ENDOPHYTIC ACTIVITY OF *BEAUVERIA BASSIANA* IN TOMATO AND ITS EFFICACY AGAINST RED SPIDER MITES (*TETRANYCHUS EVANSI*)

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Endophytic activity of *Beauveria bassiana* in tomato and its efficacy against red spider mites (*Tetranychus evansi)*

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DECLARATION

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

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DEDICATION

I dedicate this work to my mother Mrs. Respah Khavayi for the years of sacrifice, support and love. My siblings Morgan, Vincent and Pauline for your support through the happy and challenging times in life and my nephews Emmanuel and Baraka, you are a source of great joy to the family.

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LIST OF ABREVIATIONS & ACRONYMS

ANOVA	Analysis of variance
ICIPE	International Centre of Insect Physiology and Ecology
IPM	Integrated Pest Management
RSM	Red Spider Mites
SAS	Statistical Analysis System software
SNK	Student-Newman-Keuls means separation test
SDA	Sabouraud dextrose agar
GLM	General Linear Model
BCA	Biological control Agent

ABSTRACT

The tomato red spider mite, *Tetranychus evansi* Baker and Pritchard, is an important pest of tomatoes in Kenya. Acaricides commonly used in excessive amounts by farmers have led to cases of resistance as well as widespread residues in the food chain and the larger environment. There is need to seek alternative control measures such as the use of endophytes. In this study, laboratory and greenhouse experiments were carried out to screen for *Beauveria bassiana* isolates that could establish as endophytes in tomato varieties and control red spider mites. Five *Beauveria bassiana* isolates (ICIPE 10, ICIPE 35, ICIPE 273, ICIPE 279 and ICIPE 283) were evaluated for their ability to endophytically colonize three tomato varieties namely, Cal J, Kilele and Anna. *B. bassiana* isolates were cultured on Sabouraud Dextrose Agar (SDA) for three weeks and harvested in 10 ml sterile distilled water in 20-ml universal bottle containing 0.01% Tween-20 and glass beads. A concentration of 1×10^{9} conidia ml^{-1} was prepared from the stock concentration and seeds sterilized and soaked for two hours. Control seeds were soaked in sterile distilled water containing 0.01% Tween-20 for two hours and planted in sterile soil in the growth chamber for four weeks and in the screenhouse for six weeks. Out of the five isolates, only ICIPE 35 was able to colonize up to 20% of leaves, stems and root area of the three tomato varieties and persisted for 4 and 6 weeks in the growth chamber and screen-house, respectively. In the growth chamber, ICIPE 35 was able to increase growth parameters i.e. plant height, fresh shoot and root weight by 50% in the tested tomato varieties compared to the control. When endophytically colonized tomato plants were infested with the red spider mite, *Tetranychus evansi*, the mite density was up to 4 times lower in endophyte treated plants than that of non-inoculated plants. In addition, assessment of plant damage associated with red spider mites attack in *B. bassiana* enhanced plants revealed less damage compared to the controls. The mechanism of control for the endophytic *B. bassiana* was analyzed by determining the production of lipases and protease from the three tomato varieties that produced clear zone of inhibition and also formed lipid crystals which lacked in the control plates. . The specific lipases and protease were however not analyzed as part of this study. The results of this study suggests that *B. bassiana* has the potential to establish as an endophyte in tomato, enhances plant growth and reduces mite infestation. Therefore, use of *B. bassiana* as an endophyte could complement existing control measures for the management of red spider mites in tomatoes.

CHAPTER ONE

INTRODUCTION

1.1 Importance of tomato

Tomato, Solanum lycopersicum (Solanales: Solanaceae), is a vegetable of economic importance worldwide (Rice, 1987; van Dam et al., 2005). Tomato production in sub-Saharan Africa has increased over the years to over 200 million metric tons in 2014 and occupied an area of approximately six million hectares (FAO, 2015). Tomatoes are grown as a cash crop by small, medium and large-scale farmers (Morrison et al., 1989). The crop is grown in backyards for home consumption as well as open fields and controlled environments (van Dam et al., 2005). Recently, there has been more emphasis on tomato production as a nutritional security crop, source of employment and income to rural resource poor farmers (Barrón and Rello, 2000).

Most varieties of tomato are adapted to a wide range of temperatures but the optimum being between 21°C- 24°C. At temperatures below 10°C and above 38°C physiological damaged is observed and significant yield reduction (Heuvelink, 2018; Rice, 1987). Light intensity affects the colour of the leaves and the fruit (Gould, 2013). Tomatoes grow well in fertile, deep, well-drained sandy loam soils, with a pH 6 (van Dam et al., 2005).

1.2 Tomato production constraints

Tomato production in Kenya has been on the decline by approximately 80% (Ochilo et al., 2018), due to environmental stresses, declining soil fertility, poor crop management and low quality seeds (KHDP, 2008). Pest and disease pressures have also increased, reducing the yields (Ochilo et al., 2018). Diseases of economic importance are late blight caused by *Phytophthora infestans*, early blight caused by *Alternaria solani*, bacterial canker caused by *Corynebacterium michiganense* and

bacterial wilt caused by *Ralstonia solanacearum* (Varela, 2003). The arthropod pests of economic importance include the red spider mites (*Tetranychus evansi.)* Baker & Pritchard (Acarina: Tetranychidae) (Letourneau and Goldstein, 2001), African bollworm, (*Helicoverpa armigera)* Hübner. (Lepidoptera:Noctuidae), Whiteflies, (*Bemisia tabaci)* Gennadius (Hemiptera: Aleyrodidae) and (*Aphis gossypii)* Glover (Homoptera: Aphididae). Common species of spider mites in Kenya include (*Tetranychus evansi)* Baker and Pritchard, (*Tetranychus urticae)* Koch and (*Tetranychus cinnabarinus)* Boisduval (Knapp, 2002). The population build-up of spider mites is favored by hot and dry tropical weather conditions (Varela et al., 2003). However, *T. evansi* is rated among the most damaging pest in tomato production worldwide (Gotoh et al., 2010).

Tetranychus evansi was first reported in Brazil (Boubou et al., 2012; Furtado et al., 2006), but it spread to parts of East and Central Africa where it is causing serious losses to tomato and other solanaceous crops. Prophylactic chemical control has been widely used to manage the pest which has resulted to environmental pollution, chemical residues in edible produce, as well as health-related problems to growers and consumers (van Dam et al., 2005). To manage pest populations in tomato crop, sustainable methods are being considered. Among these methods is the use of entomopathogenic fungi (Chandler et al., 2000; Maniania et al., 2008).

The most common mode of infection of entomopathogens such as *B. bassiana* is spore deposition on the insect cuticle (Vega et al., 2008). They are generally applied through inundative sprays which pose few challenges as they are sensitive to solar radiations and relative humidity which affects the products efficacy (Vega et al., 2009). These have necessitated the need for development of an economical delivery system that ensures the sustainable use of important bio-control agents in agricultural production. The delivery of the bio-control agents in a systemic mode into the host plant, avoids the effects of the ambient environment and may offer a desirable solution to the utilization of the Biological Control Agents. These approach

commonly known as an endophytic association has been noted to occur in many plants (Posada and Vega, 2006; Powell et al., 2009).

Endophytic association is a relationship in which non-pathogenic microorganisms spend part or whole of their life in a host without eliciting a pathogenic response (Faeth and Fagan, 2002). Mostly they colonize plant parts and spread within the host plant (Wilson, 1995). In addition they promote plant growth and development and thus protect the plants against pathogenic and herbivorous pests (Vega et al., 2009). Fungal endophytes have also been reported to deter feeding, oviposition and performance of sap sucking insects such as thrips (Muvea et al., 2014) and leaf mining insects (Akutse et al., 2013). However, known entomopathogenic fungi such as *Beauveria bassiana* that exogenously colonizes plant root rhizosphere has been delivered endophytically in crops such as banana to control banana weevils (Akello et al., 2007). *Beauveria bassiana* does not occur naturally as an endophyte, but has been artificially introduced in plants and reported to elicit a response similar to known endophyte in control of insect pests and plant diseases (Bing and Lewis, 1991; Ownley et al., 2004b; Posada and Vega, 2006). This study generally aimed at evaluating the prospects of utilizing *B. bassiana* as an endophyte in management of red spider mites in greenhouse tomato production.

1.3 Problem statement

The yield losses associated with *T. evansi* damage are estimated to be up to 90% making it one of the most damaging pests of tomato (Saunyama and Knapp, 2003). Control is mainly by synthetic acaricides that have led to the target pest developing resistance as well as widespread chemical residues in the food chain and the larger environment. This has resulted to animal poisoning and devastating effects to non target insects especially pollinators such as the honey bee (Suchail et al., 2001). There is need therefore to develop safe and cheap biologically based control alternatives. *Beauveria bassiana* is an entomopathogenic fungus known to directly parasitize over 200 different species of insects (Li, 1988). The delivery of the fungal

inoculum include through sprays of the crop, soil drenching, and lately by auto-dissemination (Vega et al., 2008). Recently, the delivery of *B. bassiana* through the crop systems as an endophyte has been tested (Meyling and Eilenberg, 2007). The endophytic approach was due to the limiting effects of the ambient environment on the use of this fungus(Vega et al., 2009). Entomopathogenic fungi are sensitive to sunlight, high temperatures, soil amendments, organic material and moisture levels (Faria and Wraight, 2001). *Beauveria bassiana* grows well between 19-30 ^{o}C but is adversely affected by temperatures above 37 °C and fungal establishment is reduced following application when temperatures are high (Bugeme et al., 2008; Gold et al., 2003). This limits the use of sprays since the fungi lose their persistence under unfavorable environmental conditions (Hallsworth and Magan, 1999). In addition spray applications need repeated applications which increase the cost of production. The use of *B. bassiana* as an endophyte would protect it from these conditions and once established within the tomatoes it might offer the most suitable and sustainable protection against the RSM.

1.4 Justification of the study

Chemical and cultural control methods have been widely used to control the red spider mites (Takafuji et al., 2000). There is a worldwide concern about the negative effect of chemical acaricides, which have resulted in the development of resistance in mites, adverse ecological events, affecting beneficial fauna, and accumulation of residues in the environment (Yuan et al., 2010). In addition chemicals are very expensive and frequently unavailable at subsistence level (Saunyama and Knapp, 2003). Entomopathogenic fungi are being considered as alternatives to synthetic acaricides. *Beauveria bassiana* has been reported to be effective as a bio pesticide against *T. evansi* under laboratory and greenhouse conditions (Wekesa et al., 2006; Wekesa et al., 2005a). Moreover, *B. bassiana* can endophytically colonize tomatoes (Powell et al., 2009) and be protected from abiotic and biotic stresses. Subsequently, *B. bassiana* as an endophyte will promote growth and help reduce the use of chemical pesticides thereby minimizing health risks and environmental pollution.

This study aimed at assessing the potential of using *B. bassiana* as an endophyte against the red spider mites in tomatoes.

1.5 Research objectives

1.5.1 Overall Objective

The overall objective of the study was to evaluate the prospects of *B. bassiana* as an endophyte, assess its effects on the plant growth as well as determine its efficacy in control of red spider mites in tomatoes

1.5.2 Specific objectives

The specific objectives of the study were to:

1) Assess endophytic colonization of *B. bassiana* on tomato plants and determine its effects on growth

2) Determine persistence and colonization of *B. bassiana* in screen house grown Tomato

3) Evaluate the efficacy of *B. bassiana* in endophytically-colonized tomato plants against *Tetranychus evansi*

4) Determine the mode of action involved in *T. evansi* control by endophytic *B. bassiana*.

1.5.3 Research hypotheses

The research hypotheses of this study were;

1) *Beauveria bassiana* can grow endophytically within the tomato plant and can be isolated from tissues by plating on selective media

2) *Beauveria bassiana* can persist and colonize the tomato plant, persistence is dependent on time

3) Isolates of endophytic *B. bassiana* exhibit high efficacy against *Tetranychus evansi*, reducing its damage on plants and adult populations

4) Endophytic *B. bassiana* produces hydrolytic enzymes that enhance tomato resistance to *T. evansi*

CHAPTER TWO

LITERATURE REVIEW

2.1 The red spider mite

The tomato red spider mite, (*Tetranychus evansi*), is an important pest in tomatoes and other solanaceous plants(Boubou et al., 2012). The wild tomato species e.g. *Lycopersicon hirsutum* f. *glabratum* C.H. Mull., is reported to be a source of resistance genes (Williams et al., 1980), which can be incorporated through breeding. Currently there is no commercial variety known to be resistant to *T. evansi* (Smith Meyer, 1996*)*. The pest is believed to have originated is South America and spread to Africa and Asia (Knapp et al., 2003). In Africa it was first reported from tobacco in Zimbabwe (Blair, 1989) and it spread to other countries such as Congo, Ethiopia, Zambia and Tanzania through trade of solanaceous crops. In Kenya it was first reported around the year two thousand by International Centre of Insect Physiology and Ecology after sampling tomato plants from Mwea Irrigation scheme (Knapp et al., 2003).

Tetranychus evansi belong to the order Acari, suborder Prostigmata subfamily Tetranychoidea and to the family Tetranychidae (Smith Meyer, 1996). The description was given by Baker and Pritchard 1960 from plant samples collected from Mauritius.

2.2 Biology and life cycle of red spider mite

The red spider mite undergoes complete metamorphosis, egg, larvae, nymph and adult stages (Brødsgaard and Albajes, 1999)**.** Males are straw colored, slender bodies at the pointed rear end with long legs and usually smaller than the females (Smith Meyer, 1996). Adult females are approximately 0.3- 0.5mm long, oval orange red with a dark blotch on each side of the body and lay more than 100 eggs during their 30 day life cycle on the host plant preferably on the underside of the leaf (Smith

Meyer, 1996). Unfertilized females may give rise to males only which sometimes occurs in nature (Blair, 1989; Smith Meyer, 1996), while mated female produce both male and female offspring (Meyer and Craemer, 1999).

The eggs when 1st laid are deep orange, successive eggs are lighter and colour fades until they become colorless and transparent, they may sometimes be covered with a web to regulate humidity during the hot climate and protect them from predators (Cranham and Helle, 1985). The eggs hatch after approximately 7 days at a temperature of 25°C- 30°C (Zhang, 2003). Larvae are round bodied pink in color, are larger than the eggs and have only three pairs of legs (Smith Meyer, 1996). They feed for a few days then molt into the first and second nymphal stage which both have four pairs of legs and are red in color (Bonato, 1999). They both last for approximately 6 -10 days (Smith Meyer, 1996).

The nymph feed, rest, and molt into the adult stage (Smith Meyer, 1996). The development from egg to adult takes 13.6, 7, 9.8, 7.8 and 6.3 days at 21, 25 26, 31 and 36°C (Bonato, 1999; Zhang, 2003). The minimum temperature for growth generally is 10 °C and few eggs are laid approximately 80 eggs, while the optimum temperature is 34 °C with more eggs laid 120-250 eggs (Smith Meyer, 1996). Reproduction can continue throughout the year as long as the environmental conditions are favorable (Knapp, 2002; Meyer and Craemer, 1999).

Adult females can live for approximately 30 days while males 25 days. The males mature early and are able to locate the female until they emerge for copulation to take place (Qureshi et al., 1969; Smith Meyer, 1996).

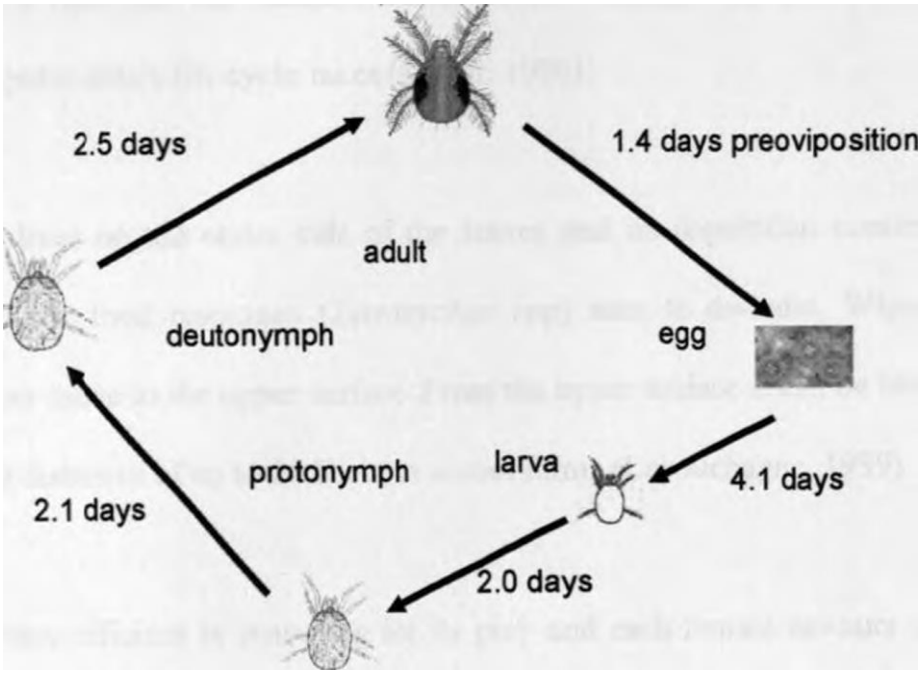

Plate 2.1: Red spider mite life cycle. Source: Bonato, 1999

2.3 Host range and pest status

Tetranychus evansi mostly infests solanaceous crops e.g. tomato, eggplant, potato, pepper and weeds such as nightshade (Knapp et al., 2003; Maniania et al., 2008). It has also been reported on plants of other families, including sweet potatoes, citrus, cut flowers cucumber and French beans (Maniania et al., 2008).

The damage by *Tetranychus evansi* is mainly caused by adults which pierce the young leaves of host plants and sucking out the fluids from plant cells (Zhang, 2003). They prefer the underside of leaves but in severe cases they can be found on both leaf surfaces as well as other parts of the plant such as the fruits and stem (Knapp, 2002). The feeding causes yellow spots to appear on the leaves which later enlarge spreading to larger areas hence reducing the photosynthetic area of the plant (Meyer and Craemer, 1999). Heavy infestation may lead to yellowing or bronzing

appearance which results in premature leaf drop. Old leaves are also affected and sometimes webbing may be seen all over the plant as an orange colouration (Knapp et al., 2003). The webs may also form clusters which can easily be dispersed by wind (Qureshi et al., 1969).

Dry and hot weather conditions favour the spread of the red spider mites and in cases where the plant manages to produce fruit it appears speckled and small which reduces its market value (Meyer and Craemer, 1999).

Plate 2.2: *Tetranychus evansi* and its corresponding symptoms upon infestation of tomato fruit (Source: http://www.dpi.nsw.gov.au October 2013)

2.4 Control options

2.4.1 Cultural control

Cultural control is valuable in preventing the establishment of *T. evansi* and can help reduce pest populations. Hot and dry conditions favour population increase of red spider mites (Qureshi et al., 1969). A jet of water forcefully applied from mist jets can reduce the relative humidity making the conditions unfavorable for reproduction and also knock them off; however this method is only effective under low pest

population (Craemer et al., 1998; Varela, 2003). Good cultural practices such as pruning, weeding and removing the debris, produce vigorous tomato plants, which have reduced breeding sites (Zhang, 2003). The use of clean planting stock, sanitation, crop rotation and strict quarantine measures are cultural methods universally advocated to reduce pest status in most crops (Varela, 2003). Plant nutrition through the addition of nitrogen which makes plants more succulent or the deficiency of potassium has been known to increase the population of *T. evansi* (Watson, 1964). Regulating the nitrogen content of the soil can help reduce the buildup of red spider mites.

2.4.2 Chemical control

Farmers especially in Africa largely depend on expensive synthetic miticides to control *T. evansi*, but pesticide applications are frequently ineffective due to cases of resistance which requires the farmer to use high amounts of miticides (Maniania et al., 2008). The excessive and inappropriate use of miticides has resulted in ecological problems, affecting non-target organisms and posing health risks to farmers and consumers (Wekesa et al., 2008). High costs have also limited the utilization of chemical control measures especially for the small scale peasant farmers (Varela, 2003).

Effort has been made to develop acaricides that eradicate the resistance species of red spider mites, but it has been unsuccessful. There is need to use narrow based rather than broad based acaricides and at the recommended doses to avoid cases of resistance (Craemer et al., 1998; Varela et al., 2003).

The webs produced by the red spider mites at times make the chemical application difficult since the webs protect the mites on the lower surfaces of the leaves where the numbers are high. The dense foliage where webs occur also hinders spray penetration hence it’s effectiveness (Craemer et al., 1998).

2.4.3 Biological control

Biological control is the use of living organisms to control insect pests, mites and diseases. It may include the use of predators, parasites or pathogens. Classical biological control involves the introduction of natural enemies of invasive species in order to regulate pest populations (Hajek and Delalibera, 2010). The introduction of natural enemies especially predatory mites of the family Phytoseiidae e.g *Neoseiulus californicus* and *Phytoseiulus persimilis* (Bugeme et al., 2008) have not been successful in the control of *T. evansi*, especially in Africa when the populations are high (Maniania et al., 2008; Zhang, 2003). The staphylinid beetles (*Oligota* spp) are known to feed on *T. evansi* but due to their delicate larvae and pupation which occurs in the soil makes rearing hard and hence they have not been effectively utilized (Varela et al., 2003).

Inundation biological control involves the use of lethal microbial pathogens to the pest, or the pathogen may produce a lethal toxin that is harmful to the pest (Eilenberg et al., 2001). Viruses cause diseases in a few spider mites e.g the citrus red mite and the European red mite. Among bacteria, *Bacillus thuringiensis* has been shown to be toxic to *T. urticae* (Wu and Guo, 2003). Pathogens of *Hirsutella thompsonii* and *Neozygites floridana* are also known to offer effective control against red spider mites (Chandler et al., 2000). Entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* have gained considerable attention as biological control agents for most agricultural pests (Li, 1988), and have been effective in the control of red spider mites in the laboratory and screen house (Bugeme et al., 2008; Maniania et al., 2008; Wekesa et al., 2005b). Previous studies have shown that isolates from these two fungal species are highly virulent suggesting potential for their utilization in the management of the red spider mite in tomato, *Tetranychus evansi* (Maniania et al., 2008; Wekesa et al., 2005b).

Several application methods have been used for application of *B. bassiana* ,e.g. spraying directly onto the pests or mites, endophytes and dry fungal spores (OWNLEY et al., 2004a; Powell et al., 2009; Tefera and Vidal, 2009; Vega, 2008; Wekesa et al., 2005a). When applied as spores dispersal and infection is by conidia, which germinate and penetrate the insect cuticle or gut wall (Compendium, 2004). The spores then germinate and grow directly through the cuticle to the inner body of their host. The fungus proliferates throughout the insect's body producing toxins and draining the insect of nutrients, eventually killing it. After the insect dies, the fungus produces an antibiotic (oosporein) that enables it to outcompete intestinal bacteria. Eventually the entire body cavity of the insect becomes filled with fungal mass (Gold and Messiaen, 2000).

Conditions of low relative humidity, soil moisture, extreme high and low temperatures, and sunlight have a great influence on the establishment of *B. bassiana* in the field (Lingg and Donaldson, 1981). This limits the use of sprays as well as dry fungal substrates.

Isolates of *Beauveria bassiana* have been tested as a biopesticide against *Tetranychus evansi* and found to be highly virulent suggesting potential for their utilization in the management of the tomato red spider mite, *Tetranychus evansi* (Wekesa et al., 2005b). There is need to seek an effective delivery method for the application of *B. bassiana* into plants such as the use of endophytes.

Beauveria bassiana can endophytically colonize a number of plant species (Brownbridge et al., 2012) which enables it to be used for the control of plant pests and diseases (Ownley et al., 2004b). This can reduce the application costs as well as protect it from the harsh environmental conditions.

2.5 Endophytes

The term endophyte refers to interior colonization of plants by micro-organisms mostly bacteria and fungi that live most of their life inside of the plant tissues

without eliciting any pathogenic symptoms (Faeth and Fagan, 2002). Endophyte-defense in plants lies in the synthesis of defensive alkaloids, which are determined by a combination of environmental conditions, plant and endophyte genetic factors (Vega, 2008). Alkaloids released by endophyte infected plants are capable of deterring insect feeding or reducing insect performance (Faeth and Fagan, 2002). *Beauveria bassiana* produces a variety of secondary metabolites which are believed to play an active role in it's biocontrol activity (Ownley *et al.*, 2008).

Initial work on entomopathogenic endophytes was conducted using *B. bassiana* in an aqueous and granular formulation on maize to suppress European corn borer. Results indicated that there was reduced tunneling achieved (Vega et al., 2008). *B. bassiana* has also been reported as an endophyte in opium poppy seeds (Quesada-Moraga et al., 2006), tomato (Powell et al., 2009) and cotton (Gurulingappa et al., 2011), among other crops.

Most fungal endophytes such as *B. bassiana* have been reported to reduce feeding, oviposition and performance of sap sucking insects such as thrips in onions (Muvea et al., 2014). In cotton endophytically colonized by either *B. bassiana or Lecanicillium lecanii* there was reduction in feeding and reproduction by *Aphis gossypii* (Gurulingappa et al., 2010).

2.6 *Beauveria bassiana* as an Endophyte

B. bassiana occurs naturally in corn, cotton and the jimson weed and endophytic isolates of the fungus have been isolated using the plating techniques on selective media (Doberski and Tribe, 1980). However in most plants such as tomato (Powell et al., 2009), it is capable of establishing as an artificial endophyte and this may play a role in its ability to control insect pests, and plant diseases (Posada and Vega, 2006). Another possible role for these endophytes could include plant growth promotion as well as impact on tritrophic interaction (Akello et al., 2007; Vega et al., 2008).

Beauveria bassiana produces secondary metabolites some of with have antibiotic properties while others determine virulence. They are controlled by genetic and cellular regulatory mechanisms (Safavi, 2012) and include beauvericin, bassianin, oosporein, bassianolide, beauverolides, beauveriolides, tenellin, (Crespo et al., 2008). Beauvericin exhibits antibacterial activity and has moderate insecticidal properties (Gupta et al., 1991; Strasser et al., 2000). There is little information about bassinolide but it is known to be toxic to lepidopterans (Charnley, 2003).

Oosporein is a red pigmented is a widespread secondary metabolite of soil-dwelling fungi including *B. bassiana* (Michelitsch et al., 2004). It's production enables *B. bassiana* to compete against the natural bacteria within the insect gut. The presence of this compound is mostly occurs after infection and *B. bassiana* is within its proliferation phase; the insect sometimes may show a pink or reddish color and this enables it to sporulate on insect cadaver without causing competition (Strasser et al., 2000). Tenellin are yellow pigments which promote cell lysis (Vey et al., 2001).

Beauvolide and beauvirolide toxic effects have not been fully demonstrated against target insects but are involved in changing the insects immune system (Xiao et al., 2012). These toxins may also act as virulence factors by facilitating the fungus to colonize and use an insect as a food source and preventing subsequent invasion by secondary invaders (Strasser et al., 2000). Other mechanisms of control in pests and diseases by *B. bassiana* may include antibiosis, competition and induced systemic resistance (Vega et al., 2008).

The use of *B. bassiana* as endophyte in the management of insect pests is beneficial since it would require little inoculum and also be protected from the environmental constraints (Akello et al., 2009), and hence it can stay in the plant for a longer period of time offering protection against pests and diseases.

Beauveria bassiana also forms an endophytic association with opium poppy, *Papaver somniferum* L. (Quesada-Moraga et al., 2006). Spraying *B. bassiana* conidia on leaf surfaces, resulted in the recovery from all treated leaves and also in pieces obtained from newly formed leaves. Laboratory and field studies also showed that *B. bassiana* can colonize leaf tissues of date palm (*Phoenix dactylifera* L.) for up to 30 days post inoculation (Gómez-Vidal et al., 2006).

In another study carried out in coffee seedlings, *B. bassiana* was able to establish as an endophyte and isolated from the stem, roots and the leaves using culturing techniques (Posada and Vega, 2006). Wagner and Lewis (2000) confirmed the establishment of *B. bassiana* as an endophyte in corn. In cocoa (*Theobroma cacao* L.*)* seedlings *B. bassiana* has been reported to be endophytic and recovery from the various plant parts was by culturing techniques (Posada and Vega, 2005).

Similarly when wheat leaves were colonized by either *B. bassiana* or *Aspergillus parasiticus* there was a reduction in the growth rate of *Chortoicetes terminifera* nymphs. In another study on cotton reduction in feeding and reproduction by *Aphis gossypii* on cotton endophytically colonized by either *B. bassiana* or *Lecanicillium lecanii* (Gurulingappa et al., 2010). Endophytic *B. bassiana* in banana significantly reduced larval survivorship of banana weevil, *Cosmopolites sordidus*, resulting in 42–87% reduction in plant damage (Akello et al., 2008).

Among members of the Solanaceae family, *Beauveria bassiana* was able to establish as an endophyte of potatoes through foliar applications and was recovered in most of the plant parts (Jones, 1994). Therefore, there is likelihood for it to establish in other members of this family such as peppers, nightshade and tomatoes. Also, *B. bassiana* has been detected inside tomato seedlings following seed treatment with the fungus. The endophytic association provided protection of tomato seedlings from damping-off disease (Ownley et al., 2004b)

In most studies conducted with endophytic *B. bassiana*, no adverse or ill effects have been reported to result from the association between the fungus and the host plant (Akello et al., 2007). Few studies have been done using *B. bassiana* as an endophyte to control *T. evansi* in tomatoes in Kenya.

CHAPTER THREE

ENDOPHYTIC COLONIZATION AND EFFECTS OF *BEAUVERIA BASSIANA* ON GROWTH OF TOMATO PLANTS

3.1 Introduction

Tomato, *Solanum lycopersicum* (Solanales: Solanaceae) production in the tropics is mainly limited by red spider mites (RSM). The RSM damage is even more pronounced and difficult to control in protected production (Zhang, 2003) and losses of up to 90% in tomato are reported (Saunyama and Knapp, 2003). The market driven demand for pesticides free tomatoes has necessitated the search for environmentally safe alternatives for pest control (Bernard and Bernard, 2010).

Entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* have been tested for biological control with promising results (Ownley et al., 2008). Beside antagonistic activity against plant pathogens, entomopathogenic fungi are also known to promote plant growth (Vega et al., 2009). The application of *B. bassiana* as a bio pesticide however has been limited by abiotic and biotic factors (Hallsworth and Magan, 1999). There is need to search for efficient and cost effective delivery methods of *B. bassiana* such as the use of endophytes to maximize results in field and greenhouse conditions.

Beauveria bassiana has the ability to colonize plants endophytically. For example previous reports have indicated that endophytic colonization of *B. bassiana* in cassava (Greenfield et al., 2016) and tissue cultured banana plants (Musa sp.) (Akello et al., 2009) significantly increased growth in comparison to non-inoculated control plants.

The objectives of this study were (i) to determine endophytic colonization of *B. bassiana* isolates on different tomato varieties and (ii) to evaluate the effect of *B. bassiana* colonization on the growth parameters of tomato varieties

3.2 Materials and methods

3.2.1 Fungal cultures

Five isolates of *Beauveria bassiana* (ICIPE 10, ICIPE 35, ICIPE 273, ICIPE 279 and ICIPE 283) were obtained from the ICIPE's Arthropod Germplasm Centre, Nairobi, Kenya (Table 3.1). The isolates were cultured on Sabouraud Dextrose Agar (SDA) medium amended with 0.05 g chloramphenicol antibiotics to minimize bacterial contamination and incubated in a Heraeus Incubator (Heraeus Holding GmbH, Hanau, Germany), for three weeks at 27°C in complete darkness (Inglis et al., 1996), to allow for sporulation.

3.2.2 Inoculum preparation

Three week old fungal culture plates were gently scrapped using a sterile blade to remove the conidia that were suspended in 10 ml sterile distilled water in 20-ml universal bottle containing 0.01% Tween-20 and glass beads. The conidial suspension was vortexed for 5 minutes to homogenize the conidial suspension. Conidial counts were done using an improved Neubauer Hemacytometer (Goettel and Inglis, 1997). A final concentration of 1×10^{9} conidia mL^{-1} based on preliminary studies was used for the inoculation of tomato seeds. The viability of conidia was assessed prior to bioassays by spread-plating 0.1 mL of 3×10^{6} conidia mL^{-1} onto 90-mm Petri dishes containing Sabouraud Dextrose Agar (Goettel and Inglis, 1997). The plates were incubated at 25 ± 2°C and were examined after 16-20 hours under the compound microscope (× 40 magnifications) for conidia germination. Conidia were considered viable when germinated on a SDA media, the germ tube was twice the diameter of the conidium. The viability tests were replicated four times. In viability tests, over 90% of conidia of all the tested isolates germinated (Table 3.1).

Table 3.1: *Beauveria bassiana* isolates used in this study and percentage of germination 21 Days After Inoculation (DAI) on Sabouraud Dextrose Agar

Isolates	Locality (country)	Source	Germination (%) X ± S.E
ICIPE 279	Mbita (Kenya)	Soil	94 ± 2.4
ICIPE 273	Kericho (Kenya)	Coleopteran larvae	96 ± 2.8
ICIPE 283	Mauritius	Soil	94 ± 3.2
ICIPE 35	Kenya	Coffee borer	96 ± 2.8
ICIPE 10	TRO campus (Kenya)	Soil	95 ± 2.6

plates at 25 ± 2° C.

Plate 3.1: Cultures of *Beauveria bassiana*

3.2.3 Tomato plants

Tomato, *Solanum lycopersicum* (varieties Cal-J, Kilele and Anna) used in this study were raised in the screenhouse at the International Centre of Insect Physiology and Ecology (ICIPE). Cal-J is a determinate open pollinated variety that is highly preferred by the red spider mites but very common among rural subsistence farmers; Kilele and Anna are indeterminate new popular hybrids among commercial farmers in Kenya.

3.2.4 Seed inoculation and colonization

Seeds of the three cultivars were surface-sterilized in 70% ethanol for 1 min and then in 1.5 % sodium hypochlorite solution for 3 min. They were washed three times with sterile distilled water and blot dried on sterile paper towels to remove the excess water. The last rinse water was plated out to assess the effectiveness of the surface sterilization procedure. Inoculation was done by soaking approximately 70 seeds in 10-ml conidial suspension of each isolate containing 0.01% Tween-20 at the concentration of $1x10^{9}$ conidia ml^{-1} for 2 hours. Mixtures were hand stirred at a 30 minutes interval until the seed were uniformly coated (Powell et al., 2009). Control seeds were soaked in sterile distilled water containing 0.01% Tween-20 (wetting agent) for 2 hours.

The seeds were then removed and placed in pots of size 80mm x 70mm (size 3) filled with sterilized soil and then placed on plastic trays (plate 4). The soil (mixture of red soil and sand in the ratio of 2:1) was sterilized in an autoclave for 2 hours at 121°C and allowed to cool for 72 hours prior to planting and no microorganism was assessed after sterilization. Seedlings were thinned to three per pot after germination and were watered once per day. Each pot received 3ml of hoagland solution every week.

There were eight replicates for each of the five isolates and their respective controls; the experiment was repeated twice and laid out in a completely randomized design. The trays containing the seedlings were kept in the growth chamber at 25 ± 2°C, 70% RH and a photoperiod 12:12 L: D for 28 days weeks. The endophytic colonization by *B. bassiana* on tomato plants was examined after two weeks after inoculation through destructive sampling of one tomato plant per pot. Plants were carefully removed from the pots and washed with tap water and surface-disinfected by immersing them in 70% ethanol for fifteen seconds, followed by three minutes in 1.85% sodium hypochlorite and rinsed three times in sterile distilled water. To determine the effectiveness of the disinfecting process, the final rinse water was plated on SDA media. Plants were dried on sterile paper towels and tissues from leaves, shoots and roots were cut into 4 mm^2 sections with a sterile scalpel and five of them were placed on Petri dishes containing SDA medium amended with 20g oatmeal agar, 5g yeast, 0.6g Dodine and 5mg chloramphenicol (Plate 4). Four plates were cultured for each tissue (roots, shoots and leaves) and for each treatment including the control. They were kept at room temperature (25°C) for two weeks and *B. bassiana* was identified based on morphological characteristics which include hyphal growth, and spore appearance (Plate 5). For each plant part, percent colonization was calculated as number of sections exhibiting *B. bassiana* outgrowth per total number of sections (Fisher & Petrini, 1987).

Plate 3.2:. Trays containing the tomato seedlings grown in the growth chamber

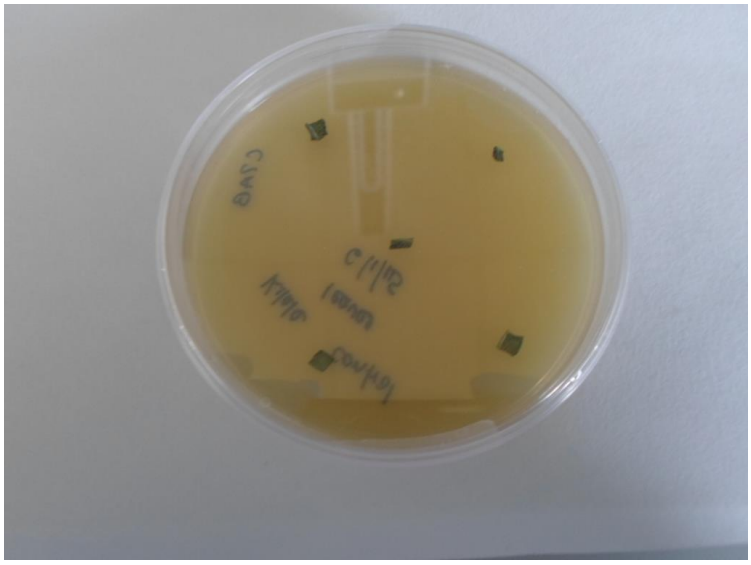

Plate 3.3: Tomato plant parts on selective SDA medium

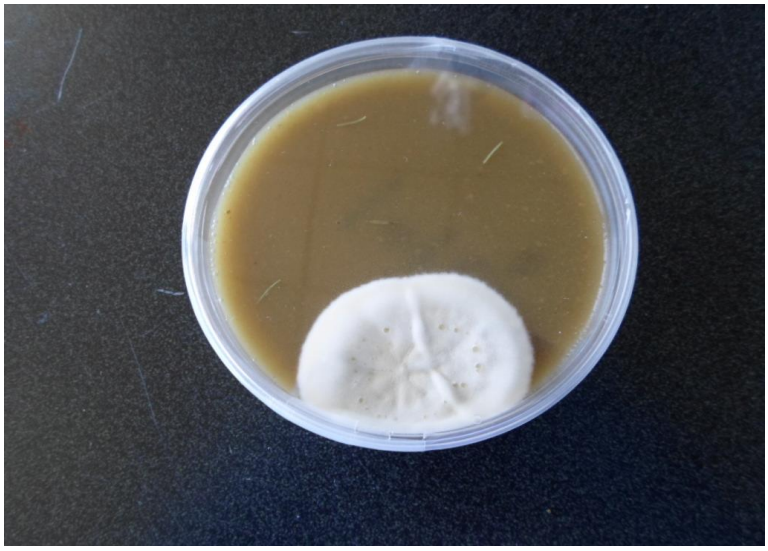

Plate 3.4: Endophytic B. bassiana ICIPE 35 from tomato plant parts on selective SDA medium

3.2.5 Effect of endophytic colonization with *B. bassiana* on plant growth

To evaluate the effect of endophytic colonization by *B. bassiana* on plant growth parameters, plant height, number of leaves, fresh and dry shoot and root weight were measured after 28 days which was the termination of the experiment. Plant height i.e. the distance from the base of the plant to the youngest leaf axil was measured using a ruler and numbers of leaves were counted. Fresh and dry shoot weights, fresh and dry root weights were also determined at the end of the experiment using a measuring scale. Dry weights were obtained by drying the shoots and roots in a hot air oven at 70°C for 72 h.

3.3 Data analysis

Colonization frequency (CF) was calculated as described by (Fisher and Petrini, 1987). The percentage fungal colonization data was transformed using the angular transformation method (arcsine square root) before being subjected to ANOVA and

whenever there was a significant treatment effect, then means were compared using SNK (Student-Newman-Keuls) test (Institute, 1999). At harvest, plant height, number of leaves, fresh shoot and root weight, dry shoot and root weight were measured. Where variance homogeneity could not be assumed, all plant growth parameters were subjected to ANOVA using the non-parametric procedures Tests were performed at 5% level of significance. All the analyses were performed using SAS (8.0) software.

3.4 Results

3.4.1 Endophytic colonization of B. bassiana on tomato varieties

B. bassiana isolate ICIPE 35 was the only one that colonized root, stem and leaves of all the tomato varieties. However, there were significant differences in levels of colonization among tomato varieties on stem ($F = 7.39$, $df = 10$, $p < 0.001$), roots ($F = 5.74$, $df = 10$, $p < 0.001$) and leaves ($F = 6.60$, $df = 10$, $p < 0.001$) (Figure. 3.1). There was no colonization of plant parts of Anna, Cal J and Kilele varieties under control treatment.

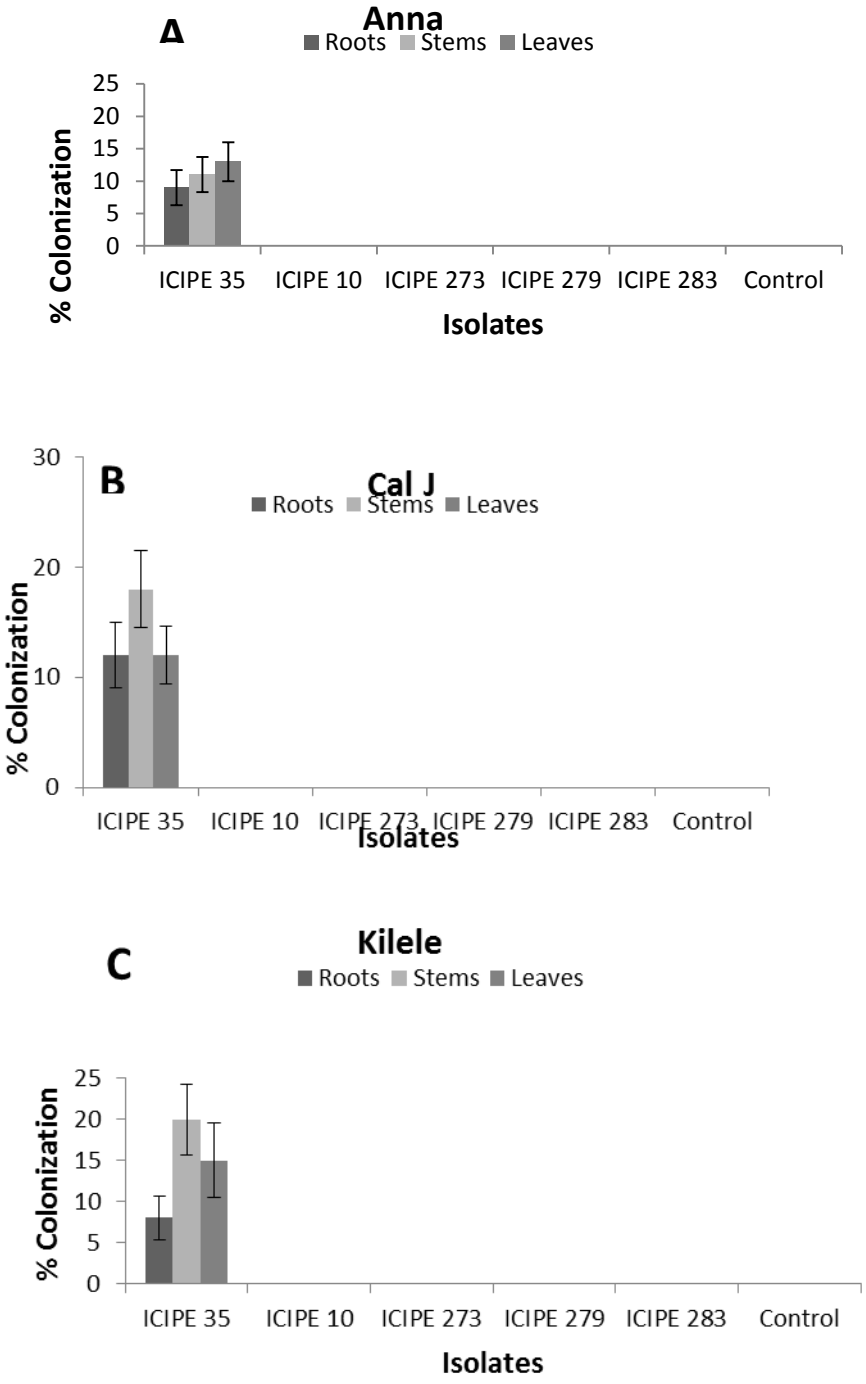

Figure 3.1: Colonization of different parts of tomato varieties by *Beauveria bassiana* isolate ICIPE 35 (A) Anna F1 hybrid (B) Cal J open pollinated (C) Kilele F1 hybrid

3.4.2 Effect of endophytic colonization by B. bassiana on plant growth

Endophytic colonization by *B. bassiana* isolate ICIPE 35 significantly increased the plant growth, number of leaves for tomato plants compared to the respective controls ($p < 0.05$) in all the tested varieties. Similarly, the, dry shoot and root weight were not significantly different between the treated tomatoes and respective controls while there were no significant differences between the treatments ($p = 0.91$) for the dry root weights (Table 3.2).

Table 3.2: Effect of *B. bassiana* colonization on plant growth parameters of different tomato varieties

Varieties	Plant height (cm)		Number of leaves		Fresh shoot weight (g)		Fresh root weight (g)		Dry shoot weight (g)		Dry root weight (g)	
	B. bassiana	control	*B. bassiana*	control	*B. bassiana*	control	*B. bassiana*	control	*B. bassiana*	control	*B. bassiana*	control
Kilele	10.61 ±0.37a	8.03 ±0.41b	9.41 ± 0.46a	6.18 ± 0.56b	0.78 ± 0.08a	0.30 ± 0.03b	0.07 ± 0.02a	0.04 ± 0.01a	0.07 ± 0.01a	0.03 ± 0.04a	0.03 ± 0.01a	0.01 ± 0.05a
CalJ	8.13 ± 0.51a	5.23 ± 0.53 b	10.37 ± 0.51a	6.06 ± 0.93b	0.50 ± 0.08a	0.20 ± 0.04b	0.10 ± 0.02a	0.03 ± 0.04b	0.05 ± 0.01a	0.03 ± 0.05a	0.03 ± 0.01a	0.01 ± 0.01a
Anna	14.7 ± 1.2 a	7.93 ±0.3b	12.53 ±0.78a	10.5 ±1.10b	1.40 ± 0.10a	0.40 ± 0.02b	0.20 ± 0.02a	0.05 ± 0.01b	0.12 ± 0.02a	0.09 ± 0.03a	0.03 ± 0.01a	0.03 ± 0.01a

*different lowercase letters refer to means (± SE) that are significantly different within rows ($P < 0.05$, SNK test; student t test was used to compare treatment and control

3.5 Discussion

In the present study, five isolates of *B. bassiana* were tested for endophytic colonization and only ICIPE 35 was able to colonize the different plant parts of the three tomato varieties. Interestingly, this particular isolate also exhibited different levels of colonization within the same tomato variety. But the reason why the other four fungal isolates did not colonize any of the tomato variety was not investigated further in this study since our focus was for an isolate that could colonize the three tomato varieties. However, the fact that ICIPE 35 was re-isolated in several plant parts means that it is able to be translocated within the plant and can provide protection against target pests. The variation in colonization in the various plant parts can be attributed to the fact that *B. bassiana* as an endophyte can display preferential tissue colonization within the various tomato varieties (Behie et al., 2015).

Variety and plant part-related colonization observed in this study suggests that colonization can be variety-specific. This implies that a variety and its various plant parts is an important factor to consider during deployment of endophytes. This is corroborated by Akutse et al., (2013) who reported variation in colonization of the roots, stems, and leaves of *Phaseoulus vulgaris* and *Vicia faba* through soaking of seeds in *B. bassiana* conidial suspensions. In this study, ICIPE 35 colonized all the three tomato varieties and was able to be isolated from the stem and leaves of these varieties for up to four weeks after seed inoculation in tomato. ICIPE 279 had been known previously to endophytically colonize *Vicia faba* and *Phaseolus vulgaris* (Akutse et al., 2013) but it could not establish in the tested tomato varieties together with several other isolates.

The plant growth parameters increased in *B. bassiana*-colonized tomato plants with variety Anna having the highest parameter values, an indication that the fungus may possess plant growth promoting ability. Several studies have reported the ability of fungal entomopathogens to promote plant growth (Dara et al., 2016; Jaber & Enkerli, 2016). For instance, *Vicia faba* plants colonized by *B. bassiana* following foliar

inoculation had increased plant height, leaf pair number, fresh shoot and root weight (Jaber & Enkerli, 2016). On the other hand, (Elena et al., 2011) reported significant increase of tomato plant height, root length, shoot and root dry weight when seedlings were inoculated with isolates of *Metarhizium anisopliae*.

The mechanisms underlying the positive effect of endophytic *B. bassiana* on plant growth were not examined as part of this study but it is postulated that this could be as a result of phytohormones produced by the fungus that enhances bioactivity of the plants as well as the production of active metabolites by endophytes (Jaber & Ownley, 2017; Ownley et al., 2010)

CHAPTER FOUR

ENDOPHYTIC COLONIZATION AND PERSISTENCE OF *BEAUVERIA BASSIANA* ON TOMATO PLANTS

4.1 Introduction

The effectiveness of any introduced biological control agent as an endophyte depends on its ability to establish and spread within the host or the elicited reaction distributed from the site of infection to other parts of the plant (Castrillo et al., 2009; Shoresh et al., 2010). Successfully established endophytes are reported to promote growth, offer protection to the plant against biotic and abiotic factors (Shoresh et al., 2010). In addition, the persistence of these effects within the host plant determines the efficacy of the control agent (Brownbridge et al., 2012).

Previous studies have demonstrated that *B. bassiana*, can endophytically be established in several crops (Vega et al., 2008) and they include ironwood (Bills & Polishook, 1991), Jimsonweed, potato (Jones, 1994), maize (Wagner & Lewis, 2000), cacao (Posada and Vega, 2005), date palm (Gómez-Vidal et al., 2006), opium poppy (Quesada-Moraga et al., 2006), banana (Akello et al., 2007), coffee (Posada et al., 2007), sorghum (Tefera and Vidal, 2009), wheat, cotton, tomato, bean, pumpkin (Gurulingappa et al., 2010), jute (Biswas et al., 2012), and radiate pine (Brownbridge et al., 2012).

Even though *B. bassiana* is known to have ability to exist in plants as an endophyte, the persistence in the plant tissues is not known. This study therefore aimed at investigating the endophytic potential and persistence of a Kenyan *B. bassiana* isolate, ICIPE 35, in three tomato cultivars (Cal J, Kilele and Anna) in the screen house.

4.2 Materials and methods

4.2.1 Fungal culture

Beauveria bassiana isolate ICIPE 35 was obtained from the ICIPE's Arthropod Germplasm Centre, Nairobi, Kenya and sub-cultured on Sabouraud Dextrose Agar (SDA) medium amended with 0.05 g chloramphenicol antibiotics to minimize the bacterial contamination. It was subsequently incubated for three weeks at 27°C (Inglis et al., 1996).

4.2.2 Inoculum preparation

Conidia were gently scrapped using a sterile blade from three weeks old *B. bassiana* cultures as per the protocol described in Chapter 3.

4.2.3 Tomato plants

Tomato plants, (*Solanum lycopersicum* varieties Cal-J, Kilele and Anna) that were used in this study were raised in the screen house in ICIPE. Seeds used in the study were obtained from Syngenta company distributors and germinated in the screen house.

4.2.4 Seed inoculation and colonization

Seeds of Cal-J, and two hybrids (Kilele and Anna) cultivars were surface-sterilized and inoculated as per the protocol described in Chapter 3. The seeds were then removed and sown in plastic pots (13cm x 10cm) filled with sterilized soil (mixture of red soil and sand in the ratio of 2:1) was sterilized in an autoclave for 2 hours at 121^0C and allowed to cool for 72 hours prior to planting. The sterilization process ensured complete removal of non desirable soil microorganisms. The pots containing the seedlings were kept in the screen house at 28 $\pm 2^0$C and 70% - 80% RH for 6 weeks. The endophytic colonization by *B. bassiana* on tomato plant was examined as per the protocol described in Chapter 3 and placed on petri dishes containing SDA

medium (Plate 5). Four plates were cultured for each tissue (roots, shoots and leaves) and for each treatment including the control. They were allowed to sporulate for two weeks in the laboratory and then *B. bassiana* identified based on its morphological characteristics (Plate 6). For each plant part, percent colonization was calculated as number of sections exhibiting *B. bassiana* outgrowth per total number of sections (Fisher and Petrini, 1987).

4.3 Data analysis

Colonization frequency (CF) was calculated as described by Fisher and Petrini, (1987). The proportion of fungal colonization per plant part and persistence which were expressed as percentages was arcsine square root transformed before subjecting to ANOVA using the procedure Generalized Linear Models in SAS. Whenever treatment effects were significant, then means were compared using SNK (Student-Newman-Keuls) (Institute, 1999). All tests were performed at 5% level of significance. All the analyses were performed using SAS (8.0) software.

4.4 Results

4.4.1 Endophytic colonization of B. bassiana on tomato varieties

Beauveria bassiana isolate, ICIPE 35, colonized roots, stems and leaves of all the tomato varieties in the screen house. However, there were no significant differences in levels of colonization among parts of the three tomato varieties i.e. stems ($F = 1.7$, $DF = 2$, $p = 0.18$), roots ($F = 2.0$, $DF = 2$, $p = 0.12$) and leaves ($F = 0.28$, $DF = 2$, $p = 0.75$) (Figure 2). For example, in Anna, there was no significance difference in colonization among the various plant parts, in leaves 4.5%, compared to stem 5% and roots 3.3%. Colonization was slightly higher in the leaves 5.8% compared to the stem 2.9% and roots 3.3% in Cal J variety. In Kilele, colonization was highest in leaves 4.8% and followed by stem 3.4% and roots 1% (Figure 4.1). There was no colonization of plant parts of Anna, Cal J and Kilele varieties under control treatment.

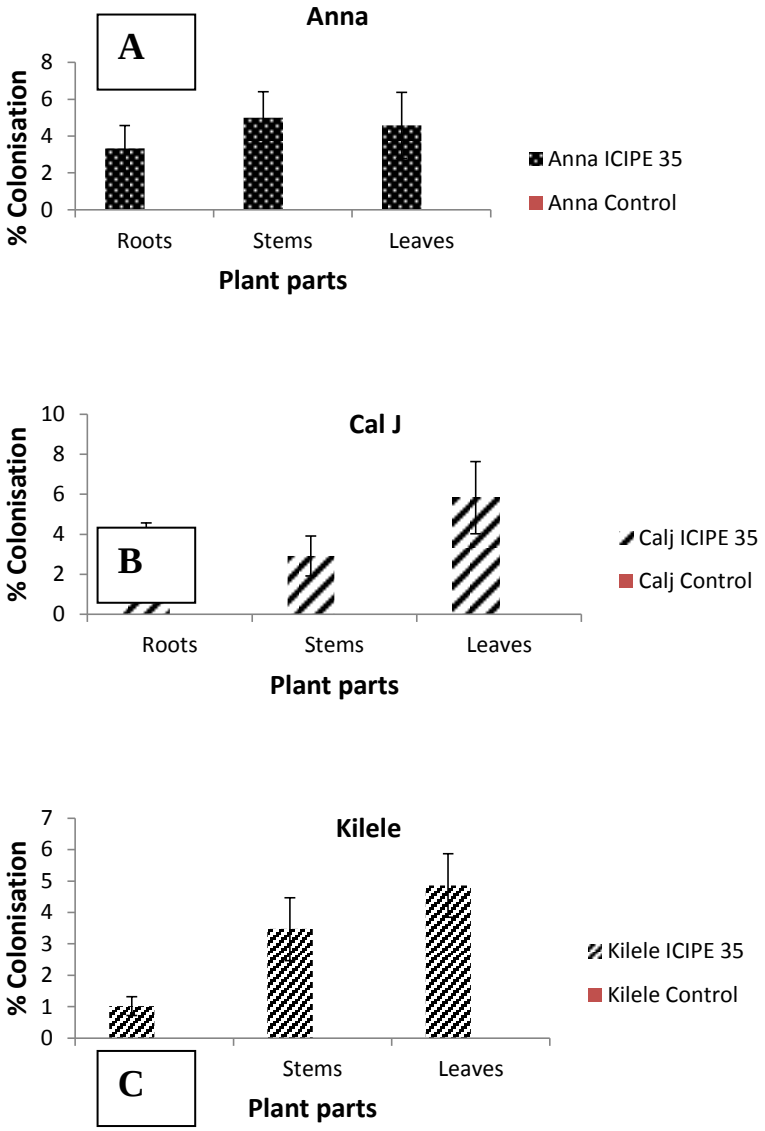

Figure 4.1: Colonization of different parts of tomato varieties by *Beauveria bassiana* isolate ICIPE 35 in the screen house (A) Anna F1 hybrid (B) Cal J open pollinated (C) Kilele F1 hybrid

There was interaction between the variety and treatment for the various plant parts at 2, 4, 6, weeks after inoculation were insignificant, roots (F= 2.3, df = 2, P = 0.10), stems (F = 1.8, df = 2, P = 0.17) and leaves (F = 0.34, df = 2, P = 0.71). Despite the general decline in percentage colonization overtime, the rate of decline was not different among the various plant parts for all the tested varieties i.e. stem (F = 1.7, df = 2, P = 0.18), roots (F = 2.0, df = 2, P = 0.12) and leaves (F = 0.28, df = 2, P = 0.75).

Percent colonization was generally high for week 2 compared to week 4 and 6, however there were no significant differences for plant roots for week 2, 4 and 6. For stems week 6 was significantly different from week 2 and 4 which were not different, while for the leaves week 2 was significantly different from week 4 and 6 which were not different (Figure 4.2).

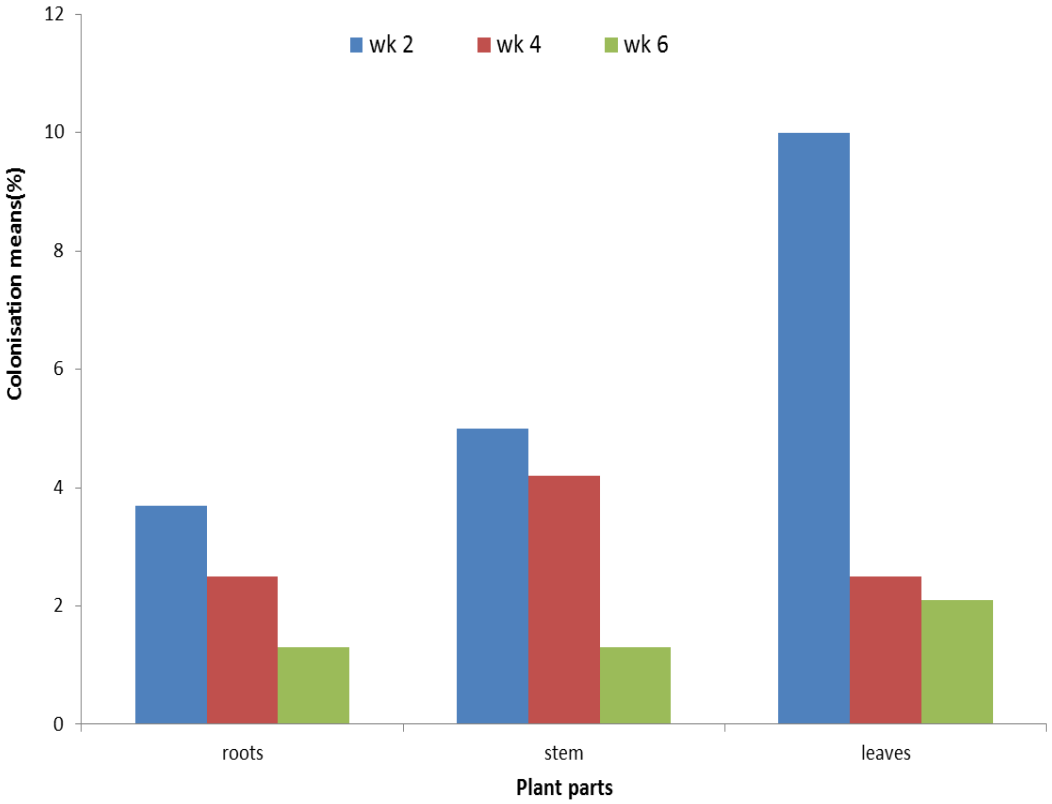

Figure 4.2: Persistence of different parts of tomato varieties by *Beauveria bassiana* isolate ICIPE 35

4.5 Discussion

Beauveria bassiana isolate ICIPE 35 was able to colonize root, stem and leaves of Cal-J, Kilele and Anna tomato varieties in the screen house. Levels of colonization differed among the various plant parts in the various tomato varieties and this could be attributed to the external environment or the biological differences within the plant tissues (Bayman et al., 1997). These results compare with Muvea et al. (2014),

who found that there were differences in the level of colonization of different plant parts by fungal isolates (Muvea et al., 2014). Similar results were also reported on french beans and faba beans (Akutse et al., 2013), and coffee (Posada et al., 2007).

The fact that the fungus was found in several parts of the plant means that it was able to spread within the plant. This is important especially if there is vertical transmission of the endophytes through the seeds so that subsequent generations may not require seed dressing (Schardl et al., 2004). Colonization of plants by entomopathogens can take several pathways which all depends on the fungi and the plant in question; however systemic spread has been advocated by most authors (Bing and Lewis, 1991). This supports previous studies that attributed passive movement of the fungal hyphae within the xylem and vascular tissues eliciting a systemic reaction (Bing and Lewis, 1992).

Colonization of Anna variety was slightly higher in the stem compared to leaves, Kilele and Cal J varieties, leaves had a higher colonization compared to the other plant parts. It is possible that the leaves provided a suitable environment for the establishment and survival of *B. bassiana* compared to the other plant parts in these two varieties (Fisher et al., 1992). Previous studies have shown that most endophytic fungi are highly adapted to particular conditions present in a given plant organ (Carroll, 1988; Fisher et al., 1992). This may be probably due to micro ecological and physiological conditions existing in the different tomato plant tissues, which confer varying survival degrees for *B. bassiana* (Bayman et al., 1997).

Although the various parts of the tomato varieties were colonized 2 weeks after inoculation a decrease was noted by week 6 and by week 8 where no colonization was observed in all the plant parts. This means that additional *B. bassiana* can be applied through soil drenching or spraying so that it offers more protection to the tomatoes against RSM. The rate of decline was faster in leaves followed by the stems and lastly the roots, which could be probably due to no multiplication within the plant rhizosphere or inhibition of *B. bassiana* germination or growth (Quesada-

Moraga et al., 2006). Similar results were observed in cocoa plants where *B. bassiana* established as an endophyte but did not persist beyond two months (Posada and Vega, 2005) and in pine trees where endophytic *B. bassiana* declined over a period of 9 months (Brownbridge et al., 2012) .

Decline in colonization of the various plant parts may also be caused by the plant response to the endophytic fungus, other fungi which occur naturally within the plant and also the expansion of plant parts (Posada et al., 2007). The culturing method used may also have reduced the fungal endophytes within the plant hence the need for improved methods like molecular tools for detection (Saikkonen et al., 2010).

The delivery of *Beauveria bassiana* as an endophyte bypasses the limitations imposed by their direct use especially foliar spray where they are affected by UV radiation, varying condition of temperature and humidity that frequently reduces conidial viability (Vega et al., 2008). Once established in the plant system, these endophytes provide plant protection against pests, lower operation costs and guarantees efficacy because the fungus is protected against abiotic factors and for this reason, this delivery method is more advantageous.

This study demonstrated that *B. bassiana* isolate ICIPE 35 can colonize tomato plants and persist up to 6 weeks in the screen house from seed inoculation and this can be used to complement existing control measures for the RSM. It also implies that there is need to increase inoculum in the soil or use *B. bassiana* as a spray to increase protection to the plant until harvesting period.

CHAPTER FIVE

EFFECTS OF ENDOPHYTIC *BEAUVERIA BASSIANA* ON RED SPIDER MITES INFESTATION AND DAMAGE ON TOMATO PLANTS

5.1 Introduction

Tomato (*Solanum lycopersicum*) is grown worldwide and is preferred due to it's nutritional value and economic importance in most parts of the world (Heuvelink, 2018; Rice, 1987). In Kenya fresh tomatoes are preferred to canned tomato paste and production is mainly for home consumption as well as for export. Over the years production has declined due to among other factors pests and diseases (Varela, 2003). Greenhouse and field production is greatly hampered by arthropod pests such as leafminer *Tuta absoluta* Meyrick and red spider mite (RSM), *Tetranychus evansi* Baker & Pritchard, which is an important exotic pest in the production of tomato and other solanaceous crops (Knapp, 2002). Synthetic chemical control that is widely used for controlling RSM is hazardous to man and environment (van Dam et al., 2005) .

Beauveria bassiana has gained considerable attention as a biological control agent for agricultural pests (Chandler et al., 2000; Maniania et al., 2008). However, conventional delivery mechanism such as spraying for this fungus has limited its successful utilization. Alternative delivery methods are needed; which include using the fungus as an endophyte so as to increase efficiency. Most endophytic fungi have a continuum of plant-fungal relationships which coexist and they play an important function of protection from abiotic stresses, pests and diseases as well as enhancing growth (Saikkonen et al., 2010).

Beauveria bassiana has been reported to be parasitic to the leaf miner, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) (Akutse et al., 2013), stemborers *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) (Cherry et al., 2004), European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Bing

and Lewis, 1991), coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae) (Vega, 2008) and banana weevil *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) (Akello et al., 2007). The negative effects on feeding and reproduction performance of various insects pest (Akutse et al., 2013; Mutune et al., 2016). The objectives of this study therefore were (i) to determine plant damage by RSM on endophytically colonized tomato plants by *B. bassiana* ICIPE 35, and (ii) to assess the effect of endophytic colonization on *T. evansi* on tomato plants.

5.2 Materials and methods

5.2.1 Tomato plants

Tomato, *Solanum lycopersicum* (varieties Cal-J, Kilele and Anna) used in this study were raised as described in chapter four.

5.2.2 Red spider mite culture

Tetranychus evansi was obtained from a regularly regenerated colony reared on tomato variety Cal-J, which were replaced before the old tomatoes dried up at ICIPE in an insectary with temperature of 25 ± 2 ^{o}C, 60 – 70% Relative Humidity and a photoperiod of 12:12 h L: D. The initial culture of mites had been collected from tomato plants in Mwea Irrigation Scheme, Kenya, in 2001 (Plate 7).

Plate 5.1: Rearing of the RSM on Cal J in the insectary

5.3 Effect of endophytic colonization on red spider mite infestation (RSM)

These experiments were conducted at ICIPE, Duduville Campus in Nairobi, Kenya. Tomato seeds were surface-disinfected and inoculated with *B. bassiana* isolate ICIPE 35 as per the procedure described in chapter 3. The seeds were sown in pots (13cm x 10cm) with sterilized soil and placed in a screen house for 3 weeks before introducing RSM (Plate 9). Three-week old tomato plants (top and lower leaves) were artificially infested with 10 adult mites of (8 male and 2 female) using a camel hair brush. Mites were allowed to establish on the plant for 10 days. Each treatment consisted of two tomato plants per pot replicated 8 times in completely randomized design. The population of adult *T. evansi* were counted at 17 and 24 days after the mites had established under a dissecting microscope. The leaf area of each leaf was determined using a leaf area meter (Li-COR Model Li-3100, Japan) to establish mites per cm^2.

Plate 5.2: Tomato plants at 2 weeks in the screen house before introducing RSM

Plate 5.3: Tomato plants at 3 weeks in the screen house after introducing RSM

Plate 5.4: Tomato plants at 6 weeks in the screen house at termination of the experiments

5.4 Assessment of plant damage

A leaf from each of the eight replicates of the 3 tomato varieties were harvested and placed in plastic containers and covered with perforated lids (Plate 11), moist cotton wool was placed at the bottom, to maintain the relative humidity of approximately 50% which is the minimum required by red spider mites for development. There were 8 replications for each variety and its respective controls. Ten adult red spider mites were introduced and the containers covered for 72hrs. The temperature for the room was regulated by the sealed windows of the room and lighting which was maintained at 26 ^{o}C – 28 ^{o}C. Leaves were then removed and assessment of plant damage done based on a scale developed by Hussey and Scopes (1985), where 1 = no damage, 2 = 1-15%, 3 = 20-30%, 4 = 35-50%, 5 = 55-70% and 6 = 80-100% of

leaf damage. The experiment was laid out in a completely randomized design and replicated two times.

Plate 5.5. Endophytically colonized leaves of tomatoes with moist cotton wool in perforated plastic containers

Plate 5.6: Plastic containers containing tomato leaves and RSM in the laboratory

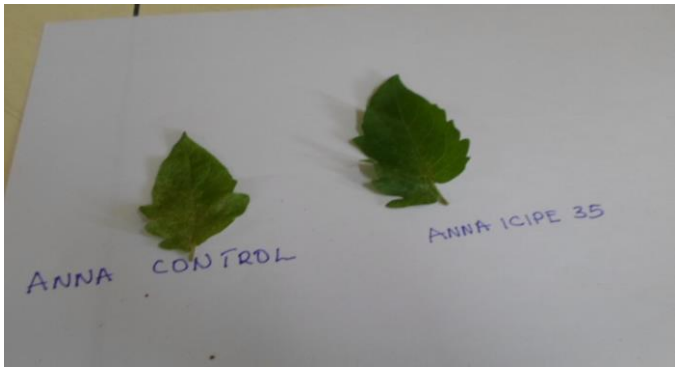

Plate 5.6. Endophytic *B. bassiana* leaves of Anna and it's control

Plate 5.7:. Endophytic *B. bassiana* leaves of Cal J and it's control

Plate 5.8. Endophytic *B. bassiana* leaves of Kilele and it's control

5.5 Data analysis

Data on plant damage were analyzed using the SAS statistical package before applying ANOVA analysis followed by Student Newman Keuls test to separate the means. Population counts of mites for the screen house experiment were log transformed to normalize data and transformed values were subjected to ANOVA. Student's t-test was used to compare treatments and control. All tests were performed at 5% level of significance. All data were analyzed using the SAS statistical package version 8 (1999).

5.6 Results

5.6.1 Effect of endophytic colonization on red spider mite density

Endophytically inoculated tomato plants had few red spider mites as compared to untreated controls which recorded high populations almost 4-5 times (Figure 5). The mite density/leaf cm^2 was significantly lower on the tomato varieties inoculated with ICIPE 35 as compared to the controls ($F= 3.50$, $df=2$, $p = 0.034$) at week 1 and week 2. In variety Anna, mite density decreased in the second week in both endophytic and control treatments. The mite density also decreased in the Cal J control in the second week while for the treated plants, the mite density remained low. In variety Kilele, mite density did not vary significantly over time for both endophytic and control treatments (Figure 5.1). Variety Kilele colonized by ICIPE 35 had the lowest number of RSM for the first week as compared to Anna and Cal J varieties. Results for the second week Cal J had the highest number of mites compared to Anna and Kilele.

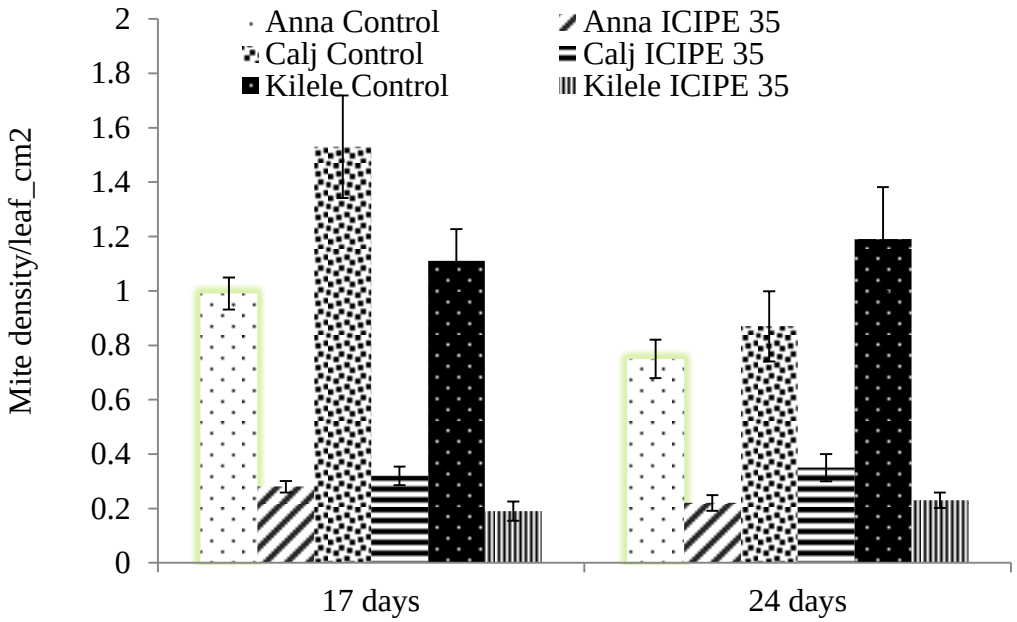

Figure 5.1: Red spider mite density/leaf cm^2 at 17 and 24 days post- inoculation

5.6.2 Effects of Beauveria bassiana ICIPE 35 -inoculated tomato plants on T. evansi plant damage

The plant damage did not differ between the treated endophytic tomato varieties leaves (P= 0.06, F= 37.49, DF= 5) (Figure 5.2). The plant damage was significantly lower in all the fungus-inoculated plants compared to their controls. There was a significant difference between the tomato varieties treated with *B. bassiana* and their respective controls (P= < 0.0001, F= 37.49, DF= 5). The implication of these results is that mites fed more on the controls which lacked the fungus compared to the endophytic tomato varieties.

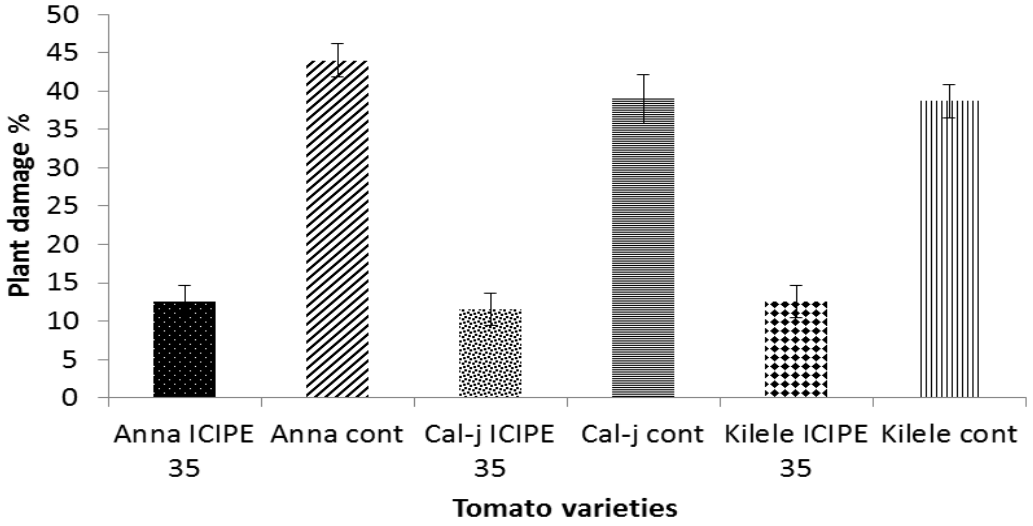

Figure 5.2: Effects of tomato varieties inoculated with *Beauveria bassiana* ICIPE 35 on feeding of adult *Tetranychus evansi* after 72 hours. Bars denote means ± one standard error (P<0.05).

5.7 Discussion

The tomato plant inoculated with *B. bassiana* had reduced adults of red spider mites as compared to their respective controls. Adult mortality noted and the reduction in RSM population may have been attributed to the presence of *B. bassiana* in the tomato plant tissues as well as the production of toxins. Our study compares with an experiment conducted with *B. bassian*a and *Cnostachys rosea* whereby there was a reduction in survival of adult *Hypothenemus hampei* (Vega et al., 2008). Similarly when *B. bassiana* was applied at the whole leaf stage to corn as a foliar, it reduced tunneling by the populations of *Ostrinia nubilalis* (Hübner) (Bing and Lewis, 1991).

The sub lethal effects involved in reduced populations were not studied at this level; however endophytic *B. bassiana* ICIPE 35 may have accumulated mycotoxins inside the tomato plants which reduced the feeding, damage as well as adult populations of RSM (Bing and Lewis, 1991; Cherry et al., 2004). The range of toxins produced by endophytic *B. bassiana* in the tomato varieties was not determined as part of this

study. Another explanation for the reduced RSM could be associated with too much energy expend on the production of lytic enzymes, including proteases, lipases that help to offer protection against infectious agents. These enzymes have been known to have antifungal activity either individually or synergistically in mixtures with antibiotics (Chapter 6) (Handelsman and Stabb, 1996).

Studies conducted in colonized date palm by endophytic *B. bassiana* revealed the induction of proteins related to plant defense and stress response which could play an active role in protection against insects, pathogens and abiotic stresses (Gómez-Vidal et al., 2009). The endophytic relationship may have caused *B. bassiana* to be primed which in turn confers systemic acquired resistance in the tomato varieties leading to reduced plant damage by the adult red spider mites (Conrath et al., 2006). Our results compare with (Gurulingappa et al., 2010; Vega et al., 2008), who reported less damage in endophytically colonized host plants. Similarly, these results agree with previous studies by (Muvea et al., 2014; Powell et al., 2009), who reported less insect pest damage on endophytic onions and tomato, respectively.

When the dead RSM from endophytic tomatoes were observed, no mycosis was evident suggesting that death could be as a result of feeding deterrence or antibiosis (Bing and Lewis, 1991). Studies done by other authors have also revealed that dead insects recovered from endophytically-colonized plants exhibit no signs of fungal infection (Akutse et al., 2013; Cherry et al., 2004). Several mechanisms may thus be involved in coffering protection to pests in crop plants by endophytic *B. bassiana*.

The plant damage was less in *B. bassiana* endophytically colonized tomato varieties than their control. This can be due to the reduced number of adults emerging hence less damage to the plants. The slight variations in the various tomato varieties in terms of damage percent may be due to the endophytic ability of *B. bassiana* in association with the host plant species (Akutse et al., 2013; Jaber, 2015).

In conclusion, this study shows that *B. bassiana* isolate ICIPE 35 has the potential to establish endophytic relationship in tested tomato varieties and reduce damage by red spider mites as well as the density. The tomato varieties of Cal-J, Kilele F1 and Anna F1 had less plant damage compared to their respective controls. Further studies are warranted for the tested colonized tomato plants during the cropping season under field conditions. This is important especially if there is vertical transmission of the endophytes through the seeds so that subsequent generations may not require seed dressing.

The plant defense ability exhibited by *B. bassiana* by reducing mite populations in tomato require more understanding on the mechanisms that suppress herbivory so as not to compromise on human and animal safety as was the case with Epichloë which protects grasses against nematodes but is also toxic to vertebrates (Schardl et al., 2004). More research should also be done to assess the mode of entry, distribution, and morphological state of the fungus inside the tomato plants.

CHAPTER SIX

MODE OF ACTION OF ENDOPHYTIC *BEAUVERIA BASSIANA*

6.1 Introduction

The mechanisms utilized by entomopathogenic fungi against insect pests are variable and sometimes not clearly understood. The mechanisms may however include antibiosis, competition, parasitism and induced systemic resistance (Vega et al., 2009). Direct parasitism can occur through the hydrolytic activity of extracellular enzymes such as protease and lipase which penetrate the plant cell wall and aid to hydrolyze the epidermis of the insect body (Burke and Cairney, 2002; Ownley et al., 2010; Petrini et al., 1992; Smith et al., 1981). However, information available indicate that not all isolates produce toxins and limited information is available on the type of toxins produced. The toxins aid in colonization and utilization of food substrates with the host (Fravel, 1988; Genthner et al., 1994).

In most cases, several mechanisms may be involved by a biocontrol agent against a specific insect pest or pathogen for example *B. bassiana* produces a host of antibiotics and has the ability to hydrolyze chitin. If the antibiotics from this fungus hydrolyses chitin, an important component in the integument of insects, this may lead to their death. Some endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites that have insecticidal activity (Bing and Lewis, 1991; Gurulingappa et al., 2011). Others produce phytochemicals that have antimicrobial properties and therefore offers protection to the plants against pathogenic diseases(Ownley et al., 2004b).

A clear understanding of the mode of action involved in biological control of *T. evansi* by endophytic *B. bassiana* is important for successful application in the field. The objective of this study therefore was to determine the production of extracellular enzymes such as lipase and protease by endophytic *B. bassiana* isolates on solid

medium so as to understand the mechanism of action involved in the biological control of RSM on tomato varieties (Chapter 5).

6.2 Materials and methods

This study was conducted at ICIPE laboratories, Nairobi, Kenya. *Beauveria bassiana* ICIPE 35 was obtained from the ICIPE's Arthropod Germplasm Centre, Duduville, Nairobi, Kenya and cultured using the procedure described in Chapter 3.

6.2.1 Seed inoculation and colonization

Seeds of cultivars Cal-J, Kilele F1 and Anna F1 hybrid were surface-sterilized and inoculated as per the protocol described in Chapter 3. The seeds were then removed and placed in pots with sterilized soil and allowed to grow in the screen house for 6 weeks. The endophytic colonization by *B. bassiana* on tomato plants was examined and cultured as per the protocol described in Chapter 3. For each plant part, percent colonization was calculated as number of sections exhibiting *B. bassiana* outgrowth per total number of sections (Fisher and Petrini, 1987).

Endophytic *B. bassiana* isolate ICIPE 35 established on the three tomato varieties was evaluated for extracellular secretion patterns of lipases and proteases. The detection of extracellular enzymes produced by fungi was carried out as per the method described by Hankin and Anagnostakis (1975). It entails the use of solid media for the detection of enzyme production by fungi. The specific procedures for lipases and proteases are described below.

6.2.2 Extra Cellular Enzymes Assay

6.2.2.1 Lipid Test

To test for the production of lipases, one week-old cultures of endophytic ICIPE 35 isolated from the tomato varieties of Anna, Kilele and Cal J were grown on SDA media for one week and was point inoculated on lipid (Tween 20) containing

medium as the primary source of carbon. A 3 mm cork borer was used to remove a disc of agar from the middle of the lipid plates and the hole was replaced by a similar sized mycelial agar discs of the endophytic ICIPE 35 from each of the tomato varieties. The medium components for the lipid test were as follows: peptone - 1 g, yeast extract - 0.1 g, agar 18 g, Tween 20 - 10 mL (autoclaved separately from the rest of the medium), distilled water - 990 mL, the pH was adjusted to 6.0 before autoclaving and pouring. A positive test for lipase enzyme was the occurrence of precipitated fatty acid crystals around the colony (plate 16) (Gessner, 1980; Hankin and Anagnostakis, 1975). The plates were measured daily for 7 days.

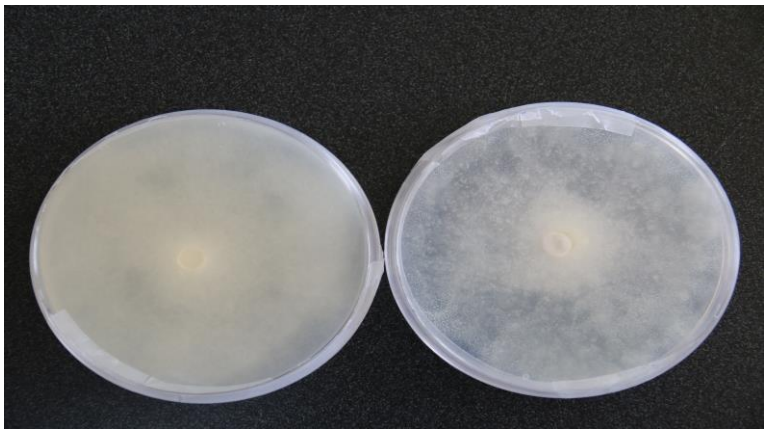

Plate 6.1: Lipase enzyme activity of tomato varieties on solid medium, control (Left) no precipitation and treatment (Right) with precipitation of fatty acids crystals around the fungal colony.

6.2.2.2 Protein test

The medium used to detect protease enzyme activity contained gelatin as the protein substrate (Hankin and Anagnostakis, 1975). A 3mm cork borer was used to remove a disc of agar from the middle of the gelatin plates and the hole was replaced by a similar sized mycelial agar discs of the endophytic ICIPE 35 from each of the tomato varieties. The medium consisted of nutrient agar plus 0.4% gelatin at pH of 6. An 8% solution of gelatin in water was sterilized separately and added to the nutrient agar at

the rate of 5mL per 100 mL. After incubation, plates were flooded with an aqueous saturated solution of mercuric chloride which precipitates protein. A clear zone around colonies indicated the presence of protease. The clear zone was assessed at the end of 7 days period (plate 17)**.** The diameters of the clear zone and of fungal colonies was measured, and the difference between the areas of the clear zone and the fungal colony calculated to provide an estimate of the levels of enzyme production by the different isolates (Alves et al., 2002).

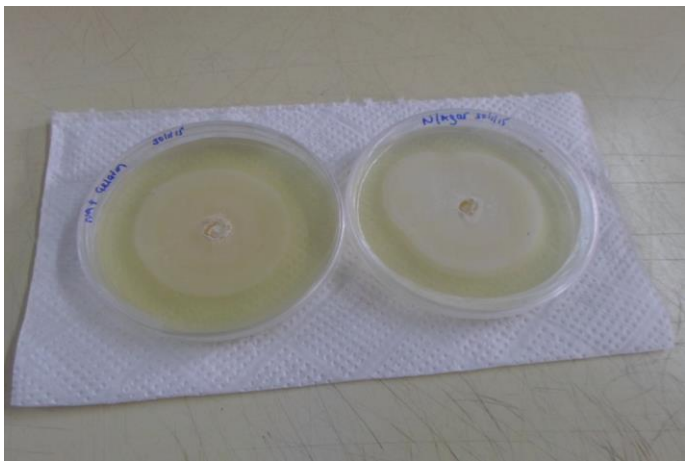

Plate 6.2: Protease enzyme activity on solid medium, treatment (Left) with a clear zone around the fungal colony and control (Right) no clear zone around the fungal colony.

Ten Petri dishes were used for the isolate and enzymes; five with medium amended with the substrate and five without the substrate (controls). The medium in the control plates comprised of each of the above-mentioned ingredients except the enzyme-specific substrate. All petri dishes were incubated for 1 week under laboratory conditions. Cultures were examined for the presence of a clear zone (halo) and the precipitation of fatty acids crystals around the fungal colony. The diameters of the clear zone and precipitation of fatty acids crystals were measured, and the difference between these areas and the fungal colony calculated to provide an estimate of the levels of enzyme production (Alves et al., 2002).

6.2.3 Data analysis

For the enzyme production assays, statistical analysis was performed on the averages of the size of the clear zones to test whether there were any significant differences among the tomato varieties (calculated from the difference in size between the fungal colony and the clear zone) using one-way ANOVA.

6.3 Results

The lipase activity was evident by the formation of lipolytic enzymes which was seen as a visible precipitate due to the formation of crystals of the calcium salt of the lauric acid liberated by the lipase enzyme or as a clearing of such a precipitate around a colony due to complete degradation of the salt of the fatty acid. The diameters of the visible precipitates did not differ among the tested tomato varieties (Figure 6.1) (P= 0.96). No visible precipitates were observed in the control plates in which the endophytic fungus was grown without the enzyme substrates (Plate 16).

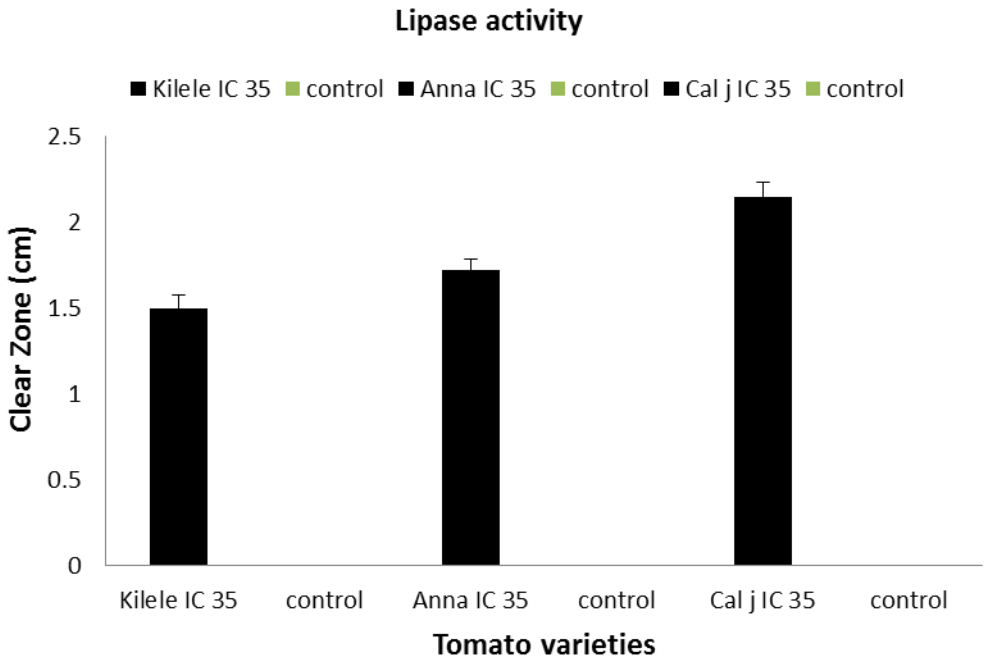

Figure 6.1 Lipase enzyme activity index of endophytic IC 35 on three tomato varieties of Kilele, Anna and Cal J, SNK test ($P < 0.05$) was used to compare different enzymatic activity.

Among the tested tomato varieties of Cal J, Anna and Kilele, differences existed between the endophytic tomatoes and their respective controls, however there was no significant differences among the varieties (Figure 6.2). Measurements of the clear zone for the protease enzyme activity were done at the end of the incubation period after flooding with a saturated solution of mercuric chloride. The diameters of the clear zones did not differ among the tested tomato varieties (P= 0.68). No clear zones were observed in the control plates in which the endophytic fungus was grown without the enzyme substrates (plate 17).

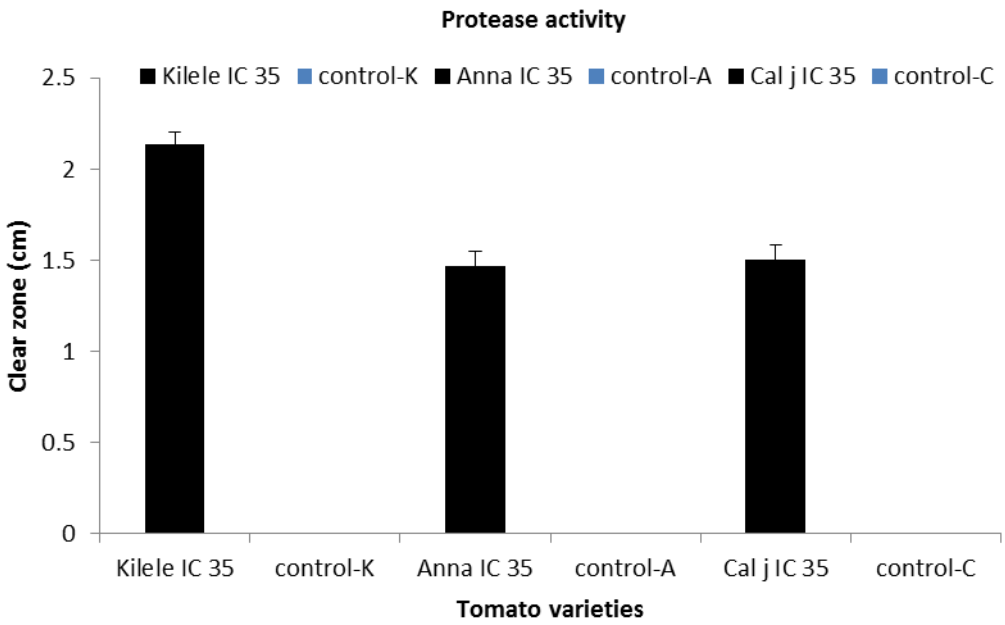

Figure 6.2: Protease enzyme activity index of endophytic IC 35 on three tomato varieties of Kilele, Anna and Cal J. SNK test ($P < 0.05$) was used to compare different enzymatic activity

6.4 Discussion

This study demonstrated that endophytic *Beauveria bassiana* ICIPE 35 was able to produce both lipases and proteases in all the tested tomato varieties of Cal J, Kilele and Anna. These enzymes trigger defensive mechanisms that could have reduced the reproduction of red spider mites in the tested tomato varieties as evidenced by the low numbers in the treated tomatoes compared to their respective controls. These cuticle degrading enzymes when combined with mechanical force aid fungi penetrate the cuticle (Campos et al., 2005).

Extracellular enzymes produced by *Beauveria bassiana*, include protease and lipase; they are believed to play a key role in cuticle hydrolysis, i.e the degradation of fatty molecules and proteins that comprise the insect body (Hallsworth and Magan, 1996). Enzyme production and pathogenicity has been found to have positive correlation

(Kaur and Padmaja, 2009) and lack of these enzymes in some strains of *B. bassiana* may delay the infection process in certain insect species (Bidochka and Khachatourians, 1990). These enzymes have antifungal activity either individually or in mixtures with antibiotics (Handelsman and Stabb, 1996; Spadaro and Gullino, 2005).

All the tested endophytic colonized tomato varieties of Cal J, Kilele and Anna were able to produce protease and it's activity according to some studies, precedes the action of chitinases (Smith et al., 1981). The production of protease need to be secreted early since proteins are the major components of insect cuticle and they play an important role in killing the insect since it is able to degrade the fatty particles of the insect body and therefore is very crucial in pathogenesis and virulence (Campos et al., 2005). A wide range of proteases has been identified and they include trypsin, chymotrypsin (Bidochka and Khachatourians, 1990).

Lipases were produced in all the tested tomato varieties tested. According to (Silva et al., 2005) the enzyme lipase plays a very important role in the insect infection process by *Beauveria bassiana*. Lipases are essential to catalyze the hydrolysis i.e. breakdown of ester bonds in lipids , fats and waxes which constitute part of the insect body and in the process they offer nutrients to the fungus (Belcarz et al., 2005). Extracellular lipases enzyme target the exoskeleton of insects.

The production of protease and lipases could be partly responsible for the reduced number of adult RSM in the treated plants (Chapter 5), since the insect cuticle is mainly composed of proteins and lipids (Belcarz et al., 2005; Kaur and Padmaja, 2009). These results have demonstrated that endophytic ICIPE 35 in tomato varieties of Cal J, Kilele F1 and Anna F1 produces extracellular enzymes on solid media and they play a key role in the degradation of the insect cuticle by digesting the starch, proteins and lipids respectively in the RSM body. This can be used as part of an integrated program in the control of red spider mites in tomatoes.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General discussion

Chemical control has been widely used by farmers but cases of resistance and environment contamination as well as direct effects to human and non-target organism have been on the rise. Although *B. bassiana* has been successfully used for the control of RSM in the field conditions (Chandler et al., 2000), environmental conditions have limited the wider application. The use of *B. bassiana* as an endophyte can therefore provide an alternative method and there is need to search for strains that provide increased colonization rates and persist for long period of time inside the plant tissues.

In this study, colonization varied among various varieties and plant parts. The above ground parts having a higher colonization than the below ground parts, the variation in colonization rate could be due to the fact that fungal endophytes display preferential tissue colonization within their plant hosts. Similar results were reported by (Behie et al. 2014) and they concluded that particular conditions present in a given plant part may also influence colonization. For optimum colonization of tomato plants by *B. bassiana*, it is critical to know the concentration and seed soaking time that will adequately improve colonization. ICIPE 35 was also able to promote growth within the tested tomato varieties without any detrimental effects; Anna variety recorded the highest values for plant height, number of leaves and fresh shoot weight compared to Kilele and Cal J varieties respectively.

Most studies have speculated that higher colonization of plant parts will increase the persistence and antagonistic effects of endophytic *B. bassiana* against insect pests and RSM. The experiments on persistence revealed that *B. bassiana* can persist up to 6 weeks in the screen house in the tested tomato varieties. Persistence within the tissues decreased with time due little multiplication within the plant tissues

(Brownbridge et al., 2012) and colonization persisted more in the leaves and stems for Anna and Kilele, while for Cal J persisted longer in the stem compared to the leaves and the roots. The prolonged presence of endophytic *B. bassiana* inside the plant tissues is important to offer protection throughout the growing season.

The presence of endophytic *B. bassiana* led to a significant reduction in the adult population of RSM and consequently reduced plant damage, Anna variety had the highest damage reduction of 31%, Cal J 27% and Kilele 26% respectively. Another possibility is that *B. bassiana* has been shown to secrete an array of low molecular weight secondary metabolites which include oosporein, beauvericin, bassianolide and beauveriolide, some of which may contain toxins, or have insecticidal, antifeedant, antimicrobial or deterrent properties.

Extracellular enzymes target the external structure of arthropods, in our study tested isolate ICIPE 35 was able to establish as an endophyte and produce protease and lipase enzymes in the tested tomato varieties of Anna, Kilele and Cal J. The production of Protease and lipase may be partly responsible for the reduced numbers of RSM (chapter 5) since the cuticle of insects and mites is composed mostly of proteins and lipids. The association of other several modes of action like antibiosis, induced resistance, priming of proteins as well as direct parasitism may all have played an important role in the reduction of the adult RSM populations. Studies done by several authors reveal that insects and mites infected with entomopathogenic fungi eat less than healthy ones during the disease incubation period (Blanford and Thomas, 2001; Ekesi, 2001) leading to decreased damage to crops . The accumulation of toxins among other factors may have contributed to reduced feeding and damage to the plant parts.

This research has demonstrated that *B. bassiana* isolate ICIPE 35 can establish as an endophyte in the tested tomato varieties of Anna, Kilele and Cal J, promote growth, persist for up to 4 weeks in the growth chamber and 6 weeks in the screen house and consequently result in reduced damage to the plants by RSM. In addition, it was able

to produce extracellular enzymes such as lipase and protease that played a role in reducing adult RSM populations; hence these results can be used for further trials in the field to supplement existing measures for controlling the RSM.

7.2 Conclusions

1) Endophytic *B. bassiana* isolate ICIPE 35 colonized and enhanced growth in the tested tomato varieties of Anna, Kilele and Cal J in the growth chamber.

2) *Beuveria bassiana* isolate ICIPE 35 was also able to colonize and persist inside tomato varieties of Anna, Kilele and Cal J for up to 6 weeks in the screen house.

3) Endophytic *B. bassiana* ICIPE 35 significantly reduced adult RSM populations and consequently plant damage to tomato plants in the screen house.

4) Mode of action utilized by endophytic *B. bassiana* ICIPE 35 included the production of extracellular enzymes of protease and lipase in all the tested tomato varieties among other factors.

7.3 Recommendations

1) *Beauveria bassiana* enhanced growth in the tested tomato varieties and therefore be tested in other tomato varieties in Kenya where it can offer protection against RSM.

2) Colonization and persistence is important since it ensures that the tomato plants are protected for long against RSM, the search for more strains of *B. bassiana* that colonize and persist long in the tomato plants is required.

3) Although endophytic *B. bassiana* ICIPE 35 significantly reduced adult RSM populations and consequently plant damage to tomato plants in the screen house, there is need for field testing ensure adaptability and efficacy under uncontrolled conditions.

4) Additional information on the mode of action is needed to ascertain the other factors involved apart from extracellular enzymes and produced toxins.

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APPENDICES

Appendix I: Sabouraud Dextrose Agar Sigma Limited

Mycological peptone	10g/litre
Dextrose	40g/litre
Agar	15g/litre