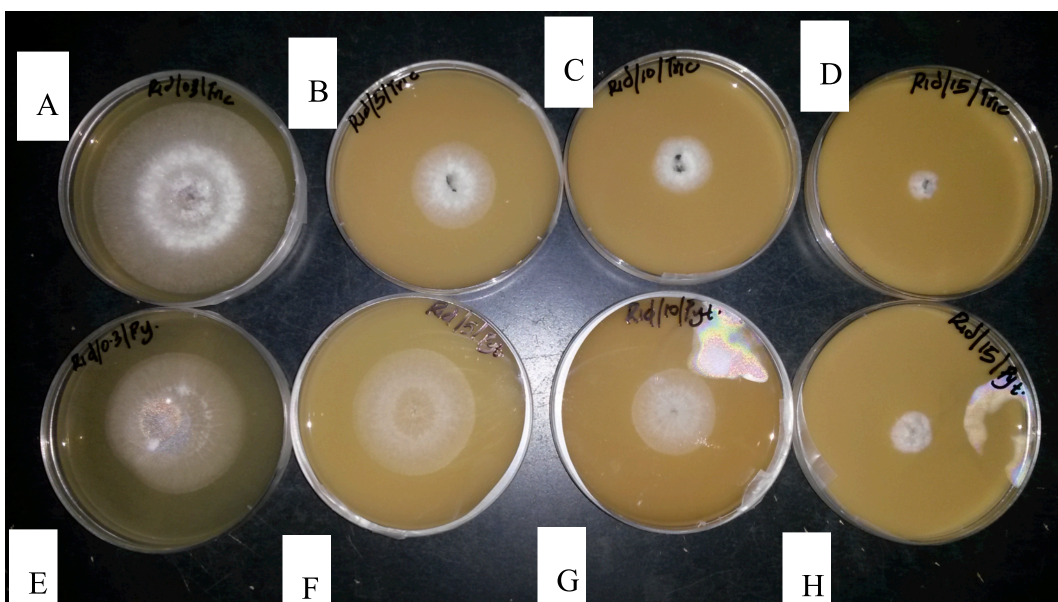

Plate 4.2: The growth *Pythium aphanidermatum* and *Trichoderma. asperellum* in different conc. of mefenoxam (0.3, 5, 10 and 15 gm/l)

The labels used image 4.2 are explained below:

A: Growth *T. asperellum* in media amended with 0.3 g/l of Mefenoxam.

B: Growth *T. asperellum* in media amended with 5 g/l of Mefenoxam.

C: Growth *T. asperellum* in media amended with 10 g/l of Mefenoxam.

D: Growth *T. asperellum* in media amended with 15 g/l of Mefenoxam.

E: Growth *P. aphanidermatum* in media amended with 0.3 g/l of Mefenoxam.

F: Growth *P. aphanidermatum* in media amended with 5 g/l of Mefenoxam.

G: Growth *P. aphanidermatum* in media amended with 10 g/l of Mefenoxam.

H: Growth *P. aphanidermatum* in media amended with 15 g/l of Mefenoxam.

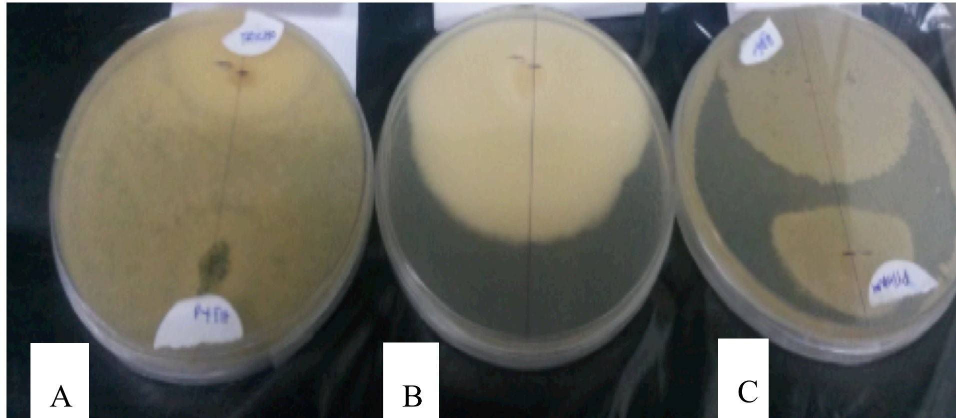

Image 4.3: Inhibition of *Pythium aphanidermatum* by *Trichoderma asperellum* and *Bacillus subtilis* in growth media not amended with any

The key below explains the labels used in the image 4.3:

A: Growth of *P. aphanidermatum* in presence of *T. asperellum*

B: Growth of *P. aphanidermatum* in the control plate

C: Growth of *P. aphanidermatum* in presence of *B. subtilis*.

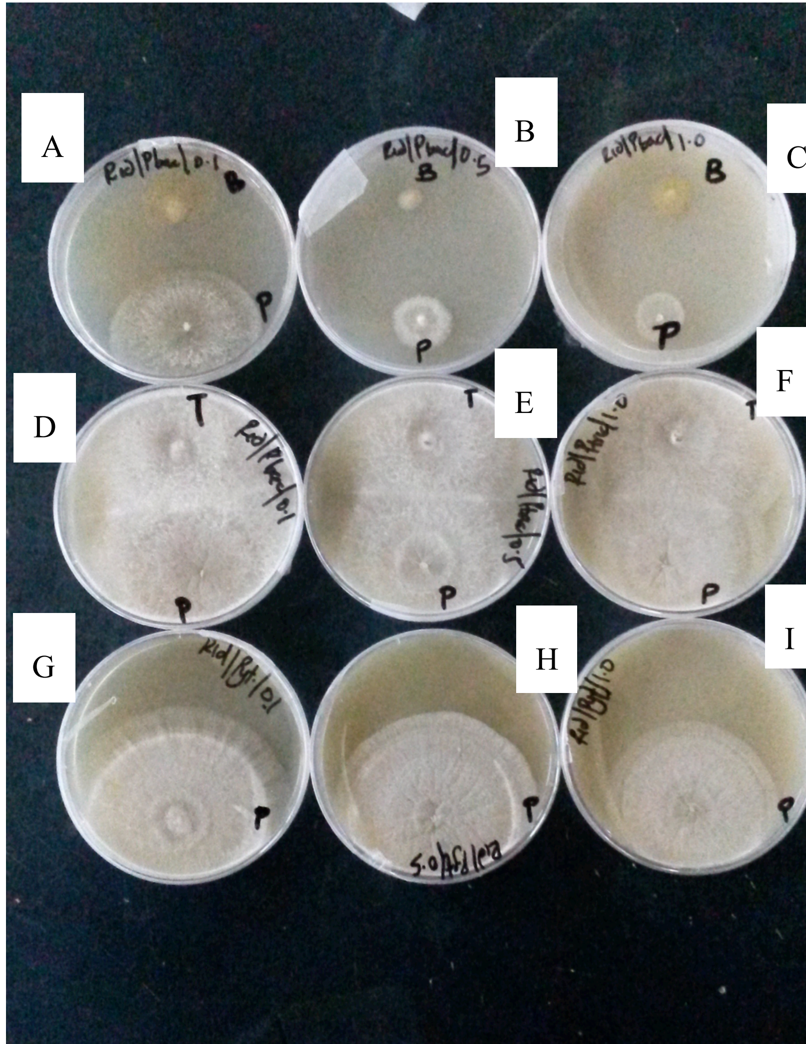

Plate 4.4: The picture shows the effect of different conc. Mefenoxam (0.1, 0,5 and 1 gm/l) on the antagonism of *Pythium aphanidermatum* (P) by *Trichoderma asperellum* (T) and *Bacillus subtilis* (B).

The key below explains the labels used in the image 4.4:

A: Inhibition of *P. aphanidermatum* growth by *B. subtilis* in media amended with mefenoxam at 0.1 g/L.

B: Inhibition of *P. aphanidermatum* growth by *B. subtilis* in media amended with mefenoxam at 0.5 g/L.

C: Inhibition of *P. aphanidermatum* growth by *B. subtilis* in media amended with mefenoxam at 1 g/L.

D: Inhibition of *P. aphanidermatum* growth by *T. asperellum* in media amended with mefenoxam at 0.1 g/L.

E: Inhibition of *P. aphanidermatum* growth by *T. asperellum* in media amended with mefenoxam at 0.5 g/L.

F: Inhibition of *P. aphanidermatum* growth by *T. asperellum* in media amended with mefenoxam at 1 g/L.

G: Growth of *P. aphanidermatum* in media amended with mefenoxam at 0.1 g/L.

H: Growth of *P. aphanidermatum* in media amended with mefenoxam at 0.5 g/L.

I: Growth of *P. aphanidermatum* in media amended with mefenoxam at 1 g/L.

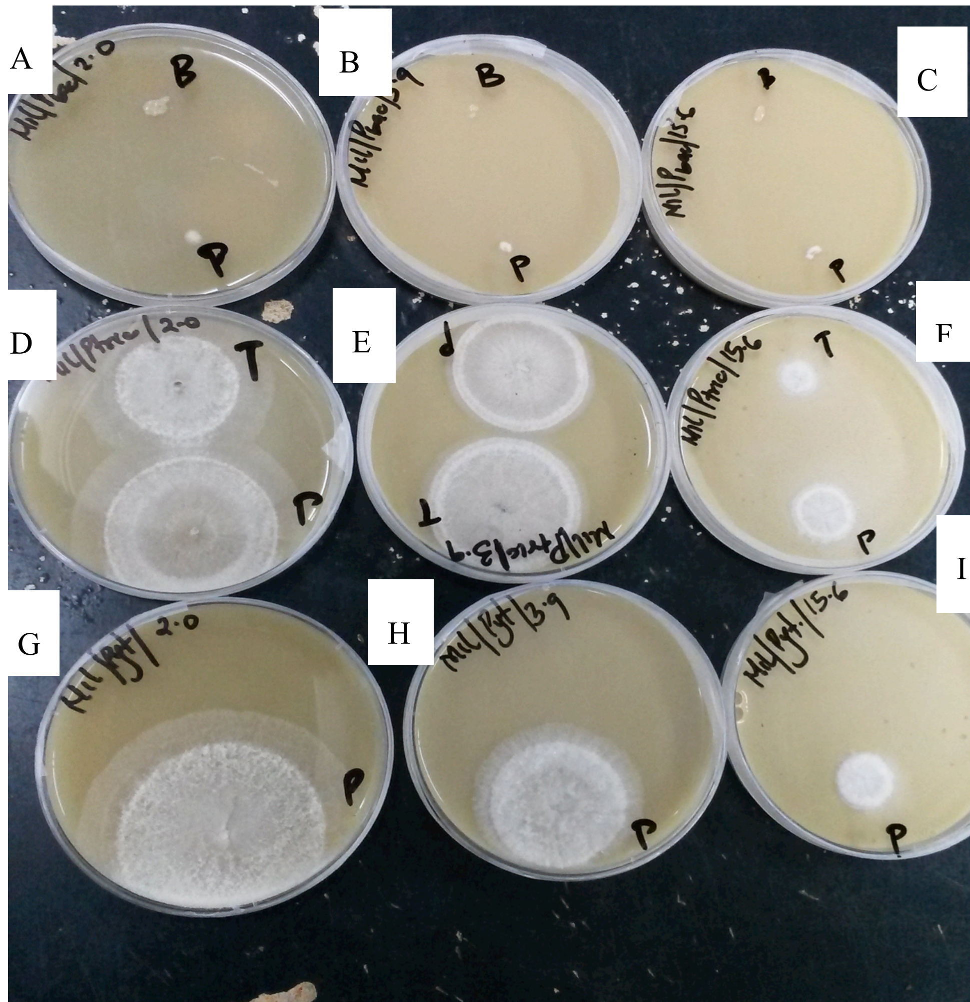

Plate 4.5: The picture shows the effect different conc. Propined/cymoxanil (2.0, 3.9, 15.6 gm/l) antagonism of *Pythium aphanidermatum* (P) by *Trichoderma asperellum* (T) and *Bacillus subtilis* (B).

The key below explains the labels used in the image 4.5:

A: Inhibition of *P. aphanidermatum* growth by *B. subtilis* in media amended with cymoxanil/Propineb at 2.0 g/L.

B: Inhibition of *P. aphanidermatum* growth by *B. subtilis* in media amended with cymoxanil/Propineb at 3.9 g/L.

C: Inhibition of *P. aphanidermatum* growth by *B. subtilis* in media amended with cymoxanil/Propineb at 15.6 g/L.

D: Inhibition of *P. aphanidermatum* growth by *T. asperellum* in media amended with cymoxanil/Propineb at 2.0 g/L.

E: Inhibition of *P. aphanidermatum* growth by *T. asperellum* in media amended with cymoxanil/Propineb at 3.9 g/L.

F: Inhibition of *P. aphanidermatum* growth by *T. asperellum* in media amended with cymoxanil/Propineb at 15.6 g/L.

G: Growth of *P. aphanidermatum* in media amended with cymoxanil/Propineb at 2.0 g/L.

H: Growth of *P. aphanidermatum* in media amended with cymoxanil/Propineb at 3.9 g/L.

I: Growth of *P. aphanidermatum* in media amended with cymoxanil/Propineb at 15.6 g/L.

4.2 Storability of *Bacillus subtilis* and *Trichoderma asperellum* isolates coated on tomato seeds

Reduction in the concentration of *B. subtilis* and *T. asperellum* was observed within a week of storage. The rate of *B. subtilis* and *T. asperellum* reduction varied with the BCA coating concentration used. A steep reduction of over 90% was observed in 2 weeks for seeds coated with 10^{13} CFU/ ml. A gradual reduction in the BCA concentration was recorded at 10^{9} CFU/ ml coating concentration (*T. asperellum;* 60.4%, *B. subtilis*; 36% in two weeks). The slowest reduction in the concentration was however observed at 10^{6} CFU/ ml coating concentration (*T. asperellum;* 10.9%, *B. subtilis*; 26.7% in two weeks). The percentage concentration of the *B. subtilis* at any given week differed significantly depending on the coating concentration. Significant difference (P=0.05) in percentage concentration after a given time was also observed among the *T. asperellum* coating concentrations (figure 4.3).

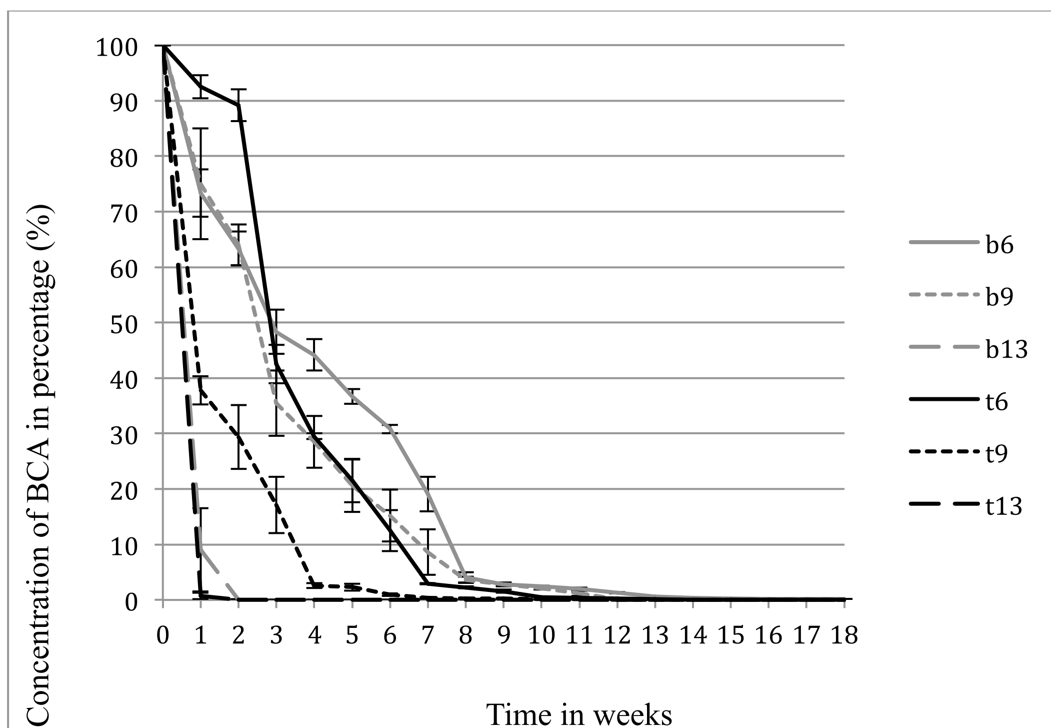

Figure 4.3: Percentage change in *Bacillus subtilis* and *Trichoderma asperellum* concentration (cfu/ml) with time on coated tomato seeds. The values and standard errors are means of 4 plates replicated 3 times pooled over 3 experiments. In the figure legend b stands for *B. subtilis* while t stands for *T. asperellum*. The number after each indicates the concentration for example b6 stands *B. subtilis* of 10^6 CFU/ ml concentrations.

4.3 Control of *Pythium aphanidermatum* damping off of tomato using *Bacillus subtilis* and *Trichoderma asperellum* coated seeds

Coating seeds with either *B. subtilis* or *T. asperellum* in *P. aphanidermatum* inoculated media resulted in a significant reduction in the percentage pre-emergence damping off compared to the treatment with uncoated seeds. The difference in the pre-emergence damping off between the seedlings coated with *B. subtilis* and *T. asperellum* was not significant (Table 4.5).

Pythium aphanidermatum symptoms were observed between the 14th and 25th day after sowing (plate 4.6 and 4.7). Significantly high percentage of post-emergence damping-off was recorded in the uncoated seedlings growing in *P. aphanidermatum* inoculated media compared to the coated seedlings ($P \leq 0.05$). The seedlings coated

with *B. subtilis* had significantly lower post-emergence damping off infection compared to those coated with *T. asperellum* with 15.3%.

Table 4.5: Efficacy of *Bacillus subtilis* and *Trichoderma asperellum* on seedling damping off caused by *Pythium aphanidermatum*.

Treatment	% Pre-emergence	% Post-emergence
B. subtilis	22.81 ±2.16[b]	10.87, ±0.013[c]
-ve control	37.12 ±1.52[a]	63.9, ±1.25[c]
T. asperellum	21.04 ±2.01[b]	15.3, ±0.02[b]

Same letter in each column indicate no significance difference in the values. The significance level is at the 0.05. % Pre-emergence refers to the % of seedlings that succumbed to damping off before emergence from the coco peat. % Post-emergence refers to the % of seedling that succumbed to damping off after germination above the coco peat surface

Plate 4.6: The picture showing high damping off disease incidence in the control treatment compared to the *Bacillus subtilis* and *Trichoderma asperellum* treatments.

Key for plate 4.6

A: *B. subtilis* treatment with no damping off.

B: Control treatment with damping off diseased seedlings.

C: *T. asperellum* treatment with no damping off.

Plate 4.7: The picture showing the girdling (➡) of the stem of the seedling not coated with any Biological control agent (control treatment seedling). *Trichoderma asperellum* and *Bacillus subtilis* coated seedling have no girdling of the stem

Key for plate 4.7

A: Control treatment seedling with damping off diseased symptoms.

B: Damping off disease free seedling of *T. asperellum* treatment.

C: Damping off disease free seedling of *B. subtilis* treatment.

4.4 Effect of the interaction between fertilizer and the biological control agents on tomato seedling growth

Seedlings coated with either of the bio control agent and fertilized regularly had a significantly higher dry mass by the 28th day after sowing compared to fertilized seedlings but non-coated seedlings (table 4.6). A significant difference was also observed between the dry masses of coated seedlings grown with fertilizers with *B. subtilis* dry mass higher than *T. asperellum* (P=0.001). No significant difference was

recorded between the dry masses of coated and uncoated seedlings grown without fertilizers.

Table 4.6: Effect of coating tomato seeds with *Trichoderma asperellum* and *Bacillus subtilis* on total dry mass of tomato seedlings

Treatment	Dry Mass (g)	
	With fertilizer	Without fertilizers
B. subtilis	2.32a ±0.019	0.10a ±0.006
T. asperellum	1.77b ±0.013	0.08a±0.008
Non-coated	1.63c ±0.006	0.08a ±0.011

Same letter in each column indicate no significance difference in the values. The significance level is at the P=0.05

CHAPTER FIVE

DISCUSSION

5.1 *In vitro* efficacy of *Bacillus subtilis* and *Trichoderma asperellum* isolates against *Pythium aphanidermatum*

The study shows that *B. subtilis* 01 and *T. asperellum* 900 have the ability to inhibit *P. aphanidermatum* growth *in vitro*. The growth inhibitory effects of the two BCAs on the pathogen were not significantly different ($p<0.05$). The level of pathogen growth inhibition by *B. subtilis* and *T. asperellum* has been shown to be significantly higher (68% and 69% respectively) than the control. Similar findings on the antagonism of fungal plant pathogens by *Trichoderma* spp. have been reported (Howell, 2003; Benítez et al., 2004; Zivkovic et al., 2010). *Bacillus subtilis* have also been shown to reduce the growth of *Pythium* spp. in onions seedlings (Monte, 2001; Korsten, 1993). However presence of synthetic pesticides interferes with the growth of the BCAs hence their ability to inhibit the growth of *P. aphanidermatum* (Lucas et al., 2004; Paine et al., 2011).

The sensitivity of *B. subtilis, T. asperellum* and *P. aphanidermatum* to mefenoxam and cymoxanil /propined was shown to increase with fungicide concentration. It was however established that *T. asperellum* and *P. aphanidermatum* has moderately higher sensitivity to the fungicides compared to the *B. subtilis,* which was established to have lower sensitivity to the two fungicides. These results therefore suggest that the presence of mefenoxam at concentration of (1.49 g/L) will inhibit 50% of *T. asperellum* growth, which is slightly lower than concentration that cause the same result in *P. aphanidermatum* (1.71 g/L). The concentration that causes 50% inhibition of *B. subtilis* growth was however observed to be high (37.47 g/L) compared to the other two concentrations. This therefore means that the concentration of mefenoxam that can efficiently control *P. aphanidermatum* may have detrimental effects on the growth of *T. asperellum,* however *B. subtilis* can survive the concentration. The similar effect of cymoxanil/propined on the BCAs was observed but for this fungicide

P. aphanidermatum is more sensitive (3.75 g/L) to the pesticide compared to *T. asperellum* (3.34 g/L).

The observed compatibility of *B. subtilis* with the two pesticides is potential for consideration in integrated pest management of *P. aphanidermatum* (Stark et al., 2004). In this study it was also shown that *B. subtilis* was able to retain its ability to inhibit *P. aphanidermatum* growth even in presence of the synthetic pesticides, however the ability was slightly lowered (0.3 g/L of mefenoxam: 51%) compared to its inhibitory ability in pesticide free media (61.6%). *Trichoderma asperellum in vitro* ability to inhibit the pathogen was however greatly reduced by the presence of the two pesticides (0.3 g/L of mefenoxam: 9.7%; 2 g/L of cymoxanil/propined: 4.4%) compared to inhibition in pesticide free media (62.5%). The results of the *in vitro* study of efficacy of the BCA against the *P. aphanidermatum* obtained from this study indicates that both *B. subtilis* and *T. asperellum* have the ability to reduce the growth of *P. aphanidermatum* however the presence of synthetic pesticides (mefenoxam and cymoxanil/propined) reduces this efficacy. The efficacy of *T. asperellum* is greatly reduced by the pesticides presence but *B. subtilis* is less sensitive to the pesticide hence retains most of its efficacy.

5.2 Storability of *Bacillus subtilis* and *Trichoderma asperellum* isolates coated on tomato seeds

The results of the study shows that the concentration of *B. subtilis* and *T. asperellum* isolates used as seed coats declined from the first week of storage. However the reduction in the BCA count varied with the coating concentration. Dressing of seeds with 10^{13}cfu/ml of BCA resulted in a rapid reduction of biocontrol concentration (*B. subtilis;* 99.93% and *T. asperellum*; 99.97% in 2 weeks) compared to the BCA coating concentration of 10^{6} CFU/ ml and 10^{9} CFU/ ml. The observed BCA reduction trends resulted in variation in the time taken by the BCA applied to the seed to decline to the lowest effective concentration of 10^{4} CFU/ ml (Harman *et al.*, 1980b). The rapid reduction of the concentration of the *B. subtilis* and *T. asperellum* could be attributed to increased water activity due to wet coating (Hong et al., 2005). The increased water activity leads to higher physiological activity of the BCA, which in turn may reduce the shelf life of the spores (Connick et al., 1996). Previous studies

reported a similar trend in the storability of the BCAs used as seed dress (Hong et al., 2005; Friesen et al., 2006).

It is therefore important to time application of the BCA on the seeds to coincide with the time of seed coating so as counter the short shelflife. *Bacillus subtilis* and *T. asperellum* applied onto the seed surface at 10^6 CFU/ ml should be utilized before the 7th week after application.

5.3 Control of *Pythium aphanidermatum* damping off of tomato using *Bacillus subtilis* and *Trichoderma asperellum* coated seeds

The significantly ($P \leq 0.05$) lower percentage pre-emergence damping-off of the seeds coated with either *B. subtilis* or *T. asperellum* under high disease pressure suggests that coating of seeds with *B. subtilis* or *T. asperellum* is effective against *P. aphanidermatum* damping-off. The results of this study corroborates with those obtained by Khare and Upadhyay (2009) in which 72.0 % of seedlings coated with *T. viride* 1433 were not affected by pre-emergence damping-off. An application of *T. harzianum* and *T. koningii* conidia to pea seeds reduced the incidence of pre-emergence damping-off by 50% and 66.7% respectively (Lifshitz *et al*., 1986). Several studies have also documented the efficacy of seed coating in reducing cases of post-emergence damping-off (Adekunle, 2006; Abdelzaher, 2004; Berger et al., 1996; Mukhopadhyay et al., 1986, Harman et al., 1980b).

The observations from the present study indicate that seeds coated with *B. subtilis* have significantly ($P \leq 0.05$) fewer cases of post-emergence damping-off compared to those coated with *T. asperellum.* This suggests a possibly higher efficacy of *B. subtilis* in controlling *P. aphanidermatum* on tomatoes when compared to *T. asperellum.* The variation could be due to the mechanism through which *B. subtilis* or *T. asperellum* antagonize the pathogen. The ability of *B. subtilis* to compete favourably with germinated *Pythium* oospores for soluble carbon and nitrogen sources from root exudates have been reported to greatly reduce the establishment of *Pythium* (Weller, 1988). *Bacillus subtilis* has also been shown to have the ability to produce antibiotics and other metabolites against *Pythium* (Haas & Défago, 2005; Paulitz & Elanger, 2001).

5.4 Effect of the interaction between fertilizer and the biological control agents on tomato seedling growth

The significantly (P=0.001) high dry mass of the seedling coated with *B. subtilis* or *T. asperellum* and grown with fertilizer compared to the non-coated grown with fertilizer indicates a positive interaction between fertilizer and coated seeds in promoting growth (p=0.001). This suggestion is furthered by the significantly low dry mass of coated seedlings grown without fertilizer. These observations indicate that a combination of seed coating and fertilizer application enhances the growth of the seedlings. Rhizospheric microorganisms have been reported to increase the uptake of nutrients by the plant and subsequent increase in plant growth (Douds et al., 2005). The arbuscular mycorhhizal fungi have been observed to increase the plant uptake of potassium, nitrogen and zinc leading to increased plant growth (George, 2000). The present data confirms the increase of plant growth in seedlings coated with biological control agent and grown with fertilizer as compared to non-coated seedlings grown under similar conditions

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The *in vitro* studies indicated the effectiveness of the BCA in slowing the growth of the pathogen. An decrease in growth of *P. aphanidermatum* by 68% and 69% achieved in *B. subtilis BS01* and *T. asperellum TRC900* plates compared to the control is an indication of the their effectiveness. However, the presence of synthetic pesticides was suggested to potentially influence the growth and efficacy of the BCAs.

The study also demonstrated that *B. subtilis* isolates BS01 and *T. asperellum* isolates T900 applied onto the seed and stored at 25 °C should be used before the 7th week. The BCA applied onto wet seeds with a concentration of 10^6 CFU/ ml should not be stored beyond 7 weeks. However this still depends on the initial coating concentration.The study demonstrated that the applications of *B. subtilis* isolates BS01 and *T. asperellum* isolates T900 as a seed dress in tomatoes can significantly ($P \leq 0.05$) suppress seedling damping off caused by *P. aphanidermatum*.

Finally, the study demonstrated that the use of BCAs was demonstrated to result to an increase in the growth of the tomato seedlings. Seedling growth in *B. subtilis* coated seeds was significantly higher ($P \leq 0.05$) with total dry mass of 2.32 g recorded compared to mass of non-treated ones (1.63g).

6.2 Recommendations

This study recommends that:

I. Coating of tomato seeds with *B. subtilis* BS01 and *T. asperellum* T900 at 10^6 CFU/ml be considered for prevention of damping off.

II. Seeds coated with *B. subtilis* and *T. asperellum* at a concentration of 10^6 CFU/ml should be utilized before the 7th week of storage.

III. Further research to be done on the interaction of fertilizer with *B. subtilis* and *T. asperellum* in plant growth promotion and on techniques to increase the shelf life of *B. subtilis* and *T. asperellum* used as seed coating.

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