EVALUATION OF ENTOMOPATHOGENIC EFFECTS OF THE FUNGI, *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* FOR BIOLOGICAL CONTROL OF *RHIPICEPHALUS* (*BOOPHILUS*) *DECOLORATUS*

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN APPLIED VETERINARY PARASITOLOGY

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DECLARATION

I declare that this thesis has not been presented for a degree in any other university and is my original work.

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DEDICATION

To my colleagues and friends, this dissertation is dedicated to you for your support.

Thank you.

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LIST OF ABREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
ESM	Error standard mean
ICIPE	International Centre of Insect Physiology and Ecology
ILRI	International Livestock Research Institute
IPM	Integrated Pest Management
EPF	Entomopathogenic fungi
L: D	Light-darkness photoperiod proportion
RH	Relative humidity
TBD	Tick-borne diseases
ECF	East coast fever
SAS	Statistical analysis system
SDA	Sabouraud dextrose agar
SE	Standard error
SNK	Student-Newman-Keuls

ABSTRACT

Rhipicephalus decoloratus causes heavy losses economically in livestock annually. Currently, control of ticks is done using acaricides but use of other alternatives such as biological control is being studied. Twelve fungal isolates; seven *Metarhizium anisopliae* and five *Beauveria bassiana* obtained from ICIPE Arthropod's germplasm were pretested for their germination potential and conidial viability, results of which indicated that their viability ranged from 96.6 % for ICIPE 644 to 100% for ICIPE 9, ICIPE 7 and ICIPE 718. The mortality of fungal isolates that was pathogenic to amitraz-susceptible and amitraz-resistant strains of *R. decoloratus* larvae, ranged from 10.0 to 100% and 12.1 to 100%, respectively. For the selected fungal isolates, their LT_{50} values ranged between 2.6 to 4.2 days in amitraz-susceptible strains and from 2.8 to 3.9 days in amitraz-resistant strains. Amitraz-susceptible strain showed LC_{50} values of between 0.4 ± 0.1 and 200.0 ± 60 x 10^3 conidia ml^{-1} while, amitraz-resistant strains had LC_{50} values ranging from 0.1 ± 0.1 to 200.0 ± 31.0 x 10^3 conidia ml^{-1}. ICIPE 7, *M. anisopliae* outperformed the other eleven isolates and was selected for amitraz compatibility and field studies. It was also shown to be compatible with amitraz. In the field study, four treatments; control, *M. anisopliae* alone, amitraz alone and combination of *M. anisopliae* and amitraz were applied on cattle. All the treatments significantly reduced tick numbers significantly on day 7, day 14, day 21 and day 28 in comparison with the control. Results of the present study demonstrated the potential value of fungal pathogens for tick control; further research is needed to determine the association of biological products with chemical products and ICIPE 7 should be improved for use as a myco-acaricide and it can manage the amitraz resistant strains of *R. decoloratus*

CHAPTER ONE

1.0 INTRODUCTION

In the tropics and subtropics, ticks and the diseases they transmit hinder livestock productivity (Walker *et al*., 2003). In East Africa, *Rhipicephalus decoloratus, R. appendiculatus* and *Amblyomma variegatum* are the common devastating tick species and their main host is cattle; but rarely horses and sheep where their life-cycle is not completed (Walker *et al*., 2003). Anaplasmosis and babesiosis are tick borne diseases and their causative agents are *Anaplasma marginale* and *Babesia bigemina,* respectively and *R. decoloratus* tick is the main vector. Anaplasma *marginale* and *B. bigemina* infections lead to high production losses and death in cattle (Melendez, 2000; Stuen *et al*., 2003). Due to lack of successful vaccination against these diseases, the only alternative is to control the vectors.

Currently, control of ticks is done mainly by use of synthetic tickicides like amidine group of acaricides, synthetic pyrethroids and organophosphates (Pound *et al.,* 2009). Toxicological, environmental and other effects have resulted due to heavy reliance on these acaricides (Ducornez *et al*., 2005 and Schulze *et al.,* 2005). Resistance of ticks to acaricides of different groups has occurred to a higher extent leading to exploration of other methods of controlling ticks (Castro-Janer *et al.,* 2010). One of the methods being taken to control tick resistance to acaricides is the application of entomopathogenic fungi (EPF) (Maniania *et al.,* 2007). Biopesticides in combination with *M. anisopliae* have been seen as a viable option for commercial purposes in tick control (Faria and Wraight, 2007). An incorporated approach in

managing tick resistance to acaricides using a combination of acaricides and EPF could tackle these setbacks but this should be explored first before they are combined. For example, amitraz was previously reported to hinder the growth of *B. bassiana* (Alizadeh *et al.*, 2007).

The objectives of the present research were to test if *B. bassiana* and of *M. anisopliae* were virulent against *R. decoloratus* strains; covering the amitraz-resistant and amitraz-susceptible strains in order to select the most virulent isolate, to assess if the selected most virulent isolate was compatible with acaricide and the efficacy of this isolate on on-host ticks in the field.

1.1 Hypothesis

1.1.1 Null hypothesis

1) Entomopathogenic fungi cannot be used to control acaricide-resistant strains of *R. decoloratus* and are not compatible with amitraz.
2) *Metarhizium anisopliae* and *B. bassiana* are not effective on *R. decoloratus*
3) ICIPE 7 and amitraz are not compatible
4) ICIPE 7 cannot be effective in controlling *R. decoloratus* in field evaluation trials

1.2 Objectives

1.2.1 General objective

To determine the entomopathogenic effects of the fungi, *M. anisopliae* and *B. bassiana* as a bio control agent of *R. decoloratus* in Kenya.

1.2.2 Specific objectives

1) To determine the effectiveness of *M. anisopliae* and *B. bassiana* on *R. decoloratus*
2) To determine the pathogenicity of the selected pathogenic fungal isolates to *R. decoloratus* strains
3) To determine the compatibility between *M. anisopliae* ICIPE 7 and amitraz in controlling *R. decoloratus* strains
4) To determine the effectiveness of *M. anisopliae* ICIPE 7 in controlling *R. decoloratus* in the field

1.3 Justification

Control of ticks is by application of ixodicides but frequent uses have disadvantages such as tick resistance, ecological pollution and they are harmful to foodstuffs. Ecological distress and unsafe use of the ixodicides has prompted the exploration of other ways of controlling ticks such as use of natural methods (Chandler *et al.*, 2000). Ixodicides are costly to small scale farmers in Africa although they manage to lower ticks to some extent. Biological control of ticks using EPF is being considered as one of the alternatives to reduce frequent applications of synthetic chemical acaricides and management of tick resistance to acaricides. A number of mycoinsecticides have been developed and commercialized in different parts of the world (Faria and Wright, 2007), except in Africa. *Rhipicephalus decoloratus* is one of the most important vectors in cattle; however its vulnerability to *M. anisopliae* is not investigated in Kenya yet. Therefore, the current study on biological control was aimed at getting an appropriate tick control option which will lower the rate of application of ixodicides and also lower the management of tick borne diseases (TBDs).

CHAPTER TWO

2.0 LITERATURE REVIEW

In Kenya and globally, ticks are the main source of economic distress in cattle industry. Diseases caused by ticks are generally spreading the entire globe (Jongejan and Uilenberg, 2004). Their management depends mainly on acaricides. However, owing to an increasing tick resistance to acaricides (Jonsson *et al.*, 2000), ecology and human protection; the advancement in exploring other methods that are safe to the environment, probably one which is natural such as use of entomopathogens like fungus. These methods are naturally harmless to the environment, cheaper than ixodicides with no incidences of persistence of ticks after their application (Polar *et al.*, 2005; Zimmermann, 2007). Entomopathogenic fungi have demonstrated that they can be used for the management of ticks (Samish *et al*., 2004) and (Maniania *et al.*, 2007).

2.1 Tick biology

Ticks are external parasites of mammals, reptiles and birds. They belong to the Phylum Arthropoda; class Acarina that consist of families: Ixodidae (hard ticks), Argasidae (soft ticks) and Nutalliellidae (Norval *et al.*, 1992). The major behavioral difference between ixodids and soft ticks is that ixodids take many days to take blood meals while soft ticks take the blood meal within a short time of less than 60 minutes. Hard ticks also differ from argasid ticks in the number of nymphal stages, with soft ticks' nymphs moulting twice while ixodids' nymphs moult once.

2.2 Morphology of ixodid ticks

The body of tick comprises two body parts, gnathosoma (capituli and basis capitulum) and idiosoma (coxae, reproductive organs and spiracular plates). In unfed female hard ticks, the conscutum covers the anterior one third of the dorsal side; the rest of the body not covered by the scutum is called the alloscutum. In male hard ticks; the scutum covers the whole dorsal part of the tick (Walker *et al.,* 2003). The outer hard covering of the tick (scutum) is where muscles attach. The eyes are on the sides of the scutum for the genus of tick with eyes. Mouthparts are located anteriorly and they have two chelicerae, segmented palps with four segments and hypostomal teeth (Walker *et al.,* 2003). Chelicerae are used for penetrating into the skin epidermis of the host. Hypostome teeth and the cement secreted by tick's salivary glands assist the ticks when attaching to the skin of the host (Sonenshine, 1991).

Rhipicephalus decoloratus (one host tick) mouthparts have small palp segments II and III. Hypostomal teeth are in columns located on the ventral surface of the hypostome and are in 3+3, arranged in two sets on either side of the midline but in other species such as *R. microplus* they are in form of 4+4 columns. Male ticks possess adanal and accessory adanal shields. In *R. decoloratus,* the adanal shields possess spurs which may or may not be seen from the dorsum (Walker *et. al.,* 2003). Caudal process may be present in males but festoons are lacking.

2.3 Direct effect of ticks on the host

Ticks attach to the host to suck blood leading to annoyance and damage of the skin when biting the host, which lowers the value of the hides and skins, reduction in weight gain and loss of blood due to the feeding of ticks, as well as secondary infections in the parasite fixation site

(Gates and Wescott, 2000). Ticks can cause severe dermatitis, reduce body weight gains and milk yield, and also create sites for secondary invasion by pathogenic organisms (Gates and Wescott, 2000). Tick paralysis is as a result of severe ascending flaccid motor paralysis due to toxins introduced by some ticks during feeding. Ticks are the main vectors of TBDs that affect cattle (Jongejan, 2007).

2.4 *Rhipicephalus* (formerly *Boophilus) decoloratus*

Rhipicephalus decoloratus is called the blue tick because it's blue in colour when fully fed (Plate 1) and belongs to the Order Ixodida and Family Ixodidae. Blue tick is very common and is widely distributed in many parts of Africa, requires only one host, mainly cattle and it is the principle vector of *Babesia bigemina*, the causative agent of babesiosis (De Vos and Potgieter, 1994). Blue ticks are host specific but can also feed on other animals such as horses, donkeys, sheep, goats and wild ungulates but infection will only occur if the ticks have been infesting on their specific host.

Plate 1: Female (left) male (center) (Afrivip) adults ticks and larva of *R. decoloratus* tick (right)

2.5 Geographic distribution of *R. decoloratus* in Africa

Rhipicephalus decoloratus is mainly found in areas where cattle graze in savanna and temperate climates with a wide distribution in Africa especially south of the Sahara but does not occur in drier parts of Africa such as Namibia, South Africa and Botswana (Plate 2).

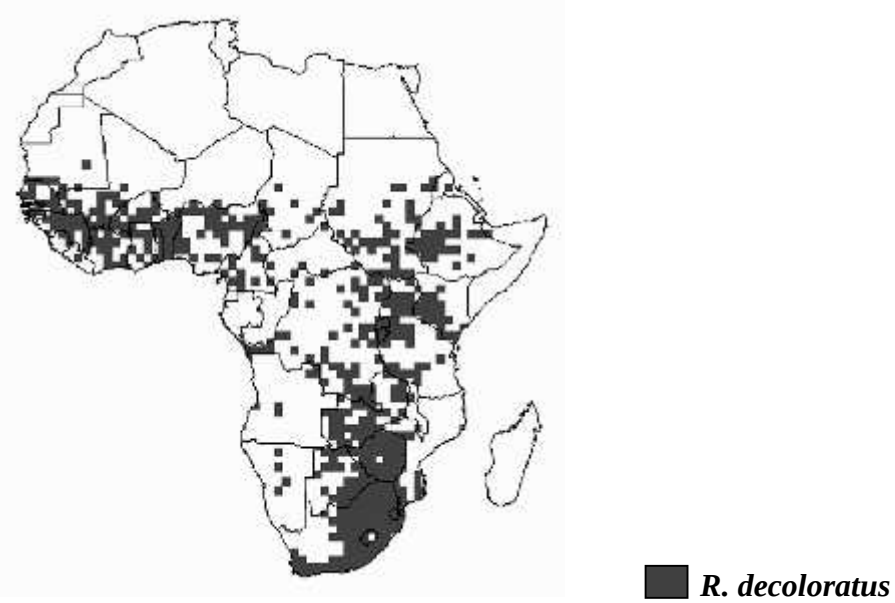


Plate 2: Distribution of *R. decoloratus* in Africa (Walker *et al.*, 2003)

2.6 Life cycle of ixodid ticks

The life cycle of hard ticks have four stages: the egg, larva with three pair of legs, nymph and an adult with four pair of legs, respectively (Sonenshine, 1991). Ixodid ticks demonstrate various stages of development in their life cycles therefore being classified as one-host ticks, two host ticks or three-host ticks (Bedford, 1934). One host tick for example, *R. decoloratus* requires only a single host for complete development where they suck blood and mate. The adults change

position on the host for mating. After mating, the female tick sucks blood until they are engorged and detaches from the host to lay eggs on the ground. The female tick can lay more than 10,000 eggs and finally it dies. The eggs are laid and they hatch to larvae if climatic conditions are favorable. The larvae hatch after some few weeks and attach onto the grass to wait for a suitable host to attach. After attaching on to the host, they feed and molt to nymph which then moults to an adult. This life cycle is fast and takes three weeks for the tick to feed and two months to lay eggs and larvae to develop. It occurs in *R. decoloratus* and *R. microplus* ticks (Estrada-Pena *et al.*, 2004; Walker *et al.*, 2007).

The two-host tick developmental life cycle requires two hosts where the larvae attach to the first host to suck blood and moult into nymphs in the same host, feed, engorge and drop off the host. When environmental conditions are suitable, they moult to adult tick and these adults wait for another host to attach to and feed to complete their life cycle. This type of life cycle occurs in *R. evertsi evertsi* (Latif and Walker, 2004).

In three-host tick life cycle, three hosts are required for completion of the life cycle (Sonenshine, 1991) where an engorged adult female detaches from the host to lay eggs and then dies. The eggs hatch to larvae which attach on to the vegetation to wait for a suitable host. While feeding, partial metamorphosis occurs inside the skin of the larva and they then moult into nymphs whose integuments harden within a few days before hatching. After feeding, the nymph molts to adult and the female adult mates on the host with the adult male. The female then falls off to the ground to lay eggs and dies. The male is left still attached onto the host for some time and will also die (Walker *et al.*, 2003). This type of life cycle occurs in ticks such as *A. variegatum.*

2.7 Life cycle of *R. decoloratus*

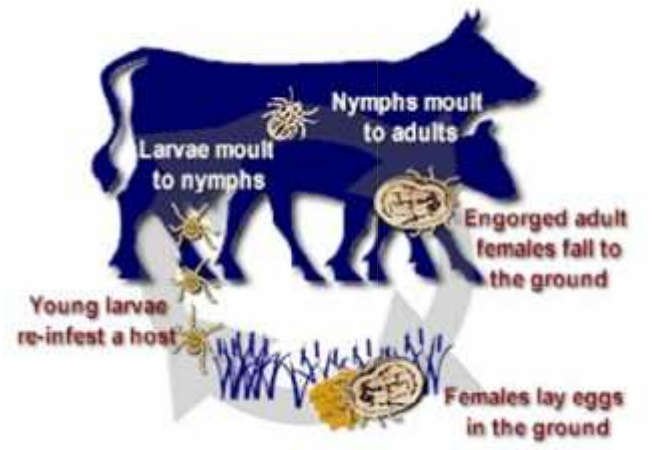


Plate 3: Life cycle of one-host tick (Junquera, 2007)

2.8 Tick behaviour

Most ixodids search for their suitable hosts by attaching on the grass and foliage with their first pair of coxae stretched in order to grab any host passing over. Ixodid ticks are able to sense the host by substances such as smell produced by the host; they also sense the shades of the passing host. These adaptations may be used when searching for efficient tick control methods (Sonenshine, 1991).

2.9 Tick habitat

Ticks of all species are adapted to a single and often highly specific habitat in which they can survive (Sonenshine, 1994). The eggs or larvae are the developmental stages of tick's life cycle that stay longer in the environment. However, their life cycles depend on different environmental conditions which they need to be protected from them in order to survive (Estrada-Pena and

Thuiller, 2008). In the environment, ticks are at a risk of drying out, starving, freezing, predisposed to predators like ants and pathogens like fungi during molting and questing. Type of habitats that a species will be found in is limited by these adverse factors and thus determines their geographical distribution (Estrada-Pena *et al.*, 2004). The accessibility of the host and flora has a lot of effect on how different species of ticks are widespread (Cumming, 2002). *Rhipicephalus decoloratus* are tropical ticks and usually occur in savanna regions with habitat of wooded grassland used as cattle pasture. They require high temperatures and therefore tend to tolerate drought.

2.10 Important tick-borne diseases of cattle and their impact on livestock industry

The most important diseases transmitted by ticks (vectors) occur in most parts of the globe mainly in the tropics and subtropics (Jongejan and Uilenberg, 2004). These vectors lead to high production losses and the diseases they transmit cause death in animals (Graf *et al.*, 2004). Their economic impact can be expressed as death, decrease in production, high cost of tick control and quarantine (Norval *et al.*, 1992). Africa has 186 million heads of cattle and ticks and TBDs remain the most serious constraint to livestock production. In every year, the cost of controlling ticks and treating the diseases they transmit in small scale farmers is between US$ 2.5 to US$ 25.0 per cow but it depends on the area and production systems (Pegram, 2001). The brown ear ticks transmit *Theileria* parasites, the causative agents of East Coast fever (ECF), in cattle in Eastern and Southern Africa (Norval *et al.*, 1992). Other examples include *annulata*, causative agent of tropical theileriosis in Mediterranean region, the Middle East and Asia. Theileriosis

causes cattle death in sub-Saharan Africa, and is therefore the major constraint in livestock keepers in the tropics.

Heartwater is transmitted by *Amblyomma* ticks and affects cattle, shoats and wild ruminants leading to high losses in non-indigenous breeds of cattle (Allsopp, 2015). The disease occurs in Africa and Caribbean regions.

Rhipicephalus decoloratus transmit *Anaplasma marginale* parasites that cause Anaplasmosis which is a less acute disease but infection in adults may lead to anemia and 50 % of the animals may die. *Babesia bigemina* parasites transmitted by *R. decoloratus* are the causative agent of Babesiosis (red water) in cattle and may cause up to 30 % mortality while *Babesia bovis* cause 70-80 % mortality (Vincent and Ring 2009). Babesiosis occurs in tropical areas and causes high death rates in exotic animals.

2.11 Tick control methods

Ticks and their effects can be controlled by using various methods that include the use of chemicals, vaccination, pasture management, breeding cattle for tick resistance and biological control.

2.11.1 Chemical control

Currently the main method of controlling ticks is by use of acaricides as a dip, spray or pour-on. They are in the classes of organophosphates, amidines, synthetic pyrethroids and macrocyclic lactones (Piesman and Eisen, 2008).

Acaricide resistance by ticks is on the rise due to increased frequency in the application of acaricides (Jonsson *et al*., 2000). The development of tick resistance to amidine group is on the rise and has been detected in *R. microplus* (Rodríguez-Vivas *et al.,* 2006; Davey *et al.,* 2008; Singh *et al.,* 2014) in Mexico, Australia and India. Resistance of *R. microplus* to amidines has been detected in Zambia (Muyobela *et al.,* 2015) and existence of amitraz-resistant strain of *R. decoloratus* has also been reported in Kenya (Hatta *et al.,* 2013).

2.11.1.1 Use of acaricides

The main methods of controlling ticks have been by use of acaricides as they may reduce their numbers within a short time but continual usage of these acaricides has led to the development of tick populations that are resistant to these chemicals (Raynal *et al*., 2013) making it impossible to control them.

2.11.1.2 Acaricide resistance

Acaricide resistance refers to the capability of a population of a tick to continue existing even after application of the chemical at the required dosage or more but at the rate which they are acceptable. Resistance can be acquired implying that they are inherited and the chemical has not been sensitive with subsequent applications (Meyer *et al.,* 2012). Tick resistance may be shared among different acaricides which have the same mode of action or ticks may be resistant to one or many acaricides that act differently (Sammataro *et al*., 2005; Bielza *et al.*, 2007).

2.11.1.3 Mode of action of amitraz and mechanism of its resistance

Amitraz is a triazapentadiene compound and is classified in amidine group of acaricides. It has been effectively used to control ticks in cattle for more than thirty years (Jonsson and Hope, 2007), however, tick resistance to amitraz has been detected (Mendes *et al.*, 2013). Amitraz has toxic effects on octopamine receptor for the neuromodulator. In bioassays where synergy is demonstrated (Ducornez *et al.*, 2005; Gong *et al.*, 2013), modification of the target site of P450 cytochrome monooxygenase is involved. Chen *et al*, 2007 and Corley *et al.*, (2013) reported the molecular basis of target site resistance, where two nucleotide substitutions in octopamine receptor in the resistant strains of ticks resulted in amino acids that were different from all the susceptible strains. These mutations proved that an altered target site was the probability that resistance to amitraz only occurs in amitraz-resistant ticks (Guerrero *et al*., 2012; Pohl *et al*., 2012) but the exact mechanism of resistance to amitraz is not yet known.

2.11.2 Use of vaccines

Ticks during feeding produce antigens which facilitate the acquisition of blood from their hosts and that can activate the production of antibodies in the hosts against internal organs of the ticks (da Silva Vaz *et al.*, 1998). The antigens are used for production of vaccines against ticks (Imamura *et al*., 2008), for example the vaccine against *R. microplus* which was based on Bm86 molecule associated with the gut of the tick, induced the production of antibodies directed against a critical protein in the tick gut, this was developed in Australia (Willadsen *et al.*, 1995). In a related development, Boué *et al*., (1998) developed a vaccine (Gavac™) in Cuba, which was

effective against *R. microplus* but its efficacy against other species of ticks could not be guaranteed.

2.11.3 Pasture management

Planted pastures are much less attractive for ticks to thrive better than on natural pastures. In areas where animals are zero-grazed, hay can bring in ticks that can transmit disease causing pathogens. Allowing the pasture to rest and rotating the pastures have been shown to lower the one host tick populations of *R. microplus* on dairy farms in Australia and also three-host ticks such as *Hyalomma anatolicum anatolicum* and argasid ticks (David, 2005). These methods do not control ticks effectively because unfed nymphs and adults survive longer in the environment (David, 2005). Tropical legumes (stylosanthes) and some grasses such as *Melinis minutiflora* and *Brachiaria brizantha* kill larvae and have anti-tick effects. Clearing the pastures may eliminate different life cycle stages of some ticks but can lead to soil erosion which is dangerous to the environment.

2.11.4 Tick resistant breeds of cattle

Keeping cattle which are genetically resistant to ticks is a good method of controlling ticks (De Castro and Newson, 1993). Resistant breeds such as Sanga and Zebu which are local breeds from Asia and Africa have high resitance to exposure of hard ticks but exotic breeds are very susceptible to tick infestations. Breeding the local breeds for resistance is a good method of controlling ticks. Tick resistance is heritable and breeding selected cattle for resistance can therefore be improved (David, 2005).

2.11.5 Biological control of ticks

Control of ticks and other vectors using natural enemies such as predators, nematodes, parasitoids and pathogens are other methods being explored. These natural enemies are safer to the environment, cheaper than acaricides with no likelihood of resistance occurring (Polar *et al.*, 2005; Zimmermann, 2007).

2.11.5.1 Predators

Many predators such as spiders, ants, beetles, birds and reptiles frequently eat ticks and reduce their numbers naturally (Samish and Rehacek 1999) but there is limitation for their use unless their numbers are markedly increased for them to be able to control ticks. This could lead to high population of unwanted species in the environment (Symondson *et al.,* 2002).

2.11.5.2 Parasitoids

The dominant order among entomophagous insects, *Hymenoptera,* was one of the efficient methods of controlling pests but very few of these parasitoid species have an effect on ticks. The most widespread parasitoid of ticks is *Ixodiphagus hookeri* (propagated from wasps) and is mostly widespread in Asia, Africa, North America and Europe (Hu *et al*., 1993). A study done in Kenya indicated that 50 % of parasitoids found in Kuja river basin in Kisii County and 70 % in Transmara areas could parasitize ticks (Mwangi *et al.,* 1994).

2.11.5.3 Tick traps

Tick traps incorporated with Neem (*Azadirachta indica*), cake extracts (0.6% of azadirachtin) and an attraction aggregation- attachment pheromone (AAAP), 1-octen-3-ol and CO_2 was able to

trap ticks (Maranga *et al.*, 2003). An integrated use of semiochemicals, such AAAP and kairomone and an entomopathogenic fungus in an inoculation device (trap), can attract specific ticks to fungus in pheromone-baited trap and therefore reduce the need to control on-host of ticks (Maranga *et al*., 2006).

2.11.5.4 Entomopathogens

Entomopathogens include entomopathogenic fungi, nematodes, bacteria, protozoa and viruses. They are known to cause natural diseases in arthropod populations (Roberts and St. Leger, 2004). Entomopathogens are widespread in the natural environment and may be produced in large quantity, formulated and applied like acaricides for tick control (Shah and Pell, 2003). They can be released into the environment for control of arthropods including ticks through inundative and augmentative releases (Lacey and Goettel, 1995).

2.11.5.4.1 Bacteria

Several bacteria such as *Proteus, Bacillus* and *Cedecea* are pathogenic to ixodids; *Proteus mirabilis* is pathogenic to *Dermacentor andersoni* ticks; *Bacillus thuringiensis* is pathogenic to *Argas persicus, Hyalomma dromedarii, Ixodes scapularis* (Samish and Rehacek 1999). *Bacillus thuringiensis* also attacks *Amblyomma hebraeum, Hyalomma marginatum and R. evertsi evertsi* and apparently causes the Blackening disease of *R. decoloratus*. *Cedecealapagei* is pathogenic to *R. microplus*. Although most of these bacterial species have been reported to be virulent to ixodids *in-vitro* (Samish and Rehacek 1999), there has been no attempt to develop them as microbial biocontrol agents because they have to be ingested by the host first for them to have any effect on ticks (Maniania *et al*., 2007).

2.11.5.4.2 Nematodes

Heterorhabditidae and Steinernematidae families of nematodes have been known to parasitize ticks (Zhioua *et al.,* 1995; Samish *et al.,* 1996). The immature nematodes penetrate the ticks' cuticle through their openings or through ingestion (Zhioua *et al*., 1995). Within the haemocoel of the tick, nematodes release bacteria that attack and kill the tick in 24-72 hours (Zhioua *et al*., 1995). The infectivity of these nematodes is mainly to the adult female ticks due to ease of entry through the genital aperture or due to the thinness of their outer covering (Samish and Glazer, 2001).

In-vitro experiments have shown that these nematodes can kill fully fed *R. annulatus* female sticks (Samish and Glazer, 1992; Samish *et al*., 1996; Samish and Rehacek 1999).

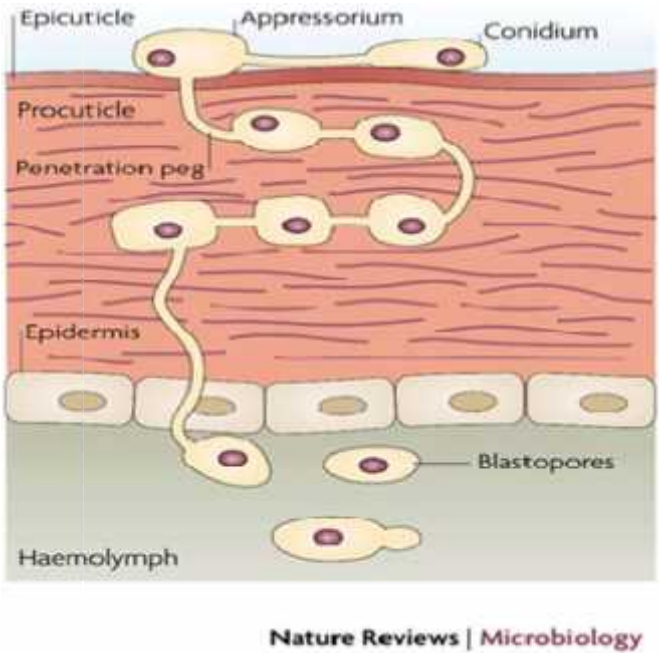
2.11.5.4.3 Entomopathogenic fungi

Entomopathogenic fungi are common in the environment and have broad host spectrum and can penetrate their host cuticle directly to occur unlike other entomopathogens which require to be ingested first (Maniania *et al.,* 2007). More than 700 fungal species have been discovered but only a few are being used for biocontrol (Samish *et al.,* 2004; Maniania *et al.,* 2007). Most common fungal pathogens of arthropods that are widely studied for biological control of ticks belong to the hyphomycetous fungi (Asco *Bacillus* mycota) and include *Beauveria, Metarhizium, Isaria, Hirsutella, Lecanicillium, Culicinomyces, Tolypocladium* and *Nomuraea*. Although they have shown good potential in the laboratory, results from the field have been generally inconsistent (Hombostel 2005; Alonso-Diaz *et al*., 2007). The control of ticks by use of these entomopathogenic fungi has few limitations such as harsh environmental conditions and sweat

from the animals which can influence their pathogenicity (Polar *et al*., 2005). In any successful infection of the host, viability of conidia is crucial especially during the penetration of the tick's cuticle where the fungus germinates and forms germ tube (Schrank and Vainstein, 2010).

2.11.5.4.3.1 Mode of infection of entomopathogenic fungi

There are many steps in infection process: Infection starts with fungal spores contacting and then sticking to the host's cuticle. These spores require polar cuticle from the host, enough nutrients and a hydrophobic surface for their formation (Wang *et al.,* 2005). After the formation of these structures, the fungus enters into the tick's cuticle where pressure exerted by appressorium and enzymes such as chitinases, lipases, esterases and proteases are involved (Wan, 2003). Then the fungus develops within the tick where yeast-like blastospores, hyphal bodies or protoplasts begin to grow (Plate 4). The tick finally dies due to lack of enough nutrients, obstruction of organs and toxicosis (Wraight *et al.,* 2007). The fungus then comes out of the dead tick as hyphae, and spores form on the surface of the tick.



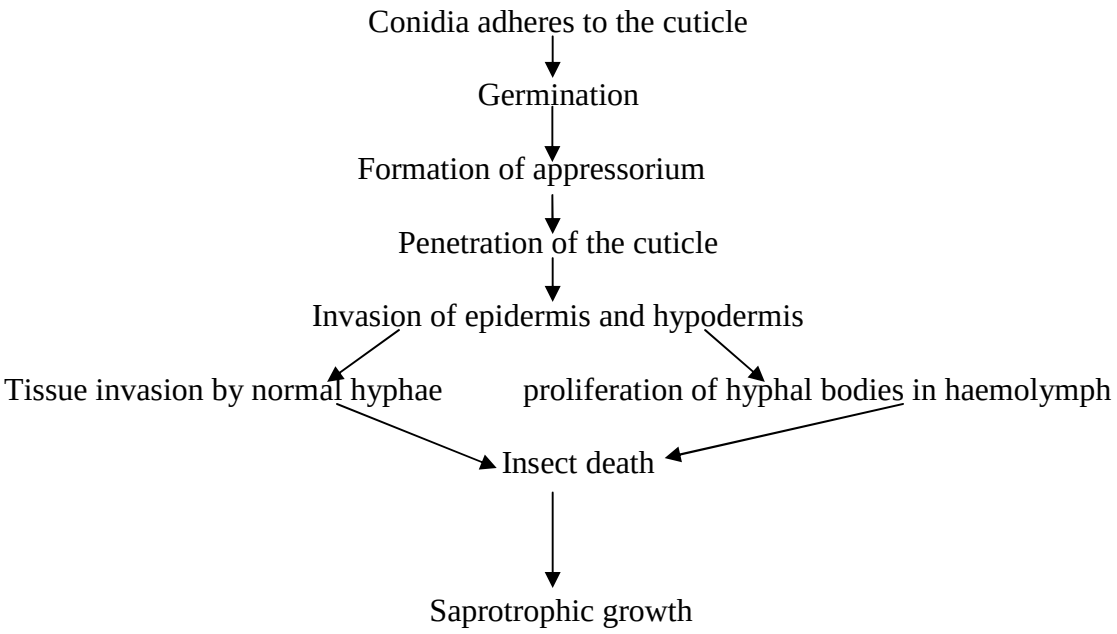


Plate 4: Infection cycle of entomopathogenic fungi (Matthew *et al.*, 2007)

2.11.5.4.3.2 The life cycle of entomopathogenic fungi

The fungal spores geminate into mycelia which produce spores. Most of the entomopathogenic fungi life cycle has two phases: the yeast like budding phase which is a dimorphic mode of growth occurs inside the host and has been observed in *B. bassiana* (Alves *et al.*, 2002) and a normal mycelial growth phase which occurs outside the host. If there is no specific host for *B. Beauveria*, it goes through asexual vegetative life cycle that involves germination, growth of filaments and formation of sympoduloconidia. If the suitable host is available, *Beauveria* goes back to the pathogenic life cycle. The conidia germinate on the surface of the host cuticle and penetrate the tick directly. After penetrating the cuticle, it acquires the yeast-like phase, forms hyphae, which flows in the haemolymph and proliferate by budding. Following the death of the host, the fungus goes back to the saprotrophic stage where hyphae are formed. The life cycle of *M. anisopliae* in liquid medium have been demonstrated by Uribe and Khachatourians, (2008).

2.11.5.4.3.3 Cultivation of fungi

Beauveria bassiana colonies have been grown in Potato Dextrose Agar medium and they demonstrated a lightly coloured and fluffy to powdery mycelia on the surface of the media (Kwon-chung and Bennet, 1992). *Metarhizium anisopliae* grown on a liquid medium produced yellowish green or olive green mycelia on surface (Plate 5). A lot of fungi have been grown on other media such as Potato Carrot Agar (Bridge *et al.*, 1993) and Sabouraud's Dextrose Agar (Kaaya, 1989).

Plate 5: White colored colonies of *B. bassiana* (left) and green color colonies of *M. anisopliae* (right)

2.11.5.4.3.4 Role of white rice in fungal mass production

When producing a lot of fungi, a physical support for aerial conidial (infective propagules) growth is required. Cereal or by-product of cereals such as wheat bran or maize, rice, millet are commonly used as substrate (Lomer and Lomer, 2009). A good substrate should have a high surface area to volume ratio with the individual particles separated to allow spaces in between the particle for aeration and conidial formation. Therefore, broken white rice is the ideal substrate as the particles are small thereby providing a large surface area and they remain separate from each other after autoclaving and inoculation (Lomer and Lomer, 2009).

2.11.5.4.3.5 Identification of fungus

Different molecular techniques have been used for identification on different entomopathogenic fungus based on polymorphism of DNA using random amplified polymorphic DNA-polymerase

chain reaction (RAPD-PCR) technique (Glare, 2004). Use of RAPD markers is based on detection of DNA polymorphism where short general primers anneal to unspecified regions in the template DNA. A specific fingerprint can be constructed for characterizing different isolates of fungus or for genetic stability testing of an individual isolate. Restriction fragment length polymorphism (PCR-RFLPs) was formerly used for characterizing species of *Beauveria* and *Metarhizium* (Bidochka *et al.*, 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Source of ticks

Two weeks old unfed larvae of amitraz-susceptible strain M001/13 were obtained from International Livestock Research Institute (ILRI) Nairobi, Kenya and amitraz-resistant strain of *R. decoloratus* M446/12 from Acarology Laboratory, Directorate of Veterinary Services, Kabete, Kenya.

3.2 Acaricide

The synthetic acaricide, used in the experiments was amitraz commercialized under the brand name Triatix®, Cooper K-Brands Ltd formulated in 12.5% emulsion concentration and 87.5% inert ingredients and was obtained from Coopers K-Brands Limited, Nairobi. For the field experiment, amitraz was used at the recommended field usage rate of 250 ppm. The *R. decoloratus* resitance to amitraz had been detected in Acarology Laboratory, Kabete and was also confirmed through molecular techniques by molecular cloning of octopamine receptors at the Agriculture and Bio-oriented Research Organization Animal Mamoru-Kenin, Japan (Hatta *et al.*, 2013).

3.3 Source of fungal isolates

In the present study, seven *M. anisopliae* and five *B. bassiana* isolates were used. They were obtained from the ICIPE's Arthropod Germplasm Centre with their origin and place of isolation as presented in (Table1).

Table 1: Source of isolates used in the experiment and their year of isolation

Fungal species	Isolate	Locality (Country)	Source	Year of isolation
Metarhizium anisopliae	ICIPE 41	Lemba (Democratic Republic of Congo, (DRC)	Soil	1990
	ICIPE 74	Mtwapa (Kenya)	Soil	1990
	ICIPE 68	Matete (DRC)	Soil	1990
	ICIPE719	Machakos (Kenya)	Soil	2013
	ICIPE 9	Matete (DRC)	Galleria	1990
	ICIPE 91	Senegal	Locust	2003
	ICIPE 7	Rusinga Island (Kenya)	*Amblyomma variegatum*	1996
Beauveria bassiana	ICIPE279	Kericho (Kenya)	Soil	2005
	ICIPE609	Meru (Kenya)	Soil	2008
	ICIPE676	Kenya	Soil	2008
	ICIPE644	Mauritius	Unknown	2007
	ICIPE718	Mbita (Kenya)	*Amblyomma variegatum*	2013

3.4 Assessment of the effectiveness of *M. anisopliae* and *B. bassiana* isolates against *R. decoloratus*

Before the bioassays were done, it was important to test and confirm if the conidia were still viable.

3.4.1 Conidial viability testing

Seven *M. anisopliae* and five *B. bassiana* were grown on SDA medium which was put in petri dishes of 90 mm diameter. They were then stored at 25 ±2^{o}C in complete darkness in an incubator.

Conidia (0.1 ml) was titrated to 1× 10^{6} conidia ml^{-1} and spread-plated on petri dishes containing SDA media where a 2 × 2 cm sterile microscope cover slip was placed on the surface of each petri dish and were later incubated at 25 ± 2^{o}C, 85 ± 5 % RH in complete darkness and germination was checked after 20 hours. The conidia that germinated were counted to determine their percentage germination whereby a germinating germ tube was twice the diameter of the propagule. From the area covered by each cover slip under the light microscope (400×), one hundred spores were counted randomly (Goettel and Inglis, 1997).

3.4.2 Susceptibility test of *R. decoloratus* strains to selected pathogenic fungal isolates

For each of the twelve tested fungal isolates, ten test larvae for each strain of *R. decoloratus*, amitraz-resistant and amitraz-susceptible were sprayed by using Burgerjon's spray tower (Burgerjon, 1956) with 10 mls of 1 ×10^{9} conidia ml^{-1}. Sterile distilled water mixed with 0.05% Triton X-100 was used to spray the control. In a 90 mm petri dish moistened with filter papers,

the sprayed larvae were then put in and petri dishes sealed with parafilm to avoid larvae from escaping. Each test was replicated four times and the petri dishes were incubated at 25 ± 2ºC and 85 ± 5 % RH, checked every day for seven days and ticks that were found dead were removed and dipped in solution containing 2.5% sodium hypochlorite and 70% ethanol in order to sterilize them. They were then rinsed two times with sterile distilled water and then for mycosis to occur on the surface of the cadavers, they were put into 90mm petri dishes that were moistened with filter papers.

3.5 Determination of pathogenicity of selected pathogenic fungal isolates to *R. decoloratus* strains

3.5.1 Dose-response mortality test

Fungal isolates that were found virulent in the pathogenicity tests include four *M. anisopliae* (ICIPE 91, ICIPE 41, ICIPE 7 and ICIPE 719) and two *B. bassiana* isolates (ICIPE 279 and ICIPE 718) were further tested for dose-response mortality against larvae of amitraz-resistant and amitraz-susceptible strains of *R. decoloratus*. The stock solution was serially diluted to get 1.0×10^8, 10^7, 10^6, 10^5conidia ml^{-1} concentrations. Test ticks were transferred to petri dishes which had moistened filter papers. Ten larvae were used in each of the four replicates and Burgerjon's spray tower was used to spray them with the conidial solution as explained in section 3.4.2. The larvae in the control were sprayed with 0.05% Triton X-100 and were monitored for seven days. To calculate the mean lethal time (LT_{50}) and mean lethal concentrations (LC_{50}) values in each replicate, the probit analysis method for correlation data described by Throne *et al*. (1995) was

used. ANOVA was used to compare the means which were separated using Students Newman's Keuls test.

3.6 Compatibility of ICIPE 7 and amitraz

Based on the mean mortality results, mean lethal time and mean dose response results, *M. anisopliae* isolate ICIPE 7 was selected for further studies including compatibility with amitraz. The following parameters were assessed to evaluate the compatibility: Conidial germination, mycelial dry weight, radial fungal growth and spores production.

3.6.1 Conidial germination

Concentrations of 1, 2, 4 and 8 % of amitraz were added to 50 ml of cooled (45^{o}C) SDA which was added 0.1 ml of 1 x 10^{6} spores ml^{-1}conidial of *M. anisopliae*. The control received an aliquot of sterile distilled water mixed with 0.05 % Triton X-100 but had no amitraz. Petri dishes were incubated at 25 ± 2^{o}C for 20 hours and the percentage germination of the conidia was determined by the method of Nana *et al.,* 2012.

3.6.2 Assessment of mycelia dry weight

Conidium (0.1 ml) of *M. anisopliae* was titrated at 1 × 10^{6} conidia ml^{-1} and to obtain mycelial mats. It was spread-plated on SDA media and incubated for three days at 25 ± 2°C (Nana *et. al.*, 2012). Mycelial mats that did not sporulate were cut off using 4 mm diameter cork borer into round agar plugs. Each of the agars was placed at the center of 90 mm diameter petri dishes containing different concentrations of 1, 2, 4 and 8% of amitraz and a control containing clean SDA agar. The petri dishes were incubated at 25 ± 2 °C in complete darkness for 7 days. Mats of

mycelia were cut with sterile spatula and placed in sterile petri dishes containing filter paper. The initial weight of the filter paper was recorded. The Petri dishes were placed in hot air oven at 50 °C for 30 minutes and the final weight of the mycelial mat with the filter paper taken. The difference between the final and initial weight was the dry weight of mycelium (Nana *et al.*, 2012).

3.6.3 Radial fungal growth

As demonstrated in section 3.6.2, agar plugs were obtained. Each agar plug was placed in a new SDA media mixed with control and different concentrations of 1, 2, 4 and 8% amitraz at the center, sealed with parafilm, placed upside down and incubated in complete darkness at 25 ± 2° C. The radial growth of the fungus was measured using two cardinal diameters which was previously drawn on the bottom of each petri dish as a reference through two orthogonal axes (Plate 6) and data was collected on day 3, 6 and 9 (Nana *et al.*, 2012). Four replicates were used.

Plate 6: Two cardinal diameters previously drawn on the bottom of each petri dish through two orthogonal axes

3.6.5 Spore production assessment

Agar plugs attained as in section 3.6.2 were placed in the middle of SDA plate containing different concentrations of amitraz of 1, 2, 4 and 8 % and then incubated at 25 ± 2°C for one week. Mycelial mats that sporulated were cut using a 4 mm diameter cork borer into round agar plugs and were put into universal bottles each which had 10 ml of sterile distilled water mixed with triton water (0.05% Triton X-100). They were vortexed for five minutes. To determine the concentration of conidia, an improved Neubauer counting chamber was used for counting (Nana *et. al.,* 2012). Four replicates were done in each concentration of amitraz.

3.6.6 Evaluation of compatibility of ICIPE 7 with amitraz

The compatibility of amitraz with ICIPE 7 was calculated based on the T values in relation to vegetative growth (VG) and sporulation values (SP) and to the % control: T = [20 (VG) + 80 (SP)]/100. Therefore, compatibility was classified as very toxic if T values are between 0 and 30; toxic if T values were between31 and 45; moderately toxic with T values of 46 to 60 and compatible if the T values were above 60 (Nana *et al.,* 2012).

3.6.7 Virulence of ICIPE 7 on *R. decoloratus* strains

Three weeks old sporulating culture of ICIPE 7 were obtained by scrapping the surface of the culture and the scrapping put into universal bottles containing sterile distilled water mixed with 0.05% Triton X-100 to make concentration of 1.0×10^8 conidia ml^{-1}. Ten mls of amitraz at concentrations of 62.5 and 125 ppm were added to the culture. The universal bottles containing glass beads were vortexed for 5 minutes to obtain a homogenous suspension. Controls were

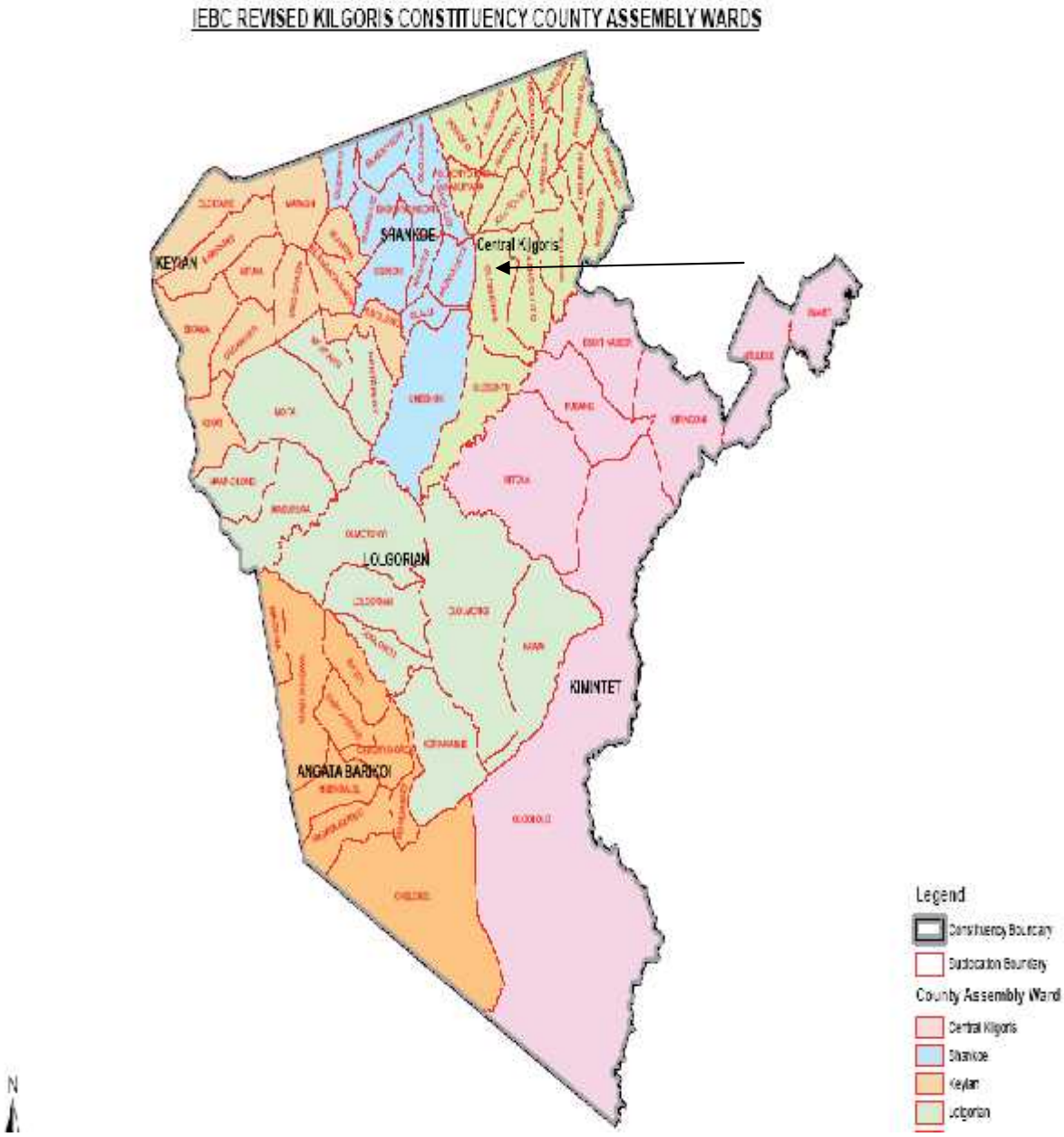
prepared by mixing sterile distilled water with 0.05% Triton X-100. The larval stages of amitraz resistant and amitraz susceptible strains of *R. decoloratus* were sprayed with the prepared treatments using the Burgerjon's spray tower (Burgerjon, 1956). For each of the four replicates, ten larvae were used for each strain of the ticks and incubated at 25 ± 2^{o}C with 75% RH and mortality data was collected daily for 7 days.

3.7 Field evaluation of efficacy of ICIPE 7 against *R. decoloratus*

3.7.1 Trial site

The trial was carried out in the Transmara Sub-county of Narok County, Kenya at a private farm situated about 12 km from Kilgoris town (Figure 1): 1^{o}00'S, 34^{o}53'E, 1716 m above sea level (GPS reading). Approximately 0.81 hectares paddock was the study area although animals grazed any where freely in the 52.6 hectares that constituted the farm. The red oat grass, *Themeda triandra* was the predominant vegetation. The grass was between 5 cm and 25 cm in height. The trial was done from October to November 2014 during the rainy season. High abundance of *R. decoloratus, R. appendiculatus* and *A. variegatum* characterized this period (Walker *et al.,* 2003). Acarology laboratory data has reported tick resistance to acaricides including amitraz in this area. During the trial period, the prevailing climatic conditions were recorded (Appendix 4).

Map 1 Transauthority, Kenya



Map 2 Kilgoris constituency, 2011

Figure 1: Maps 1 and 2 showing Narok County and Kilgoris, the study area

3.7.2 Test animals

A mixture of 25 steers and heifers were selected based on their weight and age (approximately 150-175 kg; 1.5 years old) from the main herd of cattle comprising Maasai Zebu and Sahiwal crosses. These animals were dewormed and provided with mineral supplements as well as being provided with diagnostic and clinical services during the pre-trial, trial and one month post-trial periods. To become infested with ticks, they were allowed to graze for two weeks on pasture and were free from chemical interventions of any kind to prevent tick infestation. Total whole body tick counts that included (*R. appendiculatus, R. decoloratus* and *A. variegatum*) for the pre-trial animals were done on the first day of the experiment. The counts were used to rank the animals from highest to lowest number of ticks. A homogenous sample of 20 cattle was selected from the initial group of twenty five cattle having attained the mandatory 150-250 ticks per animal as per the trial protocol. Five animals were eliminated because three had very low tick counts and the other two, one was pregnant and the other was very aggressive.

A stratified randomized complete block design method was used to allocate the twenty cattle to the four treatment groups; (i) Controls (water + 0.05% Triton X-100 + 15% canola oil); (ii) Fungus; (iii) Amitraz (recommended concentration) and (iv) Combination of amitraz (0.1%) and fungus (1×10^8 conidia ml^{-1}). The four highest ranked animals formed groups 1, 2, 3, 4, the next four highest animals formed group 4, 3, 2, 1, then 1, 2, 3, 4 up to the last animal with the lowest tick counts. The animals were identified with different ear tags colour according to their groups which were allocated to different treatments (Table 2 and Plate 7). Only *R. decoloratus* were counted during the trial period.

Table 2: Treatment groups with their identifying colour codes

Group 1 (Control) Red		**Group 2 (Fungus) Yellow**		**Group 3 (Amitraz) Green**		**Group 4 (Fungus + Amitraz) Blue**	
Ear tag no.	Tick counts	Ear tag no.	Tick counts	Ear tag no.	Tick counts	Ear tag no.	Tick counts
1	442	1	442	1	410	1	531
2	431	2	697	2	321	2	407
3	280	3	282	3	271	3	270
4	723	4	273	4	457	4	584
5	278	5	421	5	687	5	350

Group 1: Red

Group 2: Yellow

Group 3: Green **Group 4: Blue**

Plate 7: Treatment groups showing the coloured ear tags

3.7.3 Fungal mass production and formulation

Metarhizium anisopliae isolate ICIPE 7 was selected for the field trial. It was mass produced on broken white rice according to the techniques described by Lomer and Lomer (2009). The inoculated flasks were placed on shaker-incubator for three days at around 150 rpm and temperature between 25 and 30 °C (Plate 8).

Plate 8: Blastospores cultured in liquid medium in a 250 mls Erlenmeyer flasks maintained in a shaker at 150 rpm and 30^{o}C

Five hundred grams bag of sterilized rice was mixed with 50 ml of broth prepared 3 days ago, put in Milner bags and incubated for three weeks at 23-27ºC with relative humidity of between 35% and 60% during which sporulation occurred (Plate 9).

Plate 9: Mass production of *M. anisopliae* on rice substrate in Milner bags (Nana *et al.*, 2010)

After sporulation of the fungus from outside of rice grains, the bags were opened up to let the fungus and rice grains dry for about 5 days (Plate 10).

Plate 10: Culture of *M. anisopliae* on rice substrate in plastic basin undergoing drying at room temperature (Nana *et al.*, 2010)

The dry rice was poured in a metal sieve and the conidia obtained was allowed to dry up to 5% moisture content then stored at 4-6ºC in a fridge for two weeks before being used for the field trial. Conidial viability was tested as described in 3.4.1 and after 18-20 hours over 85% of conidia had germinated. Conidial suspension was titrated at 1×10^8 conidia ml^{-1} and formulated in emulsifiable formulation ratio of Silwet-L7, 0.25 ml and half liter mineral oil (canola oil) mixed in10 liters of water.

3.7.4 Application of treatments

Four treatment groups comprising five animals per group used in the present study were brought to the crush and sprayed with their respective treatments using a knapsack sprayer. Treatments

were applied once a week for four weeks. Except in amitraz treatment where individual cows were sprayed with 5 litres (standard practice in the region) two litres of suspension were sprayed per animal in a crush. Animals were sprayed starting with the head including the ears, lower parts moving to the flank, back and belly then front legs and axillae for both sides before finishing with tail switch. By the end of the spray treatment all cattle were thoroughly wetted (Plate 11).

Plate 11: Application of treatments using a hand sprayer

3.7.5 Assessment of efficacy of treatments

After each spray application, whole body tick counts of naturally infested cattle were conducted on days 0, 3, 5 and 7. Ticks were counted on the 3 regions on each animal (Plate 12). Head (head, ear, neck, the dewlap to the point of the sternum), shoulder (outer and inner foreleg from point of the sternum back to the start of the fore belly) and the back (ribs, tail and tail switch, udder and scrotum and hind legs) with much attention on blue ticks predilection sites like back, upper legs, neck, shoulder, dewlap and belly (Walker *et al.*, 2003). The treatment groups were grazed

separately to avoid rub off with the other groups and from the neighboring animals throughout the trial period. The proportion of ticks infected with fungus was determined by, collecting 8-10 ticks from each animal in each treatment group and they were put in sterile petri dish and kept at room temperature (25-27^{o}C) for 10 days. Mortality was recorded and dead ticks removed and put in petri dish which had damp filter paper for the fungus to growth on the surface of dead ticks.

Plate 12: Tick counting in situ

The effectiveness of the different experimental groups was established as the percentage reduction of the population of ticks by comparing with the negative control population (Bittencourt *et al.,* 2003).

Percentage efficacy=

$$\frac{\text{(Average population in negative group) − (Average population in treatment group)}}{\text{Average population in negative group}} \times 100$$

3.7.6 Conidial persistence on cattle

During tick counting on day 0, 3, 5 and 7, the three body regions namely head, shoulder and back were swabbed using cotton bud which was transferred to a universal bottle and was mixed with 0.05% triton water. This was placed on a vortex for 5 minutes to break the aggregated spores. Conidia (0.1 ml) was titrated to 1×10^6 conidia/ml and spread platted on SDA medium in 90 mm petri dish and 2 cm x 2 cm sterile microscope cover slips put on the media and then incubated at 25 ± 2^oC. Germination was determined after 20 hours by random counting germinated conidia from hundred spores whose germ tubes were twice the diameter of the propagule seen using the light microscope at x 400 magnification (Goettel and Inglis 1997).

3.7 Data analysis

Percentage mortality in controls was corrected using Abbott's formula (Abbott, 1925) and standardized using arcsine transformation (Sokal and Rohlf, 1981). Analysis of variance (ANOVA) was done at significance level of 95% using PROC GLM. Means were separated as a post-ANOVA procedure ($p<0.05$) using Student-Newman-Keuls analysis. The test values of lethal concentration and lethal time were calculated using the probit analysis for data correlation (Throne *et al.,* 1995) and compared using ANOVA ($p<0.05$) while separating their means using Student-Newman-Keuls test.

Alves *et al.,* (1998) formula for classifying the toxicity levels of compounds was used in classifying the compatibility of fungus with amitraz depending on how toxic they were to the fungus *in-vitro*. Analysis of the germination, mortality and radial growth data were done using ANOVA and statistical analysis for scientists (SAS) (2001). To standardize the mortality percentages in controls, Aborts formula was used. A 2-way analysis of variance was used for randomized test design. Post hoc analysis was done using the Tukey test and a value was significant if p value was less than 0.05.

CHAPTER FOUR

4.0 RESULTS

4.1: Screening of *M. anisopliae* and *B. bassiana* for their efficacy against amitraz-susceptible and amitraz-resistant *R. decoloratus*

4.1.1 Conidial viability

Conidial viability was done by tallying the conidia whose germ tubes were twice the propagules' diameter (Plate 13).

Plate 13: Germinating (A) and non-germinating (B) spores of *M. anisopliae* (magnification x 40)

Conidia viability ranged from 96.6 to 100% for the different twelve fungal isolates used in the study (Table 3).

Table 3: Percentage (%) germination of the isolates

Fungal species	Isolate	Percentage (%) germination
Metarhizium anisopliae	ICIPE 41	98.8 ± 0.8
	ICIPE 74	96.6 ± 2.7
	ICIPE 68	96.6 ± 2.7
	ICIPE 719	97.3 ± 5.2
	ICIPE 9	100.0 ± 0.0
	ICIPE 91	98.5 ± 1.0
	ICIPE 7	100.0 ± 0.0
Beauveria bassiana	ICIPE 279	97.2 ± 2.2
	ICIPE 609	97.6 ± 1.7
	ICIPE 676	97.2 ± 2.2
	ICIPE 644	96.6 ± 5.4
	ICIPE 718	100.0 ± 0.0

4.1.2 Susceptibility test

No larval mortalities in both amitraz-resistant and amitraz- susceptible tick strains were recorded in the controls. Mean mortality of between 10.0 and 100 % amitraz-susceptible and between 12.1 and 100% in amitraz-resistant strains respectively were caused by the fungal isolates. There were no significant differences among fungal isolates ($P>0.05$) except ICIPE 676 and ICIPE 74, where t-value and p-value were significantly different ($P<0.05$), (Table 4). The dead ticks that mycosed

were an indication that the death was due to entomopathogenic fungal infection.

Table 4: Percentage mean mortality rates of *M. anisopliae* and *B. bassiana* against amitraz-susceptible and amitraz-resistant *R. decoloratus* larvae

Isolates		Mortality (%)		t-value	p-value	df
		Amitraz-susceptible	Amitraz-resistant			
	Controls	0.0 ± 0.0	0.0 ± 0.0	0	0	0
M. anisopliae	ICIPE 41	100.0 ± 0.0	100.0 ± 0.0	0	0	0
	ICIPE 74	10.0 ± 1.0	100.0 ± 0.0	89.55	0.0001[a]	77
	ICIPE 68	20.0 ± 8.2	10.0 ± 5.8	0.99	0.1614	70
	ICIPE 719	83.3 ± 20.8	100.0 ± 0.0	0.80	0.4245	76
	ICIPE 9	10.6 ± 6.0	12.1 ± 5.8	0.17	0.4289	77
	ICIPE 91	96.7 ± 5.7	100.0 ± 0.0	0.57	0.5644	78
	ICIPE 7	100.0 ± 0.0	100.0 ± 0.0	0	0	0
B. Bassiana	ICIPE 279	100.0 ± 0.0	93.3 ± 5.8	1.15	0.2516	78
	ICIPE 609	22.5 ± 9.6	17.5 ± 5.0	0.46	0.3229	58
	ICIPE 676	62.5 ± 9.6	30.0 ± 10.8	2.24	0.0137[a]	76
	ICIPE 644	15.0 ± 5.8	12.5 ± 9.6	0.22	0.4122	64
	ICIPE 718	100.0 ± 0.0	100.0 ± 0.0	0	0	0

[a] ***Significantly different by Student t-test (P<0.05)***

4.2 Evaluation of the pathogenicity of amitraz-resistant and susceptible strains of *R. decoloratus* to selected virulent isolates of *M. anisopliae* and *B. bassiana*

The mean LT_{50} and LC_{50} values for the virulent fungal isolates following treatment of unfed larvae of amitraz-resistant and amitraz-susceptible tick strains of *R. decoloratus* are shown in Table 5 and 6, respectively.

Table 5: Mean LT_{50} values of selected *M. anisopliae* and *B. bassiana* in unfed larvae of amitraz-susceptible and amitraz-resistant *R. decoloratus*

Lethal Time $_{50}$ in days				t-value	p-value	df
Isolates		Amitraz-susceptible	Amitraz-resistant			
	Controls	-	-	-	-	-
M. anisopliae	ICIPE 91	3.3 ± 0.4	3.9 ± 0.3	1.2	0.234	72
	ICIPE 41	3.0 ± 0.3	3.2 ± 1.2	0.2	0.872	43
	ICIPE 7	2.6± 0.3	3.1 ±0.5	0.8	0.394	63
	ICIPE 719	4.2 ± 0.4	2.8 ±0.5	2.1	0.031[a]	74
B. bassiana	ICIPE 279	3.0± 0.3	3.3 ±0.7	0.4	0.694	52
	ICIPE 718	4.1 ± 0.3	3.0 ± 0.3	1.4	0.154	52

[a] ***Significantly different by Student t-test (P<0.05)***

The mean LT_{50} values of the selected isolates varied between 2.6-4.2 days in amitraz-susceptible strains and between 2.8-3.9 days in amitraz-resistant strains. There were no significant

differences between the fungal isolates except ICIPE 719 which had a p-value of 0.031 was significant (Table 5).

Table 6: LC_{50} values of selected *M. anisopliae* and *B. bassiana* in unfed larvae of amitraz-susceptible and amitraz-resistant *R. decoloratus*

Mean LC_{50} (x 10^3 conidia ml^{-1})				**t-value**	**p-value**	**df**
Isolates		**amitraz-susceptible**	**amitraz-resistant**			
	Controls	-	-			
Metarhizium anisopliae	ICIPE 91	3.9±0.1	100±6.2	5.5	0.022[a]	58
	ICIPE 41	50.0±6.0	79.0±6.0	0.9	0.337	58
	ICIPE 7	0.4±0.1	0.3±0.2	0.6	0.455	58
Beauveria bassiana	ICIPE 279	200±60.0	200±31.0	0.0	0.999	58
	ICIPE 718	31.0±8.0	0.1±0.1	257.3	0.001[a]	58

[a] Significantly different by Student t-test (P<0.05)

The LC_{50} values varied between 0.4 ± 0.1 x 10^3 and 200.0 ± 60 x 10^3 conidia ml^{-1} and between 0.1 ± 0.1 x 10^3 and 200.0 ± 31.0 x 10^3 conidia ml^{-1} in amitraz-susceptible and amitraz-resistant strains, respectively. There was no significant difference between amitraz-susceptible and amitraz-resistant strains, except with isolate ICIPE 91 and isolate ICIPE 718 ($P<0.05$) (Table 6).

4.3 Compatibility of amitraz and ICIPE 7

4.3.1 Conidial viability test

In the conidial viability test, approximately, 98% of conidia germinated.

4.3.2 Effects of amitraz on average conidial germination of ICIPE 7

The mean percentage germinations of conidial spores of ICIPE 7 in the various concentrations of amitraz are shown in Table 7.

Table 7: Effects of amitraz on average conidial germination of ICIPE 7

Treatments					
Replicates	**Control**	**1 %**	**2 %**	**4 %**	**8 %**
R1	100	100	95	95	98
R2	95	97	97	99	93
R3	100	100	95	93	94
R4	98	96	100	96	97
R5	98	95	99	94	99
Mean	**98.2 ± 0.9[a]**	**97.6 ± 1.0[a]**	**97.2 ±1.0[a]**	**95.4 ± 1.0[a]**	**96.2 ± 1.2[a]**
SD	2.05	2.302	2.28	2.302	2.59
ESM	0.917	1.03	1.02	1.03	1.156
F value	1.17				
P value	0.35				

[a] same letters following each other are considered not significant by using Tukey test (P < 0.05)

The mean conidial germination of ICIPE 7 in different concentrations of amitraz ranged between 95.4% and 97.6% as compared to the control which attained a high percentage of 98.2%. The conidial germination decreased with higher concentrations of amitraz in ICIPE 7 but different amitraz concentrations had no significant effect on germination of conidia.

4.3.2 Amitraz effects on mycelial dry weight of ICIPE 7

The mean mycelial dry weight of *M. anisopliae* isolate ICIPE 7 in various concentrations of amitraz are as shown in table 8 below.

Table 8: Dry weight of mycelia

Treatment	**Control**	**1%**	**2%**	**4%**	**8%**
R1	0.4546	0.4274	0.3984	0.3848	0.6931
R2	0.5331	0.3476	0.4821	0.6284	0.2454
R3	0.4846	0.4805	0.3933	0.3421	0.3421
R4	0.5775	0.4727	0.4174	0.4153	0.3002
R5	0.4946	0.6384	0.6894	0.3421	0.5839
Mean	**0.50888**	**0.47332**	**0.47612**	**0.42254**	**0.43294**
SD	0.047526	0.106322	0.12437	0.11916	0.194625
ESM	0.021254	0.047549	0.05562	0.05329	0.087039
Mean	0.51±0.0[a]	0.47±0.1 [a]	0.48±0.1 [a]	0.42±0.1 [a]	0.43±0.1 [a]
F value	0.37				
P value	0.82				

[a] same letters of means indicates no significance as it is by Tukey test (P < 0.05)

In comparison with the control, different concentrations of amitraz did not affect the mycelial dry weight.

4.3.3 Amitraz effects on radial growth of ICIPE 7

The average radial growth of ICIPE 7 in various concentrations of amitraz are shown in Table 9

Table 9: Mean radial fungal growth

Amitraz concentrations	**Colony diameter (mm)**		
	3 days	**6 days**	**9 days**
Control	[a] 9.2±0.4	[a] 17.4±0.5	[a] 27.6±2.5
SDA+1%	[a] 9.0 ±0.4	[a] 16.2±0.5	[a] 25.4±2.0
SDA+2%	[a] 8.6±0.2	[a] 15.4±0.7	[a] 24.0 ±3.1
SDA+4%	[b] 6.8±0.2	[a] 12.8±1.8	[a] 22.8±3.9
SDA+8%	[b] 5.6±0.2	[b] 11.2±1.8	[a] 17.4±2.4
F value	23.5	4.3	1.78
P value	0.01	0.01	0.17

Same letters following each other in the columns are not significantly different p< 0.05

The colony diameter ranged from 9.2 ± 0.4 mm in the controls to 8.6 ± 0.2 mm at the concentration of 2% of amitraz and the difference was not significant. However, high

concentrations of amitraz (4% and 8%) had negative effect on radial growth of *M. anisopliae* on day 3 post inoculation. Reduction in radial growth was only observed at the higher concentration of amitraz (8%) on day 6 post-inoculation. There were no significant effects of different concentrations of amitraz on radial growth on day 9 post-inoculation (Table 9).

4.3.4 Spores production

The mean conidial concentrations of ICIPE 7 for the various amitraz concentrations are shown in Table 10.

Table 10: Mean spores production

Amitraz concentration	**Yield (10^8 conidia m^{-1})**
Control	[a] 8.7 ±0.3
SDA+1%	[a] 8.2 ±0.3
SDA+2%	[a] 8.5 ±0.5
SDA+4%	[a] 7.5 ±0.3
SDA+8%	[b] 7.2 ±0.5
F value	2.83
P value	0.05

Same letter following each other indicates no significance by Tukey test (P < 0.05)

In comparison with the control, the different concentrations of amitraz did not affect the spores' production except at higher concentration of amitraz where significant effect was observed (Table 10).

4.3.5: T values calculated to show the compatibility of amitraz and ICIPE 7

The T values calculated to show the compatibility of amitraz and ICIPE 7 are as shown in Table 11 below.

Table 11: T values calculated to show the compatibility of amitraz and ICIPE 7

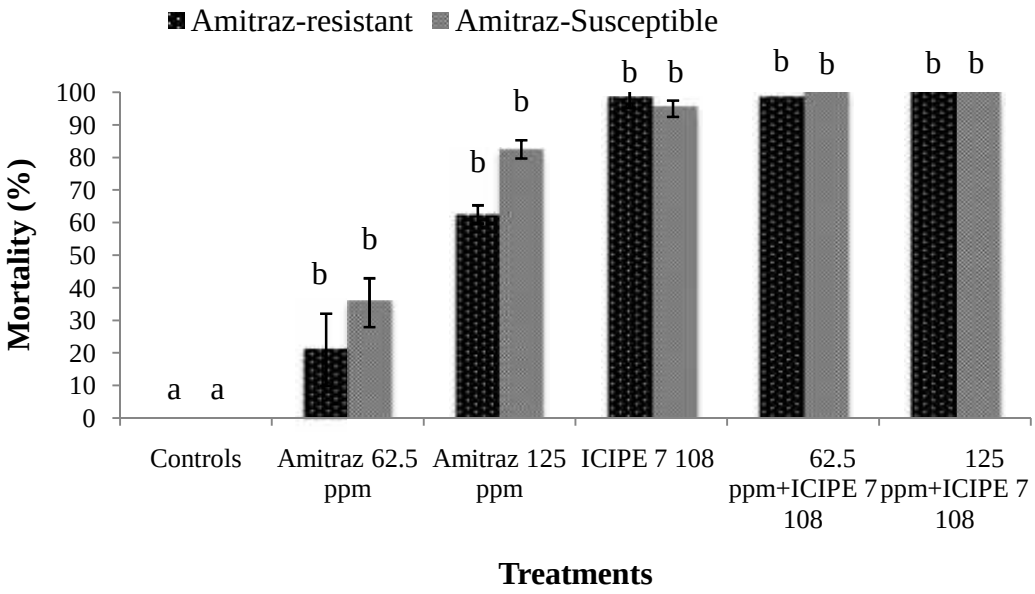
Amitraz concentration	*M. anisopliae*	
	T values	**Classification**
SDA+1%	94.2	HC
SDA+2%	96	HC
SDA+4%	84.1	C
SDA+8%	81.2	C

HC-highly compatible C-compatible

Amitraz was highly compatible with the fungus at tested concentrations of <2% and compatible at concentrations of >4% (Table 10) within a period of 7 days. The "T" values indicate that amitraz was highly compatible with *M. anisopliae* at the concentration of 1 and 2%, while compatible at the concentrations of 4 and 8% (Table 11).

4.3.6: Determination of the virulence of ICIPE 7 in combination with amitraz against amitraz-resistant and amitraz-susceptible strains of *R. decoloratus* larvae

The average death of larvae of *R. decoloratus* treated with ICIPE 7 in various concentrations of amitraz is shown in Figure 2.



[bb] same letters indicate no significant by ANOVA (P<0.05)

Figure 2: Mean mortality of amitraz-resistant and amitraz-susceptible strains of *R. decoloratus* in formulated amitraz and ICIPE 7

The controls had no tick mortality in amitraz-susceptible and amitraz-resistant strains of *R. decoloratus* but the mortality was high in amitraz-susceptible strain treated with amitraz alone at

the concentrations of 62.5 ppm and 125 ppm, respectively; but the difference was significant only at the concentration of 125 ppm ($F_{1, 8}$=53.576; P=0.001) with 62.5 % and 82.5 % in amitraz-resistant and amitraz-susceptible strains respectively. On the other hand, *M. anisopliae* ICIPE 7 applied at the concentration of 10^8 conidia ml^{-1} caused mortality of 95% in both amitraz-resistant and amitraz-susceptible strains. Combination of amitraz at concentration of 62.5 ppm with *M. anisopliae* at 10^8 resulted in mortality of 95% and 97.5% in both amitraz-resistant and susceptible strains, respectively while, combination of amitraz at concentration of 125 ppm with fungus caused mortality of 100% in both strains.

4.4 Field evaluation of effectiveness of ICIPE 7 in the control of *R. decoloratus*

The mean climatic data of the study site during the study period was: mean maximum temperature of 22.7^{o}C; mean minimum temperature of 13.8^{o}C; 86.5% RH at 06.00 am; 57.3% RH at noon; and 37.2 mm rainfall (Department of Meteorology, Kenya) (Appendix 4).

4.4.1 Mean total tick counts per treatment

The mean total tick counts after weekly application of treatments from the predilection sites were as shown in figure 3 below. The overall tick counts were high in dewlap region followed by the back regions due to the fact that there were the predilection sites for the *R. decoloratus* ticks whereas, the head region which had no ticks (*R. decoloratus*) was not its predilection site. Dewlap region maintained high tick numbers as compared to the back region.

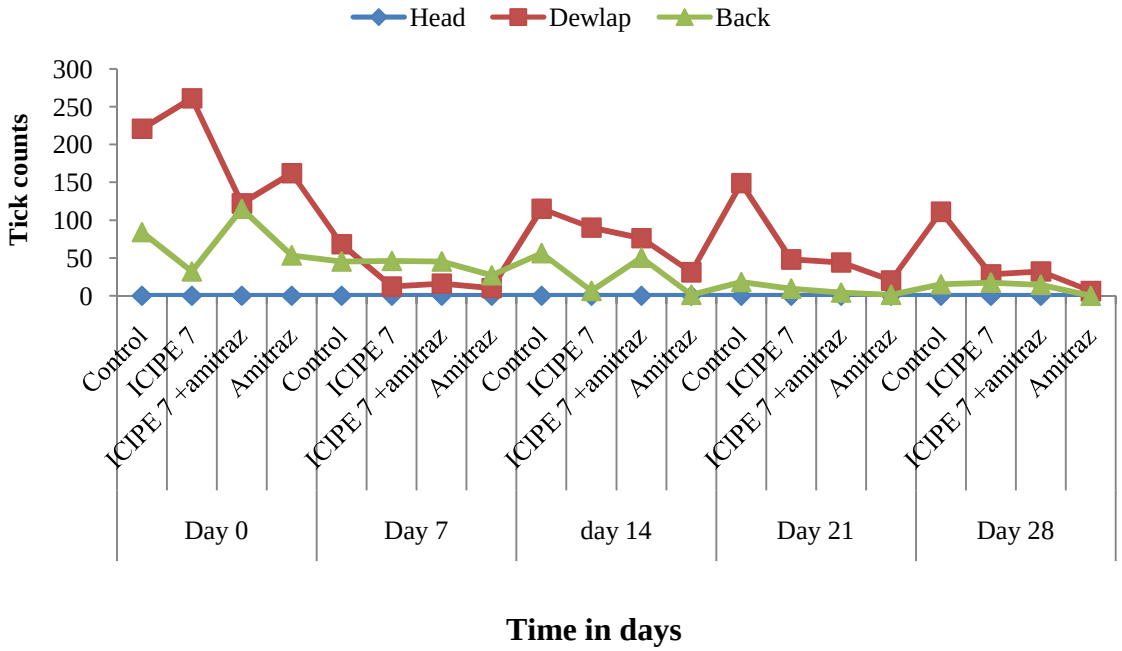


Figure 3: Mean total tick counts per treatment

4.4.2 Mean tick counts from three body regions

The mean tick counts for the three body regions per treatment are as shown in Figure 4 below. The total tick counts was high at the start of treatment in all the treatments and these decreased on day 7 with dewlap having high tick loads in the control followed by ICIPE 7 and ICIPE 7 + amitraz and increased on day 14 with the same trend in treatments but the counts decreased up to day 28 except in control which maintained high tick counts, amitraz maintained low tick counts throughout the treatment days (Figure 4).

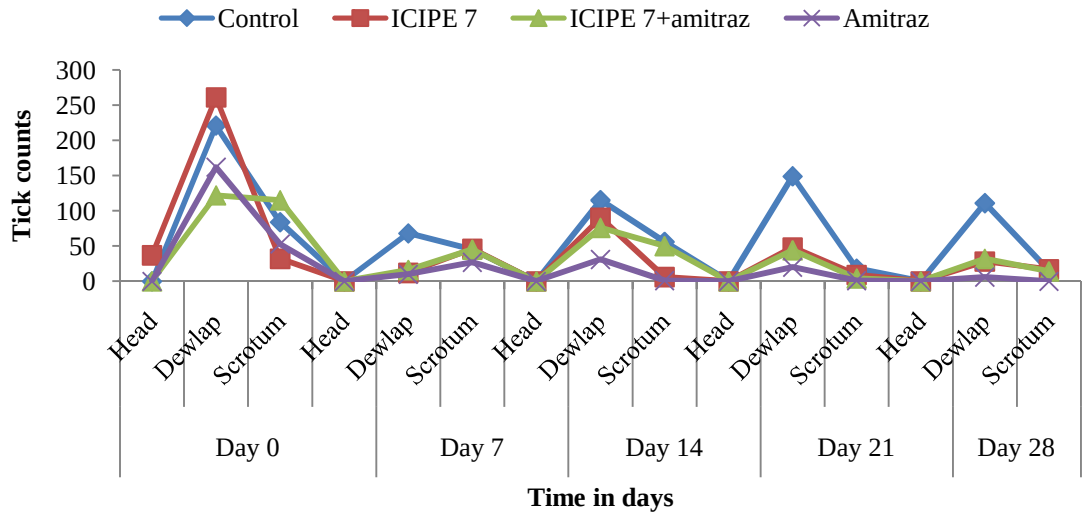
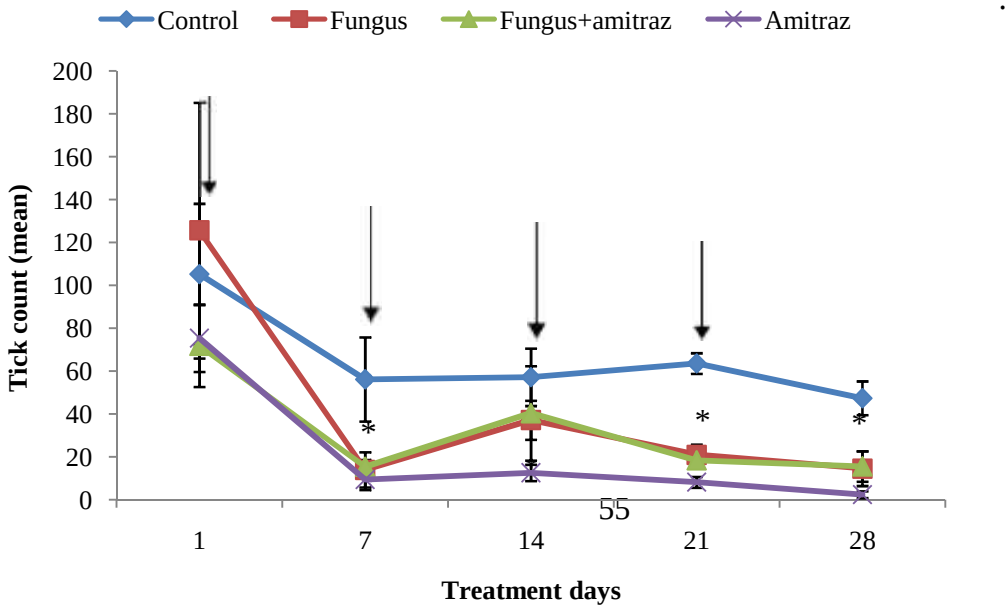


Figure 4: Mean tick counts in three body regions per treatment

4.3.3: Overall mean total tick counts on animal body after weekly application of different treatments

The mean total tick counts after weekly application of the three treatments are shown in Fig. 5 below



Arrow above indicates the dates of application of treatments and means were significantly different ($p < 0.05$).

Figure 5: Overall mean tick counts on animal body after weekly application of different treatments

Before application of treatments, the number of ticks was considerably high in all the treatments and varied between 71.8 and 125.6 and was not significantly different ($F_{3, 8}$=0.4986; P=0.6885). Application of treatments significantly reduced the number of ticks on all the sampling days: day 7 ($F_{3, 8}$=3.917; P=0.0284), day 14 ($F_{3, 8}$=9.090; P=0.0275), day 21 ($F_{3, 8}$=37.971; P=0.0001) and day 28 ($F_{3, 8}$=8.170; P=0.0016) as compared to the control (Fig. 5). No significant differences were observed between the treatments ($F_{3, 8}$=3.917; P=0.0284), except on day 14 when tick reduction was highest in amitraz alone (Fig. 5). Ticks that were collected from cows at different sampling days and brought to the laboratory succumbed to fungal infection.

4.4.3 Assessment of efficacy

After application of treatments, the efficacy ranged from 57.5% to 74% on day 7 but it reduced for fungus and fungus combination with amitraz on day 14, but it was high in amitraz due to significantly high tick reduction. On day 21 and 28, difference between the treatments was significant due to higher percentage efficacy in fungus and amitraz due to significant tick reduction but reduced in fungus combination with amitraz due to high tick counts (Fig. 6).

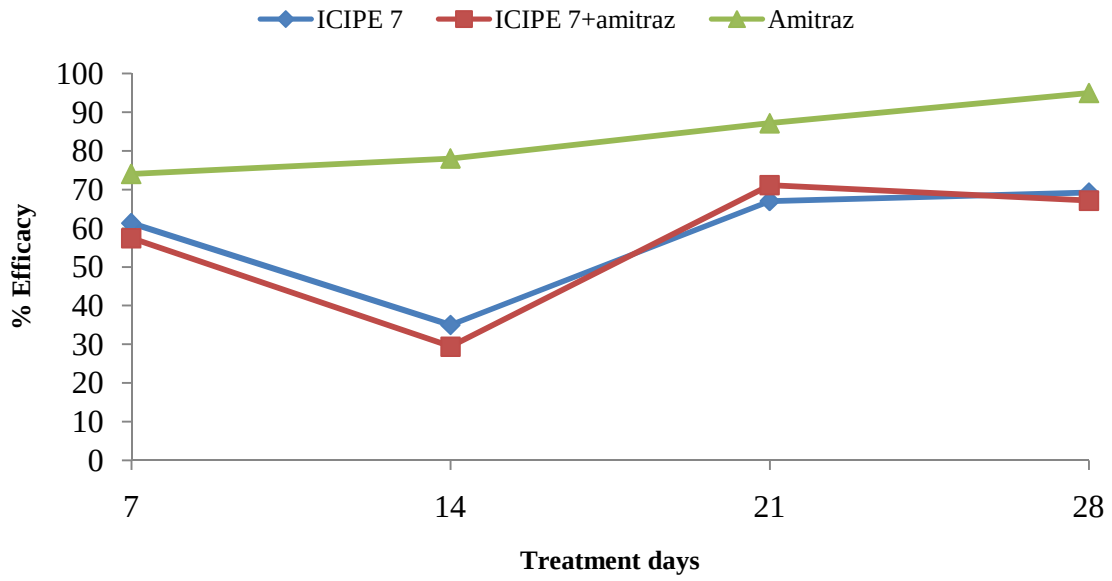


Figure 6: Percentage efficacy of different treatments

4.4.4 Mean percentage mortality of *R. decoloratus* following treatment with ICIPE 7 and amitraz

The mean percentage mortality of *R. decoloratus* after treatment with ICIPE 7 and amitraz are as shown in Fig. 7.

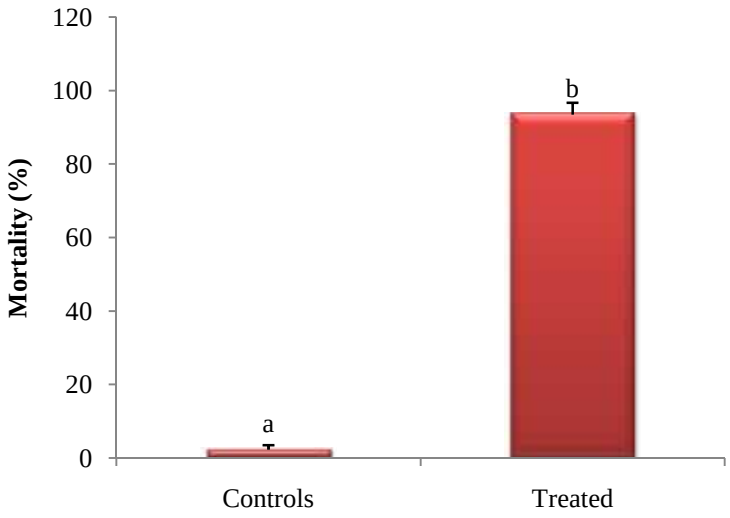


Figure 7: Mean percentage mortality (± SD) of *R. decoloratus* following treatment with ICIPE 7 and amitraz

Treatment groups attained mortality of 93.5% as compared to the control which attained 2.3% mortality indicating a high significance difference between them (Fig. 7).

4.5 Conidial persistence on cattle

The percentage conidial germination (counts) based on the body swabs are shown in Table 12**.**

Table 12: Conidial persistence on cattle (% germination)

	Day 3				Day 5				Day 7			
Cow ID	**Ribs**	**Spine**	**Ear**	**Udder/ Scrotum**	**Ribs**	**Spine**	**Ear**	**Udder/ Scrotum**	**Ribs**	**Spine**	**Ear**	**Udder/ Scrotum**
1	88	98	100	96	76	81	83	69	64	71	70	70
2	77	93	100	100	81	75	71	80	59	71	77	66
3	91	88	100	99	65	81	70	77	72	56	68	69
4	74	79	90	95	81	73	70	91	60	69	65	71
5	81	77	100	88	63	85	82	70	58	64	82	71
Mean	**82.2**	**87**	**98**	**95.6**	**73.2**	**79**	**75.2**	**77.4**	**62.6**	**66.2**	**72.4**	**69.4**
SD	7.19	8.97	4.47	4.72	8.67	4.9	6.67	8.91	5.73	6.38	6.95	2.07
ESM	3.22	4.01	2	2.11	3.89	2.19	2.99	3.98	2.56	2.85	3.11	0.93

The mean germination of the swabbed cow surfaces was high on day 3 which had p-value of 0.0052 and was considered very significant relative to day 5, where the mean germination had decreased (p-value was 0.6391) and, on day 7 the mean germination slightly reduced (p-value was 0.726), which was not significant ($p>0.05$).

Table 13: Percentage germination means per cow surface

Cow surfaces	Percentage germination (%)		
	Day 3	Day 5	Day 7
Ribs	82.2	73.2	62.6
Spine	87	79	66.2
Ear	98	75.2	72.4
Udder/Scrotum	95	72.4	69.4
F-value	6.236	0.576	2.814
p-value	0.005	0.639	0.726

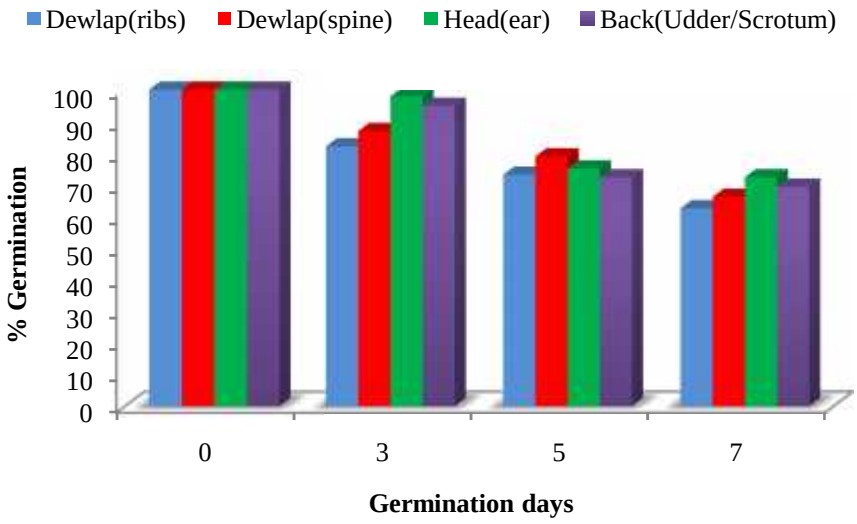


Figure 8: Mean percentage conidial germination for different cow surfaces

Conidial germination was 100% on the day of application of treatments (day 0). The conidial germination from the various regions were as follows; ear (98%), udder/scrotum (95%), spine (87%) and ribs (82.2%) on day 3 after treatment but these indicated no significant difference ($P<0.05$). On day 5, conidial germination from different cow surfaces were as follows; spine 79 %, ear 75.2%, ribs 73.2% and udder/ scrotum with 72.4% but they were not significant as p - value was greater than 0.05. On day 7, there was no significance in conidial germination from different cow surfaces as the ear maintained a higher spore germination of 72.4%, udder/scrotum 69.4%, spine 66.2% and ribs had the lowest germination of 62.6% (Table 13 and Fig. 8).

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

Different developmental life cycle phases of ticks may be infected by entomopathogenic fungi leading to the death of the ticks as demonstrated through *in vitro* tests by Polar *et al*., (2005). During the initiation of the infection process of the ticks by the fungus, the germination of the conidia is a crucial step for the fungus to penetrate into the cuticle of the tick (Schrank and Vainstein, 2010). In this study, the conidial germination of the screened isolates ranged from 96.6 to 100% after 20 hours which was in agreement with results obtained by Schrank and Vainstein (2010) indicating that for the fungus to infect the ticks successfully, germination of the conidial is important.

The present study indicated that *R. decoloratus* larvae were highly susceptible to 50% of the twelve isolates indicating possibility of their use as tick control agents. There were some differences in percentage mortalities and time taken for ticks to die after infection with the fungal isolates. Also, different fungal isolates did not discriminate if the tick strains were resistant to amitraz or not. Fungal infection of ticks was confirmed by the formation of conidial spores on the ticks. For the infection of ticks to be effective, significant amount of spores are needed to challenge the tick defense mechanism and proliferation inside the host. In the present study, only two isolates (ICIPE 7 and ICIPE 718) originated from the host while others were from the soil and other arthropods, indicating that the source of fungus has no effect on its pathogenicity.

The EPFs' pathogenicity against ticks of various species including *R. decoloratus* has been reported. For instance mortality of 40-50% in adult *R. decoloratus* by *M. anisopliae* and *B. bassiana* in the laboratory was reported by Kaaya and Hedimbi (2012).

The mean LT_{50} values in amitraz-susceptible strain varied between 2.6-4.2 days and in amitraz-resistant strain between 2.8-3.9 days of the six isolates. These LT_{50} values were used to select ICIPE 7 as the best biocontrol agent with the shortest LT_{50} and it had also a high mortality. The results indicated that slightly over 40% of the isolates of *M. anisopliae* tested were highly pathogenic against *R. decoloratus,* suggesting a high potential for this isolate to be used for tick control. Moreover, the LC_{50} values were variable and had high range of virulence. Four of them had values within $0.4 \pm 0.1 \times 10^3$ and $200.0 \pm 60 \times 10^3$ conidia ml^{-1} in amitraz susceptible and within $0.1 \pm 0.1 \times 10^3$ and $200.0 \pm 31.0 \times 10^3$ conidia ml^{-1} in amitraz resistant strains. The results suggested that *M. anisopliae* (ICIPE 7) could be used as a biocontrol agent mainly due to their high pathogenicity to tick larvae and shortest lethal time on average.

The conidial viability for the compatibility test was approximately 98% and this affected positively the compatibility of ICIPE 7 and amitraz (Alizadeh *et al.*, 2007). The present study indicated a mean percentage conidial germination of ICIPE 7 in different concentrations of amitraz of between 95.4% and 97.6%.

Combination of amitraz and fungi (*M. anisopliae*) has not been studied much but some previous studies have shown that amitraz negatively affects germination, vegetative growth and sporulation of *B. bassiana* (Alizadeh *et al*., 2007). This is contrary to the current results that showed high compatibility of ICIPE 7 with various concentrations of amitraz did not affect the

conidial growth and dry weight of mycelia. As the concentration of amitraz increased, radial growth decreased but sporulation of the fungus was not affected. Schumacher and Poehling (2012) reported that amitraz in 1.6 ppm and 40 ppm increased the vegetative growth but 200 ppm reduced spores production by 50%. In the present study, amitraz was highly compatible with *M. anisopliae* at low concentrations and similar results were observed by Schumacher and Poehling (2012).

In the present study, reduction of ticks on cattle was observed. Reduction of ticks by 69.2% was observed in ICIPE 7; 67.1% in combination of ICIPE 7 and amitraz, and 94.9% in amitraz during the four weeks of treatment application in the field. Correia *et al*., (1998) reported no effect of *M. anisopliae* on *Rhipicephalus microplus* on cattle contrary to these observations while Castro *et al.* (1997) reported more than 50% tick reduction of *R. microplus* after spraying the animals once. On the other hand, 90% reduction was demonstrated in *R. microplus* after applying *B. bassiana* and *M. anisopliae* for five weeks (Rijo-Camacho 1996). *Rhipicephalus microplus* reduction was also demonstrated by Polar *et al*., (2005) on cattle treated with two isolates of *M. anisopliae* after treatment for 3 weeks. *Rhipicephalus microplus* reduction of 40.0 to 91.2% was achieved by weekly application of *M. anisopliae* by Alonso-Diaz *et al.,* (2007). Kaaya *et al.* (2011) on the other hand demonstrated 83% reduction of *R. decoloratus* when *M. anisopliae* (1×10^8 conidia/ml) was in oil formulation.

The field test was done in the middle of the day and this could have affected the conidial germination due to the UV-A, UV-B radiation and heat from the sun (Francisco *et al*., 2008). Higher percentage efficacies were attained when similar experiment was performed in late

afternoon Anelise *et al.*, (2015) and Alonso-Diaz *et al.*, (2007). The present study was done with temperatures of 22.7^0C and the RH was 57.3% which was within the normal range of RH of between 55 and 75 % and 25^0C (Michalaki *et al*., 2007) for *Metarhizium* growth.

Combination of *M. anisopliae* and amitraz had lower efficacy as compared to amitraz alone in the present study due to the fact that, amitraz concentration added to the *M. anisopliae* was lower than the manufacturer's recommended concentration as compared to other field experiments that indicated high efficacies with the same combination but the concentration of acaricides used for treatment of cattle was equivalent to the manufacturers' recommendation and was combined with *M. anisopliae* at 1×10^8 conidia/ml (Anelise *et al.*, 2015).

In the previous studies, ticks were shown to die after application of fungi with mycosis being observed indicating the viability of conidia in spite of the effects of environment on the host (Polar *et al.*, 2008). Mycosis was also observed in the present study on surfaces of treated ticks with ICIPE 7 and this was recovered from infected tick cadavers (Murigu *et al.*, 2016). Egg laying by engorged treated females has been observed when tests were conducted under *in vitro* conditions (Hornbostel *et al*., 2004; Rot *et al*., 2013).

With on-host experiment, animals were infested again by ticks because the tick loads were high in the fields used for grazing. Three to five days after application of treatments, the tick population went down but on day seven, more new ticks on cattle were observed. The present study showed that low doses of acaricide in combination with fungus could not enhance its efficacy. Bahiense *et al*., (2006) demonstrated that treatment of *R. microplus in vitro* with combination of fungus and acaricide at recommended doses improved the efficacy of the fungus.

5.2 CONCLUSIONS

The investigation of this study concluded that:-

- Fungal isolates screened were viable
- *Metarhizium anisopliae* and *B. bassiana* isolates were highly pathogenic on amitraz-susceptible and amitraz-resistant strains of *R. decoloratus*
- *Metarhizium anisopliae* isolate ICIPE 7 was the best and most pathogenic among the screened isolates
- ICIPE 7 in combination with amitraz as a spray reduced the tick population significantly on cattle

5.3 RECOMMENDATIONS

The following recommendations were made:-

- More research to the selected virulent fungal isolates in the laboratory and in the field is warranted to provide information for the development of fungal biopesticide that can provide excellent tick control
- Further research to determine the association of entomopathogenic fungi with chemical acaricides is necessary
- Studies to determine the effects of inclusion of additives i.e., sunscreens and antioxidants in the formulation should be undertaken as this may prolong conidial persistence and efficacy under natural field conditions
- Variables such as ambient temperature, time of day, time of year; relative humidity, animal coat length influencing the optimal performance to guarantee a consistent high level of control of all on-animal tick stages with a *Metarhizium* species based biopesticide should be studied
- ICIPE 7 should be improved for use as a biocontrol agent

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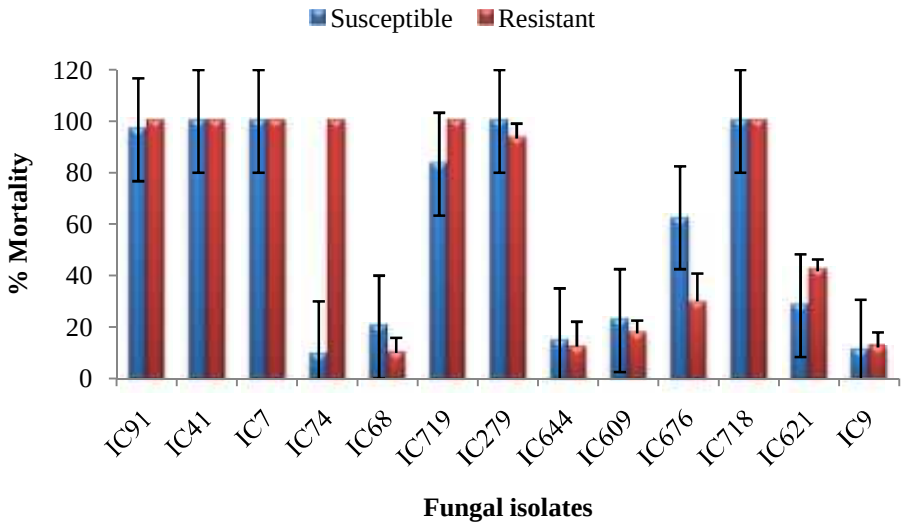
APPENDICES

Appendix 1: Virulence of *M. anisopliae* and *B. bassiana* against susceptible and resistant *R. decoloratus* ticks



Appendix 2: Pathogenicity of ICIPE 07 formulated in amitraz formulation on larval stages of susceptible and resistant strains of *R. decoloratus*

(A) Pathogenicity of ICIPE 07 formulated in amitraz formulation on larval stages of susceptible *R. decoloratus*

Treatments	DAY 0	1	2	3	4	5	6
Control	0	0	0	0	0	0	0
Amitraz	0	8.25	8.75	9.25	9.5	10	10
ICIPE 7 10^9	0	9.5	10	10	10	10	10
5µl+ICIPE 7 10^9	0	9.75	10	10	10	10	10
10µl+ICIPE 7 10^9	0	10	10	10	10	10	10
20µl+ICIPE 7 10^9	0	10	10	10	10	10	10
Treatments	Percentage mortality						
Control	0	0	0	0	0	0	0
Amitraz	0	82.5	87.5	92.5	95	100	100
ICIPE 7 10^9	0	95	100	100	100	100	100
5µl+ICIPE 7 10^9	0	97.5	100	100	100	100	100
10µl+ICIPE 7 10^9	0	100	100	100	100	100	100
20µl+ICIPE 7 10^9	0	100	100	100	100	100	100

Amitraz	MEANS	0	82.5	87.5	92.5	95	100
	SEM	0	7.5	7.5	4.7	2.8	0
ICIPE 7 10^9	MEANS	0	95	100	100	100	100
	SEM	0	2.8	0	0	0	0
5µl+ICIPE 7 10^9	MEANS	0	97.5	100	100	100	100
	SEM	0	2.5	0	0	0	0
10µl+ICIPE 7 10^9	MEANS	0	100	100	100	100	100
	SEM	0	0	0	0	0	0
20µl+ICIPE 7 10^9	MEANS	0	100	100	100	100	100
	SEM	0	0	0	0	0	0

B) Pathogenicity of ICIPE 7 formulated in amitraz formulation on larval stages of resistant *R. decoloratus*

	DAY 0	1	2	3	4	5	6	7
Control	0	0	0	0	0	0	0	0
Amitraz	0	6.25	7.25	9.25	9.25	9.75	10	10
ICIPE 7 10^9	0	9.5	10	10	10	10	10	10
5µl+ICIPE 7 10^9	0	9.5	10	10	10	10	10	10
10µl+ICIPE 7 10^9	0	10	10	10	10	10	10	10
20µl+ICIPE 7 10^9	0	10	10	10	10	10	10	10
	0	1	2	3	4	5	6	7
Control	0	0	0	0	0	0	0	0
Amitraz	0	62.5	72.5	92.5	92.5	97.5	100	100
ICIPE 7 10^9	0	95	100	100	100	100	100	100
5µl+ICIPE 7 10^9	0	95	100	100	100	100	100	100
10µl+ICIPE 7 10^9	0	100	100	100	100	100	100	100
20µl+ICIPE 7 10^9	0	100	100	100	100	100	100	100
Amitraz		MEANS	0	67.5	77.5	97.5	97.5	100
		ESM	0	11.1	8	2.5	2.5	0
ICIPE 7 10^9		MEANS	0	95	100			
		ESM	0	2.8	0			
5µl+ICIPE 7 10^9		MEANS	0	95	100			
		ESM	0	2.8	0			
10µl+ICIPE 7 10^9		MEANS	0	100				
		ESM	0	0				
20µl+ICIPE 7 10^9		MEANS	0	100				
		ESM	0	0				

5µl/l=5ppm

Appendix 3: Compatibility of ICIPE 7 and amitraz

a) Effects of amitraz on average radial growth of *M. anisopliae* isolate ICIPE 7

Day 3

Treatment	Control	1%	2 %	4 %	8 %
R1	10	9	9	7	6
R2	9	10	8	7.5	6
R3	10	10	9	6.5	6
R4	9	8	9	7	5
R5	8	8	8	6	5
Mean	9.2	9	8.6	6.8	5.6
SD	0.837	1	0.548	0.570	0.548
ESM	0.374	0.447	0.245	0.255	0.245

Day 6

Treatment	Control	1 %	2 %	4 %	8 %
R1	17	16	14	9	9
R2	19	16	18	15	18
R3	16	15	16	8	8
R4	18	18	14	16	12
R5	17	16	15	16	9
Mean	17.4	16.2	15.4	12.8	11.2
SD	1.140	1.1	1.673	3.962	4.087
ESM	0.51	0.49	0.748	1.772	1.828

Day 9

Treatment	Control	1 %	2 %	4 %	8 %
R1	29	30	35	25	23
R2	23	26	18	14	22
R3	35	18	26	35	13
R4	21	28	23	25	11
R5	30	25	18	15	18
Mean	27.6	25.4	24	22.8	17.4
SD	5.639	4.561	7.036	8.614	5.32
ESM	2.522	2.04	3.146	3.852	2.38

b) Effects of amitraz on spore production of *M. anisopliae* isolate ICIPE 7

Treatment	Control	1%	2 %	4%	8%
R1	9.39	8.31	9.29	7.54	8
R2	8.3	9.02	8.65	6.96	6.8
R3	8.75	8.75	9.76	8.6	8
R4	7.96	8.02	7.89	7.31	7.5
R5	9.23	7.01	6.78	7	5.6
Mean	8.726	8.222	8.474	7.482	7.18
SD	0.605	0.78	1.179	0.669	1.011
ESM	0.271	0.349	0.527	0.299	0.452

c) Mean mortality of amitraz-resistant and amitraz-susceptible strains of *R. decoloratus in* formulated amitraz and *M. anisopliae*, ICIPE 7

	Mean percentage mortality (%)	
Treatments	**Amitraz-resistant *R. decoloratus***	**Amitraz-susceptible *R. decoloratus***
Controls	0	0
Amitraz 62.5 ppm	20.9	35.4
Amitraz 125 ppm	62.5	82.5
ICIPE 7 10^8	98	95
Amitraz 62.5 ppm+ ICIPE 7 10^8	98	100
Amitraz 125 ppm+ ICIPE 7 10^8	100	100

Appendix 4: Field evaluation of the efficacy of ICIPE 7 against *R. decoloratus*

A) Rainfall data in millimeters and temperatures in degrees Celsius during the month of October and November 2014

OCTOBER				NOVEMBER			
Date	**Rainfall**	**Temperature**		**Date**	**Rainfall**	**Temperature**	
		Minimum	**Maximum**			**Minimum**	**Maximum**
1	Nil	15°C	27°C	**1**	1.7	14°C*	20°C
2	3.4	15°C	25°C	**2**	NIL	13°C	22°C
3	1.5	15°C	21°C	**3**	NIL	15°C	20°C
4	NIL	15°C	22°C	**4**	4.9	15°C	21°C
5	3.3	15°C	21°C	**5**	NIL	14°C	21°C
6	NIL	15°C	20°C	**6**	NIL	14°C	21°C
7	NIL	15°C	20°C	**7**	NIL	14°C	20°C
8	NIL	14°C	21°C	**8**	NIL	14°C*	21°C
9	NIL	15°C	28°C	**9**	7	13°C	20°C
10	NIL	15°C	30°C	**10**	NIL	14°C	21°C
11	NIL	15°C	21°C	**11**	NIL	14°C	22°C
12	NIL	15°C	20°C	**12**	NIL	14°C	23°C
13	NIL	15°C	20°C	**13**	NIL	15°C	24°C
14	2.7	14°C	21°C	**14**	1.5	14°C	23°C
15	1.5	15°C	21°C	**15**	NIL	13°C*	23°C
16	10.5	15°C	21°C	**16**	1.5	12°C	20°C
17	4.6	14°C	25°C	**17**	NIL	12°C	22°C
18	0.5	15°C	21°C	**18**	NIL	14°C	22°C
19	NIL	15°C	20°C	**19**	NIL	14°C	22°C
20	NIL	15°C	20°C	**20**	2.8	14°C	21°C
21	0.9	16°C	25°C	**21**			
22	4	15°C	21°C	**22**			
23	NIL	15°C	21°C	**23**			
24	1.5	15°C	20°C	**24**			
25	5.8*	16°C	22°C	**25**			
26	6.9	15°C	20°C	**26**			
27	NIL	14°C	20°C	**27**			
28	NIL	13°C	28°C	**28**			
29	NIL	16°C	21°C	**29**			
30	NIL	15°C	20°C	**30**			
31	NIL	15°C	21°C				
*		Asteric indicate the days when treatment applications were done. Temperature and rainfall data was obtained from the Hydrology section of the Trans Mara Development Project. (T.D.P) Meteorological Station, Kilgoris.					
		Trial period					

b) Total tick counts for the 25 experimental animals

Cow no.	Tick load	Cow no.	Tick load
1	723	**14**	321
2	690	**15**	282
3	687	**16**	280
4	584	**17**	278
5	531	**18**	273
6	456	**19**	271
7	442	**20**	270
8	442	**21**	170
9	431	**22**	139
10	421	**23**	86
11	410	**24**	pregnant
12	407	**25**	Very aggressive
13	350		

c) Randomized groups with colour codes

Group 1= Control			**Group 2= Fungus**			**Group 3= Amitraz**			**Group 4= Fungus+amitraz**		
Colour code: Red			**Yellow**			**Green**			**Blue**		
Ear tag	Cow no.	Tick counts	Ear tag	Cow no.	Tick counts	Ear tag	Cow no.	Tick counts	Ear tag	Cow no.	Tick counts
14	4	723	1	2	697	3	583	687	21	4	584
10	1	442	8	1	442	20	580	457	4	1	531
9	2	431	2	5	421	15	562	410	19	2	407
23	3	280	7	3	282	13	563	321	24	5	350
6	5	278	18	4	273	17	570	271	22	3	270

d) Total tick counts for all the treatment groups and days for data collection

	Week 1					2			3			4		
	Cow no.	**Day 1**	**3**	**5**	**7**	**3**	**5**	**7**	**3**	**5**	**7**	**3**	**5**	**7**
Control	**1**	63	108	29	10	144	61	71	58	41	73	67	30	37
	2	149	78	15	36	60	48	28	54	32	54	67	50	48
	3	75	28	18	2	72	49	61	46	32	60	38	44	35
	4	211	110	86	127	221	85	28	118	77	77	95	85	78
	5	28	19	24	6	67	46	98	74	62	54	46	38	39
	Total	526	343	172	181	564	289	286	350	244	318	313	247	237
	Mean	105	66.6	34.4	36.2	112.8	57.8	57.2	70	48.8	63.6	62.6	49.4	47.4
ICIPE 7	**1**	37	72	0	0	48	16	42	48	2	32	26	10	18
	2	353	46	48	45	76	58	39	8	24	29	25	23	44
	3	38	14	0	0	25	24	3	53	2	14	6	8	0
	4	64	12	4	14	56	38	58	30	21	6	23	6	11
	5	136	77	0	11	29	33	44	38	2	24	16	17	0
	Total	628	221	52	70	234	169	186	177	51	105	96	64	73
	Mean	125.6	44.2	10.4	14	46.8	33.8	37.2	35.4	10.2	21	19.2	12.8	14.6
ICIPE 7+ Amitraz	**1**	71	75	192	26	185	154	124	51	32	30	22	66	42
	2	88	24	3	13	27	17	41	20	9	16	6	10	16
	3	24	28	5	6	24	18	4	14	8	20	2	0	10
	4	134	26	3	16	50	26	27	10	6	10	8	5	10
	5	42	8	0	16	23	2	6	4	0	16	0	0	0
	Total	359	161	203	77	309	217	202	99	55	92	38	81	78
	Mean	71.8	32.2	40.6	15.4	61.8	43.4	40.4	19.8	11	18.4	7.6	16.2	15.6
Amitraz	**1**	80	10	1	21	4	4	5	2	0	6	0	0	0
	2	105	24	34	21	14	0	4	6	6	7	0	2	0
	3	109	12	16	0	1	0	12	1	2	6	0	4	4
	4	24	27	3	5	8	15	24	6	9	18	2	2	8
	5	59	7	0	0	4	4	18	2	2	4	2	0	0
	Total	377	80	54	47	31	23	63	17	19	41	4	8	12
	Mean	75.4	16	10.8	9.4	6.2	4.6	12.6	3.4	3.8	8.2	0.8	1.6	2.4

e) Tick counts on different predilection sites

Day 0					7				14			
	C	**F**	**F+A**	**A**	**C**	**F**	**F+A**	**A**	**C**	**F**	**F+A**	**A**
Head	0	0	0	0	0	0	0	0	0	0	0	0
Dewlap	221	261	122	162	68	12	16	10	115	90	76	31
Back	84	32	115	53	45	46	45	27	56	6	50	1

Day 21					28			
Head	**C**	**F**	**F+A**	**A**	**C**	**F**	**F+A**	**A**
Dewlap	0	0	0	0	0	0	0	0
Back	0	48	44	20	111	28	32	6
Head	18	9	4	1	15	17	14	0

C=Control, F=ICIPE 7, F+A=ICIPE 7+Amitraz, A=Amitraz

f) Overall total tick counts on animals' body after weekly application of treatments

Mean tick counts					
Treatments	**Day 1**	**7**	**14**	**21**	**28**
Control	105	36.2	57.2	63.6	47.4
ICIPE 7	125.6	14	37.2	21	14.6
ICIPE 7 + amitraz	71.8	15.4	40.4	18.4	15.6
Amitraz	75.4	9.4	12.6	8.2	2.4

g) Weekly total tick counts for the treatment groups

Treatments	Week 1					Week 2		
		Day 1	3	5	7	3	5	7
Control	Total	526	343	172	181	564	289	286
	Mean	105	66.6	34.4	36.2	112.8	57.8	57.2
ICIPE 7	Total	628	221	52	70	234	169	186
	Mean	125.6	44.2	10.4	14	46.8	33.8	37.2
ICIPE 7+ amitraz	Total	359	161	203	77	309	217	202
	Mean	71.8	32.2	40.6	15.4	61.8	43.4	40.4
Amitraz	Total	377	80	54	47	31	23	63
	Mean	75.4	16	10.8	9.4	6.2	4.6	12.6
Week 3					Week 4			
		3	5	7	3	5	7	
Control	Total	350	244	318	313	247	237	
	Mean	70	48.8	63.6	62.6	49.4	47.4	
ICIPE 7	Total	177	51	105	96	64	73	
	Mean	35.4	10.2	21	19.2	12.8	14.6	
ICIPE 7+ amitraz	Total	99	55	92	38	81	78	
	Mean	19.8	11	18.4	7.6	16.2	15.6	
Amitraz	Total	17	19	41	4	8	12	
	Mean	3.4	3.8	8.2	0.8	1.6	2.4	

H) Means and standard error of means (SEM)

Days		Control	ICIPE 7	ICIPE 7+ amitraz	Amitraz	p-value	f-value
1	Means	105	125.6	71.8	75.4	0.6889	0.4979
	SEM	32.9	59.6	19	15.7		
7	Means	36.2	14	15.4	9.4	0.4742	0.8758
	SEM	23.4	8.2	3.2	4.8		
14	Means	57.2	37.2	40.4	12.6	0.1886	1.795
	SEM	13.3	9.1	21.9	3.8		
21	Means	63.6	21	18.4	15.6	<0.0001	32.975
	SEM	4.8	4.8	3.3	2.4		
28	means	47.4	14.6	8.2	2.4	0.001	9.023
	SEM	7.9	8.1	7	1.6		

i) Percentage efficacy

	Percentage efficacy (%)			
Treatments	**Treatment days**			
Day	7	14	21	28
ICIPE 7	61.3	35	67	69.2
ICIPE 7+Amitraz	57.5	29.4	71.1	67
Amitraz	74	78	87.1	94.9