FORMULATION OF *Trichoderma harzianum* AND ITS COMPARATIVE STORAGE STABILITY IN DIFFERENT SUBSTRATES FOR THE MANAGEMENT OF ARMILLARIA ROOT ROT OF TEA

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DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted or presented for examination in any institution.

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RECOMMENDATION

This thesis has been submitted with our approval as supervisors according to Egerton University regulations

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DEDICATION

To my mother Mary Mutai, daughter Immanuella Chemutai Rono and husband Dominic Rono for their love and unconditional support.

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ABSTRACT

Armillaria root rot has been documented as one of the major diseases of tea (*Camellia sinensis*) that hinders successful establishment of tea when planted shortly after deforestation. Effective methods for managing the disease have not been fully developed. *Trichoderma harzianum*, a soil-borne fungus, has been demonstrated to be an effective antagonist to several species of *Armillaria*. In this study, formulation, stability in storage and viability of *T. harzianum* in selected substrates was evaluated. *Trichoderma harzianum* isolate (T_4) was multiplied by liquid fermentation and formulated in vermiculite, kaolin, wheat bran and talc substrates. Inocula of *T. harzianum* were prepared on potato dextrose agar and potato dextrose broth, and assessed for conidial yields over different time periods. The different substrates were then analyzed for compatibility with the fungal propagules by comparing dry weight, number of colony forming units and microscopic counts after one month of incubation. Stability of the formulations in storage was determined by assaying for viability of the inoculum; number of colony forming units, sporulation rates and microscopic counts every month. Effect of vegetative interaction between *Armillaria* isolate ($8KA_1$) and *T. harzianum* isolated from the different formulations on the pathogen was also evaluated. Data on differences in the tested parameters was subjected to ANOVA with the help of SAS statistical software and the means separated using LSD (P=0.05). There was a positive correlation between conidial yield and incubation period in both PDA (R^2=0.9274) and potato dextrose broth medium (R^2=0.8954). Compatibility tests revealed that all the substrates supported multiplication and viability of *T. harzianum* at levels over 10^4 CFU/g and were thus considered to be compatible with the fungus. Most formulations retained viable propagules above 10^6 CFU/g by the sixth month of storage. Kaolin formulations had the highest mean counts (6×10^6 CFU/g) followed by vermiculite (4.2×10^6 CFU/g), talc (2.1×10^6 CFU/g) and wheat bran (3.4×10^5 CFU/g). Mycelial formulations were more stable compared to conidio-mycelial and spore formulations. Vegetative interaction between *Armillaria* and *T. harzianum* revealed that *T. harzianum* taken from all the formulations antagonized *Armillaria* regardless of the period of storage. Attempts to re-isolate *Armillaria* after 20 days of interaction did not yield any viable pathogen, indicating stability of the antagonist in storage. Results from this study will enable formulation of *T. harzianum* based product whose use can be easily incorporated as a husbandry practice to manage *Armillaria* root rot in tea.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
BCA	Biological control agent
CFU	Colony forming units
DNA	Deoxyribonucleic acid
FYM	Farm yard manure
LSD	Least significant difference
MEA	Malt extract agar
PDA	Potato dextrose agar
RFLP	Restriction Fragment Length Polymorphism
SAS	Statistical Analysis Software
SEM	Scanning electron microscopy
TF	Theaflavins
TR	Thearubigins
TRFK	Tea Research Foundation of Kenya
YT	Yeast and tryptone
DMRT	Duncan's multiple range test

CHAPTER ONE
INTRODUCTION

1.1 Background information

Kenya is a major tea (*Camellia sinensis*) producer with more than 110,000 hectares of land devoted to tea. The main tea growing area is in the Kenyan Highlands, west of the Rift Valley, at altitudes over 2600m (Etherington, 1973; Anon, 1990). The plant thrives under high and evenly distributed rainfall of ≥ 1700mm annually (Anon, 2002). Tea is a major foreign exchange earner and the main source of 17 to 20 percent of Kenya's total export revenue (Anon, 2011). Tea bushes are harvested throughout the year, with the best quality being produced from January to February and in July, during the drier periods of the year (Anon, 2002). Tea production is, however, limited by a number of factors that include pests and diseases (Wilson and Clifford, 1992). Species of *Armillaria* cause root rot that damage tea plants in various regions of the country. The disease has been documented as one of the major causes of damage in tea in Kenya (Onsando *et al.,* 1997) causing substantial losses especially in smallholder farms.

Species of *Armillaria* are soil borne phytopathogens referred by various names such as oak fungus, shoestring root rot, honey mushroom, and honey agaric. The latter two refer to the color of the fruiting structure of the fungus that can sometimes be seen at the base of infected trees. The species presently described in Kenya are *A. mellea* and *A. heimii* (Otieno *et al.,* 2003a). The predisposing factors to infection of woody plants by *Armillaria* species include: drought, flooding, poor drainage, frost, repeated defoliation by insects or foliar diseases, other poor soil conditions, excessive shade, polluted air, chemical or mechanical injuries (Goodchild, 1960). The loss of fine feeder roots due to the disease deprives affected plants of sufficient nutrients and water resulting in branch dieback. The fungus can be of considerable importance in the final death of weakened trees and shrubs. Serious radial and terminal growth reduction of affected plants may occur. *Armillaria* species are commonly found in most forest soils, remnants of stumps and roots of forest trees that harbour the fungus as active pathogen or quiescent lesions that serve as the primary sources of inoculum for the infection of tea (Goodchild, 1960; Munnecke *et al.,* 1981).

The management options for *Armillaria* in tea plantations include ring barking, removal of remnant roots to pencil thickness, plastic mulching of soil amended with fresh plant residues and infestation with *T. harzianum* (Masuka, 1993; Fox, 2003). Although control can be obtained

by removing *Armillaria*-infected stumps, additional methods appear to be necessary. Recent research has focused on the management of *Armillaria* root rot by soil solarisation in combination with organic amendments and biological control with *T. harzianum.* One study on the formulation of *T. harzianum* in different substrates showed that wheat bran was the best carrier material for *T. harzianum* in terms of spore count, growth rate and fungal weight (Anon, 2010). However, this carrier is too bulky, thus the need to test other substrates.

1.2 Statement of the Problem

Armillaria species can occur in roots of various trees as a saprophytic epiphyte and sometimes as a parasite. The fungus is of economic importance in several plant species in Kenya and is a major threat to tea establishment. It can cause crop losses as high as 50% in smallholder farms but unfortunately no appropriate methods have been developed to curb the effects of the disease. Uprooting of infected trees and subsequent soil solarization are the most effective methods but most farmers are not keen on adopting them due to high cost. Some strains of *T. harzianum* are well-known antagonists of *Armillaria* and can be used as bio-control agents (BCA) for *Armillaria* root rot. However, *T. harzianum* has not been formulated and commercialized successfully for use to control *Armillaria* root rot in tea. Mycelia and spores/conidia are the reproductive forms/propagules in which the majority of fungal species grow. These dry fungal propagules can be stored and easily applied for control of the disease, but they have low viability due to damage to cellular structures of the mycelia by drying and milling.

1.3 Objectives

1.3.1 General objective

To develop a formulation of *T. harzianum* with optimal cell viability and biological activity under prolonged conditions of storage for the management of *Armillaria* root rot in tea.

1.3.2 Specific objectives

1. To compare the compatibility of *T. harzianum* with different substrates
2. To determine the stability of *T. harzianum* in storage of different formulations
3. To evaluate effect of *T. harzianum* isolated from different formulations on *Armillaria* through vegetative interactions *in vitro*.

1.4 Hypotheses

1. There is no significant difference in compatibility of the *T. harzianum* formulations with different substrates
2. There is no significant difference in the stability in storage of the *T. harzianum* formulations.
3. There is no significant effect of *T. harzianum* isolated from different formulations on *Armillaria* through vegetative interactions *in vitro*.

1.5 Justification

Recent advances in biotechnology have resulted in significant increase in the use of microorganisms as biological agents in agriculture, forestry, and environmental management. A large number of fungi are known for their specific pathogenicity to phytopathogenic fungi and insects. Many of these fungi have been subjected to scientific studies and development for commercial use as biological control agents (BCAs). One advantage of BCAs over their chemical counterparts is that their active ingredients are non-hazardous to man or the environment. BCAs can, if formulated appropriately, occupy a unique position in the market, requiring no or negligible precautions for their use. Controlling *Armillaria* by physical and chemical methods alone is at present inadequate. Biological control either alone or in integration with other methods appears necessary. *Trichoderma harzianum* is reported to be effective in inhibiting colonization of plant materials by *Armillaria* species, an indication of potential as a biocontrol agent. For better performance, it is necessary to test different substrates for their compatibility with *T. harzianum* propagules without adverse effects on viability and biological activity under prolonged conditions of storage. For commercial use, there is also need for stable formulations that can be easily applied by farmers. Developing a formulation of *T. harzianum* effective against *Armillaria* species would enable the management of *Armillaria* root rot in tea to be easily applied in farmers' fields.

CHAPTER TWO
LITERATURE REVIEW

2.1 The Tea Plant

The tea plant, *Camellia sinensis* is an evergreen shrub of the *Camellia* family that is native to China, Tibet and northern India (Barclays, 1967; Anon 1990; Wilson and Clifford, 1992; Anon, 2002). It is one of about 80 species of East Asian evergreen shrubs and trees that belong to the tea family, Theaceae (Barclays, 1967). The tea plant is cultivated in all the continents; Africa, Asia, Australia, America and Europe. It grows, in particular, close to the equator at high altitudes, but can also be grown at low altitudes (Anon, 2002).

Tea was first planted in Kenya in 1903, but it was not until the early 1920s that planting on a commercial basis was undertaken (Etherington, 1973). The tea plant reaches a height of nine meters but is kept pruned to a low, mounded shrub in cultivation. The foliage is emerald green, while the flowers are fragrant, yellow-centered, white and about four centimeters wide. Tea leaves and leaf buds are used to produce the popular beverage tea. For millenia, tea was used as a medicinal beverage because it flavoured water and seemed to help prevent sickness (Porter, 1999). There are two main varieties of the tea plant. The small leaf variety, known as *Camellia sinensis*, thrives in the cool, high mountain regions of central China and Japan. The broad leaf variety, known as *Camellia assamica*, grows best in the moist, tropical climates found in Northeast India and the Szechuan and Yunnan provinces of China. Both varieties are grown in various tea growing regions of Kenya (Wilson and Clifford, 1992). There are four main types of tea: green tea, black tea, oolong tea and white tea. The specific variety of tea plant and the way the leaves are processed after harvesting determine the type of tea that is created (Anon, 1990). Depending on whether the leaves undergo fermentation or not, the tea is black or green and produces an orange to dark red and sometimes scented infusion in the case of black tea, and yellow insipid beverage for green tea.

2.1.1 Cultivation

Tea grows under a great variety of climatic conditions ranging from equatorial to humid, temperate climate. It thrives in moist, well-drained, slightly acidic soils, and prefers partial shade. Tea is grown in an expansive region of the world between latitudes ranging from the equator to 33°S in Natal South Africa and 49°N in Georgia (Etherington, 1973) and spanning a

range of altitudes from sea level in Bangladesh to over 2600m in Kenya. The plant thrives under high and evenly distributed rainfall. A suitable climate has a minimum annual rainfall varying from 1500mm (Uganda) to 3500mm (Java). On average, economic tea production requires ≥ 1700mm annual minimum rainfall which should not fall below 50mm per month for any prolonged period (Anon, 1990; Anon, 2002). Optimum temperature for shoot growth ranges from 18°C to 30°C. Above 30°C and below 12°C, growth of the tea plant is jeopardized. Sunshine is also an important factor and the tea plant requires an average of five hours of sunshine per day.

Tea grows on a wide range of soil types developed from diverse parent rock material under high rainfall conditions. Suitable soils should be free draining, have a depth of not less than 2m, pH between 4.5 and 5.6, a texture of sandy loam to clay and good water holding capacity. During the growing season, the plant is kept pruned to a short bush because only the young, tender leaves and buds are wanted for commercial processing into marketable tea (Wilson and Clifford, 1992). Tea can be propagated from softwood cuttings rooted under mist or from seeds. Seed propagation requires no pretreatment, and grafting selected scion wood onto large root stock speeds early growth and promotes early flowering of young plants.

2.1.2 Economic Value

Tea export earnings in Kenya rose by 40.5 % from Ksh 69 billion registered in 2009 to Ksh 97 billion in the year 2010 (Anon, 2011). The improved performance was attributed to a combination of factors including improved purchasing power and increased demand for the beverage in emerging markets such as China, Iran and Turkey. Egypt is the largest market for Kenyan tea; Pakistan, United Kingdom, Afghanistan and Sudan are other top markets. Good weather conditions and a weak shilling as well as improved export volumes also contributed to the overall earnings.

2.2 Diseases of tea

A number of diseases have been recorded in tea growing countries of Africa, but only a few are economically important. Most of the diseases are caused by fungi which infect various parts of the plant (Wilson and Clifford, 1992). The attack results in general debilitation, defoliation, reduction in bush frame and sometimes death of the bush. The important diseases occurring in Africa can be categorized as leaf, stem and root diseases. Leaf diseases predominant

in the nursery include grey and brown leaf spots. Stem affecting diseases include *Hypoxylon* wood rot and branch and collar canker. *Armillaria* root rot is the common most root disease in Kenya and is the subject of this study.

2.2.1 Hypoxylon wood rot

This is a wood rotting disease caused by *Hypoxylon serpens* fungus. The inocula of the fungus sexual spores (ascospores) and asexual spores (conidia) occur on infected wood of numerous hosts of *H.serpens*. The main port of entry into host plants is through wounds such as those resulting from pruning, sun-scorch and hail. Inoculum is mainly disseminated by wind or rain splash. *H.serpens* manifestation begins with decline of the bush due to sectorial rotting and death of the primary branches (Anon, 2012). Rotten wood bears superficial irregular dark-grey to black raised patches of fructifications (stomata) of various sizes. Infected (rotten) primary branches along the paths have whitish patches of immature fructifications (stomata) of various sizes (Wilson and Clifford, 1992). The disease is managed by adherence to recommended pruning height not less than 20" inches above the ground. Sanitation at every pruning cycle is necessary to reduce inoculums. Infected branches should be surgically pruned and treated with copper oxychloride fungicide mixed with vegetable oil (Anon, 2002; Mutai, 2008).

2.2. 2 Branch and collar canker

This is a stem disease caused by a fungus *Phomopsis theae.* The primary inoculums of the fungus consist of asexual spores (conidia) produced on host tissue. The pathogen invades the stem through open wounds. Predisposing factors are deep planting, planting in gravelly soils, mulching closer to collar, wounds caused by weeding implements, fertilizer application close to the collar, low moisture status in bark and surface watering during dry weather(Anon, 2012). Chlorosis, cessation of growth, profuse flowering and canker on stem are the symptoms of collar canker. Certain clones are highly susceptible to collar canker. Preventive measures include avoiding planting of susceptible clones in gravelly soils and drought prone areas, improving organic matter of marginal soils and using plants with good root system (Mutai, 2008). Removal of affected portion by pruning to healthy wood and application of copper fungicide or spore suspension of biocontrol agents like *Trichoderma* and *Gliocladium* to cut ends are the curative measures (Anon, 2002).

2.2.3 Grey leaf spot

This is a fungal disease caused by *Pestalotia theae.* The disease is predisposed by excessive moisture and shading. The fungus infects mature leaves (maintenance foliage) of tea causing grey round/oval lesions marked with concentric zonation (Anon, 2012). Black fructifications are visually discernible on the upper surfaces of the lesions. Sometimes under favorable conditions, twig die-backs occur as resulting infections on wounds of twigs after removal of pluckable shoots from susceptible clones. Control is only necessary in the nursery and is easily accomplished by optimizing watering and shading (Anon, 2002). Shade trees should be minimized in mature plantations.

2.2.4 Brown leaf spot

Brown leaf spots are caused by *Colletotrichum cammeliae* fungi. The disease is also predisposed by excessive moisture and shading on nursery plants (Anon, 2012). The fungus infects leaves causing brown necrotic lesions starting at the leaf margins. The edges of the lesions are clearly defined and marked with concentric rings. The lesions initially appear yellow and gradually turn chocolate brown from the center outwards. Minute black fructifications appear on both sides of the lesions. Many such lesions coalesce leading to blight affecting whole leaves. The control approach of the disease is similar to that of the grey leaf spot by optimizing watering and shading (Anon, 2002).

2.3 Armillaria root rot

Armillaria root rot is found throughout temperate and tropical regions of the world. Affected plants include hundreds of species of trees (including tea), shrubs, vines, and forbs growing in forests, along roadsides, and in cultivated areas (Goodchild, 1960; Williams *et al.,* 1989; Mwangi *et al.,* 1989; Fox, 2003). The disease is caused by species of *Armillaria* which live as parasites on living host tissue or as saprophytes on dead woody material. *Armillaria* species seem to be natural components of forests, where they live on coarse roots and lower stems of conifers and broad-leaved trees (Mohammed *et al*., 1994). As parasites, they cause growth reduction, wood decay and mortality.

The species identified as causing the disease has most often been referred to as *Armillaria mellea* (Vahl: Fr.) Kummer (Wargo and Shaw, 1985). Recent research, however, indicates that several different but closely related species are involved (Otieno *et al*., 2003a). According to Fox (2003), thirty six species of *Armillaria* are known to occur in various habitats worldwide. Of

these only a few are pathogenic. Many of the species that have been described are virtually harmless saprophytes. Others such as *Armillaria mellea*, *A. ostoyae*, *A. novae-zelandiae* and *A. luteobubalina* are highly virulent pathogens while others such as *A. lutea* attack trees under stress but also can infect some susceptible healthy plants such as strawberry.

Variation in mycelium and rhizomorph morphology was observed amongst some Kenyan isolates of *Armillaria* (Gibson, 1960). According to Mwenje *et al*. (2006), in a study that used isozyme analyses, RFLP patterns and comparisons of DNA sequence data, isolates of *Armillaria* from diseased tea bushes in Kenya represent three distinct groups. The results were in agreement with previous investigations that found a variety of *Armillaria* groups in Kenya (Mwangi *et al.*, 1989; Mohammed *et al*., 1994; Otieno *et al.*, 2003a). These groups are thus referred to as Kenyan groups I, II and III. According to these authors, the Kenyan group I isolates which were previously characterized by Otieno *et al*., (2003a) represent *Armillaria fuscipes.* However, Otieno *et al*. (2003a) did not use the name *A. fuscipes*, instead referring to this group as *Armillaria heimii*. The Kenyan group II isolates of *Armillaria* were assigned the name *A. mellea* ssp. *africana* by previous investigators (Mohammed *et al.*, 1994) while the Kenyan group III isolates from tea have not been clearly assigned to any definite species.

2.3.2 Detection and diagnosis of *Armillaria* root rot

Since *Armillaria* species commonly inhabit roots, their detection is difficult unless characteristic fruiting bodies are produced around the base of the tree or symptoms become obvious at the crown or on the lower stem. Crown symptoms on conifers and broad-leaved trees vary somewhat. Generally, the foliage thins and discolours, turning yellow, then brown; branches die back and shoot and foliar growth are reduced (Fox, 2003). On large, lightly infected or vigorous trees, crown symptoms develop over a number of years. On small, extensively infected or low-vigor trees, crown symptoms develop rapidly: the foliage quickly discolours, and the tree often dies within a year (Morrison, 1981). On such trees, premature foliage loss and reduced shoot and foliar growth may not be apparent. Trees affected by prolonged drought or attacked by rodents, bark beetles, or other fungi, particularly other root pathogens, can produce crown symptoms similar to those caused by *Armillaria* species. Thus, additional evidence, often found on the roots and on the lower stem, is needed to diagnose the disease.

If *Armillaria* is present, removing the bark covering infections would expose the characteristic, white mycelial mats or the rhizomorphs that grow between the wood and the bark

(Fox, 2003). The white mycelial mats are marked by irregular, fanlike striations; hence, they are often referred to as mycelial "fans." The thick mats decompose, leaving impressions on the resin-impregnated inner bark (Williams *et al*., 1989; Worall, 2004). Rhizomorphs growing beneath the bark are flat, black to reddish brown (Morrison, 1981) and up to 0.20 inch (5 mm) wide. They have a compact outer layer of dark mycelium and an inner core of white mycelium. Rhizomorphs also grow through the soil. Except for being cylindrical and about half as wide, subterranean rhizomorphs are similar to those produced beneath the bark (Onsando and Waudo, 1989).

Mushrooms, the reproductive stage of these fungi, confirm the presence of *Armillaria*. The short-lived mushrooms may be found growing in clusters around the bases of infected trees or stumps (Fox, 2003). They are mostly abundant during moist periods (Masuka, 1993). *Armillaria* species cause white rot of infected wood. When wood first begins to decay, it looks faintly water soaked; then it turns light brown (Williams *et al.,* 1989). At the advanced stages of decay, wood becomes light yellow or white and may be marked by numerous black lines. Advanced decay is spongy in hardwoods but often stringy in conifers (Smith and Smith, 2003).

2.3.3 Disease Cycle

Species of *Armillaria* may survive as rhizomorphs and/or vegetative mycelium on/in the dead and dying wood of tree stumps and roots and may live for decades in coarse woody material (Masuka, 1993; Mullen and Hagan, 2004). From this food source, the fungus spreads to living hosts (Smith and Smith, 2003). Spread occurs when rhizomorphs, growing through the soil, contact uninfected roots or when uninfected roots contact infected ones (Munnecke *et al.,* 1981). Sometimes the fungus can be found several feet above the soil line on the trunk of dead trees several years after being killed by *Armillaria* species (Wargo and Shaw, 1985). Rhizomorphs can grow for distances of up to 10 feet (3 m) through the upper soil layers, and they penetrate the roots by a combination of mechanical pressure and enzyme action (Williams *et al.*, 1989). The growth of rhizomorphs and their ability to penetrate roots depend upon the specific fungus, the type and amount of the food source, the soil environment, and the host species (Worrall, 2004). In the cold season, mushrooms may arise from the rhizomorphs (Onsando *et al.*, 1997). Millions of microscopic whitish spores (basidiospores) produced in the mushroom caps are carried by the wind to dead stumps or injured bark at the base of living plants (Wargo and Shaw, 1985). Under favourable conditions of moisture and temperature, a few basidiospores germinate and produce a mycelium that infects the bark and later the sapwood and

cambial regions (Williams *et al.*, 1989). However, this natural fructification by production of basidiomata is rare in Africa and is limited to cooler areas. Basidiomata were found only in one tea plantation located at high altitude (2180m) in Kericho (Onsando *et al.,* 1997) consistent with the above observations. White "fans" of the mycelium develop on the sapwood, followed by the formation of rhizomorphs. Some species of the fungus or perhaps strains within species are virulent parasites while others are opportunistic and act selectively on small or weak individual plants (Munnecke *et al.,* 1981). *Armillaria* also colonizes the declining root systems of plants felled or killed by other agents (Smith and Smith, 2003).

Infection occurs when mycelium of *Armillaria* comes in contact with and adheres to young roots of a susceptible plant by means of a gelatinous secretion (Wargo and Shaw, 1985). The mycelium penetrates a root by the action of secreted enzymes that partially digest the cell walls of the young root (Masuka, 1993). The fungus then grows into the root tissue between the cells (Fox, 2003). Once a plant has been invaded, the fungus continues to ramify through the root and trunk tissues; even after the host plant has been dead for several years (Mullen and Hagan, 2004). A tree or shrub may die in one to several years after initial infection, depending on the vitality of the plant and environmental conditions (Williams *et al.*, 1989). *Armillaria* species can also pass from tree to tree via root grafts. Roots of trees under stress are most easily infected. *Armillaria* species are generally inhibited at soil temperatures above 26°C (Onsando *et al.,* 1997).

2.3.4 Damage caused by *Armillaria* root rot

Infection results in growth reduction and wood decay. Growth reduction often goes undetected or is ascribed to other agents and thus is probably underestimated (Mwangi *et al.*, 1989). Likewise, decay extends only a few feet into the lower stem and often goes unnoticed until the tree falls or is cut (Masuka, 1993). *Armillaria* species infect and kill trees that have been already weakened by competition, other pests, or climatic factors and also infect healthy trees, either killing them outright or predisposing them to attacks by other fungi or insects (Fox, 2003).

2.3.5 Physical, biological and chemical methods for management of *Armillaria*

Armillaria species are indigenous to many areas and live on a wide variety of plants and woody material and their eradication or complete exclusion is not feasible (Munnecke *et al.,*

1981). Their management is typically directed toward limiting disease buildup or reducing its impact (Morrison, 1981). The pathogen has evolved highly sophisticated mechanisms of protection against outside deleterious effects (Onsando and Waudo, 1989; Fox, 2003). These include the production of antibiotics and the formation of pseudosclerotia (Raziq, 2000). Any chemical or biological control agent would have to overcome the effect of these biochemical and resistant structures before it can eradicate the fungus.

Physical removal of inoculum by removing diseased trees and uprooting even neighbouring uninfected stumps was recommended (Goodchild, 1960) as one of the methods of managing the disease in tea plantations. Trenches over a meter deep can be dug to isolate the infected plants from healthy parts of a plantation thereby restricting the spread of *Armillaria* species. However, these methods of physical control can be laborious and often impracticable for established forest trees (Morrison, 1981; Smith and Smith, 2003). Ring-barking to girdle trees about 60 - 90 cm above the collar one year before they are felled is recommended as a method of killing trees that reduces the longevity of inoculum of *Armillaria* species in the roots (Masuka, 1993). Girdling stops downward movement of photosynthates thereby leading to depletion of carbohydrate reserves in the roots hence preferentially favouring invasion and survival of saprotrophs at the expense of the pathogen (Masuka, 1993).

Systemic and non-systemic fungicides have been used for the control of *Armillaria* species but field experiments showed that the chemicals failed to fully eradicate the rhizomorphs in soil even with as high a concentration as 10,000mg/L (Fox, 2003). Soil fumigation with carbon disulphide, methyl bromide or chloropicrin has been used to manage the disease (Munnecke *et al.*, 1981). Methyl bromide has been the most extensively used fumigant that is applied between crops because of its non-specific action and good penetrability in soil but its use in agriculture has been banned because of its adverse effects on the environment. Chloropicrin will destroy even the most resistant soil pathogens, but penetration through soil is much more difficult to achieve. Carbon disulphide can be injected at regular intervals over an infected site after removing stumps, and although it has a lower toxicity it is cheaper. It also has a high vapour pressure enabling it to penetrate deep into soil (Fox, 2003). This is a desirable attribute as *Armillaria* species have been found viable at a depth of almost 3 m. Because the currently available chemicals for controlling the pathogen are either ineffective or phytotoxic, there is need for alternative approaches to managing the disease.

Successful use of fungal biocontrol agents like *Trichoderma* species for the control of diseases caused by soil borne pathogens like species of *Armillaria*, *Rhizoctonia, Sclerotium, Fusarium, Pythium,* and *Phytophthora* in several crops have been reported (Cook and Baker 1983). *Trichoderma* species are under intensive research because of their abundant natural occurrence, biocontrol potential against fungal and nematode diseases as well as host defense inducing ability (Harman and Kubicek, 1998). Research on BCAs of *Armillaria* has been undertaken on an intensive level since 1914 using *Trichoderma*. Significant findings were made by Dumas and Boyonoski (1992) with regard to the mode of action of *Trichoderma* species. Using Scanning electron microscopy (SEM), they found that *Trichoderma polysporum,* T. *harzianum* and *T. viride* were mycoparasites on the rhizomorphs of *Armillaria gallica*. These *Trichoderma* species attacked and penetrated the melanized outer tissue of the rhizomorph and once inside they killed the *Armillaria* hyphae by coiling and direct penetration. After one week the rhizomorphs infected with each of the three species of *Trichoderma* were devoid of hyphae.

Species of *Trichoderma* particularly *T. harzianum* and *T. viride* function by antagonism, inhibition or prevention of development of rhizomorphs and mycelia (Otieno, 2002; Fox, 2003). Depending on the particular isolate of *Trichoderma* species, control may be achieved by competition, production of antibiotics, or by myco-parasitism (Tu, 1980; Klein and Eveleigh, 1998; Mohammad *et al.,* 2008). The antagonistic strains of *T. harzianum* coil around hyphae of their host *Armillaria* and enzymatically degrade their cell walls.

To achieve disease suppression, *T. harzianum* must be capable of colonizing the woody substrates or the fungal structures *in situ*. Such colonization may be preceded by other factors that weaken the resistance of the pathogen (Harman and Kubicek, 1998; Otieno *et al.,* 2003b). An effective method of disease suppression is to use the bio-control fungus in conjunction with chemical fungicides applied at reduced rates, and physical methods such as soil solarization. Solarization for 10 weeks resulted in increases in soil temperatures that reduced viability of the pathogen by up to 100% (Otieno, 2002; Otieno *et al.,* 2003c). Application of *T. harzianum* to soil surrounding inocula of *Armillaria* species consequent to solarization for five weeks caused total loss of inoculum viability. *Trichoderma* species can therefore be used as bio-control agents to manage *Armillaria* root rot. Integration of the physical, chemical and biological methods is an efficacious approach for managing *Armillaria* root rot especially targeting the pathogen in small-holder tea plantations.

2.4 Formulation of fungal preparations for managing fungal pathogens

Among the hundreds of organisms identified as potential biological control agents for plant diseases, only few have proved commercially acceptable. A fungal biocontrol preparation comprises sporulated fungal biomass and a carrier material (Churchill, 1982; Wall *et al.,* 1996). Different formulations (fungal spores and powdery preparations of fungal mycelium) have been used in the control of soil borne pathogens (Harman *et al*., 1980). A formulation with agricultural potential should possess several desirable characteristics such as: easy preparation and application, stability, adequate shelf life, abundant viable propagules, and low cost (Churchill, 1982). The formulation should be amenable for application to both phylloplane and rhizosphere, depending on the plants and pathogens to be controlled.

When mass producing a fungal bio-control agent it is essential that the strain is well maintained. It must be free from contamination and carefully conserved so that it not only remains viable in agar culture, but also retains its virulence. If fungal strains are sub-cultured too often on artificial media, they can lose their virulence (Hintz, 2010). This can be prevented by maintaining isolates for storage on agar slants, which may be kept in the refrigerator for 6-12 months. The spores from these cultures can then be used to inoculate working cultures. Shelf life is a very important parameter in the development of a formulation because most products have to be stored for long periods of time before they can be marketed and used. The market for biological control products is not only determined by agricultural aspects such as the number of diseases controlled by one bio-control product in different crops (Cook and Baker, 1983), but also by economic considerations such as cost-effective mass production, easy registration and the availability of alternative means of control including chemical fungicides. The major aspects of successful biological control technologies include the establishment of product, formulation and delivery system for microorganism that enable efficient disease control. There are two major methods of inoculum production: solid-state fermentation and liquid-state fermentation (Tabachnik, 1989; Jeyarajan, 2006).

2.4.1 Solid-state fermentation

Solid state fermentation refers to a process for fermenting microorganisms on a solid medium that provides a substrate for anchoring the microorganisms, in the absence of any freely flowing substance (Hintz, 2010). Solid state fermentation is a very common method for mass production of *Trichoderma*. Various cheap cereal grains like sorghum and millets are used as

substrates (Jeyarajan, 2006). The grains are moistened, sterilized and inoculated with *Trichoderma* and incubated for 10-15 days. *Trichoderma* produces dark green spore coating on the grains. These grains can be powdered finely and used as seed treatment or the grains can be used for enriching Farm Yard Manure (FYM) for soil application. Solid state fermentation results in a product that is generally used as it is for soil application or for enriching organic manures. This technique is suitable for small-scale production in cottage industries or at individual farmer level. The disadvantage of this technique is that it is laborious and results in a product which is bulky and prone to contamination.

2.4.2 Liquid fermentation

In liquid fermentation system, *Trichoderma* species are grown in liquid media in stationary/shaker/fermenter cultures, then formulated and used for field application. Maximum biomass of the fungus can be realized in a short time by using appropriate medium in a fermenter with aeration, agitation, temperature, pH and antifoam controls than in shake-flask cultures (Wall *et al.*, 1996). This is more suitable for industrial production of *Trichoderma* species. According to Prasad and Rangeswaran (1998), the maximum amount of biomass and viable propagules of *T. harzianum/T. viride* can be obtained within 96h of fermentation in a fermenter with aeration, agitation and temperature controls.

2.3.3 Formulations of *Trichoderma* species

Products formed from solid or semi-solid state fermentation do not require sophisticated formulation procedures prior to use. For example, grain or other types of organic matter on which *Trichoderma* is grown are simply dried, ground and added to the area to be treated (Jeyarajan, 2006). Biomass produced in liquid fermentation can either be separated from medium and concentrated or from the entire biomass with medium can be incorporated into dusts, granules, pellets, wettable powders or emulsifiable liquids (Hintz, 2010). The carrier material may be inert or a food base or a combination of both. Colony Forming Units (CFUs) of *Trichoderma* species should be a minimum of 2×10^6 CFU per ml or g on a selective medium. Pathogenic contaminants such as *Salmonella*, *Shigella* or *Vibrio* should not be present in the formulation while other microbial contaminants should not exceed a count of 1×10^4 per ml/gm (Prasad and Rangeswaran, 1998) and the maximum moisture content should not be more than 8% for dry formulation of fungi.

2.5 Delivery of *Trichoderma* for disease management

For successful disease control, delivery and establishment of *Trichoderma* to the site of action is very important. Mastouri *et al* (2010) in their trial demonstrated that treatment of tomato seeds/seedlings with *T. harzianum* enabled the plants to withstand biotic, abiotic and physiological stresses. The most common methods of application of *Trichoderma* are by seed treatment, seedling dip, soil application and wound dressing (Ramanujam *et al.*, 1999).

2.5.1 Seed treatment/ bio-priming

Seed coating with certain species of *Trichoderma* is one of the easy and effective methods of delivering the antagonist for the management of seed/soil-borne diseases. Seed is coated with dry powder/dusts of the fungus just before sowing (Mukhopadhyay *et al.*, 1992).

Seed bio-priming is treating of seeds with specific isolates of *Trichoderma* sp. and incubating under warm and moist conditions until just prior to radical emergence. This technique has potential advantages over simple coating of seeds as it results in rapid and uniform seedling emergence. *Trichoderma* conidia germinate on the seed surface and form a layer around bio-primed seeds creating an immediate barrier to potential seed diseases (Mishra *et al.*, 2001).

2.5.2 Root treatment

Seedling roots can be treated with spore or cell suspension of antagonists either by drenching the bio-agent in nursery beds or by dipping roots in bio-agent suspension before transplanting. This method is generally used for the vegetable crops and rice where transplanting is practiced (Singh and Zaidi, 2002). Root dipping in antagonist's suspension not only reduces disease severity but also enhances seedling growth in rice, tomato, brinjal, chili and capsicum.

2.5.3 Soil treatment

There are several reports on the application of BCAs to the soil and other growing media either before or at the time of planting for control of a wide range of soil-borne fungal pathogens (Baby and Manibhushanrao, 1996). Such applications are ideally suited for greenhouse and nursery. *Trichoderma* isolates for use as bio-control agents are capable of colonizing farm yard manure (FYM) and therefore application of colonized FYM to the soil is more appropriate and beneficial. This is the most effective method of application of *Trichoderma* for biocontrol, particularly in the management of diseases caused by soil-borne pathogens.

2.5.4 Aerial spraying / Wound dressing

Trichoderma viride and *Trichoderma harzianum* species have been applied successfully to the aerial plant parts for the bio-control of decay fungi in wounds on shrubs and trees (Papavizas, 1985). Aerial spraying ensures fine distribution of the bio-control fungus, thus most suitable for flowers foliar disease management. Sprays with *Trichoderma* in the field significantly reduce the incidences of *Botrytis* rot of strawberries and grapes and *Sclerotinia* head rot of sunflower (Agrios, 2005).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Experimental site

The experiments were conducted at the Tea Research Foundation of Kenya (TRFK), Kericho Plant Protection laboratories.

3.2 Preparation of *Trichoderma harzianum* (T4) inoculum

Conidia of *Trichderma harzianum* isolate (T_4) were harvested according to the method used by Mustafa *et al.* (2009), from a biologically pure culture from TRFK stock cultures using a 2 mm cork-borer and serially diluted to 10^{-4}. The initial conidial concentration was determined microscopically by counting the number in 0.1ml of the diluted sample in three replicates. One hundred milliliters of distilled water in wash bottles was used to wash the spores from each plate containing *T. harzianum* culture (Tabachnik, 1989; Hintz, 2010). The spore suspensions obtained were thoroughly mixed and 10ml of the suspension pipetted and inoculated on a carbon enriched liquid medium in Erlenmeyer flasks. The composition of the liquid medium used comprised the ingredients (g/l distilled water) in Table 1.

Table 1: Concentrations and ingredients of *Trichoderma harzianum* broth recipe

Medium	Amount (g/l)
Potato dextrose broth	2.4
Malt Extract	10.0
KH_2PO_4	1.99
Glucose	10.0
$FeCl_2.4H_2O$	0.002
$MnSO_4.H_2O$	0.002
NH_4NO_3	1.0
$ZnSO_4.7H_2O$	0.002
$MgSO_4.7H_2O$	0.2
Thiamine-HCl	0.001
KCl	0.2
Chloramphenicol	0.25
K_2HPO_4	3.99

Source: Tabachnik, 1989

The flasks were incubated on a rotary shaker at 200 rpm and temperature of 28°C. After 24 to 48 hours, the mycelia produced were homogenized in an Ultra Turrax homogenizer / blender and measured to form one-litre initial inoculum as described by Wall *et al.* (1996). The homogenized inoculum was inoculated on 10 litres of the same liquid medium in a fermenter. During the following 48 to 65 hour growth period, the pH of the medium was kept between 5.8 and 7.0, and the temperature maintained at 28°-29°C. A Silicone "Sigma" Antifoam Y emulsion was added by means of a peristaltic pump (Prasad and Rangeswaran, 1998; Hintz 2010). Growth was stopped after 48 to 65 hours when the conidial yield of about 4×10^6 conidia/ml was reached. This was determined microscopically by counting. The initial conidial yield was compared with the yields at 12, 24, 48 and 65 hours according to procedure described by Tabachnik (1989).

3.2.1 Separation of the fungal propagules

After incubation, the fungal biomass obtained was separated. Spores were harvested from the 1000 ml portion of the liquid broth by the use of a vacuum spore harvester. The mycelial propagules remained in the liquid broth after spore harvesting. Conidio-mycelial propagules were obtained by filtering out the 1000 ml portion of the liquid broth.

3.3. Inoculation of substrates

The *T. harzianum* inoculum was first produced by liquid fermentation in broth medium then inoculated into each of the solid substrates using the method described by Wall *et al.* (1996) and Hintz (2010). The two authors described the production of solid substrate inoculum of *Chondostereum purpureum* fungus, a biological control for weed trees. A two stage fermentation process was used whereby inoculum was first produced by liquid fermentation in broth medium and then inoculated into the solid substrates. The separated propagules (conidia/spore, mycelia and conidio-mycelia) were mixed with 1000 ml of sterile liquid nutrient medium and used to inoculate solid substrate contained in a fermentation vessel, that had been pre-wetted with liquid nutrient and sterilized by autoclaving (Tabachnik, 1989). This liquid culture provided an ideal inoculum for Vermiculite, Talc, Kaolin and wheat bran substrates contained in sterile bags (200 ml inoculum into each 500g bag of sterile milled substrate). The treatments were replicated three times in a 4x3 factorial design. Solid matrix fermentation was performed at temperature of 22-26°C for 4 to 6 weeks to allow adequate colonization of the substrate and the uniform material subsequently used as the active ingredient in the formulation.

In the embodiments, the fermentation was conducted in such a way that the finely particulate substrate is predominantly colonized by fungal propagules, that is, at least 50% of the particles are colonized by the propagules. This was achieved by mixing the content in the fermenter to allow the dispersion of the inoculum and colonized particles during the fermentation as described by Churchill (1982). To produce propagules that are more stable, the moisture content of the fermentation substrate was maintained between 10 and 30% (w/w). In order to increase the yield of the fungal propagules and colonization of the particles, the pH of the substrate and nutrition medium was adjusted to a range of 4-7 according to the method used by Hintz (2010).

3.4 Compatibility of *Trichoderma harzianum* with different substrates

The end of the fermentation was determined by a standard fungal biomass determination namely: dry weight, colony forming unit (CFU) determination, and microscopic observation (Prasad and Rangeswaran, 1998). Comparison was made on the compatibility of *T. harzianum* propagules (conidia/spore, mycelia and conidio-mycelia) with the above substrates. At the end of the fermentation, the particulate substrate having on its surface the fungal propagules from the fermentation step was unloaded from the fermentation vessel into a high shear blender to break up clumps. The comminuted material was then passed through a sieve to remove clumps. To determine the dry weight, 10g samples were obtained and dried in an oven at 60°C for one week and the constant dry weights determined. The numbers of colony forming units were determined using 1g samples of solid substrates. A homogeneous suspension of the sample in sterile water was serial diluted to 10^{-3} grams substrate/ml sterile water and 1 ml suspension of each dilution plated (3 plates/sample dilution) onto malt extract agar (MEA) and incubated at 25°C. Sporulation rate was determined by counting the number of spores using a haemocytometer.

3.5 Stability of *Trichoderma harzianum* formulations in storage

Formulations found to be compatible with *T. harzianum* propagules were used. To determine inoculum titre, samples of solid substrate were taken at monthly intervals after initial substrate inoculation (4 weeks to 6 months). Titre was then determined from three separate samples of 1g solid substrate. Homogeneous suspension of the sample in sterile water was serial diluted to 10^{-3} grams of substrate/ml sterile water and 1 ml suspensions of each dilution was

plated (three plates/sample dilution) onto yeast-trypton (2YT) agar plates, for determination of the number of CFUs and incubated at 25°C. Sporulation rate was determined by counting the number of spores using haemocytometer. Plates were assessed for the number of CFUs and sporulation rates after four days of growth.

3.6 Vegetative interaction between *Armillaria* and *Trichoderma harzianum*

Vegetative interaction between *Armillaria* isolate ($8KA_1$) and *T. harzianum* (T_4) was evaluated according to the method described by Onsando and Waudo (1994) and Mohammad *et al.,* (2008). The above isolates were obtained from the TRFK stock cultures

3.6.1 Preparation of tea bark extract

Two hundred grams of fresh tea root bark of 4-year-old seedling tea plants was macerated into 300mls of 95% ethanol containing 50mg Potassium meta-bisulphate, a reducing agent, to stop conversion of catechins to theaflavins (TF) and thearubigins (TR). The maceration was done using a blender for 10 minutes. The blended mixture was filtered under reduced pressure. The filtrate was evaporated at 40°C using a rotary evaporator at about 10mls, then washed with 50mls of distilled water. It was evaporated to dryness, then stored in a freezer (Onsando and Waudo, 1994).

3.6.2 Vegetative interaction

Five millimeter agar blocks containing *Armillaria* isolate ($8KA_1$) were transferred from PDA to MEA containing 0.5g/l tea root bark extract. The bark extract was meant to enhance the growth of the fungus. The cultures were allowed to grow for two weeks and about 10mm diameter. Five millimeter blocks of one day old *T. harzianum* (T_4) previously sub-cultured from the different formulations were then introduced into the MEA plates containing two-week- old *Armillaria* cultures 25mm apart. The controls consisted of single cultures of *Armillaria* sp. only, and were not inoculated with *T. harzianum.* The plates were incubated at room temperature (18-21°C). Colony diameter of *Armillaria* was measured over a 20-day period, at 2-day intervals. At the end of the period, successful re-isolation of the pathogen was recorded as (+) and the unsuccessful as (-).

3.7 Experimental design

The experiment was set up as a 4 x 3 factorial replicated three times with the factors as follows:

Factor 1: 4 substrates or carrier material

1. Talc
2. Vermiculite
3. Wheat bran
4. Kaolin

Factor 2: Propagule type

1. Conidia (spore),
2. Mycelia,
3. Conidia + mycelia

The factors were combined as shown in Table 2 and laid out in three replicates

Table 2: Substrate- propagule combinations

Substrate	Propagule type		
	Conidia/spore based	Mycelia based	Conidio-mycelium based
Talc	Conidia, Talc	Mycelia, Talc	Conidia-mycelium, Talc
Vermiculite	Conidia, Vermiculite	Mycelia, Vermiculite	Conidia-mycelium, Vermiculite
Wheat bran	Conidia, wheat bran	Mycelia, wheat bran	Conidia-mycelium, wheat bran
Kaolin	Conidia, Kaolin	Mycelia, Kaolin	Conidia-mycelium, Kaolin

3.8 Data collection and analysis

The data on fungal biomass and inoculum titre of *T. harzianum* substrate-propagule combinations were collected. The data included the conidial yield, substrate-propagule compatibility, stability of *T. harzianum* in storage and vegetative interaction of *T. harzianum* and *Armillaria* sp. isolate $8KA_1$. Co-relation and regression analysis was used to identifying the relationship between conidial yield variable and time on PDA and broth medium. A model of the relationship was hypothesized, and estimates of the parameter values used to develop an estimated regression equation. These data were also subjected to one-way analysis of variance (ANOVA) using SAS statistical package (SAS System for Windows, 2002). Mean values of all replicates were tested for significant differences by means of t-test statistic and separated by Least Significant Difference (LSD) at $P \leq 0.05$.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Conidial yield studies on PDA and on broth medium

There was a positive correlation (R^2 =0.9274) between conidial yield and time on PDA medium. The number of spores increased progressively ($P \leq 0.05$) from day five with a minimum of 0.19 x 10^9 to a maximum of 2.1 x10^9 by day 13 (Figure 1). Mustafa *et al.* (2009) in their comparative studies of different media (Potato Dextrose Agar, Waksman agar, Agar-agar, Czepak's agar and Corn Meal agar) found out that PDA was the best medium for growth of three *Trichoderma* species (*Trichoderma viride*, *Trichoderma harzianum* and *Trichoderma longibrachiatum)* in terms of growth, spore production and biomass yield.

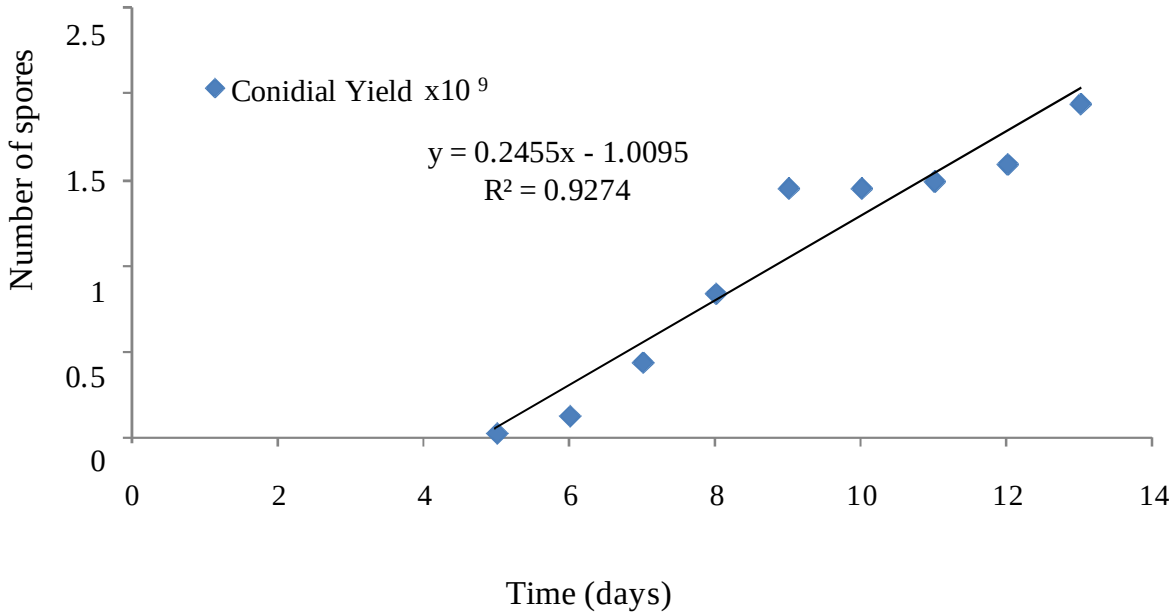

Figure 1: Mean conidial yield of *Trichoderma harzianum* on PDA

In broth medium, there was also a positive correlation (R^2 =0.8954) between conidial yield and time. The number of spores increased progressively ($P<0.05$) from day one with a minimum of 0.07 x 10^7 to a maximum of 4.17 x10^7 by day 7 (Figure 2). According to Ramanujam *et al.* (1999), maximum biomass production can be achieved within a short period of time by using appropriate medium in a fermenter with aeration, agitation, temperature, pH and antifoam controls than in shake flask cultures. Prasad and Rangeswaran (1998) revealed that the

maximum amount of biomass and viable propagules of *T. harzianum / T. viride* can be obtained within 96h of fermentation in a fermenter with aeration, agitation, and temperature controls. In the current study, the target conidial yield of about 4 x 10^6 was achieved by day 3 and the substrates were inoculated at this stage.

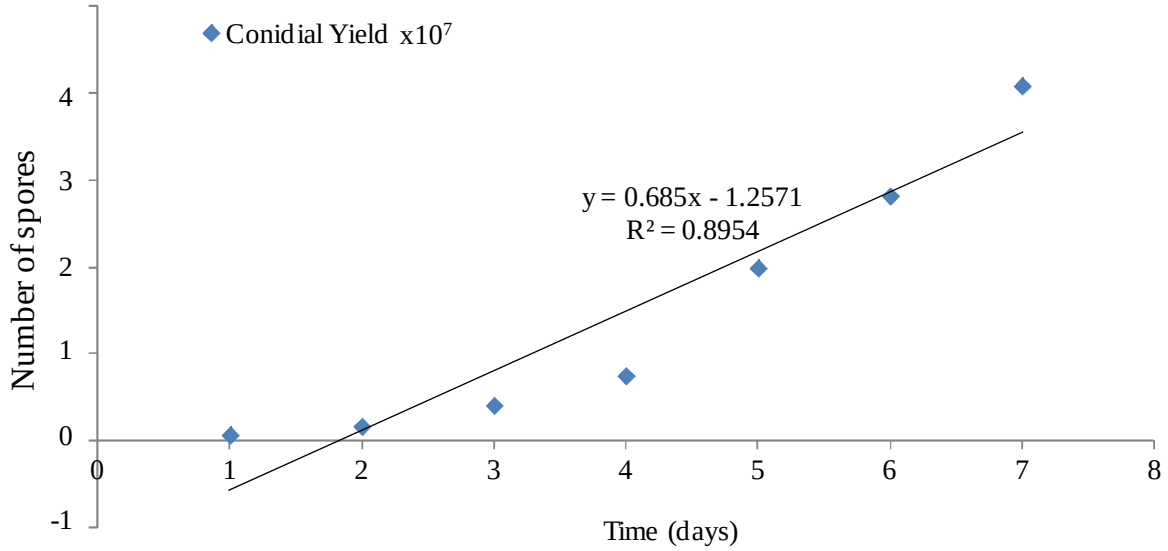

Figure 2: Mean conidial yield of *Trichoderma harzianum* on broth medium

Most current research on biological control agents is directed towards the optimum production of propagules able to exert effective field control over pathogens (Elad *et al*., 1983; Lewis and Papavizas, 1991; Andrews, 1992). Liquid cultivation has been successfully employed to produce *T. harzianum* spores (Lewis and Papavizas, 1991). The formulation of chemically defined culture media permits controlled physiological studies to identify optimal conditions and critical factors involved in fungal cell processes. Tabachnick (1989), in his patent, used a liquid broth medium for the production of *T. harzianum*. The liquid medium constituents (Table 1) comprised of a combination of Carbon sources, the Nitrogen source, buffer mixture, antibiotic and vitamin sources and a pH from about 5.8 to 7.0. Agosin *et al*. (1997) found that the highest spore longevity was found in a medium with a C: N ratio of 14 and a pH of 7.0, when most resulting spores were still alive after 45 days storage. These spores also remained viable during storage under a broad range of relative humidity values, indicating that they would be more sustainable in the field.

4.2 Substrate-propagule compatibility

4.2.1 Performance of *Trichoderma harzianum* in different substrates

Statistical analysis revealed that the dry weights, spore counts and CFU counts of *T. harzianum* in different substrates varied significantly ($P \leq 0.05$). Kaolin formulations had the highest dry weight followed by vermiculite, talc and wheatbran respectively. Spore counts also varied significantly ($P \leq 0.05$) with Kaolin formulations having the highest counts, wheat bran and vermiculite were median, and talc had the least counts. Colony counts also varied significantly ($P \leq 0.05$) with Kaolin having the highest counts followed by vermiculite, talc and wheat bran respectively (Table 3).

Table 3: Substrate Compatibility with *Trichoderma harzianum*

Substrates	Dry weights	Spore counts ($x10^8$)	Mean CFU($x10^6$)
Kaolin	7.63a	10.1111a	4.92a
Vermiculite	6.27b	5.2778b	4.55b
Talc	3.72c	2.1500c	3.06c
Wheatbran	6.23b	5.4444b	0.47d
CV %	8.85	24.6	11.37
LSD(P<0.05)	0.5099	1.365	0.357

**Means followed by the same letter are not significantly different at $P \leq 0.05$*

Dry weights, spore counts and colony counts of different propagules varied significantly ($P \leq 0.05$) with the substrates after one month of storage (Table 4). Conidio-mycelial propagules had the highest dry weights followed by the mycelial and spore propagules respectively. Conidio-mycelial propagules also had the highest spore counts followed by the spore and mycelial propagules respectively. Mycelial propagules had the highest colony counts followed by conidio-mycelial and spore propagules respectively.

Biological agents are mostly quantified in terms of CFU counts and not spore counts. This is because spore counting will give a total of all spores regardless of their viability (Jin *et al.,* 1996; Jakubikova *et al.,* 2006). CFU counts on the other hand show the actual counts of the viable spores in a biological agent. All the propagules at this stage supported over 10^8 spores per ml and over 10^6 CFU/g/ml (Table 4) and were thus considered to be compatible with the substrates. The compatibility of these substrates with *Trichoderma* propagules in the long run

was determined in the 6- month storage stability tests. This reveals that there is a difference in the compatibility of *T. harzianum* propagules with different substrates.

Table 4: Compatibility of *Trichoderma harzianum* propagules in different substrates

Propagule	Dry weights	Spore counts (x10 ^{8})	CFU counts (x10 ^{6})
Mycelia	5.98a	5.06b	3.47a
C.mycelia	6.06a	6.28a	3.19ab
Spores	5.85a	5.90ab	3.11b
CV %	8.85	24.6	11.37
LSD(P<0.05)	0.4416	0.1182	0.3095

**Means within columns followed by the same letter are not significantly different at P≤0.05*

The choice of the substrates used in the current study was specifically geared towards the use of low cost material for the production of a *Trichoderma* formulation that can support high conidial counts over 10^6 CFU/g after 6 months of storage. Jayrajan and Angappan (1998) stated that the minimum population of a fungal bio-agent in any formulation for seed/seedling treatment should be above 10^6 CFU/g. In the current study, the mean conidial counts for all the propagules were over 10^6 CFU/g/ml and spore counts over 10^8 spores /ml. These counts were achieved in month 1 of storage and thus the propagules were considered compatible with the substrates. Biological control agents should possess several desirable characteristics, including: ease of preparation and application, stability during transport and storage, abundant viable propagules and good shelf-life (Lewis and Papavizas, 1991; Andrews, 1992). One of the major limitations to the application of biological control agents (BCAs) such as *Trichoderma* species is the development of appropriately formulated products (Fravel, 2005).

There is abundant literature on the use of conventional synthetic media like glucose, cellulose, soluble starch, and molasses to produce *Trichoderma* species (Lewis and Papavizas, 1983; Gupta *et al*., 1997). However, the cost of these raw materials for commercial production of BCAs is one of the major limitations behind the restricted use. Various substrates have been successfully used to produce *Trichoderma* species by solid state fermentation. These include fine clay, peat, vermiculite, alginate, wheat bran, talc, diatomaceous earth, pasteurized soil (Boyetchko *et al*., 1998), Oil-based formulations (Batta, 2005), Coffee husk formulations

(Sawant and Sawant, 1996), Pesta granules (Connick *et al*., 1991), Press mud based formulation (Jeyajeran, 2006) and Wheat flour- kaolin (Prasad and Rangeswaran, 1998). Despite the use of alternate sources, the cost of production is still high. Some additives (protectants and carriers) have been used to increase BCA survival under adverse environmental conditions (Wraight *et al*., 2001). *Trichoderma* species can be formulated as pellets (Papavizas and Lewis, 1989), dusts and powders (Nelson and Powelson, 1988), and fluid drill gels (Conway, 1986). These substrates/carriers may be inert or a food base or a combination of both.

In this study, inert substrates (kaolin, talc, vermiculite and wheatbran) were used. The choice of the above substrates trailed down to the fact that they are harmless to the environment, inexpensive, and easily available. Preservatives were not used because excessive amounts of preservatives such as sugar and other compounds in the field application cause various problems (Panahian *et al.,* 2012). For example, for use in treatment of roots and soil, these compounds can absorb other opportunistic microorganisms such as bacteria which are likely to compete with the key organism and are able to overcome the conidia. Therefore, *Trichoderma* spores are best formulated with minerals such as zeolite, Bentonite, Vermiculite, Kaolin, Perlit, Clay and other mineral compounds that are not problematic (Boyetchko *et al*., 1998; Panahian, *et al.,* 2012).

4.3 Stability in storage of *Trichoderma harzianum* in different substrates

4.3.1 Spore counts

ANOVA tests showed that spore counts of *T. harzianum* formulations varied significantly ($P \leq 0.05$) over a 6 month period (Table 5). The mycelia, conidio-mycelial and spore propagules followed somewhat the same trend over the 6 month period. For all the propagules, month 1 had moderate mean spore counts which peaked in month 2 when the highest counts were recorded. In the subsequent months, the counts dropped (an indicator of depletion of nutrient reserves) and were lowest at month 5. Mycelial formulations had the highest mean spore counts of 4.15 $x10^9$ spores /ml, followed by the conidio-mycelial and spore formulations which had the same mean level of counts of 4.06 $x10^9$ spores /ml. It is therefore deduced that formulations of *T. harzianum* bearing mycelial propagules will yield higher spore counts compared to those bearing the conidio-mycelia and spores. The same level of counts attained from conidio-mycelial and spore propagules, reveals that there is no real significance in separating the spores from the mycelia with respect to conidial yield.

Table 5: Mean spore count s of *Trichoderma* propagules at different storage periods

Month	Mycelia	C.Mycelia	Spores
1	5.06(1.69)b	6.28(1.89)b	5.90(1.78)b
2	17.04(2.82)a	15.08(2.64)a	15.83(2.66)a
3	1.17(0.63)c	1.04(0.58)c	1.11(0.62)c
4	0.77(0.55)c	0.98(0.64)c	0.70(0.52)c
5	0.39(0.31)c	0.37(0.3)c	0.28(0.24)c
6	0.45(0.35)c	0.59(0.44)c	0.51(0.4)c
Means	4.15	4.06	4.06
C.V %	23.07	23.39	29.67
LSD (P<0.05)	0.2	0.21	0.25

**Means within columns followed by the same letter are not significantly different at P≤0.05*
**Data in parathensis are log transformed ln(x+1)*

Overall, kaolin formulations had significantly ($P \leq 0.05$) high mean counts of 7.13 $x10^9$ spores/ml followed by vermiculite at 4.5 $x10^9$ spores/ml, talc at 2.48 x 10^9 spores/ml and wheat bran 2.23 x 10^9 spores/ml. (Table 6). For kaolin formulations, conidio-mycelial propagules yielded the highest counts followed by mycelial and spore propagules respectively. For vermiculite formulations, spore propagules yielded the highest counts followed by the mycelial and conidio-mycelial propagules, respectively. For talc formulations, mycelial propagules yielded the highest counts followed by the conidio-mycelial and spore propagules respectively. For wheat bran formulations, the mycelial propagules yielded the highest counts followed by the conidio-mycelial and spore propagules respectively. This can therefore be interpreted that kaolin, and wheat bran *T. harzianum* formulations will yield higher spore counts when inoculated with conidio-mycelial propagules, compared to those bearing mycelial and spore propagules respectively. Vermiculite *T. harzianum* formulations inoculated with spore propagules will yield higher spore counts compared to those bearing mycelial and conidio-mycelial propagules.

Table 6: Mean spore counts of *Trichoderma* in different substrates

Substrate	Mycelia	C.Mycelia	Spores	Mean
Kaolin	6.8(1.47)a	7.34(1.57)a	7.25(1.47)a	7.13
vermiculite	4.66(1.1)b	4.01(1.05)b	4.83(1.14)b	4.5
Talc	2.64(0.84)c	2.45(0.86)c	2.34(0.78)c	2.48
w.bran	2.44(0.82)d	2.43(0.85)d	1.81(0.75)d	2.23
C.V %	23.07	23.39	29.67	
LSD (P<0.05)	0.16	0.17	0.2	

**Means followed by the same letter are not significantly different at $P \leq 0.05$*

**Data in parathensis are log transformed ln(x+1)*

The spore counts were significantly high ($P \geq 0.05$) in all the formulations over 10^9 spores/ml at months 1, 2 and 3. The count levels dropped to 10^8 spores/ml from month 4 to month 6. These high spore counts did not necessarily translate to the same levels of colony counts (Table 6). The mean viable conidial counts during the 6 month period were all over 10^6 CFU/g except for the wheat bran formulations which had a mean of 10^5 CFU/g.

A lot of information on the use of BCA such as *Trichoderma* species has been published but few studies have been found regarding spore production. Results for conidia production in submerged fermentation (Jin *et al.,* 1996; Verma *et al.,* 2005, 2006, 2007; Jakubikova *et al.,* 2006) were expressed as colony-forming units per milliliter (CFU/ml) after incubation on agar; thus, the results cannot be directly compared.

For solid state fermentation, some work on conidia/spore production has been published (Tewari and Bhanu, 2004; Leland *et al*., 2005; Chen *et al.*, 2005; Zhao and Shamon, 2006). Among them, only one has addressed *Trichoderma* conidia production (Tewari and Bhanu, 2004) but did not emphasize on the standard spore concentration for the formulated product. General spore quantification using a haemocytometer will give total counts of both germinating/viable and non-germinating/dead spores. In this study, CFU counts data were also taken which gave a clearer picture on the viability of conidia in different substrates, unlike spore counting.

4.3.2 CFU counts

Formulations from mycelial propagules had the highest mean counts at 3.4 x10^6 CFU/g followed by the conidio-mycelial at 2.8 x10^6 CFU/g and the spore propagules at 2.6 x10^6 CFU/g (Table 7). According to Papavizas (1985), hyphae/mycelia are the main propagules of *Trichoderma* species and cannot withstand drying and lose viability when dehydrated. Chlamydospores and conidia have been used as the active ingredients in most *Trichoderma* species based products (Harman *et al.*, 1991; Jin *et al.*, 1991; Eyal *et al*., 1997). Micropropagules of *Trichoderma* species in the form of conidia are preferred over chlamydospores and mycelial biomass because of the viability and stability in field application (Amsellem *et al*., 1999).The formulations however, did not undergo any form of drying and this is a possible explanation as to why the mycelial formulations outperformed the spore and conidio-mycelial formulations in terms of CFU and spore counts.

Table 7: Mean CFU of *Trichoderma propagules* at different storage periods

Month	Mycelia	C.Mycelia	Spores
1	34.67b	31.84a	31.06ab
2	26.83d	25.42b	23.83cd
3	30.7c	26.31b	17.95d
4	35.86b	25.47b	25.19bc
5	41.56a	29.83ab	22.72cd
6	36.97b	30.28ab	33.45a
Means	34.43	28.19	25.7
C.V %	12.78	21.37	31.88
LSD (P<0.05)	3.59	4.92	6.69

**Means followed by the same letter are not significantly different at* $P \leq 0.05$

For the mycelial propagules, the lowest counts at 2.68 x 10^6 CFU/g were recorded at month 2 and these increased progressively in month 3 and month 4, peaking in month 5 at 4.15 x 10^6 CFU/g where the highest counts were recorded. Month 1, month 4 and month 6 had more or less the same level of counts, of over 3.0 x 10^6 CFU/g. For the conidio-mycelial formulations, the lowest counts were recorded in month 2 and month 4 at 2.5 x 10^6 CFU/g. Month 1 had the highest counts (3.18 x 10^6 CFU/g) followed by month 6 (3.03 x 10^6 CFU/g), month 5 (2.98 x 10^6

CFU/g) and month 3 (2.63 x 10^6 CFU/g) respectively. For the spore propagules, the lowest counts were recorded in month 3 (1.8 x 10^6 CFU/g), month 5 (2.27 x 10^6 CFU/g) and month 2 (2.38 x 10^6 CFU/g) respectively. The highest counts were recorded in month 6 (3.35 x 10^6 CFU/g), month 1 (3.11 x 10^6 CFU/g) and month 4 (2.52 x 10^6 CFU/g) respectively.

All kaolin formulations had the highest mean counts at 6 x10^6 CFU/g followed by vermiculite (4.2x10^6 CFU/g), talc (2.1 x10^6 CFU/g) and wheat bran (3.4 x10^5 CFU/g) respectively (Table 8). For kaolin formulations inoculated with mycelial propagules the highest counts of 5.45 x10^6 CFU/g were recorded followed by those with conidio-mycelial at 4.93 x10^6 CFU/g and spore propagules at 4.90 x10^6 CFU/g respectively. The vermiculite formulations inoculated with mycelial propagules had the highest counts of 4.7 x10^6 CFU/g followed by those with conidio-mycelial at 4.61 x10^6 CFU/g and spore propagules at 3.42 x10^6 CFU/g respectively. For talc formulations inoculated with mycelial propagules the highest counts of 3.06x10^6 CFU/g were recorded followed by those with spore at 1.7 x10^6 CFU/g and conidio-mycelial propagules with 1.5 x10^6 CFU/g, respectively. The wheat bran formulations inoculated with mycelial propagules had the highest counts of 5.57 x10^5 CFU/g followed by those with conidio-mycelial (2.39x10^5 CFU/g) and spore propagules (2.27 x10^5 CFU/g), respectively. This can therefore be interpreted that kaolin, vermiculite and wheat bran *T. harzianum* formulations will yield higher CFU counts when inoculated with mycelial propagules, compared to those bearing conidio-mycelial and spore propagules respectively. Likewise, talc *T. harzianum* formulations inoculated with mycelial propagules will yield higher CFU counts compared to those bearing spore and conidio-mycelial propagules respectively.

Table 8: Mean CFU of *Trichoderma* in different substrates

Substrate	Mycelia	C.Mycelia	Spores
Kaolin	54.59a	49.37a	48.98a
Vermiculite	46.96b	46.15a	34.2b
Talc	30.59b	14.85b	17.04c
w.bran	5.57d	2.39c	2.27d
C.V %	12.78	21.37	31.88
LSD (P<0.05)	2.93	4.02	5.46

**Means followed by the same letter are not significantly different at $P \leq 0.05$*

The quality of a microbial formulation is dependent on the propagule density in the biomass and its ability to survive in nature (Harman *et al.*, 1991). Production of adequate quantities of good quality inoculum is an essential component of the bio-control program. It is worth noting that there is a reduction of population in a non-formulated treatment. This can be reasonably assumed to be attributed to lack of protection against several factors, including sunlight, temperature, humidity, leaf surface exudates, and competitors (Jones and Burges, 1998). The above storage stability tests data, spore counts and colony counts shows that Kaolin formulations were most sustaining to viability of *T. harzianum* followed by vermiculite, talc and wheat bran respectively. Mycelial propagules were also found to be more stable compared to the conidio-mycelial and the spore propagules. Therefore, the stability of the *T. harzianum* formulations in storage differs with substrates and propagule types.

Significant ($P \leq 0.05$) differences were noted in the mean colony counts of *T. harzianum* in different substrates. Wheat bran formulations had the lowest counts of 3. 41 x 10^5 CFU/g after 6 months of storage. All the other formulations (Kaolin, vermiculite and talc) had over x10^6 CFU/g mean level of counts. Jayrajan and Angappan (1998) and Ramanujam *et al.* (1999) proposed a standard of over x10^6 level of counts of CFU/g for *T. harzianum* formulations. The results were also consistent with the findings of Prasad *et al.* (2002), who also reported a conidial formulation which retained a viable propagules count of above x10^6 CFU/g even after 180 days of storage. However, our formulations did not meet the Harman and Custis (2006) standard of 5x10^9 CFU/g level of surviving conidia.

4.4 Vegetative interaction of *Armillaria* species with *Trichoderma harzianum*

There was a significant ($P \leq 0.05$) difference on the effect of *Trichoderma* isolated monthly from different substrates on *Armillaria* colony diameters up to a period of six months. By the 6th day of double plating *T. harzianum* with *Armillaria*, most of the colonies had met (Plates1 and 2). Observations of the interaction between the opposing colonies were done for a period of 20 days using light microscopy. The fast growing *T. harzianum* isolates were able to grow over the *Armillaria* cultures by day 8 of the study and completely covering the plate by day 20. *Trichoderma* was isolated from all formulations at months 1, 2, 3, 4, 5, 6 indicating stability in the substrates. *Trichoderma* isolated at these different times were paired with *Armillaria* sp. to study the vegetative interaction.

Assays for *Armillaria* viability from paired cultures were done by attempts to isolate the fungus on day 20 of the study. Successful re-isolation was recorded as (+) and non-successful as (–). Re-isolation tests of *Armillaria* on the 20th day were all negative (-) for each of the months (Plate 1 and 2). Non successful isolation indicates efficacy of *Trichoderma* against *Armillaria* (Onsando and Waudo, 1994). Since the re-isolation tests were non- successful even after 6 months of storage of the formulations, it means that *Trichoderma* was still sufficiently antagonistic to *Armillaria* and still retained its virulence, indicating stability in storage. The negative viability of *Armillaria* can therefore be attributed to the penetration of the melanised outer tissues of the rhizomorphs by *Trichoderma* which kill the *Armillaria* hyphae by coiling and direct penetration, only visible through Scanning Elecron Microscopy (Dumas and Boyonoski, 1992). After one week, the rhizomorphs infected with *Trichoderma* are devoid of hyphae. This causes leakage of the cytoplasm from the host cells resulting in their emptiness. The host cytoplasm is, apparently, utilized by the pathogen, which is capable of degrading proteins and lipids.

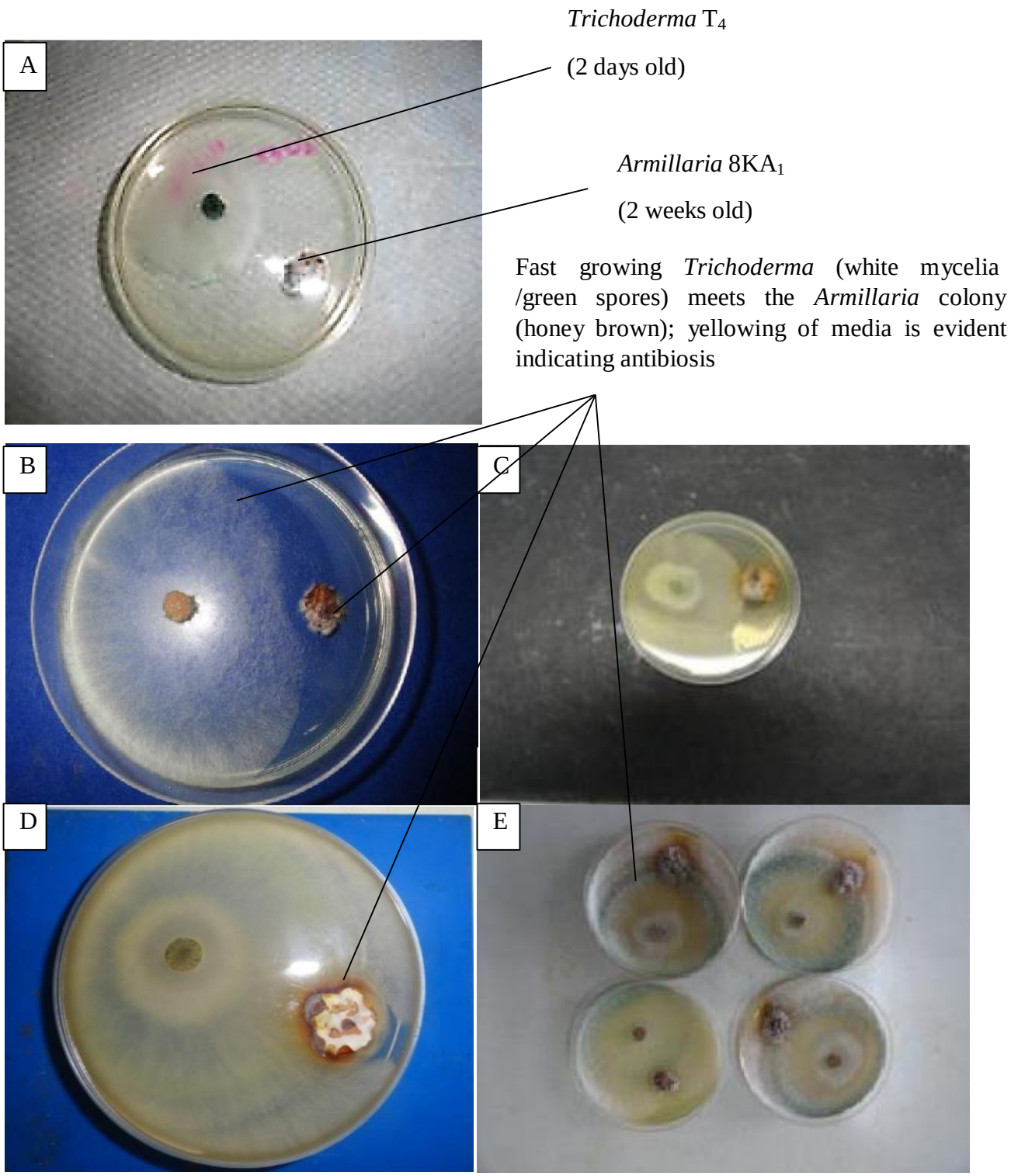

Plate 1: Vegetative interaction between *Trichoderma* and *Armillaria* after varying incubation periods; A) Day 2, B) Day 4, C) Day 6, D) Day 8, E) Day 10

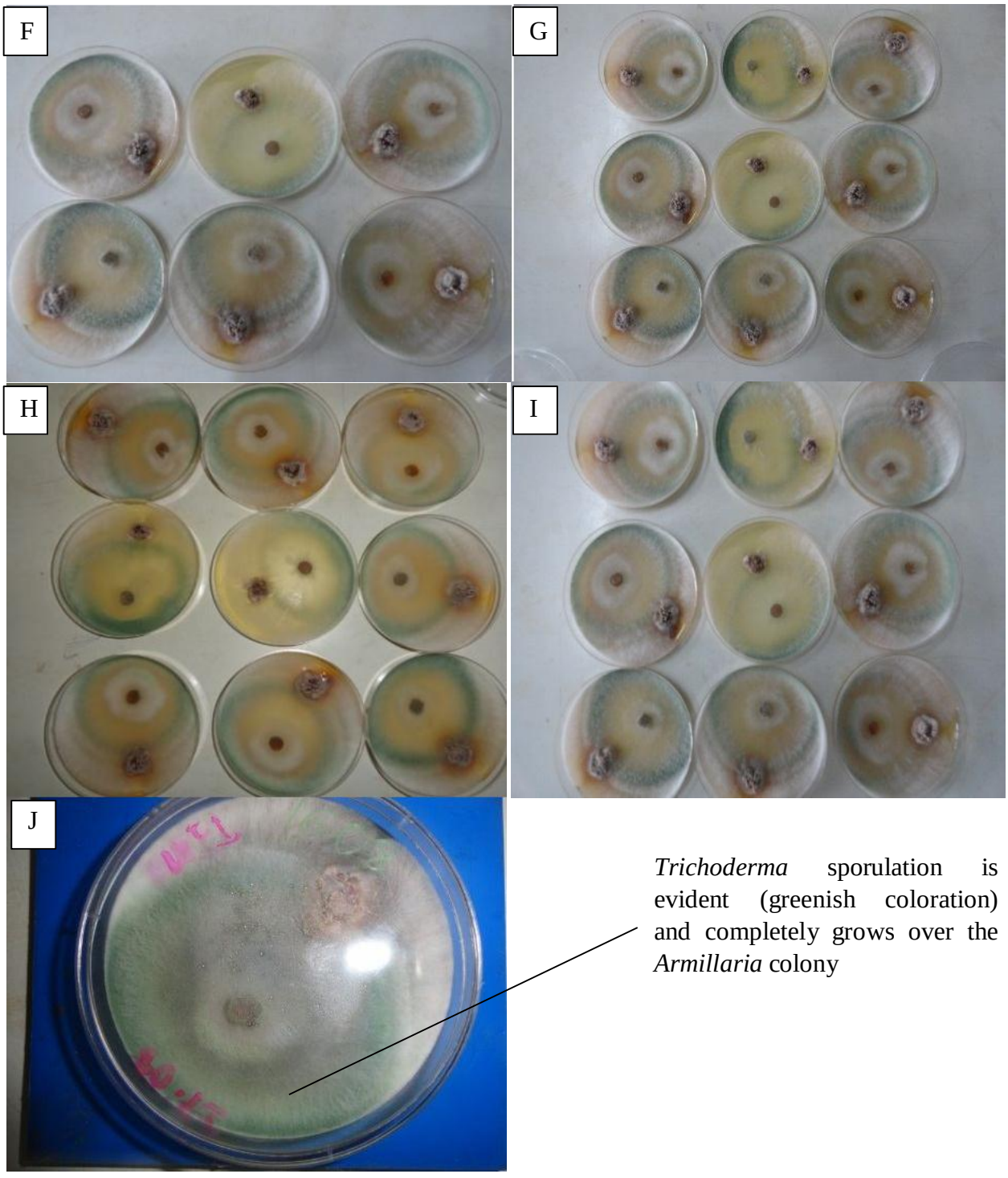

Plate 2: Vegetative interaction between *Trichoderma* and *Armillaria* after varying incubation periods; F) Day 12, G) Day14, H) Day 16, I) Day 18, J) Day 20

Results of the current study indicate that the *T. harzianum* isolate could be formulated and remain stable without losing capacity to antagonize *Armillaria* sp. at least for six months. The plates that had the least diameters of *Armillaria* sp. were considered to be exhibiting the highest inhibition due to antagonism by *T. harzianum*. Prasad *et al* (2002) also reported a conidial formulation produced by solid state formulation which retained viable propagules even after 180 days (6months) of storage. Adequate shelf life and abundant viable propagules are marketing qualities desirable for formulations with agricultural potential (Churchill, 1982).

In the six month period, the inhibition of *Armillaria* by *T. harzianum* varied significantly ($P \leq 0.05$) from the controls (Table 9). In month 1 and month 2, kaolin-*T. harzianum* formulations had the highest inhibition towards *Armillaria,* followed by the vermiculite, talc and wheat bran formulations. In month 3, vermiculite -*T. harzianum* formulations had the highest inhibition towards *Armillaria,* followed by talc, kaolin and wheat bran respectively. In month 4, kaolin-*T. harzianum* formulations had the highest inhibition towards *Armillaria,* followed by the talc, wheat bran and vermiculite formulations respectively. In month 5, talc-*T. harzianum* formulations had the highest inhibition towards *Armillaria,* followed by the vermiculite, wheat bran and kaolin formulations respectively. In month 6, talc-*T. harzianum* formulations had the highest inhibition towards *Armillaria*, followed by the wheat bran, kaolin and vermiculite formulations respectively.

The inhibition of *Armillaria* by *Trichoderma* studied in the 6 month period was not consistent. In all the formulations there were peaks of decreased inhibition and depressions of increased inhibition over the 6 month period. This means that regardless of the storage period, *T. harzianum* did not lose its capacity to inhibit *Armillaria*. Shelf life of the formulated product of a bio-control agent plays a significant role in successful marketing (Ramanujam *et al.,* 1999; Prasad and Rangeshwaran 2000).On a business angle, the *T. harzianum* formulations have 6-months flexibility as the ability to inhibit *Armillaria* is not impaired. A buyer can buy a formulation stored for 6 months and still achieve the same efficacy if he had bought a formulation stored only for 1 month. The viability and efficacy of the propagules after 6 months confirms the stability in storage of the formulations, which is an essential quality in marketing (Churchill, 1982).

Judging by the means as seen in (Table 9), Kaolin formulations had the highest inhibition rate (15.17 mm), followed by vermiculite (15.41 mm), talc (15.41 mm) and wheat bran (15.53 mm)

formulations respectively. The controls (*Armillaria* alone) had the largest diameters in all the months averaging 16.47 mm. It is concluded that the substrate used in formulating *T. harzianum* ultimately affects its efficacy, at least demonstrable *in vitro*, against *Armillaria* species.

Table 9: Mean colony diameters of *Armillaria* sp. double- plated with *Trichoderma harzianum*

	Month 1 Diameter mm	Month 2 Diameter mm	Month 3 Diameter mm	Month 4 Diameter mm	Month 5 Diameter mm	Month 6 Diameter mm
Kaolin	15.02d	14.53c	15.34c	14.92c	15.96c	15.3c
Vermiculite	15.54c	15.22b	14.77b	15.63b	15.44c	15.87c
Talc	15.82bc	15.39b	15.22b	15.36b	15.43bc	15.21c
W.bran	15.88b	15.22b	15.64b	15.38b	15.77ab	15.29b
Control	16.26a	16.65a	16.44a	16.81a	16.14a	16.51a
CV %	3.79	4.75	5.27	3.43	4.31	3.8
LSD(P<0.05)	0.3	0.37	0.42	0.27	0.35	0.3

**Means followed by the same letter are not significantly different at $P \leq 0.05$*

Armillaria is generally a slow growing fungus compared to *Trichoderma* which grows very fast. (Plate 1 and 2). The fast and vigorous growing attributes of *Trichoderma* enables it fungus, to colonize the surface of *Armillaria* colonies, similarly to other pathogenic fungi such as *Sclerotinia sclerotiorum* (Tu, 1980), *Rhizoctonia solani* (Elad *et al.*, 1983), *Rosellinia necatrix* and *Agaricus bisporus* (Cook and Baker, 1983). An ideal antagonist should produce more inoculum than the pathogen; resist, escape, or tolerate other antagonists; germinate and grow rapidly; and invade and occupy organic substrates (Raziq, 2000). *Armillaria* species have some unique abilities including production of antibiotic compounds with considerable inhibition against fungi and bacteria, and production of rhizomorphs, as highly differentiated organ with a special structure that enables the fungus to resist antagonistic effects of other organisms.

It was only possible to see the *Trichoderma* colonies growing over the *Armillaria* sp. and yellowing of the medium (Plates 1 and 2). *Trichoderma* species produce antibiotics, which change MEA medium to yellowish (Papavizas, 1985; Raziq, 2000). Some of the interaction mechanisms of the *Trichoderma* hyphae with *A. mellea* rhizomorphs only seen through Scanning

Electron Microscopy (SEM) include; pene tration of antagonist hyphae in rhizomorphs and disintegration of rhizomorph content (Dumas and Boyonoski, 1992). *Trichoderma* species produce various enzymes such as cellulases, proteases, chitinases, cellobiases, exo- and endo- glucanases. These compounds digest the components of the hyphal wall and the melanin content in outer cortex of rhizomorphs in *Armillaria* rhizomorphs, for direct penetration, degradation and lysis. The antagonistic effects of *Trichoderma* arises from various attributes, such as tolerance to changes in environmental conditions (Munnecke *et al.*,1981), ability to degrade various organic substances in soil, resistance to inhibitors and metabolic versatility, production of various toxic compounds, antibiotics and enzymes (Vandriesche and Bellows, 1996; Howell, 2003). These abilities permit *Trichoderma* to compete, mycoparasite and to antagonize many fungi such as species of *Sclerotinia, Rhizoctonia, Rosellinia* and *Botrytis* (Cook and Baker 1983; Elad *et al.,* 1983; Elad and Kapat, 1999). Studies on the effect of volatile metabolites showed significant control ability and inhibitory effect of *Trichoderma* isolates on mycelial growth of *Armillaria*, (Mohammadi and Danesh, 2000). The major volatile compound in *Trichoderma* species is 6-pentyl-α-pyrone (6-PAP), but *T. virens* produces a different spectrum of metabolites. All strains of this species seem to produce viridin and viridol, but some strains also produce gliovirin and heptelidic acid, whereas others produce gliotoxin (Howell *et al.,* 1993).

CHAPTER FIVE
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

1. Formulation of *Trichoderma harzianum* with optimal cell viability and biological activity under prolonged conditions of storage was successfully achieved. All the substrates tested were proven to be compatible to *T. harzianum* in terms of dry weights, spore counts and CFU counts after 1 month of storage.
2. After 6 months of storage, kaolin showed the highest growth of *T. harzianum* followed by vermiculite, talc and wheat bran formulations respectively. Formulations with mycelial propagules had the highest mean counts followed by the conidio-mycelial and the spore propagules. However, these propagules performed differently in different substrates. Thus the stability of the *T. harzianum* formulations in storage differs with substrates and propagule types.
3. Vegetative interaction data revealed that the *T. harzianum* isolate (T_4) could be formulated and remain stable without losing capacity to antagonize *Armillaria* sp. at least for six months. Kaolin formulations had the highest mean inhibition towards *Armillaria* followed by vermiculite, talc and wheat bran, respectively. Thus the substrate used in formulating *T. harzianum* ultimately affects its efficacy, *in vitro*, against *Armillaria* species.

5.2 Recommendations

From the results obtained, the following should be carried out so as to acquire a wholesome *Trichoderma* formulation that can retain viability under prolonged periods of storage:

1. Three substrates (Kaolin, vermiculite and talc) proven to support optimum cell viability and biological activity of *T. harzianum* after 6 months of storage should be exploited for *Trichoderma* formulation.
2. Other locally available and inexpensive inert substrates such as clay and unrefined soapstone (used to make talc) can also be tested for *Trichoderma* formulation.
3. Further work should be done on storage stability tests over 6 months with consideration of other parameters such as temperature, lighting, moisture, and pH among others.
4. Formulated products should be exploited for tea cutting/root treatment in the nursery, soil treatment prior to planting /in *Armillaria* affected fields and also for compost enrichment.

5. Field persistence trials and suitability tests on the formulations should be done for all weather conditions. This should entail applications of the products as drench and as dry applications at different rates in comparison with other products in the market.
6. Solid state production systems should be scaled up to industry production levels.
7. Large scale demonstration of the bio-control technology should be done in farmers' fields.

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