CHAPTER ONE

INTRODUCTION

1.1 Background information

Tea (*Camellia sinensis* (L) Kuntz, is an evergreen plant that grows mainly in tropical and subtropical climate (Wealth of India, 1950). Tea is Kenya's major foreign exchange earner and a source of 17 to 20% of the total Kenya's export revenue (Mekonnen *et al*., 2014). About 314,875 farmers depend on tea for their livelihood. In addition to providing an income for about 3 million Kenyans, tea planting also contributes enormously towards the environmental sink hence checking global warming (Anon, 2011a, Gesimba *et al*.,2005).

Despite the immense efforts to manage tea cultivation by Kenya Tea Development Authority (KTDA), there are several challenges being experienced by farmers, ranging from climatic changes, new harvesting technologies and of paramount importance are pests and diseases. Pests and diseases pose as the biggest challenge especially in losses incurred by farmers. The major diseases that affect tea in Kenya include *Armilleria ssp* root root, and *Hypoxylon serpens* wood rot (Anon, 2006).

Some of the devastating biotic stresses of tea include tea weevils *(Entypotrachelus meyeri*), which comprise of 27 species. The most prevalent in Kenya are tea root weevil (*Apertmentus brunneus*) Nematocerus weevil (*Nematocerus sulcalus*), Systates weevil (*Sytates sp*.) Kangaita/Kamari weevils (*Entypotrachelus meyeri*) (*Micars / Kolbe*) and Nyambene weevils (*Sprigodes mixtous*) (Anon, 2006).

Adult weevils damage tea by defoliating nursery, mature and newly established tea orchards. Severe damage is caused when they attack newly established areas and in nurseries (Benjamin *et al.,* 1968; Chen *et al.,* 2007).

Kimari/Kangaita weevils have been documented to occur throughout the tea growing areas of Kenya. Occasional isolated outbreaks occur causing variable level of damage (Sudoi *et al.,* 1999). They are polyphagus and feed on over 14 plant families (Benjamin *et al.,* 1968). Presently, management strategy against tea weevils includes spraying with Karate® (lambdacyhalothrin). Hand picking is also applied where the pest population is low (Anon, 1986), while biological control offers a great potential against the tea weevils. For instance, Sudoi *et al.* (1999), isolated entomopathogenic fungus, *Hirsutella* species which gave high mortality rate on the Kangaita weevil. Preliminary investigation at Tea Research Foundation of Kenya (TRFK) has indicated that locally isolated *Beauveria bassiana* using *Galleria* larva moth, indicated good prospects in the control of the tea weevil (*E. meyeri*) and the tobacco cricket (Anon, 2001, 2003 and 2006). Entomopathogenic fungi are interesting microbial agents for control of insects' pest (Kouassi, 2003). *Beauveria bassiana* have successfully been used to control several pests such as: -Tobacco spider mites (*Tetranychus evensi*) Pritchard (*Acinina tetraychidae)* infesting tomatoes (Wekesa, 2006). This fungus has shown success also in management of diamond back moth *Lepidiptera plutellidae* (Vandenberg *et al.,* 1998) and potato beetle *Leptimotarsa decemlinaeta*, (Wraight *et al.,* 2000). Trudel *et al*., (2007) suggested that *B. bassiana* had a potential to persist in the environment and for horizontal transmission between weevils. He proposed more investigations on its ability to control populations of white pine weevil *Pissodes strobi*.

Beauveria bassiana has proved to be competitive with chemical insectides for protection of forests and farms against pests (Zhang *et al.,* 1996). However, no proven documented report has been published on the fungal isolates variation and potential on tea weevils in Kenya.

In recent years, consumers of tea have more interest in food safety and like to buy chemical free tea. To reduce chemical use in tea cultivation, it is important to develop new disease and pests control methods that have broad and long-lasting control mechanisms that are environmentally friendly (Seneshaw *et al*., 2003).

The increased use of conventional chemical pesticides over the years has not only contributed to an increase in food production, but also has resulted in adverse effects on the environment and non-target organisms. In view of these side effects, the necessity for sustainable crop production through eco-friendly pest management technique is being largely felt in the recent times. Of the several microbial pathogens *viz.*, bacteria, fungi, viruses, protozoans and entomopathogenic nematodes reported, only a few have been studied systematically for their usefulness (Bharathi, 2005). A careful evaluation of these beneficial pathogens can lead to gainful exploitation in microbial control programs (Burges, 1998).

This research was therefore, designed to investigate the potential and effect of *B. bassiana* on tea weevils (*Entypotrachelus meyeri*), to determine the pathogenicity of the fungus and to determine the shelf longevity of the fungus harvested using different methods.

1.2 Statement of the problem:

Tea weevils contribute to reduced production through reduction of tea leaf area by feeding on the pluckable two leaves and the bud, hence reduced performance of tea through less area for photosynthesis. In addition, the tea grower incurs losses in terms of time and finances in the process of carrying out the management of tea weevils through measures instituted to control their menace. The need for green tea devoid of agrochemicals and insecticide residue for the sake of international requirement to buy our tea has posed a challenge through the use of pesticides in tea weevils' management.

Enthomopathogenic fungi have been used s*u*ccessfully to control several pests in plantations such as the brown leafhopper on rice (Aguda *et al*., 1988), *Leucenia psylid* on *Leucaenia* (Sajap, 1993) and Banana root weevil (Chen *et al*., 2007 and Lopes *et al*., 2013). *Beauveria bassiana* has been used successfully to control several pests such as: Tobacco spider mites (*Tetranychus evens*i (Tetraychidae) infesting tomatoes (Wekesa *et al*., 2006). *Beauveria bassiana* is a fungus that is found naturally in soils throughout the world and acts as a parasite on various insect species, causing white muscardine disease. An imported (Botanigard) formulation of *Beauveria bassiana* is being used in the Kenya as a biological insecticide to control a number of sucking insects including thrips, aphids and whiteflies on French beans and snow peas (PCPB, 2011). This fungus has shown success also in management of diamond back moth *Lepidiptera plutellidae* (Vandenberg *et al*., 1998) and potato beetle *Leptimotarsa decemlinaeta*, (Wraight *et al.*, 2000).

Trudel *et al.,* (2007) suggested that *B. bassiana* had a potential to persist in the environment and for horizontal transmission between weevils. He proposed more investigations on its ability to control populations of white pine weevil *Pissodes strobi.*

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1.3 Justification of the study

The most used management strategy against tea weevils is based solely on a cultural practice of hand picking when weevils are in low population and spraying with chemicals such as Karate that contains synthetic pyrethriod, lambdacyhalothrin as the active ingredient. Because environmental and regulatory concerns, research on developing alternative control strategies is warranted. It is therefore, prudent that studies for the development of alternative effective and safer control practices are developed to avoid dependency on only a few practices. Biological control that this study aims to initiate will offer better promising strategies in the future management of tea weevils. The strategy has no chemical pesticides residues levels, thereby ensuring safe and sustainable environment and enhancing consumer acceptability and confidence in Kenyan tea that is grown pesticide free. In addition, other produce/crops susceptible to weevil insects can benefits from these approaches.

The tea weevils mostly defoliate nursery stock, mature and newly established tea including the commercially harvestable shoot (Benjamin *et al.,* 1974; Chen *et al.,* 2007).

Several insect pests have been recorded on tea that affect production and prominent among which is the tea weevil. Decline in tea production has been attributed to pests such as the mites, the mosquito bug and tea weevils. Studies on seasonal distribution, on the tea weevils in Kenya are few. Therefore, studies for understanding the population dynamics of the tea weevils and developing strategies for their management need to be undertaken in order to minimize losses to tea (Dutcher *et al.,* 1985).

1.4 General Objective

To evaluate the potential of *Beauveria bassiana* as a biological control against tea weevils (*Entypotrachelus meyeri***).**

1.5 Specific Objectives

1) To determine the efficacy of *B. bassiana* isolates on tea weevil (*Entypotrachelus meyeri*).
2) To determine the biological characteristics of the *Beauveria bassiana* isolates in relation to their virulence on tea weevils.
3) To assess the efficacy of *B. bassiana* concentrations on tea weevils.

1.6 Hypothesis

1) H_{01}: There is no significant effect of *B. bassiana* isolates on tea weevil (*Entypotrachelus meyeri*).
2) H_{02}: There is no significant association between the biological characteristics of the *B bassiana* isolates and their virulence on tea weevils.

3). H_{03}: There is no significant differences between *B. bassiana* isolates concentrations on tea weevils.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin, evolution and taxonomy of tea

2.1.1 Origin and evolution

Tea is native to East and South Asia and probably originated around the point of confluence of the lands of northeast India, north Burma, southwest China, and Tibet (Rolfe *et al.*, 2003). Although tales exist in regards to the beginnings of tea being used as a beverage, no one is sure of its exact origins. The usage of tea as a beverage was first recorded in China, with the earliest records of tea consumption with records dating back to the 10th century BC (Rolf*e et al.*, 2003). It was already a common drink during Qin Dynasty (around 200 BC) and became widely popular during Tang Dynasty, when it was spread to Korea and Japan. Trade of tea by the Chinese to Western nations in the 19th century spread tea and the tea plant to numerous locations around the world (Rolfe *et al.*, 2003).

According to (Rolfe *et al*., 2003), tea was imported to Europe during the Portuguese expansion of the 16th century, at which time it was termed chá. In 1750, tea experts travelled from China to the Azores Islands, and planted tea, along with jasmines and mallows, to give the tea aroma and distinction. Both green tea and black tea continue to grow in the islands that are the main supplier to continental Portugal. Catherine of Braganza, wife of Charles II, took the tea habit to Great Britain around 1660, but it was not until the 19th century Britain that tea became as widely consumed as it is today. In

Ireland, tea had become an everyday beverage for all levels of society by the late 19th century, but it was first consumed as a luxury item on special occasion such as religious festivals, wakes, and domestic work gatherings such as quilting. Tea was first introduced in Africa in 1687 at Cape of Good Hope but in Eastern Africa it started in the 1920s (Wealth of India,1950). Monfreda *et al*., (2008) reports show that in 2003, world tea production was 3.21 million tons annually and in 2008, world tea production reached over 4.73 million tones. The largest producers of tea are the People's Republic of China, India, Kenya, Sri Lanka, and Turkey. Data generated by the Food and Agriculture Organization (FAO) of the United Nations as of January 2010 show that the world produced 3,833,750 tons of tea with Kenya producing 345,800 tones (Oya, 2012 ; FAO,2013).

2.1.2 Taxonomy

The genus *Camellia* of the family *Theaceae* consists of 82 species but *Camellia sinensis* (L) Kuntz is the most economically important. The two varieties of tea are China tea *Camellia sinensis var sinensis* and Assam tea *C. sinensis var assamica.* However, there are other hybrids, agrotypes and ecotypes of tea. When growing naturally Asssam tea is evergreen tree, from10 to 15m in height, with a large crown. The trunk is straight and the bark is brownish with grey spot. Tea consists of a tap-root, well-developed secondary roots Gesimba *et al*., 2005). .

The leaves are alternate, with short petioles, pubescent to glabrous, tender to coriaceous. The lamina is elliptic to lanceolate, 7-22cm long and 3-8cm wide. The buds, the young shoots and young leaves are often downy. Axillary flower with pedicel solitary or in

groups of 2 to 4. Calyx usually consists of 5 green sepals, which are herbaceous to slightly membranous. The white corolla consists of 6 to 8 petals joined to base of the androceium. The stamens are numerous and the anthers are yellow (Gesimba *et al*., 2005). Gynoceium with its superior ovary is very pubescent. Pollination is carried by insects and by wind. The fruit, about 2-3 cm in diameter, is loculicidal dehiscent capsule, with three loculii, containing one to three seeds. At maturity almost 12 months after fertilization, the capsule splits open and frees the seeds with a thick cotyledon that has high content of oil (Wealth of India, 1950).

The China tea is bushy growing only as high as 6m. The dark green leaves are smaller and deciduous (Rofle, 2003). The flowers are solitary Chinese *Camellia sinensis* is native to mainland China, South and Southeast Asia, but it is today cultivated across the world in tropical and subtropical regions. It is an evergreen shrub or small tree that is usually trimmed to below two meters (six feet) when cultivated for its leaves. It has a strong taproot. The flowers are yellow-white, 2.5–4 cm in diameter, with 7 to 8 petals (Rofle, 2003).

Camellia sinensis plant, has a cross-section of the flower (lower left) and seeds (lower right).The leaves are 4–15 cm long and 2–5 cm broad. Fresh leaves contain about 4% caffeine. The young, light green leaves are preferably harvested for tea production; they have short white hairs on the underside. Older leaves are deeper green (Anon 2011a). Different leaf ages produce differing tea qualities, since their chemical compositions are different. Usually, the tip (bud) and the first two to three leaves are harvested for processing. This hand picking is repeated every one to two weeks. Tea is made from the

young leaves and the unopened leaf buds of tea plant, *Carmelia sinensis* (L) o. Kuntz (Anon 2011a). The leaf is rich in the alkaloid caffeine which contributes to 4 % of the dry matter in the young leaf. The leaf also consists of polyphenols which contribute to 1/3 of dry matter of the young leaf (Harler, 1956). These substances are oxidized through controlled oxidation normally called fermentation and then condensed to form bodies having tanning products.

2.1.3 Ecology

The best tea is grown a relatively cool climate at high altitude up to 2400m but yield will be affected, below 1000m the quality of tea is far less appreciated. Rainfall is crucial for crop success and should be plentiful with a minimum of 1300-1400mm evenly distributed within the year. During dry seasons a minimum of 50mm per month. Air temperature plays an important role in the growth and yield of tea (Gesimba *et al*., 2005).. Temperatures of between 12^0-13^0 C and above 30^0 C hinder shoot development. Soil temperatures also affect the shoot growth; the hours of sunlight are also important and should remain at least 5hours a day (Raemaeker, 2001).

Tea growth requires a relative humidity between 70-90%. Violent wind harms tea as they lower the moisture degree of air. Tea thrives well in acid soils which are deep at least 2m, well structured, and well drained with high levels of minerals and well developed humus containing horizon. Most tea soils have a pH of 4.0 to 5.0 (Raemaeker, 2001).

2.1.4 History and Tea Growing Regions in Kenya

Tea was discovered more than 2000 years ago in China, but is naturally distributed in the whole of Asian Monsoon regions (Barnejee, 1992) and was first introduced into Kenya by a settler farmer G.W Caine in early 1903 (Gesimba *et al*., 2005). Seeds were obtained randomly through open pollinated natural hybrids between the Assam and China varieties from Assam region of North East India (Barnejee, 1992). The introduced Manipuri hybrid seed was planted in Limuru in 1904. The raised seedlings become the source of seed for future planting hence the first commercial plantation was not established until 1924 (Watts, 1999). The earlier industry was dominated by colonial settler who was the only ones with access to seed. In 1960, Special Crops Development Authority (SCDA) was established to promote the cultivation of the crop within the small-holder agricultural sub-sector (Watts, 1999).

This evolved later to become the Kenya Tea Development Authority (KTDA) whose main function was to facilitate the expansion of tea cultivation into the native lands.

The Great Rift Valley divides the country into two tea growing regions almost asymmetrically and defines the two growing regions (Gesimba *et al*., 2005. To the East of Rift are tea areas within and around the slopes Aberdare Highlands that's in Kiambu,Limuru, Thika Nyeri, Maragwa and Othaya, areas at the foot of Mt. Kenya mainly Kerugoya , Embu and Meru (Nkubu, Chuka, Chogoria among other areas) tea growing region and around/within the Nyambene hills, areas between Mikinduri and Maua (Gesimba *et al*., 2005). The West of the Rift is defined by the Mau Escarpment and the areas are the Nandi, Kericho highlands, Mt Elgon Region (Kapsakwony and Kapsara

near Kitale) and Kisii highlands. Tea grows on the slopes of the highlands within the altitude of between 1500 and 2700 metres above sea level (Gesimba *et al*., 2005.

Tea is mainly grown in the lower Highland sub-zone one (LH1) and a small tea growing area is on the Coffee-tea zone, upper midland one (UM1) which are normally related to be marginal area for lucrative tea growing. They include the area of upper Meru that is the Chuka , Nkubu Chogoria area , the Tombe Ogembo , Kiamokama area in Kisii and Chavakali/vihiga-Mudete KTDA catchment of Kakamega (Anon, 2003).

2.1.5 Importance of tea

The seeds of *Camellia sinensis* and *Camellia oleifera* can be pressed to yield tea oil, a sweetish seasoning and cooking oil that should not be confused with tea tree oil, an essential oil that is used for medical and cosmetic purposes, and originates from the leaves of a different plant. Kenyan tea has been found to contain a lot of antioxidants (Anon, 2011a). Tea polyphenols lower heart ailments and tumors; the catechin cream from tea can prevent skin cancer. These catechins also prevent diabetes by inhibiting glucose uptake by inhibiting the sodium dependent glucose transporter mechanism. The antiviral and antibacterial proportions of tea reduce infections of respiratory tract and the lungs. It has been reported that 8-12 cups of tea taken daily can reduce pulmonary diseases and raise the metabolic rates (Jain *et al*., 2006). Tea leaves contain more than 700 chemicals, among which the compounds closely related to human health are flavanoides, amino acids, vitamins (C, E and K), caffeine and polysaccharides Dona *et al.,* (2003). Biological studies have shown that the quality determinants of tea, especially catechins and phenolic acids are associated with medicinal properties such as antidiabetic, antimicrobial, anticancer, antioxidants due to presence of catechins and

antiaging activities (Khan *et al*., 2007). Tea drinking is associated with cell mediated immune responses of human body and improves growth of beneficial microflora in the intestine (Chen, 1999). It also imparts immunity against intestinal disorders, protects cell membranes from oxidative damages, prevents dental caries due to presence of fluorine, normalizes blood pressure, prevents coronary heart disease due to lipid depressing activity, reduces blood glucose activity and normalizes diabetes (Chen, 1999). Tea possesses germicidal and germistatic activities against various gram positive and gram negative bacteria in human (Chen, 1999). Several epidemiological studies as well as studies in animal model have shown that green tea can confer protection against various cancers such as those of the skin, breast, prostate and lung (Yang *et. al*., 2002). It has also been shown to be hypocholesterolemiant (Yang *et al.,* 2002) to prevent the development of atherosclerotic plaques, antibacterial, anti-inflammatory and anti-HIV activities (Yang *et al.,* 2002, Dona *et al.,* 2003 and Khan *et al*., 2007). Among age associated pathologies and neurodegenerative diseases, green tea has been shown to confer significant protection against Parkinson's disease, Alzheimer's disease and Ischemic damage. Tea plants thus prove to be a future potential as an important raw material for pharmaceutical industry.

Moreover, tea drinking has recently proven to be associated with cell-mediated immune function of the human body. Tea plays an important role in improving beneficial intestinal micro flora, as well as providing immunity against intestinal disorders and in protecting cell membranes from oxidative damage. Tea also prevents dental caries due to the presence of fluorine (Graham, 1992). The role of tea is well established in normalizing blood pressure, lipid depressing activity, prevention of coronary heart diseases and diabetes by reducing the blood-glucose activity. Tea also possesses

germicidal and germ static activities against various gram-positive and gram negative human pathogenic bacteria. Both green and black tea infusions contain a number of antioxidants, mainly catechins that have anti-carcinogenic, anti-mutagenic and anti-tumoric properties (Graham, 1992, Ferrazzano, *et al*., 2009).

Economic importance of *Camellia* is primarily due to its use as a beverage. It has received much attention for its aroma, pleasant taste and numerous medicinal benefits and has been socially and habitually consumed by people since 3000 B.C. (Lin *et al.,* 2003). Tea is a very important source of revenue for the tea producing countries; in Kenya it contributes about 26% of export earnings and 4% of the Gross Domestic Product (GDP) (Wachira *et al.,* 2004). At the house-hold level tea plant is so-called the crop of the poor, especially in the tropical mountainous areas, because even with the minimal investment required, tea can be planted and harvested weekly or each ten-day period on hard and sloping soils where the other food crops or cash crops could not grow effectively (Vo, 2007). In addition, planting tea plant on the remote mountainous areas is considered as an effective method to cover the bare sloping lands, thus provide a means of soil conservation. Cultivation of tea in the remote areas also provides many jobs to rural communities and certainly contributes to the development of local infrastructure (Owuor *et al*., 2010). Tea is served as a daily drink for two third of the world population (Muktar *et al.,* 2000). Drinking tea became a special culture ceremony in many countries (such as Japan, China and Vietnam). Tea also cannot be absent in many cultural events such as traditional New Year and wedding ceremony. Other than their use as a beverage, green leaves are also used as vegetables such as 'leppet tea' in Burma and 'Meing tea' in Thailand (Vo, 2007). Tea seed oil is used as lubricant, yet extraction from seeds is not

economical. As cakes of tea seed contain saponins, their value as fertilizer is poor and are unfit for animal feed due to low nitrogen, phosphorous and potassium content. However, they can be used successfully in the manufacture of nematicides (Vo, 2007).

2.1.6 Diseases and pests of tea

Like other monocultures, tea is subject to diseases and pests. Diseases affect the aerial parts of tea but especially the roots of tea, which is particularly susceptible to root rots, in Kenya the common serious diseases of tea include *Armillaria spp* root rot and *Hypoxylon serpens* wood rot diseases. However, there are also some other minor diseases in tea like stem canker or basal rot (*Phomopsis theae*), grey blight *(Pestalotia spp*.) and Brown leaf spot. Other uncommon diseases have been reported such as nematode damage, *Cylindrocarpon* root rot, crown gall and damping off (TRFK, 2011 unpublished report). Among all problems that affect tea, tea weevils have posed as a big problem to farmers in Kenya (Anon, 2006).

2.1.7 The Entomopathogen *Beauveria bassiana*.

Entomopathogenic fungi were among the first organisms to be used for the biological control of pests. More than 700 species of fungi from around 90 genera are pathogenic to insects. Most are found within the deuteromycetes and entomophthorales. Some insect-pathogenic fungi have restricted host ranges, for example, *Aschersonia aleyrodis* infects only scale insects and whiteflies, while other fungal species have a wide host range, with individual isolates being more specific, for example, *Metarhizium anisopliae* and *Beauveria bassiana* (Florez, 2002). Fungal species such as *M. anisopliae* and *B. bassiana* are well characterized in respect to pathogenicity to several insects and they have been used as agents for the biological control of agriculture pests worldwide. In

Colombia, about 11 companies offer at least 16 products based on the entomopathogenic fungi *B. bassiana*. These products are used not only in the coffee crop but also in other crops such as cabbage, corn, bean, tomato, potato. They are also used to treat public disease vectors (such as flies and mosquitoes) (Florez, 2002).

2.1.8 The infection process.

In contrast to bacteria and viruses that pass through the gut wall from contaminated food, fungi have a unique mode of infection. They reach the hamocoel through the cuticle or possibly through the mouth parts. Ingested fungal spores do not germinate in the gut and are voided in the faeces. The death of the insect results from a combination of factors: mechanical damage resulting from tissue invasion, depletion of nutrient resources and toxicosis (Hong, 2003)

2.1.9 Adhesion and germination of conidia

Attachment of a fungal spore to the cuticle surface of a susceptible host represents the initial event in the establishment of mycosis. For most entomopathogenic fungi host location is a random event and attachment is a passive process with the aid of wind or water. It has been found that dry spores *of B. bassiana* possess an outer layer composed of interwoven fascicles of hydrophobic rodlets. This rodlet layer appears to be unique to the conidial stage and has not been detected on the vegetative cells. The adhesion of dry spores to the cuticle has been suggested to be due to non-specific hydrophobic forces exerted by the rodlets (Boucias *et al.,* 1988). In addition, lectins, a kind of carbohydrate binding glycoproteins, have been detected on the conidial surface of *B. bassiana*. It has been also suggested that lectins could be involved in binding between conidia and the

insect cuticle. The exact mechanisms responsible for the interaction between fungal spores and the cuticle remain to be determined (Latge *et al.*, 1988).

After the pathogen reaches and adheres to the host surface, it proceeds with rapid germination and growth that are profoundly influenced by the availability of nutrients, oxygen, water, as well as pH, temperature, and by the effects of toxic host-surface compound. Generally, fungi with a broad host range germinate in culture in response to a wide range of nonspecific carbon and nitrogen sources. Entomopathogenic fungi with restricted host range appear to have more specific requirements for germination (Leger *et al*., 1989).

2.1.10 Formation of an infection structure

Entomopathogenic fungi invade their hosts by direct penetration of the host cuticle. The cuticle has two layers, the outer epicuticle and the procuticle. The epicuticle is a very complex thin structure that lacks chitin but contains phenol-stabilized proteins and is covered by a waxy layer containing fatty acids, lipids and sterols (Hackmann, 1984). The procuticle forms the majority of the cuticle and contains chitin fibrils embedded into a protein matrix together with lipids and quinones (Neville, 1984). Protein may account for up to 70% of the cuticle. In many areas of the cuticle the chitin is organized helically giving rise to a laminate structure.

In common with many entomopathogenic fungi, *B. bassiana* conidia germinate on the host surface and differentiate an infection structure termed appressorium. The appressorium represents an adaptation for concentrating physical and chemical energy over a very small area so that ingress may be achieved efficiently. Thus, formation of the

appressorium plays a pivotal role in establishing a pathogenic interaction with the host. Appressorium formation may be influenced by host surface topography and biochemical investigations indicate the involvement of the intracellular second messengers Ca2+ and cyclic AMP (cAMP) in appressorium formation (Leger *et al*., 1991).

2.1.11 Penetration of the cuticle

Pathogenic fungi need to penetrate through the cuticle into the insect body to obtain nutrients for their growth and reproduction. Entry into the host involves both enzymatic degradation and mechanical pressure as evidenced by the physical separation of lamellae by penetrated hyphae. A range of extracellular enzymes that can degrade the major components of insect cuticle, including chitinases, lipases, esterases and at least four different classes of proteases, have been suggested to function during the fungi pathogenesis(Bradfisch *et al*.,1990). The production of cuticle-degrading enzymes by *M. anisopliae* during infection structure formation on *Calliphor vomitoria* and *Manduca sexta* has been investigated by biochemical and histochemical analysis both *in vivo* and *in vitro*. Among the first enzymes produced on the cuticle are endoproteases (termed PR1 and PR2) and aminopeptidases, coincident with the formation of appressoria. N-acetylglucosaminidase is produced at a slow rate as compared to the proteolytic enzymes. Chitinase and lipase activities have not been detected (Leger *et al.,* 1989). Although the complex structure of the insect cuticle suggests that penetration would require the synergistic action of several different enzymes, much of the attention has focused on the cuticle-active endoprotease as a key factor in the process.

2.1.12 Production of toxins

There is considerable circumstantial evidence from deuteromycete pathogens for the involvement of fungal toxins in host death. The action of cytotoxins is suggested by cellular disruption prior to hyphae penetration. Behavioral symptoms such as partial or general paralysis, sluggishness and decreased irritability in mycosed insects are consistent with the action of neuromuscular toxins (Hong, 2003). *Beauveria bassiana* and *M. anisopliae* produced significant amounts of toxic compounds within their hosts. For example, the toxins Beauvericin, Beauverolides, Bassianolide and Isarolides have been isolated from *B. bassiana* infected hosts (Hamill *et al.,* 1969; Elsworth *et al*., 1977); toxins Destruxins (DTXs) and Cytochalasins have been isolated from *M. anisopliae* infected hosts. The toxins have shown to have diverse effects on various insect tissues. DTX depolarizes the lepidopteran muscle membrane by activating calcium channels. In addition, function of insect haemocytes can be inhibited by DTX (Bradfisch *et al*., 1990). Presumably, there are still many toxins that remain to be isolated from infected insects and except DTXs, their relevance to pathogenicity remains to be established.

2.1.13 Host defense systems

In order to prevent invasion by fungi, insects have evolved various defense mechanisms. The defensive arsenal of insects contains both passive structural barriers, such as the cuticle, and a cascade of active responses to pathogens that gain access to the haemocoel. These active responses include melanization, cellular reactions, humoral reaction to recognize the non-self-pathogen, and production of protease inhibitors.

Melanization: the oxidation of phenolic compounds to dihydroxyphenylalanine, typified by the production of brown or black melanic pigments, is a common feature of the

response of many insects to fungal infection. Melanin may partially shield cuticle from enzymatic attack or may be toxic to fungi. However, such protection is incomplete. The investigation from Leger *et al*., (1988) indicates that melanization is primarily an effective defense against weak or slow growing pathogens, but is ineffective against more virulent fungi.

Cellular reactions: once the cuticle and epidermis have been breached the invading fungus is faced with the defense systems of the haemolymph. The responses to mycopathogens within the haemocoel include phagocytosis, encapsulation and nodulation. However, the effect on fungal elements is uncertain. With the arbitrary injection method, Bidochka *et al*., (1987) found that haemocytes of the migratory grasshopper, *M. sanguinipes*, encapsulate viable conidia of *B. bassiana.* However, they fail to suppress conidial germination within the nodule. It was suggested that the production of toxins and extracellular proteases by *B. bassiana* could trigger the invasion of encapsulation.

Humoral reactions: in response to fungal challenge, insects elicit an acquired humoral "immunity" to subsequent infection. Recognition of "non-self" is critical to the initiation of the haemocytic defense reaction and this selective response in insects depends on a specific chemical recognition on part of the haemocytes. Serum and haemocyte cell membrane-bound lectins have been found in many insects (Mello *et al*., 1999). They could play a role in immune defense reactions since they agglutinate pathogens as well as fungi (Mello *et al*., 1999). Thus, insect serum agglutinin may function as opsonic mediating the enhanced attachment of granulocytes to the hyphal bodies (Pendland *et al*., 1988).

Production of protease inhibitors: host-produced protease inhibitors, which inhibit cuticle-degrading enzyme activities of pathogens, may contribute to insect defense systems. Such compounds have been isolated from the serum of *Anticarsia gemmatalis* larvae which were resistant to infection by *Nomuraea rileyi* (Boucias *et al.*, 1987).

2.2 Classification of *Beauveria bassiana*

The filamentous fungus *Beauveria bassiana* belongs to a class of insect pathogenic deuteromycete (imperfect fungus) *B. bassiana* (Balsamo) *Vuillemin* occur throughout the world and it has the largest host range among the fungi imperfecti. The fungus has been classified as follows: Class: Hyphomycetes Order: Hypocreales Family: Cordycipitaceae Genus: *Beauveria* Species: *B. bassiana* (Tesfaye et *al.*, 2012).

2.2.1 The life cycle of *B. bassiana*

Beauveria bassiana has a dimorphic mode of growth. In the absence of the specific insect host *Beauveria* passes through an asexual vegetative life cycle that includes germination, filamentous growth and the formation of sympodulo conidia. In the presence of its host insect, *Beauveria* switches to the pathogenic life cycle (Hong, 2003). The conidiospores germinate on the surface of the cuticle and the germinated hyphal tubes penetrate the insect's integument directly. When having penetrated the cuticle, the fungus alters its growth morphology to a yeast-like phase and produces hyphal bodies, which circulate in the haemolymph and proliferate by budding. Following the death of the host, fungal growth reverts back to the typical hyphal form (the saprotrophic stage). The ability to convert to the yeast-like phase may be a prerequisite for pathogenicity (Hong, 2003).

2.2.2 Biocontrol by *Beauveria bassiana*

It has been detected from over 700 species and also occurs in the soil as a ubiquitous saprophyte (Seneshaw *et al* .2003 and Tesfaye *et al* 2012). The different *Beauveria* strains are highly adapted to particular host insects. A broad range of *B. bassiana* species have been isolated from a variety of insects worldwide that are of medical or agricultural significance. An interesting feature of *Beauveria* is the high host specificity of many isolates. Hosts of medical importance include vectors for agents of tropical infectious diseases such as the tsetse fly *Glossina morsitans morsitans*, the sand fly *Phlebotomus* that transmits *Leishmania*, and the bugs of the genera *Triatoma* and *Rhodnius*, the vectors of Chagas' disease. Hosts of agricultural significance include the Colorado potato beetle, the codling moth and several genera of termites. In Ethiopia the ubiquitous fungus, *B. bassiana* was also isolated and identified from parts of different insects from many parts of Ethiopia including *Fura, Sekota, Wikro, Erer, Gusquam, Debremarkos, Ashengie, Tikurinchini, Metahara, Wonji-Shoa and Finchaa*. *B. bassiana* mainly infects insects belonging to Lepidoptera, Coleoptera and Hemiptera. It also occurs in Diptera and Hymenoptera (Seneshaw *et al*., 2003; Tesfaye *et al*., 2012). Furthermore, the high level of persistence in the host population and in the environment provides long-term effects of the entomopathogenic fungi on pest suppression.

In China, *B. bassiana* has been applied against the European corn borer *Ostrinia mubilalis*, pine caterpillars *Dendrolimus* spp., and green leafhoppers *Nephotettix spp*. In the Soviet Union, *B. bassiana* is produced under the trade name Boverin for control of the Colorado potato beetle *Leptinotarsa decemlineata* and the codling moth *Laspeyresia pomonella*.

Entomogenous fungi are potentially the most versatile biological control agents, due to their wide host range that often results in natural epizootics. An attractive feature of these fungi is that infectivity is by contact and the action is through penetration (Bharathi, 2005). These fungi comprise a heterogeneous group of over 100 genera with approximately 750 species, reported from different insects. Many of these offer a great potential in pest management. The most important fungal pathogens are *Metarhizium spp*., *Beauveria* spp.,*Nomuraea rileyi, Verticillium lecanii* and *Hirsutella* spp (Bharathi, 2005).

In France and Italy, where silk production was important in the 16th and 17th centuries, heavy losses of larval silkworms were experienced every year from "muscardine." In 1835, the Italian scientist Agostino Bassi de Lodi (the "Father of Insect Pathology") showed that the problem affecting the silkworms was actually caused by a fungus that multiplied in and on the body of the insect (Prior, *et al*.1988, and Li , *et al*,2001). This was the first microorganism to be recognized as a contagious agent of animal disease. Yes indeed, the first animal pathogen to be understood was of insects, not humans! The fungus was later named *Beauveria bassiana* in honor of its discoverer. The very distinctive and noticeable white mummies of affected caterpillars gave rise to the name muscardine, which is derived from the French word for the bonbons which the mummified specimens resembled. Today the term muscardine refers to an insect fungus or disease caused by a fungus (Goetell *et al.*, 2001).

Beauveria bassiana is a common soil borne fungus that occurs worldwide. It attacks a wide range of both immature and adult insects. It seems to flourish in soil and humid environments close to the ground and has been recorded from a number of coleopterans

hosts and their larvae. The fungus offers the most promise in microbial control because of its high pathogenicity for large number of hosts (Mengech *et al.*, 1995). It has even been found infecting the lungs of wild rodents, and the nasal passages of humans. There are many different strains of the fungus that exhibit considerable variation in virulence, pathogenicity and host range. It occurs in the soil as a saprophyte (Columbia encyclopedia, 2008).

The infective unit in most of the entomopathogenic fungi is a conidium or spores which when land on a susceptible host, put forth germ tubes or infection pegs from appressoria. These structures secrete a complex of cuticle degrading enzymes *viz.*, chitinases, proteases and lipases, which are capable of hydrolyzing corresponding cuticular constituents' *viz.*, chitin, protein and lipids. This facilitated the germ tube to invade haemocoel and fat bodies. The invading vegetative hyphae consumes the contents of haemolymph for its growth and metamorphosis. On exhaustion of the haemolymph content, the host insect become moribund and the fungi sporulate after death of the host (Bharathi, 2005). Undoubtedly, many pathogen enzymes are important determinants of virulence since they enable the pathogen to coexist with the changing metabolic processes associated with the host's diseased state. Leger *et al.*, (1992) cloned a gene encoding protease (*Pr1*) from *M. anisopliae* which solubilized cuticle protein to assist penetration and provided the nutrients for further growth (Leger *et al.*, 1988).

The cuticle is the first barrier to infection by fungi. Hence, rapid and direct penetration of the cuticle is important for virulence. The insect procuticle is primarily chitin fibrils embedded in a protein matrix and penetration appeared to involve both mechanical and enzymatic components.

2.2.3 Action of *Beauveria bassiana* on insects

Penetration is a stage of infection where specificity may be determined since, many pathogens are virulent after being injected into the haemolymph of an otherwise no susceptible host. Virulent isolates, however, had 10-17 times more endochitinase activity and15-28 times more exochitinase activity than virulent isolates.

Metabolites of fungal pathogens are involved in the infection process. Pigments like bio-chrome such as bassianin and tenellin or dibenzoguinones such as oosporin are responsible for colour change in the insect body. Many of the entomopathogenic fungi produce toxins which act as poisons for the insects and thereby are killed. Aerial conidia sporulated from infected or mummified cadavers are widely disseminated by wind. Splashing of rain also accounted for spreading but only for short distances. In water stagnated irrigated rice ecosystem, the fungal propagules (conidia oospores) are disseminated through irrigation water (Bharathi, 2005). As with all insect-pathogenic fungi, *Beauveria bassiana* produces spores that are resistant to environmental extremes and are the infective stage of the fungal life cycle. The spores (called conidia in this case) infect directly through the outside of the insect's skin. Under favorable temperature and moisture conditions, a conidium (singular of "conidia") adhering to the host cuticle will germinate. Kumar (1984) showed that *B.bassiana* (Balsamo) Vuillemin conidia have shorter survival in soils with high temperatures or water saturated but soils covered with vegetation enhanced the fungal survival. For *B. bassiana* isolates *in vitro* growth of most isolates is adversely affected at 10 and 15 °C. The greatest reduction at 10 °C in rates of conidial germination and colony growth, compared with other temperatures (Yeo *et al.* 2003). Relative humidity (RH) and temperature are known to be limiting environmental

factors for fungal development on insects High rates of infection and a rapid kill of insects by the hyphomycete fungi *Beauveria bassiana* and *Metarhizium anisopliae* is obtained at humidities close to saturation . Infection of bugs diminishes with *B. bassiana* at RH below 97%. Optimal temperatures for fungal development on the insect host range from 16 to 30°C for *B. bassiana* and *M. anisopliae* with a faster development at the higher temperatures (Luz *et al*., 1998).

The fungal hypha growing from the spore secretes enzymes that attack and dissolve the cuticle, allowing it to penetrate the skin and grow into the insect body. Once inside the insect it produces a toxin called Beauvericin that weakens the host's immune system. After the insect dies, an antibiotic (oosporein) is produced that enables the fungus to outcompete intestinal bacteria. Eventually the entire body cavity is filled with fungal mass. When conditions are favorable the fungus will grow through the softer parts of the insect's body, producing the characteristic "white bloom" appearance. Relative humidity must be 92% or more for *B. bassiana* to grow outside the insect. These external hyphae produce conidia that ripen and are released into the environment, completing the cycle (Li, *et al.*, 2001).

2.2.4 Biocontrol modes of *Beauveria bassiana*

In addition to infecting insects, *B. bassiana* can colonize corn plants, having the capability of living in the vascular tissue of certain corn cultivars as an endophyte. European corn borer tunneling is reduced in corn plants with the fungus. In studies in Iowa, the fungus colonized the plant when applied as a granular formulation of conidia

on foliage at whorl stage, moved internally in the plant, and persisted throughout the season to provide significant suppression of corn borers.

Beauveria bassiana is an important entomopathogenic fungus currently under development as a bio-control agent for a variety of insect pests. Although reported to be non-toxic to vertebrates, the potential allergenicity of *Beauveria* species has not been widely studied (Westwood *et. al.*, 2006). Strains of *B. bassiana* have been licensed for commercial use against whiteflies, aphids, thrips, and numerous other insect and arthropod pests. Besides silkworm, the extensive list of hosts includes such important pests as whiteflies, aphids, grasshoppers, termites, Colorado potato beetle, Mexican bean beetle, Japanese beetle, boll weevil, cereal leaf beetle, bark beetles, lygus bugs, chinch bug, fire ants, European corn borer, codling moth, and Douglas fir tussock moth. Natural enemies, such as lady beetles, are susceptible too (Cloyd *et al*., 2001). *B. bassiana* fungal formulations are being spread onto a range of vegetables, melons, tree fruits and nuts, as well as organic crops. As alternatives to chemical pesticides these agents are natural occurring and are considered to be non-pathogenic to humans (Tingle *et al*., 2003, Westwood *et al*., 2006).

Beauveria bassiana is available commercially as a microbial insecticide since *Beauveria bassiana* can now be mass produced by a fermentation process and formulated to enable the fungus to withstand ultraviolet light, and temperature and humidity extremes commonly encountered in the field. (Fargues *et al.*, 1997) studied the effect of both moisture and temperature on the infectivity of *B. bassiana* and reported that the most favorable conditions were 97 percent RH and temperature of 20°C combined with either 75 per cent, 25°C or 43 percent. Under less favorable alternating conditions (lower and

higher temperature) the amount of inoculums required for killing 50 per cent of first instar nymph was 10 or 20 times higher. However, Akello *et al.,* 2007 showed that the fungus viability against Banana weevil *Cosmopolites surdidus* was constrained by abiotic and biotic factors especially temperature, humidity and light. Maniania (1997) evaluated the formulations of *B.bassiana* on management of Maize stem borer (*Chilo partellus*). He then reported high potentiality of the fungi, and suggested that the formulation from the fungus would be an alternative biopestide over the traditional chemical pesticides (Maniania, 1993), while Castrillo *et al*., (2011) showed that the fungi was effective in the control of ambrosia beetle *Xylosandrus germanus* (Coleoptera:Curculioniodae). Wekesa, *et al.* (2006) showed that both *B.bassiana* and *Metarhizium anisopliae* isolates were capable of causing significant mortality in eggs and motile stages of *Tetranychus evansi* with eggs and the adults being the most susceptible; he proposed *B. bassiana* and *M. anisopliae* have promising control agents. Dembelio *et al.* (2010) noted a high potential of *B. bassiana* in all stages of the life cycle of red palm weevils *Rhynchophorus ferrugineus.* He further found that strains of the fungus could infect eggs, larval and adult stages of *R. ferrugineus* efficiently and transmit the disease to untreated adults. Manainia (1997) showed 5-100% mortality in stem borers *Chilo partellus* larvae and 26-86% in *Busseola fusca* and suggested the fungus potential as a strong microbial agent in control of the pests. Ngumbi *et al.* (2011) realized a high potential with a mortality rate of above 75% when he investigated the pathogenicity of *Beauveria bassiana* and *Metarhizium anisophilia* against adult *Phlebotonus duboscqi* (never-lemaire). (Liu, *et al.,* 2003*)* investigated the virulence of *B. bassiana* against plant bug, *Lygus lineolaris* and found a 70% morality, he further demonstrated that for the plant bug a concentration of 1X 10^7

conidia /ml of the fungi was effective in killing the bug. More than 80% success has been seen in the control of desert locust in Ethiopia by entomopathogenic fungi (Seneshaw *et al.*, 2003).

2.2.5 The advantages of using entomopathogenic fungi as insecticides

There are several advantages of using entomopathogenic fungi as bioinsecticides: (1) Their high degree of specificity for pest control. Fungi can be used to control harmful insect pests without affecting beneficial insect predators and non- harmful parasites. (2) The absence of effects on mammals and thus the reduction of the hazards normally encountered with insecticide applications, such as pollution of the environment. (3) The lack of problems caused to insect resistance and prolonged pest control. (4) A high potential for further development by biotechnological research. (5) High persistence in the environment provide long-term effects of entomopathogenic fungi on pest suppression make entomopathogens easier to use for control.

However, there are also a number of constraints on the use of fungi as insecticides:

(1) Two-three weeks are required to kill the insects whereas chemical insecticides may need only 2-3 hours. (2) Application needs to coincide with high relative humidity, low pest numbers and a fungicide free period. (3) Due to the high specificity additional control agents are needed for other pests. (4) Their production is relatively expensive and the short shelf life of spores necessitates cold storage. (5) The persistence and efficacy of entomopathogenic fungi in the host population varies among different insects species, thus insect-specific application techniques need to be optimized to retain long-term impacts. (6) A potential risk to immune depressive people (Hong, 2003).

2.2.6 Isolation of *Beauveria bassiana*

The success of microbial control of insect pests depends not only on the isolation, characterization and pathogenicity but also on conidia production of the microbial agents in the laboratory (Bhadauria *et al.*, 2012). Different infectious *B. bassiana* propagules can be isolated and selected for host targeting. Thus, in addition to mycelial and hyphal growth, *B. bassiana* produces a number of mono-nucleated single-cell types, including aerial conidia, blastospores and submerged conidia, which can be isolated from agar plates, rich-broth submerged cultures and nutrient-limited submerged cultures, respectively (Holder *et al*., 2007). *Beauveria bassiana* produce three single cell forms, aerial conidia, in vitro blastospores and submerged conidia in different conditions. Aerial conidia are produced on the surface of solid medium by a process of hyphal extension, formation of phialides (rachis) and spore production. Aerial conidia usually are used for biological control agents because they are relatively resistant to varying environmental conditions and can be formulated to prolong shelf life (Jiang *et al.*, 2008).

Conidia production have done both in agricultural products has well as culture media. Sabouraud Dextrose Broth (SDB) and Potato Dextrose Agar (PDA) have shown to be the best media in mass production of *B. basssiana* conidia in vitro (Bhadauria *et al.*, 2012). However, Conidia harvested from solid media and dried to reduce moisture contents and allowed to separate from substrata is preserved for long without losing power of growth and pathogenicity (Lopes *et al*., 2013). *Beauveria bassiana* has been mass-produced on different solid substrates, including sugarcane wastes, silkworm pupal powder, agar medium and steamed rice (Feng *et al*., 1994).

2.2.7 Isoenzyme variations in *B. bassiana*

Cloyd, *et al*., 2001 noted that the variation in virulence of *B. bassiana* could also be related to enzyme production and activities during the course of the penetration of the host cuticle. Isoesterase analysis can be used to identify a virulent isolate because of variation in isoesterase profiles among isolates from different insect hosts. Feng *et al.* (1994) noted that the variation in virulence of *B. bassiana* may also be related to enzyme production and activities during the course of the penetration of the host cuticle. Isoesterase analysis can be used to identify a virulent isolate because of variation in isoesterase profiles among isolates from different insect hosts and/or different geographic regions. In the laboratory, repeated subculture of an isolate sometimes leads to the attenuation of its original features such as growth, sporulation and virulence (Feng *et al.*, 1994). Virulence among isolates from different insect's hosts could arise due to different genes associated with conidiation in *Beauveria bassiana* with suppression subtractive abilities (Wu, *et al*., 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Determination of the efficacy of *Beauveria bassiana* isolates

3.1.1. Rearing of weevils

Weevils for this study were collected from Mununga tea farms in Kirinyaga County, where the invasion had been reported to be high, and Giciaro farm in Nyambene, in Meru County. The collected weevils were kept in perforated plastic containers 3000ml capacity. Small beakers planted with tea shoots were placed inside large containers for the weevils to feed on. The weevils were transported in the same containers to TRFK laboratories for rearing at $25^{o} \pm 2^{o}$C, 60-70% relative humidity (RH) and a photoperiod of 12h:12h (day: dark). The adult weevils were transferred to rearing cages 100cm×50cm×100cm for weevils to breed. The cages were made of metal frame covered by fine mosquito net, with a glass door. The rearing cages were layered with sterile soil 5cm deep covered with wet tea leaf trenches where the weevils could hibernate and breed. In the cages we placed four small beakers planted with tea shoots for the weevils to feed on. These shoots were changed after every two days. The rearing was carried out for 7 weeks to allow mature young weevils to start feeding. The efficacy trials were done using young weevils of the approximately the same age.

3.1.2 Isolation of the *B. bassiana*

The fungus was isolated by the insect bait method. Trap larvae of the waxy moth (*Galleria mellonella*) were used as insect's baits to capture entomopathogenic fungi from

soil samples collected from different localities. This trap insect technique (Bedding and Akhurst, 1975) was originally used for isolation of entomopathogenic nematodes from the soil.

A colony of *G. mellonella L*. was maintained in the laboratory at room temperatures (20^{o} C ± 3). The larvae were reared on the *Galleria* diet that consisted of 307g of maize meal, 225g honey, 45g bees wax and 90g yeast. The diet was prepared by first boiling the bees wax, and once melted it was mixed with honey. The mixture of maize meal and yeast was poured into the melted bees wax and honey. The mixture was stirred while cooking on a hot plate until it became firm and evenly mixed. The mixture was then placed on a bowl with perforated lid and left to cool overnight. The *G. mellonella* larvae were then introduced into the meal. They were then allowed to grow to maturity producing mature moth which produced new larvae.

Moist soil samples were then placed in a petri dish. Ten medium sized larvae were placed into the soil. Dishes were regularly turned in the beginning of baiting period (first week) to make the bait insect larvae to penetrate as much soil as possible while they were still vigorous. The larvae were left in the soil until the fungus growth was seen on their body. Whole infected larvae that already showed hyphal growth on their bodies were first surface sterilized with 70% ethyl alcohol prior to incubation on the Potato Dextrose Agar (PDA). This was to prevent external saprophytic fungi from growing on the dead cadaver.

The fungus was isolated from the infected *G. mellonella* moth by scrapping the moth grown mycelia using sterile scapel onto sterile filter paper, aseptically.

Plate I: Bowl with perforated lid with *Galleria* diet

(Source, Author 2016)

The scraps were then transferred into the surface of solid quarter strength PDA in Petri dishes using sterile forceps.

Three sets of the scraps was placed in each plate. The quarter strength PDA was prepared by adding 9gms of dehydrated PDA to 1 litre of distilled water, while stirring to obtain a uniform suspension. The suspension was then autoclaved at 120^{o}c at 15 Psi for 15 minutes. After autoclaving the medium was allowed to cool, to about 30-40^{o}c in lamina flow air flow cabinet. Antibiotics (streptomycin sulphate 100 mg / 1 plus penicillin 62.8mg /1) were added to the cooled medium mixed using a magnet stirrer to

make semi selective that was to inhibit bacterial growth. The selective cooled PDA media was then aseptically poured into 9 cm petri-dishes and allowed to solidify before use. After placing the fungi scraps in Petri-dishes, the plates were sealed with Parafilm tapes to avoid contamination and were then incubated at room temperature (20^{o}C). The plates were observed after 24 hours onwards for presence of fungal growth. The grown culture was confirmed by microscopic observation of the fungi.

After 4 days, the fungi were subcultured on fresh PDA plates; subsequent subculturing was done to obtain pure culture of the entomopathogenic fungi.

The larvae were placed in PDA culture medium, PDA is known to be among the best culture media that induces the best linear growth for *B. bassiana* (Meyling.., 2007). The larvae were incubated at room temperature until adequate growth of the fungus was observed. The fungus was then transferred to fresh PDA medium and incubated for 7 days under the same conditions.

The fungi that were harvested from each of the different soil samples were termed isolates. The isolates were then coded according to different places where the soils were collected. They were coded as follows: Bb ke 1-5 recently isolated from Kericho TRFK Timbil Tea fields, Bbke6a and Bbke6b isolated in 2006 from within Kericho County, BbGi 7 were isolated from assorted weevils from Giciaro farm in Nyambene hills of Meru County, BbCh c 8 and BbCh c1-5 were isolates from Chepkoilel farm in Eldoret, Uasin Gishu County and BbMu 9 were isolates from Mununga, in Kirinyaga County.

3.1.3 Single conidium suspension.

The mass of conidia produced by the pure culture was aseptically transferred to sterile universal bottles containing sterile distilled water by use of sterile wire loop. A small loopful was streaked into the surface of the fresh PDA. The plates were then incubated at 20^{o}C for 24 hours and then observed under a dissecting microscope for conidia germination.

The germinating conidia were marked out with a circle on the reverse of the plate using a marker pen, and the marked media portion was then carefully cut and aseptically transferred to fresh PDA plates using a sterile inoculating needle. The fresh inoculated plates with a single conidium isolate were then examined immediately under a dissecting microscope to confirm that only a single germinating conidium was transferred. The dishes were incubated at 20^{o} C for 24 hours (CMI, 1983).

3.1.4 Culture maintenance

The single spore cultures were subcultured on several plates of PDA media and used for bioassays after 10 days of incubation. Stock cultures for each isolate were maintained on PDA slopes in sterile universal bottles in refrigerators at 4^{o} to 5^{o}C. These slopes were prepared by dispersing 10mls of autoclaved molten PDA into tilted, screw-capped universal bottles. The slopes were incubated with agar plugs (2mm in diameter, cut from leading margins of fungal cultures on PDA using a sterile cork borer) of a single conidium isolates. These were then incubated at room temperatures for 7 days before being transferred to the refrigerator. The pure fungal cultures were covered with a sterile liquid parafilm before refrigeration (Johnston *et al*, 1983). The cultures were checked regularly for contamination (Carmichael, 1956).

3.1.5 Fungal identifications

Samples of the isolated entomopathogenic fungus were microscopically confirmed in the TRFK laboratories for identification using cultural and hyphal characteristics that it was *B. bassiana*. The fungi cultural characteristics in the isolates were evaluated macroscopically in order to categorize the fungi.

3.1.6 Mass Production of *Beauveria bassiana*

Mass production of the conidial suspensions of each isolate was produced for screening and confirmation of the fungal potency. Each isolate was inoculated in sterile pearled barley cereals in plastic fungal growth bags containing filter paper.

For each isolate, 100ml of the fungal suspension with a concentration of 1×10^7 conidia /ml was added to 200g of pearl barley and the bags sealed and placed for 14 days at 25^oC in darkness in growth chambers (Sabbahi, *et al.*, 2009). The cereals were manually crumbled within the bags every 2 days to provide aeration throughout the culture substrate.

Conidia were harvested from each bag by adding and mixing with 200ml of deionised water. The suspension was then filtered through two layers cheesecloth to remove mycelia and the barley heads. The conidia concentration was determined by using hematocytometer.

3.1.7 Formulation and Application of *Beauveria bassiana* in the green house

Beauveria bassiana conidia isolates were applied using a formulation based on Burges (1998) method. The *B. bassiana* formulation was made up of 1 % skimmed milk, 2% glycerol, 4% canola oil and 5 % clay. Oil was used because it is an excellent adhesive,

promoting contact between the active ingredient (the conidia) and the lipophilic insect cuticle while also increasing the conidia's rain-fastness on the waxy leaf surface of treated host plants (Burges, 1998). Clay was added to protect conidia against UV light (Butt, 2002). Glycerol was included due to its role as nutrient as humectant, nutrient and adhesive; whereas skimmed milk act as nutrient and humectant as well (Burges, 1998). For each treatment, water containing the required concentration of conidia was added to the final formulation. Long-term effects of the formulation ingredients on the viability and virulence of the *B. bassiana* are unknown, so the final mixing of the formulation was conducted immediately prior to application, as recommended by Goettel *et al.* (1984).

All treatments were sprayed three times at 7-day intervals and were applied as foliar sprays taking care to thoroughly wet both sides of the foliage. A CO_2-powered backpack sprayer was used at a constant pressure of 40 psi and a flow rate of 50ml/ min. (pots in the green house).

3.1.8 Evaluation of efficacy of *Beauveria bassiana* in the green house

The efficiency of *B. bassiana* treatments was evaluated by counting the number of tea weevils' adults per plants in each pot in the green house. The counts were taken a day before the first application and was followed by regular counting after 3 days until the end of the experiment. For each treatment and each sampling period, counts were performed on five randomly selected plants (Plate2). Sampled plants were identified to avoid double counts on the same plant, which may interfere with results. Dead weevils were collected and kept individually on wetted filter paper in Petri dishes at 25ºC,

90%RH in darkness for 14 days to promote fungal growth. Conidia were harvested from cadavers with a sterile cotton swab and inoculated on selective oatmeal medium (OSM) (17.5g oatmeal agar, 0.45g Cyprex (dodine), 2.5mg crystal violet, 0.2 g penicillin G, and 0.5g streptomycin in 500ml of deionized water) as described by Chase *et al.* (1986). An isolate colony was then selected and inoculated onto an SDA medium in order to assess its morphological characteristics, which were used to confirm if the fungus recovered from the insects is *B.bassiana* (Sabbahi *et al.*, 2009).

Plate 2: Perforated containers with small beakers planted with pieces of tea shoots for the adult weevils to feed on.

(Source: Author, 2016)

3.2. Fungal characteristics, colony growth rates and conidial yields

3.2.1 Determination of Biological characteristics and the *B. bassiana* isolates virulence.

Twenty-two isolates were cultured on potato dextrose agar (PDA) medium in petri dishes (90mm diameter), with three replicates in each isolate. The dishes were maintained in an incubation chamber at $25^{o} \pm 1^{o}$ C and RH$\geq$ 80%. Macroscopic morphological characteristics of each colony, including color, texture and reverse color were observed. The diameters of the colonies were measured daily for 14 days after incubation. After 14 days, a culture disc from the center of each colony was removed using a sterile 13mm diameter punch and placed into a 50ml flask with 20ml of sterile 0.05% Tween 20. Each isolate contained three flasks as replicates. The conidial suspensions of the 22 isolates were prepared by stirring for 10 minutes with a magnetic stirrer, the conidial concentrations were determined by use of a hemocytometer, with three spore counts taken for each flask culture and adjusted to 1.0×10^{6} conidia/ml with 0.05% Tween 20. To obtain the colony count, in three replicates, 0.1ml of an aqueous suspension of each isolate were spread plates on freshly prepared PDA in petri dishes the sealed with a parafilm and incubated at 25^{o}C for 16 hours. Germination rates were determined by examining 100 conidia per plate using a binocular light microscope (power 400x). A conidium was considered germinated if the germ tube was at least the length of a swollen conidium.

3.3 Assessment the efficacy of *B. bassiana* concentrations on tea weevils

3.3.1 Laboratory assessment with selected *Beauveria bassiana* isolates

To assess LC_{50} and LT_{50} of four most effective and selected isolates BbGi7a, BChc2, BbChc3 and BbKe6a four concentrations were prepared (10^5 ,10^6 ,10^7 and $10^{8)}$. Conidia of sub-cultured *B. bassiana* isolates were inoculated into 50 ml of Sabouraud Maltose Yeast (SMY) liquid broth in a 250ml Erlenmeyer flask and incubated for 2–3 d at 25 °C with rotary shaking of 200 rpm. Ten millilitres of the culture were put onto plates containing SMY solid medium and incubated for 10–15 d at 25 °C. The conidia which developed in the flask were suspended in 15ml of a solution containing 0.2 % Tween 20 and 0.89 % Sodium Chloride (NaCl), and then the conidial suspension was filtered through a 2-layered filter paper (90mm) to remove mycelial fragments and aggregated conidia. The concentration of conidia was determined by using a hemocytometer. Ten cold-anaesthetized weevils were inoculated by dipping in 10ml of conidial suspension for 5 minutes. The number of dead weevils was recorded every 24 hours. Each treatment was replicated three times and a control that was distilled water added 15 ml of 0.2%Tween 20 and 0.89% Sodium Chloride (NaCl).

Dead weevils were collected and kept individually on wetted filter paper in Petri dishes at 25^o C at 70% relative humidity (RH) in darkness for 14 days. The mortality (%) 14 days after treatment of the weevils the Average Survival Time (AST) of the weevils was calculated according to Kapan-meier survival analysis (Dembilio *et al.,* 2010). Conidia were harvested from the tea weevil with sterile cotton swabs and inoculated on selective media. Selected colonies were inoculated on SDA medium to assess the morphological

characteristics to confirm that the fungus on the insects is *B.bassiana* (Sabbahi *et al.*, 2009*;* Trudel *et al.*, 2007).

3. 4 Statistical analysis

SAS version 9.0 was used for all statistical ANOVA analyses on radial growth, sporulation, germination and weevils' mortalities Correlation analysis was used to measure the linear relationship between two variables (Bewick *et al.,* 2003). The macroscopic characteristics of the isolates were categorised the colony colour and the colony textures. Correlation analysis was also determined on the radial growth of the isolates and spore count on each isolate.

Data on insect mortality caused by the fungus was converted to percentages.

Tea weevils were treated with different isolates and the eaten areas on the tea leaves was compared between different isolates using ANOVA (α= 0.05). The cumulative weevil's mortality in each treatment was for control of mortality. The number of tea weevils with mycosis was estimated as percent proportion of dead weevils. The mean and standard error of all the replicates for mortality after seven days and mycosis were calculated and presented in tables. Mortality data were presented as percentage mortality, although actual mortality was used for statistical tests. Logit transformation (SAS) was used to estimate the lethal time to 50% mortality (LT_{50}) and lethal concentration causing 50% mortality (LC_{50}) for the selected isolates . The percentage mortalities were analyzed with SAS model with isolates, dosage and time as the main factors and isolate by dosage, isolate by day, dosage by day and isolate by dosage by time interactions. The Least

Significant Differences (LSD) was used to compare the means of the mortalities of the weevils caused by the selected *B. bassiana* isolates.

CHAPTER FOUR

RESULTS

4.1 Fungal characteristics, colony growth rates and conidial yields

4.1.1 Macroscopic cultural characteristics of the fungal isolates

Based on the macroscopic characteristics of 14- day- old culture (Table 4.1), the 22 isolates of *B. bassiana* were classified into three groups. Group 1 (Bb Ke 6a, Bb Ke 6b, Bb Ch c1, Bb Ch c2, BbCh c3, Bb Chc4, BbCh c8b and BbCh c5) produced white, villous colonies and the reverse colour was brown yellow. Group 2 (Bb Ke 1, BbKe 2, BbKe 3, BbKe 4, BbKe 9 and BbKe 5) had cream white and felty colonies with brown reverse colour, while group 3 (Bb Gi 7a-d) produced white yellow and thin powdery colonies with yellow reverse colour. The characteristics of the fungal isolates are shown in Plates 3a, b and c.

Table 4.1: Colony morphological characteristics of the tested isolates of *Beauveria bassiana*

Isolates	Groups	Colour of colony	Colony texture	Reverse Colour
BbKe6a	1	white	villous	yellow- white
BbKe6b	1	white	villous	yellow-white
BbCh c1-5	1	white	villous	yellow-white
BbCh c8b	1	white	villous	yellow-white
BbKe 1	2	Cream-white	felty	light-brown
BbKe2	2	Cream-white	felty	light-brown
BbKe3	2	Cream-white	felty	light-brown
BbKe4	2	Cream-white	felty	light-brown
BbKe 5	2	Cream-white	felty	light-brown
BbGi7a	3	white-yellow	thin-powdery	yellow
BbGi7b	3	white-yellow	thin-powdery	yellow

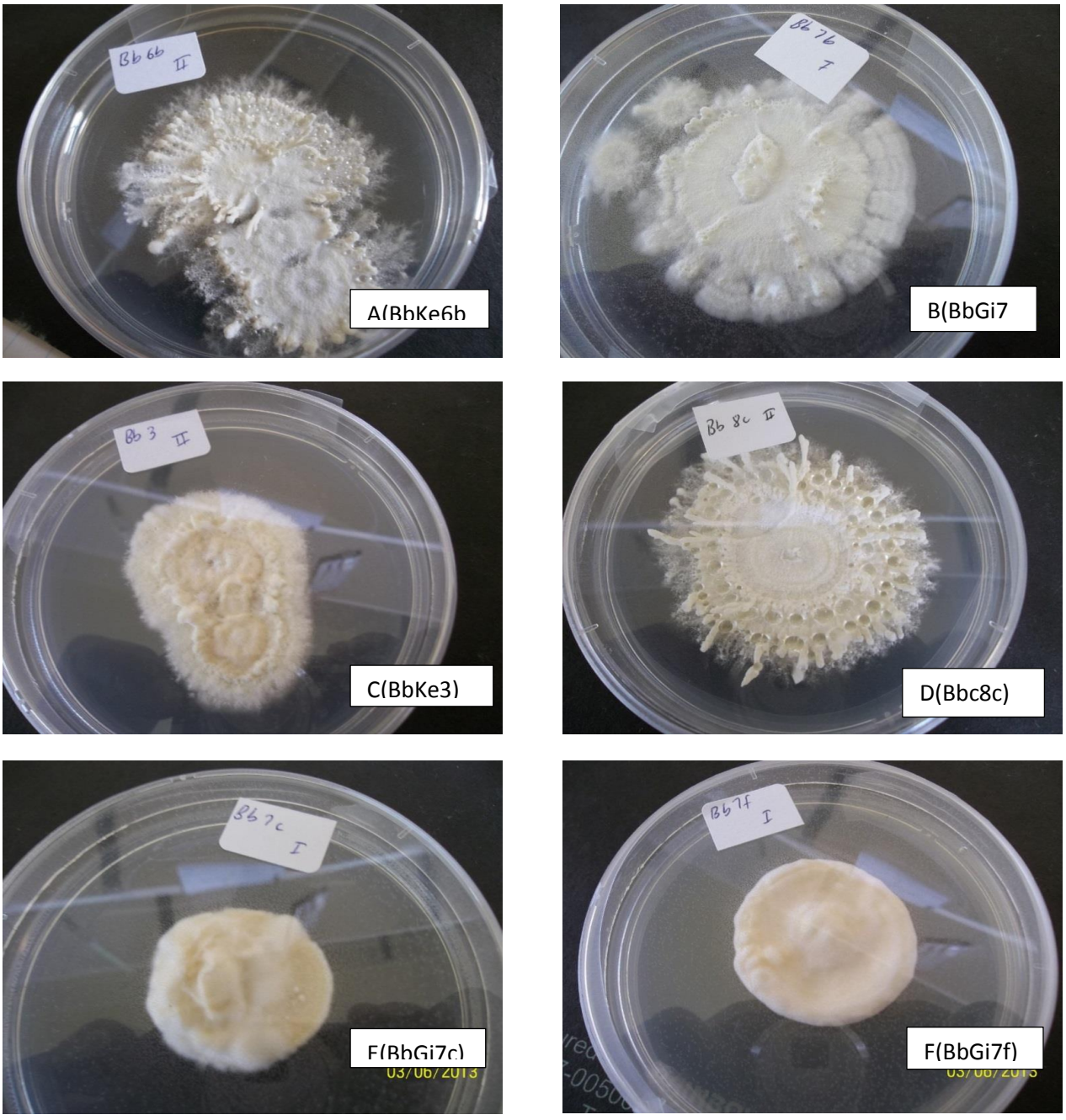


Plate 3a: Cultural morphological characteristics of some isolates A(BbKe6b), B(BbGi7d), C(BbKe 3), D(Bbc8c), E (BbGi7c), and F(BbGi7f) of *Beauveria bassiana* (Source: Author 2016)

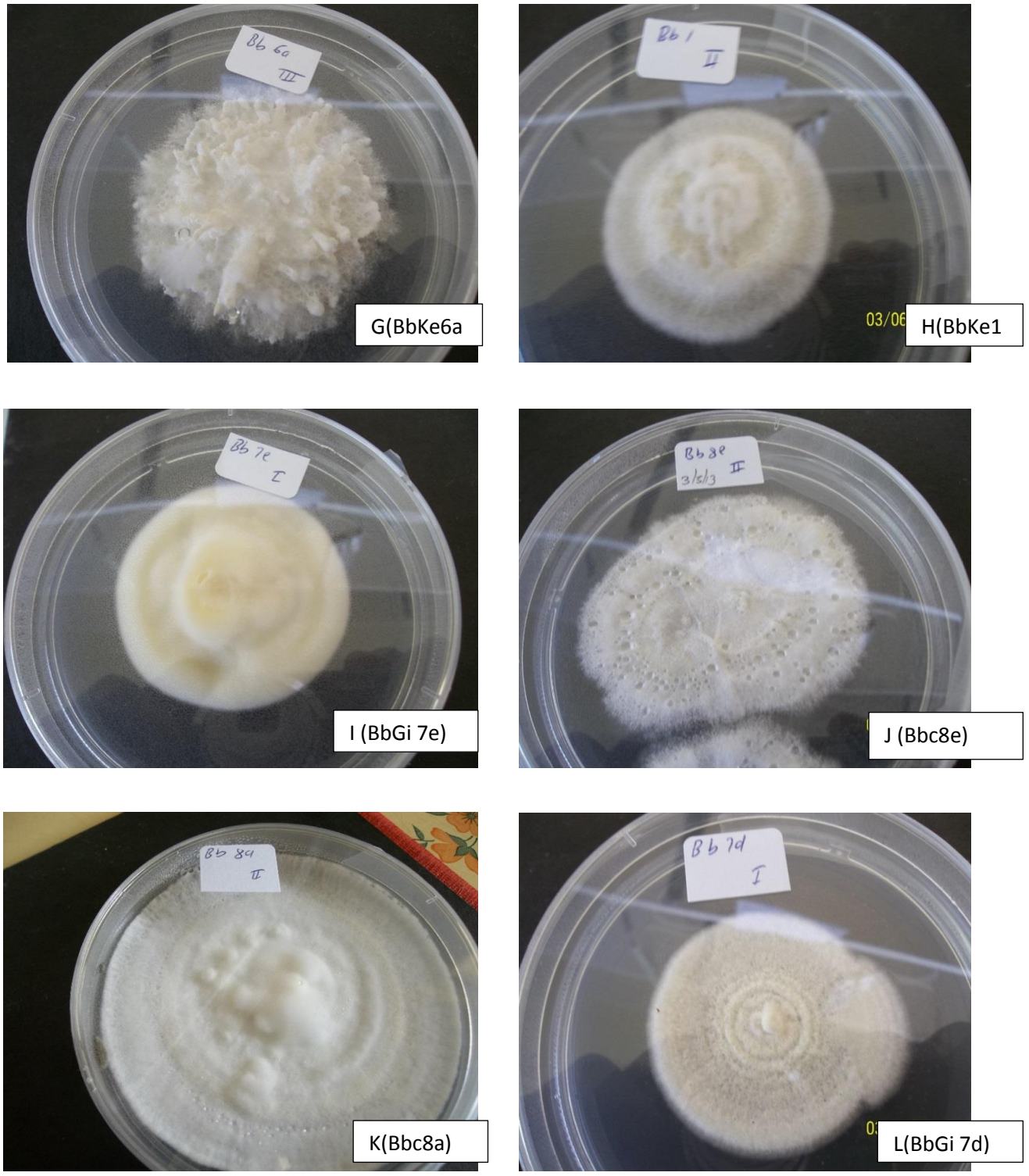
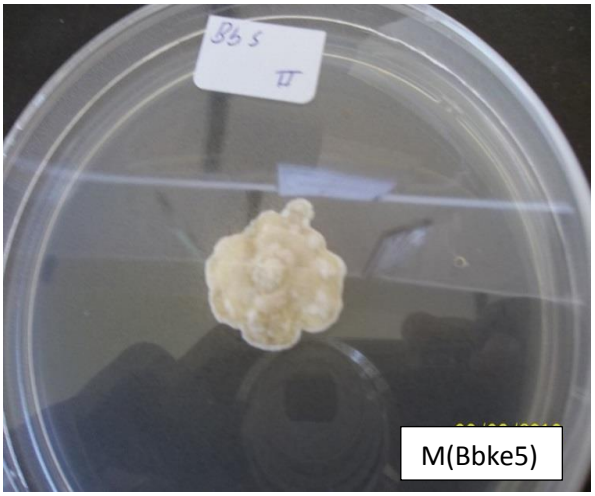
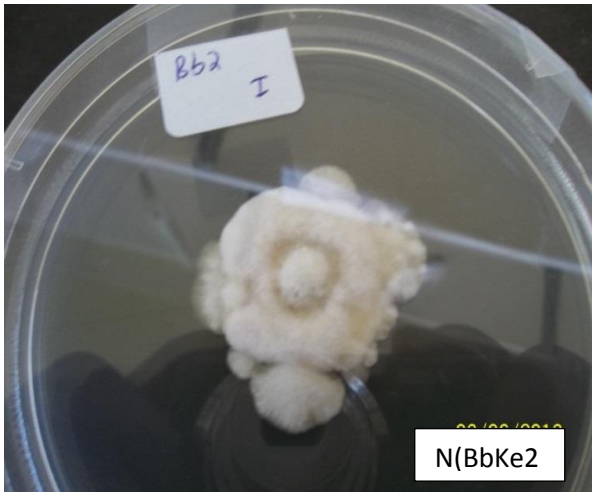


Plate3 b: **Cultural morphological characteristics of some isolates G** (BbKe6a), **H** (BbKe1), **I** (BbGi7e), **J** (Bbc8e), **K** (Bbc8a) and **L** (BbGi7d) **of *Beauveria bassiana***

(Source: Author,2016)





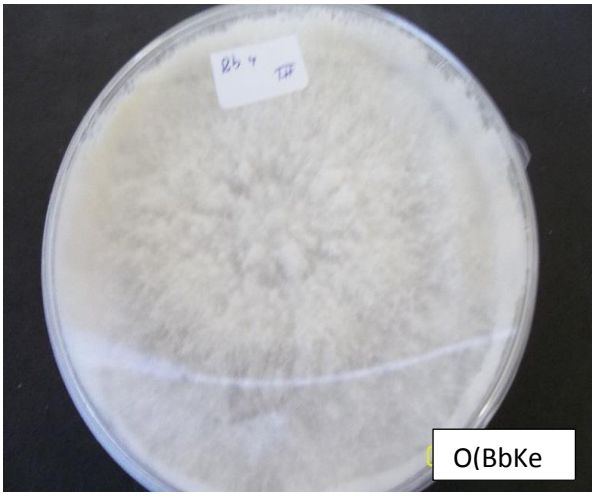


Plate 3c: Cultural morphological characteristics of some isolates M (BbKe5**), N (**BbKe2**), and O (**BbKe4**) of *Beauveria bassiana***

(Source: Author, 2016)

4.1.2 Radial growth of *Beauveria. bassiana* isolates

Isolates from Chepkoilel soils and Kericho county and Timbilil estates exhibited the highest radial diameter 51mm in Bbke4, 20.67mm in BbCh8d and 18.33mm in BbKe6a (Figure4.1a), however, there was no significant difference in the isolates growth radial diameter at $p<0.5$.

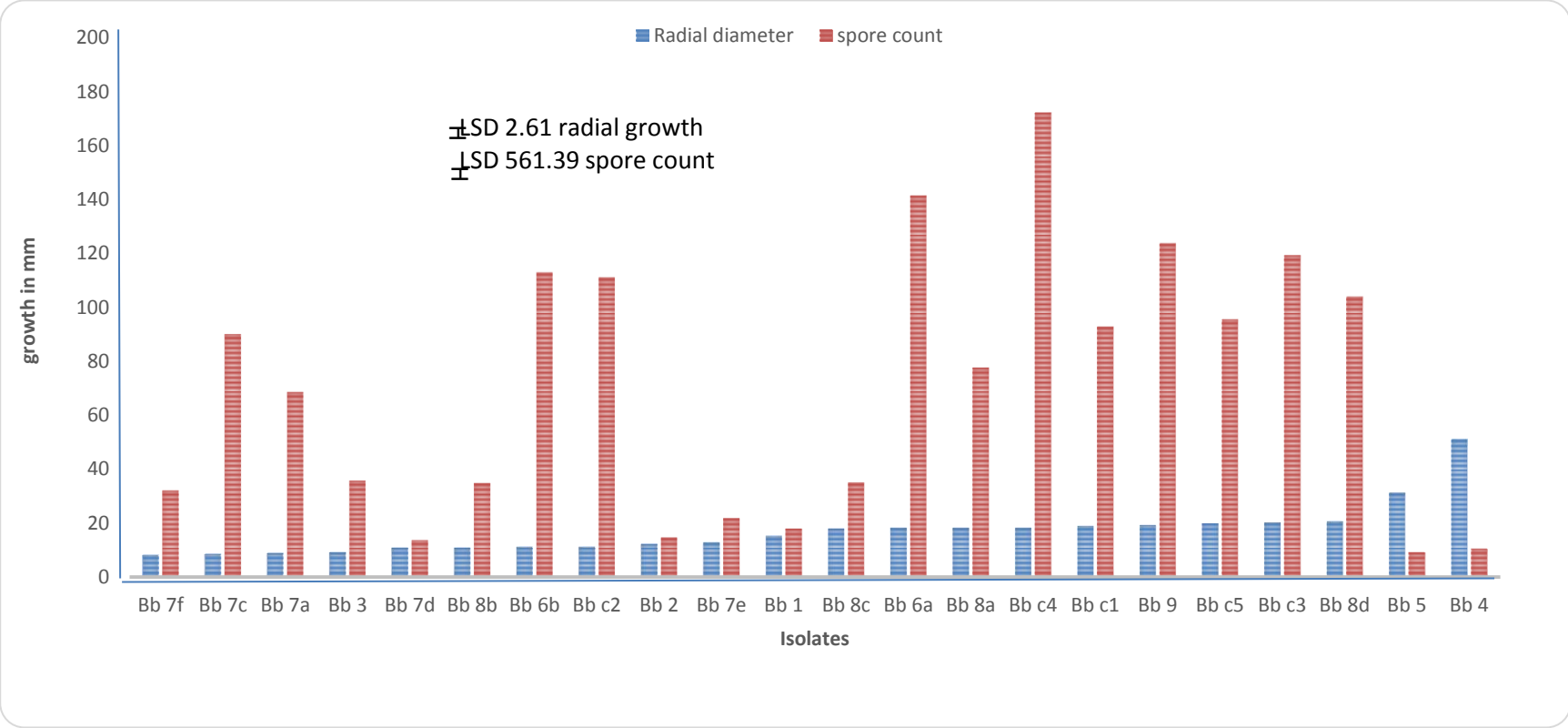


Figure 4.1a: Spore count of *B. bassiana* isolates under a light microscope and radial diameter in mm on day 7

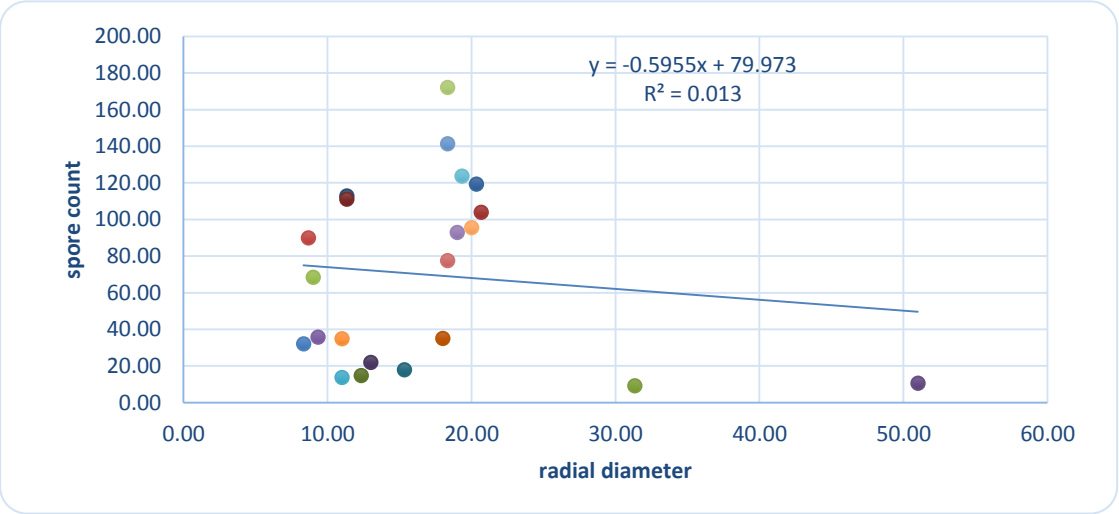


Figure 4.1b Correlation between radial diameter and spore count

There was negative degree of correlation between the radial diameter and spore count of the isolates (Figure 4. 1b).

4.1.3 Spore count

Bb Ch c4 had the highest number (17,216) of spores followed by Bb Ke 6a (14,141.71), Bb Mu 9 (12375), Bb Ch c3 (11933.30), Bb Ke 6b (11291.70) and Bb Ch c2 (11108.3), whereas Bb Ke 1, Bb Ke 2Bb Ke 2, Bb Gi 7d, Bb Ke 4 and Bb Ke 5 had the least number of spores (between 1800 and 925). Bb Ch c5, Bb Ch c1, Bb Gi 7c, Bb Ch c 8a and Bb Gi 7a had moderately high number of spores (between 9558.3 and 6858.3) while Bb Ke 3, Bb Ch c 8c, Bb Ch c 8b, Bb Gi 7f and Bb Gi 7e had moderately low number of spores (between 3583.3 and 2191.7) (Table 4.2 and Figure 4 2a).

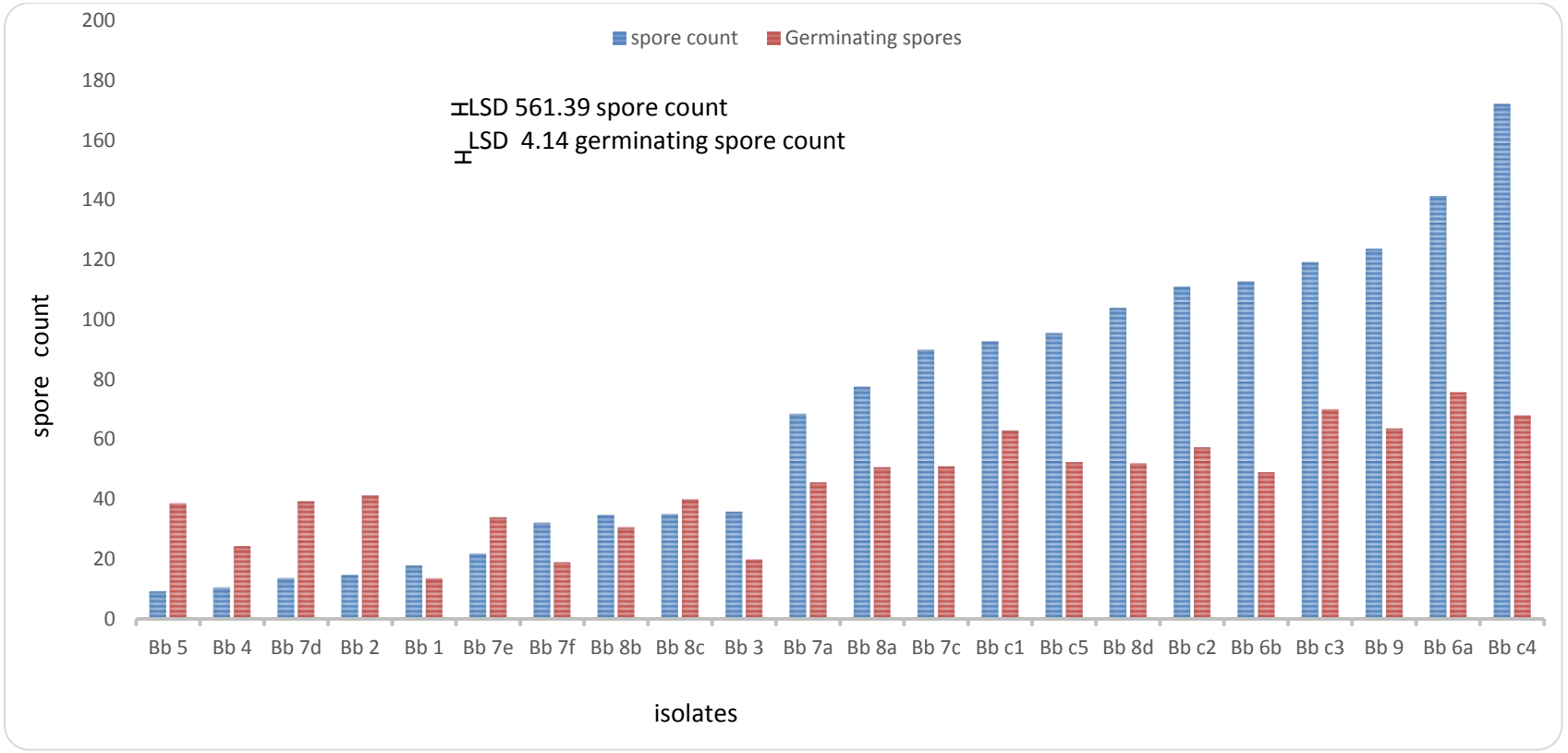


Figure 4.2a: Germination and spore count of *B. bassiana* isolates after 16hrs under light

Table 4. 2: Growth parameters of the 22 *Beauveria bassiana* isolates

Isolate	Radial growth	Mean Radial growth for 7 days (mm)	Spore count	Germinating	Non germinating	Cfu
BbKe 1	15.33d	8.05(2.04)def	1800.00ih	13.67m	86.33a	10.00(2.38)e
BbKe 2	12.33e	7.62(1.92)ef	1483.30ij	41.33g	58.67ef	3.33(1.46)e
BbKe 3	9.33fg	5.86(1.85)fg	3583.30g	20.00kl	83.33ab	3.00(1.37)e
BbKe 4	51.00a	22.05(2.74)a	1058.30j	24.33k	75.67c	11.00(2.40)e
BbKe 5	31.33b	17.86(2.67)b	925.00j	38.67i	61.33e	8.67(2.22)f
BbCh c1	19.00c	10.81(2.28)c	9291.70e	63.00d	37.00j	313.33(5.74)b
BbCh c2	11.33ef	6.24(1.92)fg	11108.30d	57.33e	42.67i	6.33(1.89)e
BbCh c3	20.33c	11.62(2.32)c	11933.30c	70.00b	30.00l	325.00(5.79)b
BbCh c4	18.33c	10.71(2.29)c	17216.70a	68.00bc	32.00lk	308.33(5.67)b
BbCh c5	20.00c	11.24(2.31)c	9558.30e	52.33f	47.67h	330.67(5.80)b
BbKe 6a	18.33c	10.19(2.23)cd	14141.70b	75.67a	24.33m	400.00(5.98)a
BbKe 6b	11.33ef	6.57(1.91)fg	11291.70d	49.00fg	51.00gh	70.00d(4.26)e
BbGi 7a	9.00fg	5.24(1.75)g	6858.30h	45.67gh	54.33fg	11.33(2.49)f
BbGi 7c	8.67fg	4.8(1.73)g	9008.30e	51.00f	49.00h	112.00(4.66)d
BbGi 7d	11.00efg	6.57(1.88)fg	1375.00ij	39.33i	60.67e	7.67(2.14)e
BbGi 7e	13.00de	7.00(2.03)fg	2191.70h	34.00j	66.00d	14.00 (2.68)e
BbGi 7f	8.33g	5.1g (1.67)	3216.70g	19.00l	81.00b	3.00(1.37)e
BbCh 8a	18.33cd	10.52(2.23)cd	7766.70g	50.67e	49.33g	277.33(5.62)bcd
BbCh 8b	11.00fgh	5.62(1.88)hij	3491.70i	30.67h	69.33d	230.00(5.42)cd
BbCh 8c	18.00d	10.24(2.04)cd	3516.70i	60.00e	60.00e	210.67(5.35)d
BbCh 8d	20.67c	11.71(2.34)c	10400.00e	52.00e	48.00g	301.33(5.67)bc
BbMu 9	19.33c	9.48(2.14)cde	12375.00c	63.67cd	36.33jk	216.33(5.37)c
CV%	9.41	8.60	4.88	5.54	4.74	7.70
LSD	2.61	0.11	561.39	4.14	4.28	0.49

**The figures in parenthesis are log transformed ln(x+1). Means followed by the same letter in the column are not significantly different at P<0.05.*

4.1.4 Germination counts

BbKe 6a (75.67), BbCh c3 (70.00), BbCh c4 (68.00), BbMu 9(63.67) and BbCh c1 (63) had more than 60 germinating spores, which were considered to be high whereas BbKe 1(13.67), BbGi 7f (19), BbKe 3 (20), Bb Ke4 (24.33) and BbCh 8b (30.67) had germinating conidia below 33 germinating conidia which were considered to be the lowest. Table 4.2. BbCh c5 (52.33), BbCh 8d5 (2), BbGi 7c (51.00), BbCh 8a (50.67), BbKe 6b (49) and BbGi 7a (45.67) had germinating conidia above 45 to 53 which was considered to be moderate. BbKe 2 (41.33), BbCh 8b (30.6), BbGi 7d (39.33), BbKe 5 (38.67) and BbGi 7e (34) had germinating conidia between 30.6 to 41.33 that was considered to be low. Figure 4.2a.

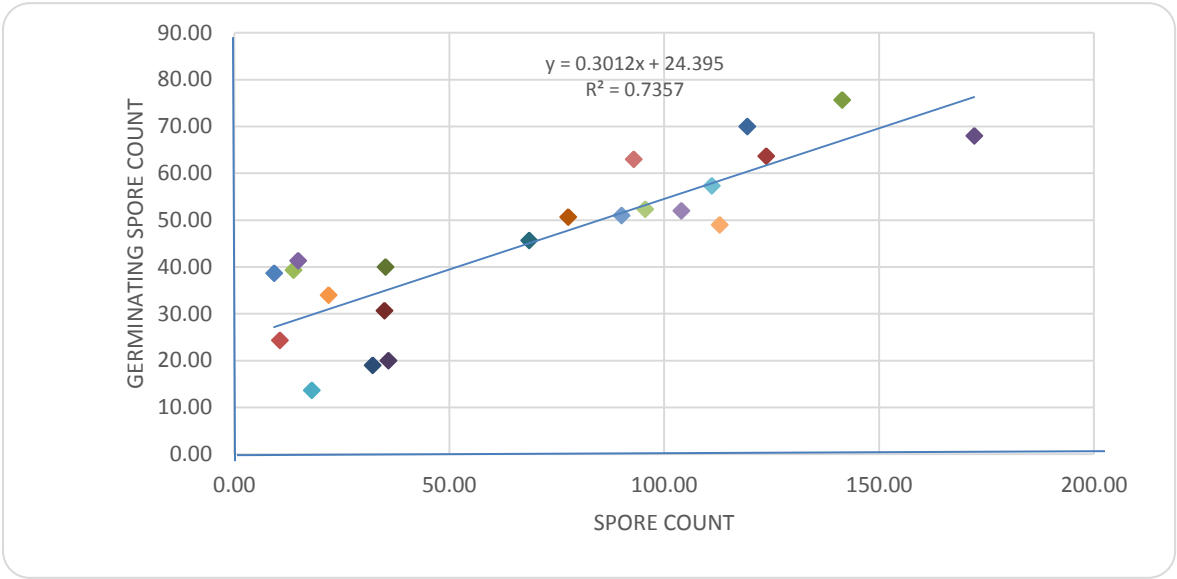


Figure 4.2b: Correlation between spore count and germinating spores

A high positive degree of correlation between the spore count and the germinating spores of the isolates was noted (Figure4.2b).

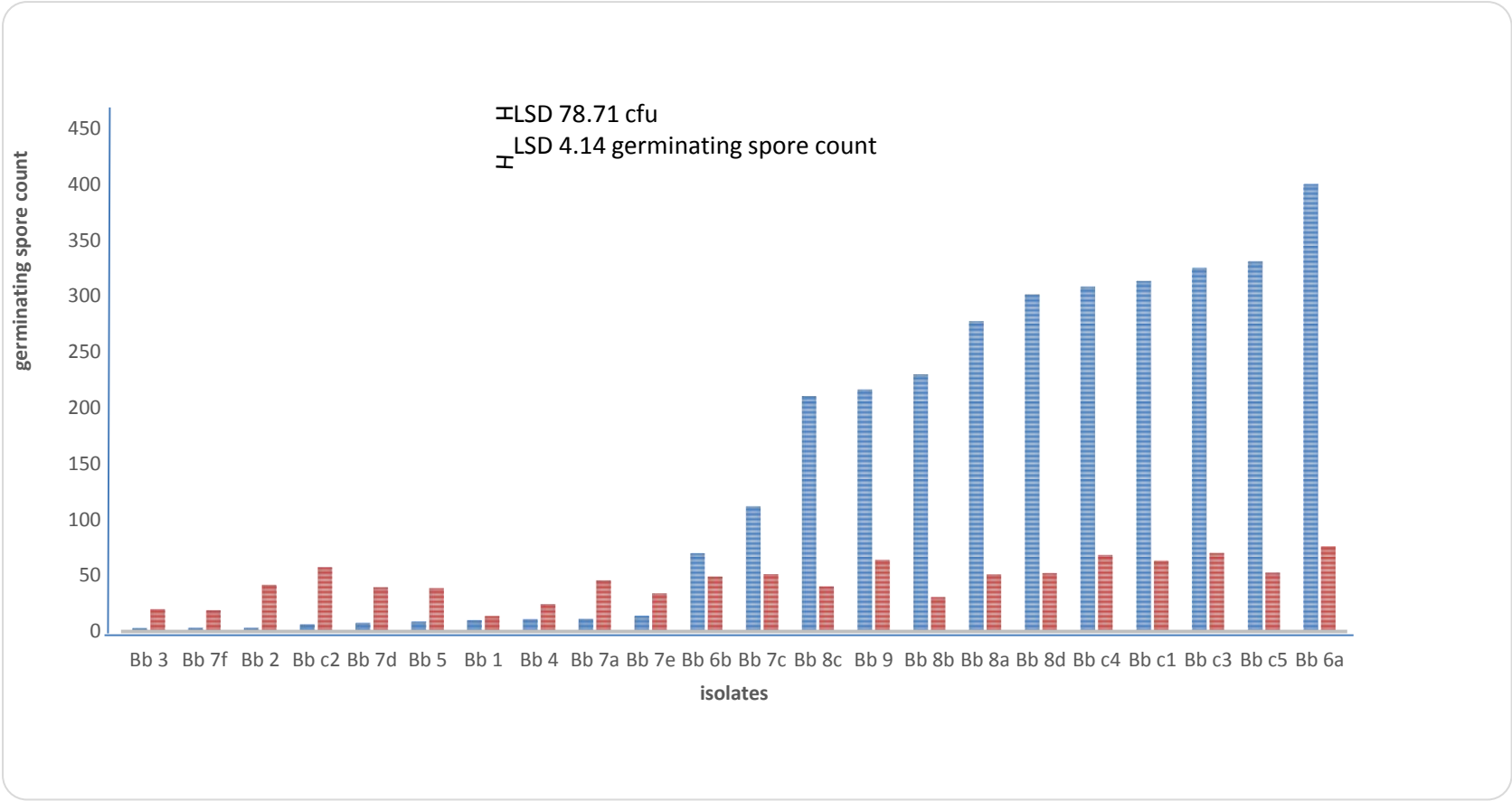


Figure 4.3a: Germination and cfu counts of *B. bassiana* isolates after 16hrs under a light microscope

4.1.5 Colony forming units (cfu)

Isolate BbKe6a had the highest number of colony forming units (400.00) while the isolate BbGi7f had the least colony forming units 3.00(1.37) (Figure4.2b). However, the colony forming units of isolates from Kericho (BbKe (1-5) and from Giciaro farm BbGi7 (a-f) were significantly different at ($p<0.5$) (Table 4.2). The isolates with the high spore germination (BbKe6a had 75.67 and BbChc3 had 70.00) also showed high colony forming units (cfus) BbKe1 had 13.67 and BBKe3 had 3.00) whereas, the isolates with low spore germination also had low colony forming units (cfus) (Figure 4.3a).

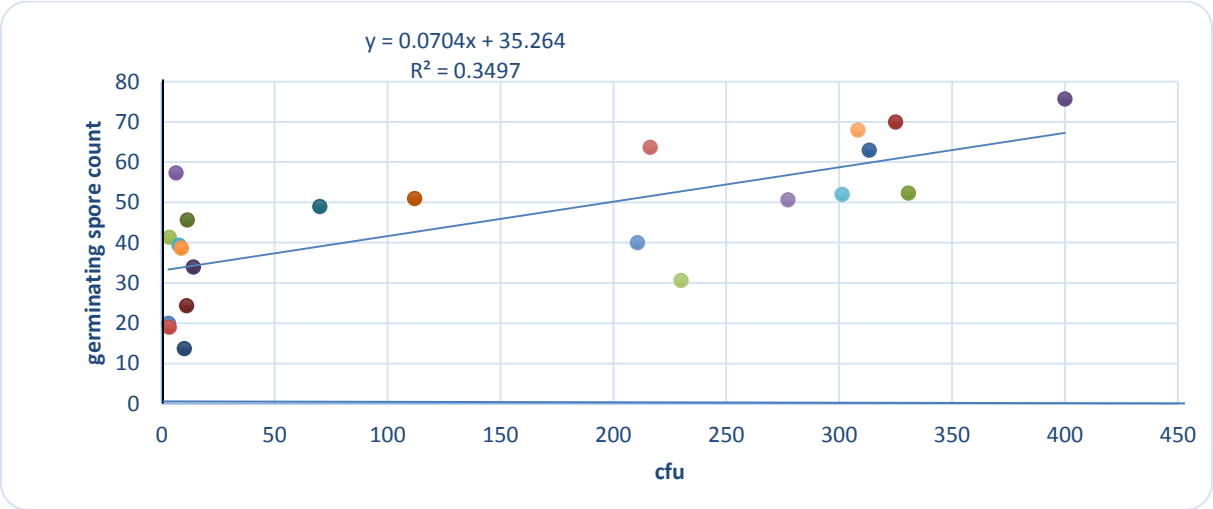


Figure 4.3b: Correlation between cfu and germinating spore counts

There was a positive correlation between the colony forming units of the isolates and the germinating spore counts (Figure 4.3b).

4.2 Determination of the efficacy of the selected isolates on the weevils

4.2.1 Effect of *B. bassiana* on population of the tea weevils

Isolate BbGi 7a had a higher insecticidal activity against the tea weevils compared to the other isolates (Table 4.3.). Results of this study also indicated that the highest insecticidal activities of the isolates ranged between the 9 days to 11 days after in inoculation of the weevils with the conidial suspensions as shown in (Figure 4.4). There was no significant difference on the concentration of the Isolates, although isolates BbKe 6a, BbCh c2, BbCh c 3 and BbGi7a all showed growth of the fungal mycelia on the dead weevils.

The four selected isolates were significantly different (LSD=2.83, $p<0.5$), while the concentrations were shown to be also significantly different (LSD=2.53, $p<0.5$). (Table 4. 3) the weevils were susceptible to the four isolates of *B. bassiana* tested.

Table 4.3: Weevils mortalities in different spore concentrations of different selected isolates

Time (Days)	Isolate		Concentrations			
			10^5	10^6	10^7	10^8
Day 1	BbGi7a		0	0	0	0
	Bb Chc 2		0	0	0	0
	Bb Chc 3		0	0	0	0
	Bb Ke 6a		0	0	0	0
	Control		0	0	0	0
Day 2	BbGi7a		13.3	23.3	16.7	13.3
	Bb Chc 2		0	3.3	3.3	0
	Bb Chc 3		0	10	16.7	16.7
	Bb Ke 6a		0	6.7	0	0
	Control		0	0	0	0
Day 3	BbGi7a		20	30	20	16.7
	Bb Chc 2		3.3	10	10	6.7
	Bb Chc 3		10	13.3	30	26.7
	Bb Ke 6a		10	10	10	3.3
	Control		3.3	0	0	0
Day 4	BbGi7a		33.3	43.3	56.7	33.3
	BbChc 2		10	13.3	20	10
	Bb Chc 3		13.3	16.7	36.7	33.3
	Bb Ke 6a		13.3	16.7	16.7	10
	Control		6.7	3.3	0	0
Day 5	BbGi7a		43.3	46.7	70	40
	Bb Chc 2		13.3	20	26.7	16.7
	Bb Chc 3		26.7	26.7	50	46.7
	Bb Ke 6a		13.3	20	20	10
	Control		6.7	6.7	0	3.3
Day 6	BbG7a		56.7	66.7	76.7	40
	BbChc 2		20	23.3	33.3	23.3
	Bb Chc 3		33.3	36.7	60	60
	Bb Ke 6a		16.7	26.7	30	16.7
	Control		10	10	0	3.3
Day 7	BbGi7a		70	83.3	90	50
	Bb Chc 2		30	30	43.3	33.3
	Bb Chc 3		40	46.7	73.3	66.7
	Bb Ke 6a		20	26.7	33.3	23.3
	Control		10	10	3.3	6.7

Weevils' mortalities in different spore concentrations of different selected isolates (contd)

Time (Days)	Concentrations				
Day 8	Isolates	10^5	10^6	10^7	10^8
	BbGi7a	76.7	93.3	96.7	73.3
	Bb Chc 2	40	33.3	50	36.7
	Bb Chc 3	43.3	53.3	83.3	73.3
	Bb Ke 6a	26.7	33.3	40	26.7
	Control	10	10	3.3	10
Day 9	BbGi7a	86.7	96.7	100	80
	Bb Chc 2	43.3	43.3	56.7	43.3
	Bb Chc 3	50	63.3	83.3	76.7
	Bb Ke 6a	30	36.7	43.3	30
	Control	10	13.3	3.3	10
Day 10	BbGi7a	86.7	96.7	100	83.3
	Bb Chc 2	50	60	66.7	50
	Bb Chc 3	56.7	70	93.3	90
	Bb Ke 6a	36.7	43.3	56.7	43.3
	Control	10	20	10	16.7
Day 11	BbGi7a	93.3	100	100	90
	Bb Chc 2	56.7	70	73.3	60
	Bb Chc 3	60	76.7	96.7	93.3
	Bb Ke 6a	43.3	50	56.7	46.7
	Control	16.7	20	10	16.7
Day 12	BbGi7a	100	100	100	96.7
	BbChc 2	63.3	80	86.7	70
	Bb Chc 3	66.7	93.3	100	100
	Bb Ke 6a	53.3	63.3	63.3	56.7
	Control	20	23.3	10	16.7
Day 13	BbGi7a	100	100	100	100
	Bb Chc 2	70	90	90	80
	Bb Chc 3	70	96.7	100	100
	Bb Ke 6a	56.7	70	73.3	63.3
	Control	20	23.3	13.3	20
Day 14	BbGi7a	100	100	100	100
	Bb Chc 2	83.3	93.3	96.7	86.7
	Bb Chc 3	73.3	100	100	100
	Bb Ke 6a	63.3	80	83.3	73.3
	Control	23.3	26.7	13.3	20
C.V (%)					29.67
LSD (p=0.05)	Isolates				2.83
	Concentration				2.53

Both the isolates and the concentrations were significantly different at (P<0.5) (Table 4.3). Although all the tested isolates caused mortality in the weevils, there was a significant variation in the efficacy among them. Higher percentages of mortality were recorded (between 100% and 53.3%) when the weevils were inoculated with different *B. bassiana* isolates compared to the low mean mortalities (between 3.3% and 26.7%) control treatment (Table 4.3). The fungal concentrations were found to be significantly different at (p<0.5) (Table 4.3).

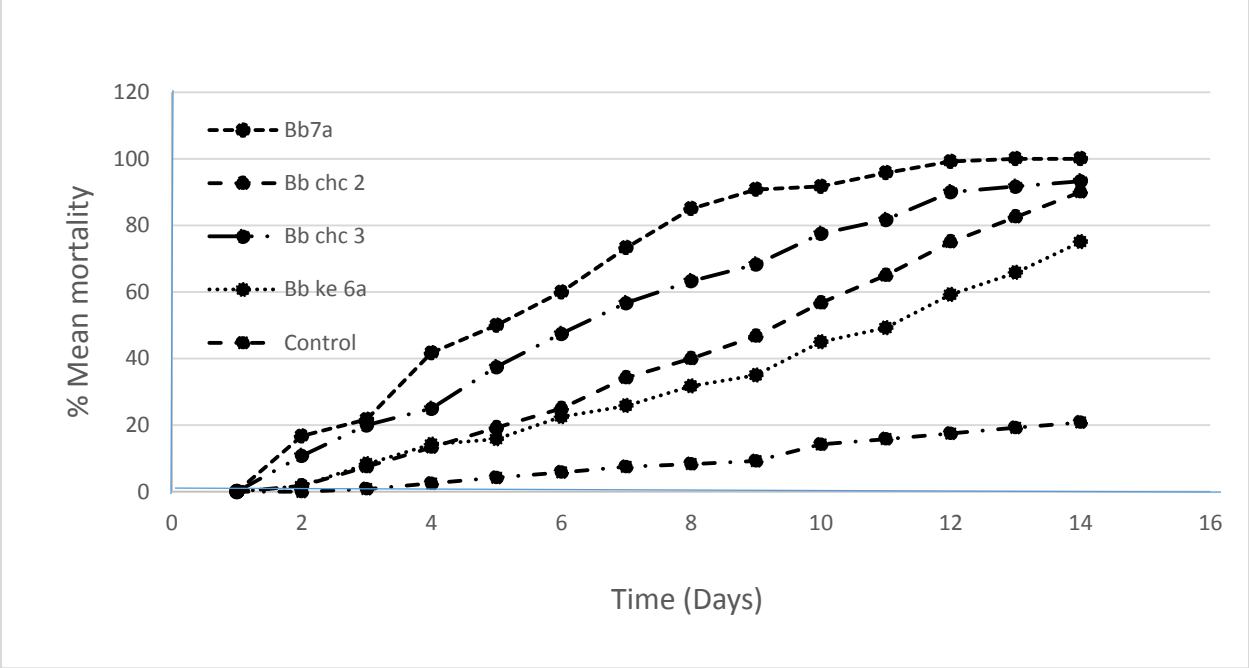


Figure 4.4 Mean mortalities of weevils in the selected *B, bassiana* isolates

The selected isolates were found to be significantly different in relation to mean mortalities of the weevils at (p<0.5) (Figure 4.4). The dead weevils were found to be covered by fungal mycelia after three days that confirmed they were killed by the entomopathogenic fungus *B. bassiana* (Plate 4).

Plate 4: Dead Cadavers of the Nyambene weevils

(Source: Author, 2016)

4.2.2 Effect of *B. bassiana* isolates on feeding of the tea weevil

Results obtained showed that there was a significance difference (P <0.5) in the different isolates in area of the leaf eaten. Isolate BbCh c3 had the largest area eaten by the tea weevils but not significantly different to leaf area eaten by the weevils in the controlled treatment. The isolate with least eaten area was BbGi 7a followed by Bb Gi7f, BbCh c2, BbCh 8c which did not differ significantly (P≤0.05) (Table 4.3).

Table 4. 4: Effect of *B. bassiana* isolates on feeding of the tea weevil (Area eaten in cm^2)

Isolates	Concentration			Mean
	10^5	10^6	10^7	
BbGi 7a	2.13	1.39	1.11	1.55a
BbCh c2	1.59	2.19	1.70	1.72a
BbGi 7f	2.30	2.00	2.23	2.18b
BbCh c3	3.78	2.02	3.48	3.09c
BbCh 8c	2.37	2.72	2.03	2.37b
BbKe 6a	2.21	1.69	2.04	1.98b
Control	2.80	2.80	2.80	2.80b
CV (%)				49.70
LSD (p≤0.05) Isolates Concentration				 1.08 NS

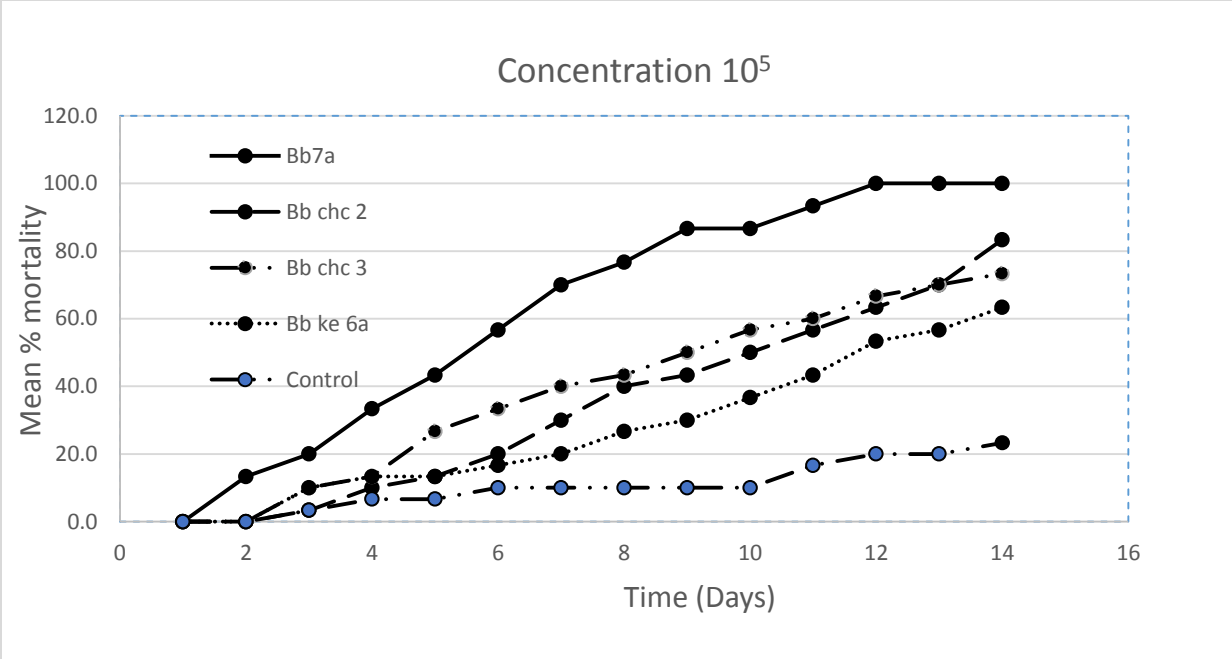


Figure 4.5a Weevils mortalities at concentration 10^5 conidia ml^{-1}

The lethal time LT_{50} all the isolates was more than 10 days except for isolate BbGi7a where the LT_{50} was 6days (Figure 4.5a).

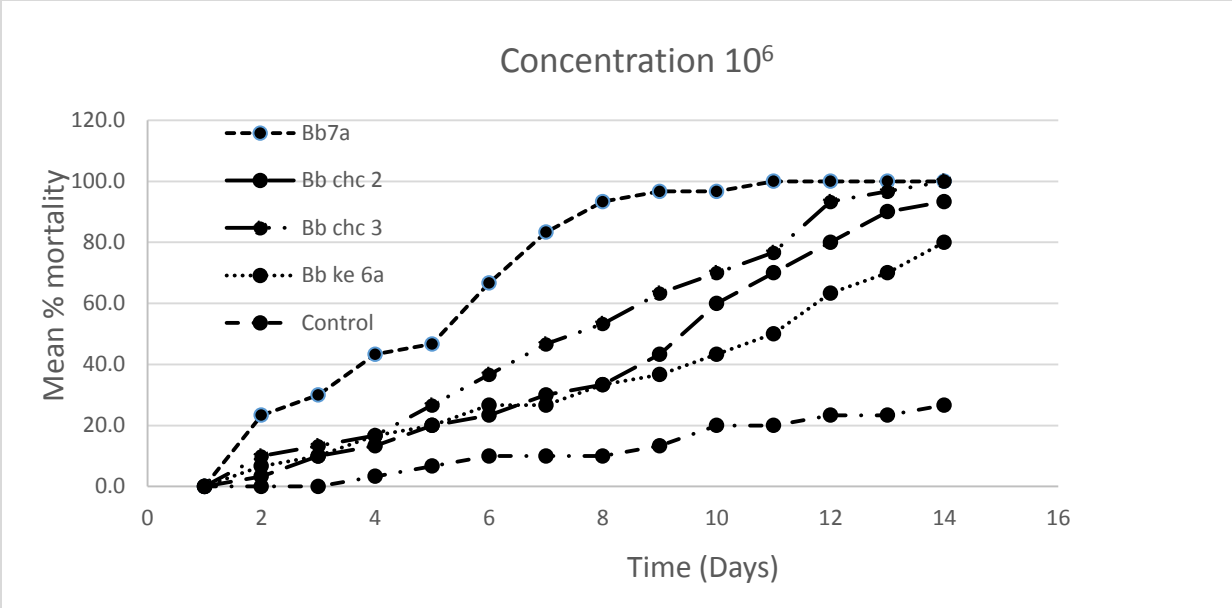


Figure 4.5b Weevils mortalities at concentration 10^6 conidia ml^{-1}

The conidial concentration of 10^6 conidia ml^{-1} gave a lethal time LT_{50} of 6days in BbGi7a, 8days in BbChc2, 10days in Bbchc3 and a LT_{50} of 11 days in BbKe 6a (Figure 4.5b).

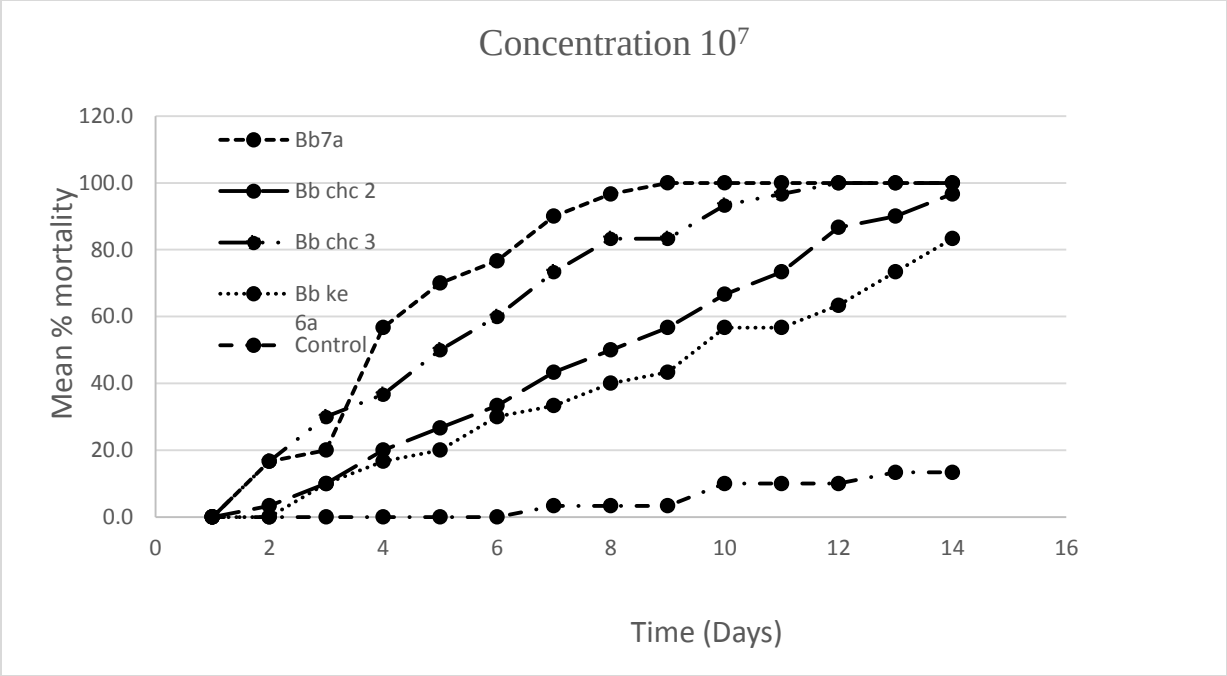


Figure 4.5c Weevils mortalities at concentration 10^7 conidia ml^{-1}

Conidial concentration of 10^7 conidia ml^{-1} gave a LT_{50} of 4days in BbGi7a, 5days in BbChc2, 7days in BbChc3 and 9days in BbKe6a (Figure 4.5c).

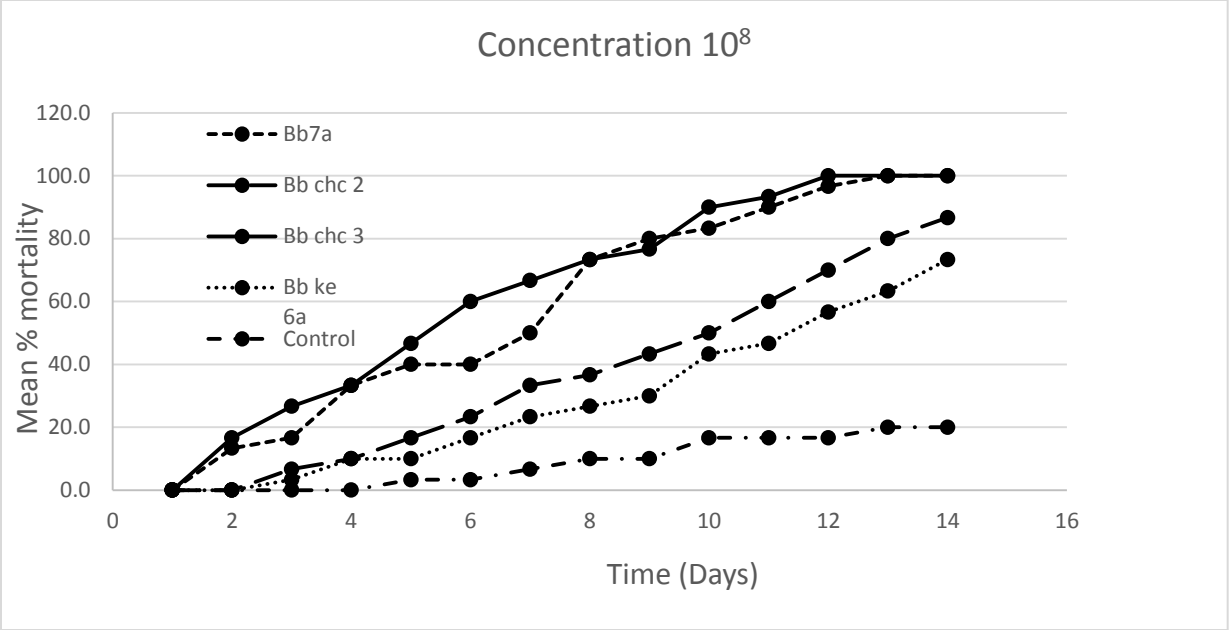


Figure 4.5d Weevils mortalities at concentration 10^8 conidia ml^{-1}

The concentration 10^8 conidia ml $^{-1}$ gave LT of 5.5days in BbGi7a, 7.3days in BbChc2, 9.7 days in BbChc3 and 11.0 in bbKe6a (Figure 4.5d). Mean percent mortality of weevils infected with (a) 10^5, (b) 10^6, (c) 10^7 and (d) 10^8 concentrations of four *B. bassiana* isolates (isolate Bb Gi7a, BbChc 2, BbChc 3, Bb Ke 6a and Control) for 14 days showed that all the isolates had potential of being biological control agents to the tea weevils. The susceptibility was dose dependent (Figure4.5a-d), the best of the isolates killed 100% of the weevils at dose of 10^7 conidia ml^{-1} by the 9th day after exposure Isolate Bb Gi7a killed over 80% of the weevils by 5^{th} day after exposure to a conidial concentration of 10^5 conidia ml^{-1} (Table 4.3). No significant differences were observed in weevil mortality between isolates BbKe 6a and BbCh c2 at all the concentrations (Table 4.3). The dose- mortality relation curves of isolates BbKe 6a and BbCh c2 were also similar (Figures 4.5a-d). However significant differences were observed in weevils mortality between isolates BbKe 6a and BbGi 7a and between isolates BbCh c2 and

BbGi7a at 10^5, 10^6 , 10^7 and 10^8 conidia ml^{-1}. There were no significant differences in the weevils' mortality between the four best isolates of *B. bassiana* at all the concentrations on the 10^{th} day. Figure (4.5). The LC_{50} value for the isolate BbKe 6a was lower than of the other isolates followed by the isolate BbCh c2, BbCh 3 and BbGi 7a. There were no significant differences in the mortalities caused by the isolates BbCh c3 and BbGi 7a. Thus, *B. bassiana* isolates BbGi 7a and BbCh c3 had the highest pathogenicity among the four isolates tested.

Table 4. 4 Summary of the isolates, dose and mean mortalities of the weevils.

Isolates	**Concentration**				**%Mean mortality**
	10^5	10^6	10^7	10^8	
BbGi7a	62.9	70.0	73.3	58.3	66.1
Bb Chc 2	34.5	40.7	46.9	36.9	39.8
Bb Chc 3	38.8	50.2	66.0	63.1	54.5
Bb Ke 6a	27.4	34.5	37.6	28.8	32.1
Control	10.5	11.9	4.8	8.8	9.0

The isolate BbGi7a showed a mean mortality of 66.1%, while the isolate BbKe6a had the least mean mortalities of 32.1%. The conidial concentration of 10^7 ml^{-1} showed high weevil mortalities BbGi7a (73.3%), BbChc2 (46.9%) BbChc3 (66.0%) and BbKe6a (37.6%) in all the selected isolates compared to the other concentrations (Table 4.4).

CHAPTER FIVE

DISCUSSIONS

Entomopathogenic fungi such as *B. bassiana* are naturally occurring biological control agents. However, no documented studies have been reported on the use of this fungi for biological control on tea weevils except the preliminary studies done at the Tea Research Foundation of Kenya (TRFK) by Sudoi *et al*., (1999). The present study evaluated the pathogenicity of 22 fungal isolates against the tea weevils. Despite the variability in virulence, the experiments clearly demonstrated that *B. bassiana* is an effective biocontrol agent against *Entypotrachelus meyeri*.

This study first considered the selection and survey on the pathogenicity of *B. bassiana* isolates on tea weevils, earlier studies have shown that the physiological characteristics of *B. bassiana*, such as conidial viability, germination speed of the conidia, spore production are related to the fungal virulence in response to environmental conditions like temperature and relative humidity. (Liu *et al.*, 2003).

There were significant differences in radial growth, germination, sporulation and colony count among the different *B. bassiana* isolates included in this study. The isolates obtained from Kericho had high radial growth but low sporulation while some had a low number of germinating conidia such as Bb Ke 4 and Bb Ke 5. Isolates from Chepkoilel were high sporulators with a high number of germinating conidia such as Bb Ch c4. These isolates also had high numbers of colony forming units. Most of those isolated from Giciaro farm in Nyambene exhibited slow growth rates e.g. Bb Gi 7a, Bb Gi 7c and Bb Gi 7f, and had low colony counts. Isolates from Mununga also showed high growth

rates, high spore count and high number of germinating conidia while one isolate from TRFK stock (Bb Ke 6a) showed high spore counts, high numbers of germinating colonies and colony counts but had a moderate rate of growth. The daily hyphal growth rates observed in this study were similar to that obtained by Varela *et al*.,(1996), Fargues *et al.* (1997), Ouedraogo *et al*. (1997), Thomas *et al*., (1997), and Vidal *et al.* (1997) (with a range between 1.0 and 6.0 mm/day). It is believed that fungal isolates with rapid germination and hyphal growth rates may have an advantage as biological control agents because host infection can potentially occur much faster (Hajek *et al*., 1994; Varela *et al*, 1996). In this study, isolates from TRFK stock, Chepkoilel, Mununga and those from Kericho had these traits thus may have an advantage as biological control agents.

Spore production by the *B. bassiana* isolates, in this study ranged between 9.25×10^2 and 1.72×10^4 conidia/ml, which was quite different from that of Varela *et al.,* (1996) where production ranged between 2.2×10^8 and 1.6×10^9 conidia/cm^2. Differences in fungal isolates and incubation temperatures (18^0 C in our study vs. 27^0C in the previous study) may have contributed to this discrepancy. High negative correlation was noted between some variables like radial growth and spore count such that isolates with a high radial growth had no relationship with spore count. A positive correlation between the cfu and germinating conidial count was noted. Isolates with high spore count had high number of germinating conidia indicating a positive correlation as well. It is evident from the results of this study that some *B. bassiana* isolates are most virulent on the tea weevil. Isolate BbGi7a had a moderate spore count number of 6858.80, and germinating units of 45.67 was found to be the most virulent on the Nyambene weevil. This observation may be attributed to the locality of the isolate which is the common habitat of the Nyambene

weevil and therefore, this may mean a local isolate is more promising as a control agent since it has adapted to the local conditions.

The study has also found out that the virulent type of isolates affects the feeding rate of the weevils. This implies that the more the *B. bassiana* penetrates the body of an insect the weaker the insect becomes hence feeds less. Therefore, the entomopathogenic fungus *B. bassiana* isolates studied could be containing diverse assemblage of genotypes and probably comprises species complexes as reported by Inglis *et al.* (2001). It is conceivable to have individual isolates or pathotypes which exhibit substantial specificity on the host range.

In the bioassay to evaluate the pathogenicity of the 22 isolates of *B. bassiana* from different hosts and sources, the virulence of the fungi towards the weevils varied greatly. All the isolates tested were able to infest the tea weevils in the laboratory, but there were significant differences at $p< 0.5$) between the isolates. Earlier studies done on other Coleopterians have shown similar patterns of differences in insect pathogenicity between different isolates of *B. bassiana* (Clarkson *et al*., 1996; Inglis *et al* 1997; Moino *et al.,* 1998). The entomopathogenic activity of *B. bassiana* was confirmed by the presence of fungal hyphae on the body of the weevils. Fungal growth was evident as early as 4days after exposure in 10^7 conidia /ml. The weevils exhibited signs of infection such as post-emergence of the fungus followed by conidiation on the surface of the cadavers, at relative humidity above 60%.

Beauveria bassiana isolates BbGi7a, BbChc2, BbChc3 and BbKe6a were identified as the most pathogenic isolates to the tea weevils, causing mortalities of 70% or more within

8days, at a conidial concentration of 10^7 ml^{-1}. The isolates had significant differences in there action on the weevils, these differences may be due to the isolates differences in virulence. Furthermore, the tea, weevils' hard cuticle acts as a barrier for fungal penetration and its thickness increases with every moulting so that difference in susceptibility of the weevils to entomopathogenic fungi (Gindin *et al.*, 2006). This has been shown in studies done on palm weevil *Rynchophorus ferrugineus (Olievier)* where 100% mortality of the adult weevils treated with *B. bassiana* Larval mortality of 82-92% was shown within 24hours in palm weevil *Rynchophorus ferrugineus (Olievier)* treated with *Beauveria bassiana* in conidial concentration of 1 X 10^8conidia ml^{-1} while on the adult weevils a 100% mortality was shown in 13 days. (Haijer *et al*, 2015). This is attributed to the lack of thick cuticle in the larvae compared to the adult.

Dose-mortality bioassays showed that mortality rates of the tea weevils were a function of the *B. bassiana* isolates conidia concentration. Mortality responses observed were proportional to the concentrations of conidial suspensions. The mortality of the tea weevils increased with increase in conidial concentration, from concentration of 10^5 with mean mortality (40.9% to 51%) to mean mortalities of(55.5% to 73.3%) in 10^7 conidial concentration , this is similar to work shown by; Mwamburi *et al.,* (2010) and Vanmachi *et a.l*, (2011). In the present study, low mortalities were observed at conidial concentrations below 10^7 conidia ml^{-1}. This was the same as observed by Haijer *et al.,* (2015) on weevils that showed 100% mortality after 13days of inoculation. For isolate BbGi7a the theoretical maximum of 100% mortality was induced at the dose of 10^7 conidia ml^{-1} at 7.8 days and therefore, no further increase was possible with time. This is acceptable for entomopathogenic fungi when used as a biological control agent since they

target reducing the pests population to 50% and above the level of effectiveness obtained in this study compares favourably well with those of Omukoko *et al. (*2014) working on other weevils. Dose –dependent mortality was demonstrated previously by Trudel *et al.* (2006) using six isolates of *B. bassiana* against the White Pine Weevil by using two methods of application, soil and branch. This dose effect of *B. bassiana* conidia concentrations on infections levels and rates may be due to the following reasons. First, it is possible that only certain conidia infest the host (Butt *et al.*, 2000). Secondly, it was possible that a certain conidia minimal number of attached conidia are needed before the weevil cuticle can be penetrated. Such positive correlations between the number of infective spores and mortalities have been obtained when using *B. bassiana* against other arthropods (Kaaya *et a.l*, 1995; Santoro *et al*, 2008). The four previously assayed isolates were selected for further bioassays. These isolates BbGi7a, BbKe6a, BbChc3 and BbChc2, which caused the greatest levels of mortality among the tea weevils. Subsequent bioassays indicated that isolates BbChc3 and BbKe6a had the lowest LC_{50} values: isolate BbGi7a had the lowest LT_{50} value and greatest slope. These findings were consistent with the earlier assays that indicated that isolate BbGi7a was the most virulent isolate. The use of *Beauveria bassiana* as a biological control agent against tea weevils would be attractive for several reasons. First, it provides an alternative to chemical management strategy of the tea weevils. Secondly, use of *B.bassiana* as a biological control agent on the tea weevils avoids the hazardous effects of insectides to the tea users and the environment. The continued use of insectides would also lead to emergence of resistant strains of the weevils, but there will be little chance of the weevils developing resistance to *B. bassiana.* The search for effective strains of entomopathogenic fungi

should include natural isolates from the target insect because isolates may have higher virulence than those from unrelated hosts (Mainania, 1992). However, a major limitations to the development of fungi for insect control is the lack of readily available formulation technology for improved shelf life, persistence, efficacy and the field targets.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1). The study demonstrated that the isolates BbGi 7a isolated from Giciaro farm in Meru County had the highest efficacy against the Tea weevil *E. meyeri* followed by BbCh c 3 isolated in University of Eldoret soils in Uasin Gishu county. The isolate Bb Ke 6a isolated from Timbilil farm in Kericho County had the least efficacy.

2). From the study based on the macroscopic characteristics the 22 isolates were classified into three groups, Group 1 (Bb Ke 6a, Bb Ke 6b, Bb Ch c1, Bb Ch c2Bb c 3, Bb Chc4, Bb c 8a and BbCh c5), produced white, villous colonies and the reverse colour was brown yellow , group 2 (Bb Ke 1, BbKe 2, Bbke 3, BbKe 4, BbKe 9 and BbKe 5) had cream white and felty colonies with brown reverse colour and group 3 (Bb Gi 7a-f) produced white yellow and thin powdery colonies with yellow reverse colour. However, these categories did not reflect the isolates virulence. Group 3 and group 1 exhibited the highest virulence.

3). Study demonstrated that the conidial concentration of 10^7 conidia ml^{-1} gave the highest mean mortality in all the tested isolates from 73.3 % in BbGi7a to 37.6% in BbKe6a, while isolates concentration of 10^5 condia ml^{-1} had the lowest mean mortalities of the weevils from 62.9% in BbGi7a to 27.4% in BbKe6a.

6.2 Recommendations

From the findings of this study, it is evident that *Beauveria bassiana* alone could not effectively manage the weevils; therefore the following recommendations are put forward:

1. There is need to incorporate other entomopathogenic fungi together with *B. bassiana*, which could bring out better results, in mind here is conidium production by three species of insect pathogenic fungi especially the ones isolated from the same soils *B.bassiana* is isolated from.
2. Although cultural classification of the isolated *B.bassiana* was done though macroscopic morphological characteristics of the conidia there is need to isolate more of the fungi isolates and characterize them by use of the modern molecular genetics, by use of DNA markers. This could also determine the reason of the fungi virulence, by isolating the virulent genes from the most virulent isolates and perhaps intrude the genes to other isolates.
3. This study was carried out *in vitro*, it is advisable that more studies be done especially in the field where the weevils invade so as to determine the field conditions on the efficacy of theses fungi in the normal environment of the weevils.
4. Rather than the dipping method that was done to infect the weevils, more study on the effects of other methods of infection could be studied to derive the best method of infecting the tea weevils.
5. The ecology, mode of feeding and breeding of these weevils seemed rather complicated, It is suggested that more studies on this area be done to understand

the weevil better in the Kenyan perspective and therefore be in a better position to determine its better mode of control.

6. Detailed study could also be embarked on the cultivars of tea that are preferred in feeding by the tea weevils, in combination with the fungi to manage the menace through bicontrol with tea breeding.

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