POTENTIAL OF METARHIZIUM ANISOPLIAE IN THE MANAGEMENT OF TOMATO BORER (*Tuta Absoluta*) INFESTING TOMATO

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DECLARATION

This thesis is my original work, and it has not been presented for any award degree or diploma in any other university.

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DEDICATION

To my dear parents, Juliana Muhongo Mulama and Ernest Mulama Ingosi, for laying down their all to give me a quality education.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA:	Analysis of Variance
LC50:	Lethal Concentration 50
IPM:	Integrated Pest Management
ICIPE:	International Centre of Insect Physiology and Ecology
CFU:	Colony forming unit
CV:	Correlation of Variation
PDA:	Potato Dextrose Agar
TDTA:	(3E, 8Z, 11Z)-3,8,11-tetradecatrienyl acetate
EPN:	Entomopathogenic Nematodes
IPPC:	International Plant Protection Convention
J1:	First Juvenile
NPnEO:	Nonylphenol ethoxylate
N:	Population size
USD:	United States Dollar

GENERAL ABSTRACT

Greenhouse experiments were conducted consisting of 5 treatments: *Metarhizium anisopliae* (6.0 x 10^3 cfu /ml) formulated with Nonylphenol ethoxylate, *Metarhizium anisopliae* (6.0 x 10^3 cfu /ml) conidia alone, Nonylphenol ethoxylate alone, compared to a standard pesticide having Indoxacarb 85g/L and Emmamectin benzoate 15g/L. The field experiment consisted of M*etarhizium anisopliae* (6.0 x 10^3 cfu /ml) in Nonylphenol ethoxylate, *Metarhizium anisopliae* (6.0 x 10^3 cfu /ml) conidia alone, Nonylphenol ethoxylate alone and a standard pesticide Indoxacarb 85g/L with Emmamectin benzoate 15g/L.

Rio grande variety had the largest leaf area in the two seasons but this was comparable with that recorded for M82, Eden, Cal J and Moneymaker. The evaluated tomato varieties retained viable conidia of *Metarhizium anisopliae* on their leaves but Rio grande variety significantly ($p<0.05$) retained the most colonies. Adjuvants, Nonylphenol ethoxylate 15% and Tween 80 significantly ($p<0.05$) increased the radial growth of *Metarhizium anisopliae* ICIPE 69 and ICIPE 78 isolates, compared to control whereas liquid soap significantly ($p<0.05$) prevented the radial growth of *Metarhizium anisopliae* ICIPE 69 and ICIPE 78 isolates at all concentrations when compared to control.

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The findings of the laboratory assays show that *Metarhizium anisopliae* significantly ($p<0.05$) caused mortality to *Tuta absoluta* larvae. One hundred percent (100%) mortality of Tuta larvae was achieved within 36 hrs of treatment of larvae treated with *Metarhizium anisopliae* 1.2×10^6 cfu /ml. *Metarhizium anisopliae* 1.2×10^3 cfu /ml , 1.2×10^4 cfu /ml and 1.2×10^6 cfu /ml did not differ in effect 60hours after treating the larvae. In the greenhouse experiment, no differences were noticed in the population of larvae in the different treatments except in the 8th week where *Metarhizium anisopliae* & NPnEO recorded the least mean population of larvae and was significantly ($P<0.05$) lower than control but comparable with the rest of the treatments. The resultant yield recorded show that the standard pesticide, Indoxacarb 85g/L and Emmamectin Benzoate 15g/L, significantly ($p<0.05$) had the highest yield compared to control but it was comparable to the second highest yield recorded in *Metarhizium anisopliae* & NPnEO. Control had the most larval population, most damaged tomatoes and lowest yield recorded which were significantly ($p<0.05$) different from the treated tomatoes. In the open field, Indoxacarb 85g/L and Emmamectin Benzoate 15g/L had the least mean population recorded which was significantly different ($p<0.05$) from control but not from *Metarhizium anisopliae* and Nonylphenol ethoxylate

15% treatment with the second lowest population. The resultant yields were significantly ($p<0.05$) higher compared to control with the least damage percentage of the fruits in both Indoxacarb 85g/L and Emmamectin Benzoate 15g/L and *Metarhizium anisopliae* and Nonylphenol ethoxylate 15% treatments. *Metarhizium anisopliae* can be used to manage *T. absoluta* under field and greenhouse conditions and that Nonylphenol ethoxylate 15% and Tween 80 as adjuvants can be used to formulate and facilitate distribution of the conidia and enhance growth for fast establishment on the crop.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Tomato (*Solanum lycopersicum* L) is horticulture crop valued for its fruit. It can be used as food and as a commercial crop. The tomato plant has botanical characteristics such as fleshy fruit, a sympodial shoot, and compound leaves. It belongs to the large Solanaceae family. Within Kenya, tomato is ranked second among other vegetables in terms of value and production next to irish potato (Sigei *et al.*, 2014). It contributes 14% of the total vegetables produced and 6.72% of the total horticultural crops (Ochilo *et al.*, 2019).

The Food and Agriculture Organization (FAO) reported in 2021 global land area under tomato cultivation was 5.03 million hectares which produced 180.8 million kilos (FAO, 2021). The leading tomato producer in the world is China producing 62.9 million kilos of the total worldwide production(FAO, 2021). It is followed by India, the United States of America, Turkey, and Egypt (FAO, 2021). A land area of 1.6 million ha is used to cultivate tomatoes in Africa. These yielded 21.7 metric tons of tomatoes in 2019. The top tomato producer in Africa is Egypt with an average production of 6.8 million kilos in 2019 followed by Nigeria, Tunisia, and Morocco. Kenya only produces 0.2 % of the tomatoes produced globally (FAO, 2021).

Production of the tomatoes can be done either in an open field or under greenhouse conditions. Production under field conditions accounts for 95%, while greenhouse production contributes 5% of the total tomato produced in the country. Kenya is ranked sixth among the tomato-producing countries in Africa. The total production is estimated to be 397,007 tones. Kirinyaga, Kajiado, and Taita Taveta are the major tomato-producing counties in Kenya (Geofrey*et al.*, 2014). Abiotic factors, pest and diseases are the major group of constrains that affect tomato production (Ochilo *et al*, 2019). The main abiotic constrains facing tomato production are water availability and soil fertility(Karuku *et al.,* 2017).

1.2 Problem Statement

Tomato (*Solanum lycopersicum L*) is a popular vegetable in Kenya (Sigei *et al.,* 2014). Tomato is grown for income generation and used for food (Ochilo *et al.*, 2019). However productivity of tomato is affected by pests and diseases (Ochilo *et al*, 2019). *Tuta absoluta* the main pest affecting tomato production. It can cause up to 100% yield loss (Assinapol, 2020). *Tuta absoluta* is an invasive pest from South America (Tropea *et al.*, 2012). Direct losses of tomato yield can be incurred through further reduction in production rate when the pest gains entry into a new area (Venkatramanan *et al.,* 2019). Even though some management measures have been developed, the cryptic nature of *Tuta absoluta* makes it challenging to manage in places where the pest has been reported (Biondi *et al.,* 2018). The pest is reportedly causing indirect effects and has affected farmers by increasing cost of production (Hill *et al*., 2019). In Kenya, the impact of *Tuta absoluta* and other invasive pests on the well-being of people is primarily felt in the rural area, where there are people depend mainly on agriculture (Shackleton *et al.,* 2019).

Small scale farmers prefer the use of chemical pesticides for its management. This choice, although feasible, is threatened by the ability of *Tuta absoluta* to develop chemicals resistance (Peris *et al.*, 2018). The Brazilian population of *Tuta absoluta* has shown resistance to abamectin, cartap, and permethrin (Siqueira *et al.,* 2000).

Similarly, some *Tuta absoluta* populations in Greece also exhibited resistance to diamide pesticides (Roditakis *et al.,* 2015). In Argentina *Tuta absoluta* populations have shown resistance to deltamethrin because of pesticide selection pressure (Lietti *et al.,* 2005). The resistance experienced in some geographical regions can be dispersed into new areas through pest movement from a part that has resistant populations to new places (Campos *et al.,* 2015). To minimize resistance, other management practices like the use of entomopathogenic fungi should be developed for integrated pest management. Studies have confirmed that pesticides can be associated with the cause of various diseases like cancer, leukemia, and asthma. The integration of entomopathogenic fungi like *Metarhizium anisopliae* in the management practice will help in reducing exposure to pesticides.

1.3 Justification

Tomato (*Solanum lycopersicum L*) is a significant vegetable in Kenya (Sigei *et al.,* 2014). Tomato is cultuvated as a cash crop and used for food (Ochilo et al., 2019). Tomato consumption reduces the risk of having some diseases like cancer (Salehi *et al.,* 2019).More than 30 % of tomato farmers in Kenya have reported the effects of *Tuta absoluta* on their farms (Ochilo *et al.*, 2019). The pest has a very rapid dispersal mechanism as it can drift with the help of wind spreading to new areas (Tonnang *et al.*, 2015). This type of dispersal renders quarantine measures ineffective. The pest also has a very high reproductive capacity allowing it to quickly build up populations beyond the economic threshold level within a short period (Tropea *et al.*, 2012). Trading of infested tomato fruits has also aided the fast spread of the pest. The ability of the problem to survive and adapt to changes in the ecological conditions and feed on multiple crop hosts, including weeds, make it difficult to control (Illakwahhi *et al.*, 2017). Yield losses inflicted by *Tuta absoluta* are up to 100% when the conditions are conducive for the pest, and proper management methods are not implemented (Assinapol, 2020). Presence of the pest causes diversion of capital from meeting production costs to putting in place management strategies; it also raises tomato production costs through increased exposure to chemical pesticides due to the increased amount of pesticides needed to manage the pest (Aigbedion-Atalor *et al.,* 2019). Efficacy of entomopathogenic fungi like *Metarhizium anisopliae* is not widely tested. Although, the fungus is reported to attack both eggs and the larvae of the *Tuta absoluta* (Tadele and Emana, 2017). *Metarhizium anisopliae* has also been shown to effectively manage diamond back moth *(Plutella xylostella)* (Shehzad *et al.*, 2021). More studies need to be done in Kenya to confirm the potential of *Metarhizium anisopliae* in management of Tuta absoluta. Other *Metarhizium* species have also been successfully isolated from other Lepidopteran pests like larvae of *Denrolimus* species. Therefore, further studies need to be done to confirm the efficacy of *M. dendrolimatilis* in the management of *Dendrolimus* and other related pests (Chen *et al.,* 2017). Studies have been done to determine the effect of leaf growth on the retention and distribution of *Metarhizium anisopliae* conidia on plant leaves (Inyang *et al.,* 1998). This study aims to precisely evaluate the effect of tomato leave growth on the retention of *Metarhizium anisopliae* conidia. This study seeks to assess the effect of *Metarhizium anisopliae* in the management of *Tuta absoluta* to reduce losses associated with the pest. The findings will contribute to the available options for managing the pest. Determination of the influence of adjuvants on the growth of *Metarhizium anisopliae* will also inform the best formulations possible for effective Tuta management.

1.4 Objectives of the study

1.4.1 Broad objective

To contribute to sustainable management of *Tuta absoluta* through use of environmentally friendly biopesticides for improved tomato productivity.

1.4.2 Specific objectives

The specific objectives of the study were:

I. To determine the effect of tomato varieties on the regular application of *Metarhizium anisopliae* conidia.

II. To assess the effect of adjuvants on Metarhizium *anisopliae* growth.

III. To evaluate the efficacy of *Metarhizium anisopliae* in managing *Tuta absoluta* infesting tomato.

1.5 Hypotheses

I. Regular application of *Metarhizium anisopliae* is not affected by tomato leaf morphology.

II. *Metarhizium anisopliae* colonies growth is not affected by selected adjuvants, Nonylphenol ethoxylate 15%, Tween 80 and liquid.

III. The application of *Metarhizium anisopliae* is not effective in managing of *Tuta absoluta*.

CHAPTER TWO: LITERATURE REVIEW

2.1 Economic importance of *T. absoluta*

The East African region is estimated to lose between 91.0 million USD and 101.1 million USD annually due to *Tuta absoluta* invasion. Kenya is estimated to be losing between 59.81 million USD and 66.51 million USD annually (Pratt *et al*., 2017). This loss is higher than that incurred by its neighbors, estimated at 3.41 - 3.81, 26.51- 29.5 1 and 1.21 -1.31 million USD in Ethiopia, Tanzania and Uganda, respectively (Pratt *et al.* 2017). In economies like Nepal, where tomato production is mainly for meeting the domestic needs, only 1% is exported, the effects of *Tuta absoluta* infestation result in a magnified direct economic impact. In such cases, the pest causes tomato prices to increase by very high margins, up to 32% (Venkatramanan *et al.*, 2019).

Some growers cannot access export markets because of *Tuta absoluta* measures of restriction and some countries have systems that help curbing the spread of *Tuta absoluta* into their geographical region. The International community (IPPC) concerned with plant health and spread of pests has an international standard (ISPM) that mitigates the spread of the pest. These measures aim to ensure fair trade among countries while containing the spread of agricultural pests and diseases (Garnas *et al.,* 2016). For example, after the pest risk assessment was done in some developed countries, the developed states prohibited the import of Solanaceae plants from developing countries for planting within their territories. Other strict conditions like refrigeration, screening and secure packing of fruits have been made mandatory for tomatoes imported from countries that report pest infestation in addition to the fruit not having any pest damage (Potting *et al.,* 2013).

In places where *Tuta absoluta* has been established, tomato farmers prefer to use synthetic pesticides to manage the pest (Peris *et al.*, 2018). According to Biondi *et al.* (2012) spinosyns have an impact on non- target beneficial arthropods. Although products containing spinosad compounds manage *Tuta absoluta* and other arthropod pests, spinosad has a low effect on predators but very lethal to parasitoids. Spinosad interferes with larval development in predator and parasitoid arthropod species. It also causes physiological effects on the vital body functions of natural enemies such as lifespan, immune system, and reproduction. When pollinators, like bees, are exposed to spinosad they get tremors that lead to paralysis and death.

2.2 Tomato production practices

Tomato production can be done in an open field or under greenhouse conditions. The seeds take around four days to germinate at an optimum temperature ranging from 26 – 32^0C. Tomato seedlings can be obtained through grafting to have clean planting materials without diseases (Khah *et al.,* 2006). Soil-borne diseases are significantly managed when grafted tomato seedlings are used compared to planting the seeds directly (Rivard and Louws, 2008). When tomatoes are transplanted into a more profound depth, they produce bigger fruits than those transplanted into shallow holes. This is influenced by the early establishment of the seedlings when the planting depth is deep. Deep planting can also lead to more fruit production (Vavrina *et al.,* 1996). Management practices influence tomato yield in the field include tillage, fertilization and cover cropping. Tillage increases nitrogen uptake by the plant, consequently increasing fresh fruit production (Yaffa *et al.,* 2000). Tomato production under greenhouse conditions enables all-year-round productivity in all regions. The greenhouse designs used for tomato production depend on the location's environmental conditions (Brugger *et al.*, 2004). Lighting, humidity, and ventilation are factors that influence the design of the greenhouse (Hemming *et al.,* 2009; Hemming *et al.,* 2016).

2.3 Nutritional benefits of tomatoes

Tomato fruits contain carotenoid lycopene. The lycopene compounds are the health component of tomatoes (Dawid, 2016). Tomato consumption reduces risks of developing some diseases in humans (Salehi *et al.,* 2019). According to Burton and Reimers (2011) tomato consumption can reduce the risk of diseases such as osteoporosis, cognitive dysfunction, ultraviolet light-induced skin damage, developing cardiovascular diseases and some cancers. Living and non living factors, limit tomato production directly or indirectly, in different regions. A combination of humidity and high temperature provides a conducive environment for pathogenic fungi to thrive. High temperature and low humidity provide a climate conducive to insect pests like thrips and *Tuta absoluta* to thrive. The diseases and pests consequently hinder tomato production (Anastacia *et al.,* 2011).

2.4 Biology of *Tuta absoluta*

Tuta absoluta is in the class: Insecta; family : Gelechiidae, with its specific epithet as *Tuta absoluta* (Gebremariam, 2015). The adult moth strategically chooses sites for laying eggs for better chances of larvae development. Although the adult moth can lay eggs on any part of the tomato plant, it prefers the apical or median area of the plant canopy. The female adult likes laying eggs on the lower side of the leaf, but some eggs can be deposited on the upper surface of the leaf. Oviposition can be done on the apical main stem, especially before fruiting (Torres *et al.,* 2001; Cherif *et al.,* 2013).

Tuta absoluta undergoes a complex metamorphosis. The stages include larva, pupa, and adult. The eggs are oval – cylindrical, cream-colored, and small. The eggs are about 0.2 mm in diameter and 0.4mm in length (Sanda *et al.,* 2018). The early instars of the larvae are white or cream with a black head. They then turn green to pink with a brown head. The prothoracic shield becomes pale. The pupae are less than 6 mm in length; they form between rolled tomato leaves or in the soil (Srindhar *et al.,* 2014). The adult moth has a body length of 5- 7 mm (Visser *et al.,* 2017). Male *Tuta absoluta* have black spots on the wings and have brown to silver genitalia which is ovate shaped. They have a pair of segmented filiform antennae (Figure 2.1).

The male reproductive organ has a broad ovate-shaped gnathos with a digitate valve within a medial hump and constriction. The vinculum is deeply excavated medially and has a pair of trapezoid-shaped processes with curved tips (Srindhar *et al.,* 2017).

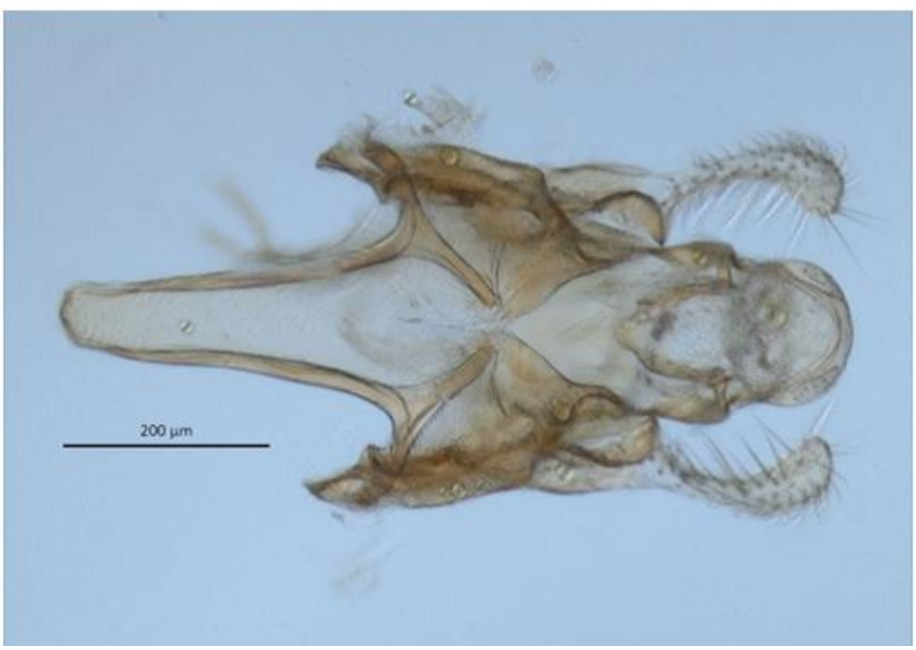

Fig 2. 1: *Tuta absoluta* male genitalia (Visser *et al*., 2017)

The larvae burrow into the leaves for feeding or disperse from theoviposition site after hatching. It feeds on the mesophyll tissues below the cuticle and epidermis of the leaves. The larvae can also mine into the apical buds, stalks, and fruits (Savino *et al.,* 2012). The feeding leads to conspicuous blotches on the leaves and pinhole size holes on fruits. Dark frass can be seen in the mines after the larvae have finished feeding (Srindhar *et al.,* 2017).

The larvae undergo four larval stages; the larval length range from 0.6 mm to 8 mm. The first larval stage appears creamy, while the fourth stage is green or pinkish (Tropea *et al.,* 2012). One lateral and another ventral band are visible on the head of the larvae. After completing the four larval stages, pupation occurs in the soil. The pupation process may last for 10 to 11 days before the adult emerges (Sanda *et al.,* 2018). Morphologically, the adult appears to be sickle brown or silver-color with spotted wings. The length of the body may range from 5 to 7 mm long, while the wing length ranges from 8 mm to 10 mm. *Tuta absoluta* undergoes complete metamorphosis. It takes 24 to 30 days to complete the metamorphic stages.

Tuta absoluta can lay 250 to 300 eggs during its life as a mature adult. The pest can have up to twelve generations annually (Retta and Berhe, 2015). Female adults release sex pheromones that attract males for mating during the reproduction process. *T. absoluta* has effective survival mechanisms which enable it to overcome harsh environmental conditions and chemical pesticides. It pupates in the soil. Hence the high temperature from the sun and chemicals from pesticide application cannot affect their development. The eggs are safely laid under the leaf for protection against predators and exposure to harsh conditions. *T. absoluta* adults can fly for a considerable distance, and the spread is mainly aided by wind and humans. Humans can contribute to its movement by exchanging infested products (Zekeya *et al.*, 2016).

2.5 Spread of *Tuta absoluta*

Tuta absoluta is a pest native to Central America. In 2007, *it* was reported in Spain, and in 2008 in the Mediterranean region (Desneux *et al.,* 2011). In a span of 5 years, the pest colonized a geographical spread of approximately 400 km in the Mediterranean region. This rapid spread was attributed to the trading of tomato fruits (Tropea *et al.,* 2012). In Africa, Morocco first reported the pest between 2007 and 2008. Other North African countries including Sudan, Niger and Senegal reported it between 2008 and 2012. The pest entered Eritrea, Ethiopia, and Kenya in 2013 (Mansour *et al.,* 2018). Tanzania and Nigeria reported it in 2014 and 2015, respectively. According to modeling information published by Guimapi *et al.* (2016) the pest will spread and cover the entire continent successfully. *Tuta absoluta* is established even in the southern part of Africa except for Madagascar and Mauritius. By 2017, *Tuta absoluta* was reported in 41 of the 54 African countries (Figure 2.2). Although the pest had been reported in some countries, there were no details of when it entered some African countries (Mansour *et al.,* 2018).

After surveying thirty-five farms and two markets in twelve districts from six provinces in Zambia, some regions, like the central province, showed a very high level of infestation by *Tuta absoluta* (Luangala *et al.,* 2016). In 2016, samples of the *Tuta absoluta* moth were trapped using pheromone traps on the border between South Africa and Mozambique (Visser *et al.*, 2017).

Fig 2. 2: Map showing the spread of Tuta absoluta in Africa (Source: Mansour *et al*., 2018)

2.6 Management of *Tuta absoluta*

2.6.1 Monitoring the pest population

The first step towards managing *Tuta absoluta* is assessing the pest population within the tomato crop. In an open field, *Tuta absoluta* eggs can be assessed through geostatistical analysis. This method entails characterization and development of spatial distribution maps. An effective pest management strategy, can be developed with information from these distribution maps (Martins *et al.*, 2018).

2.6.2 Cultural control methods

Intercropping is used as a cultural practice in pest management to increase vegetation diversity in a given area by growing two or more crops simultaneously in the same field. The variety of crop affects the damage densities by reducing the pest immigration rate into a field and the rate at which the pest spreads in area. The rate of a pest entering and scattering from a site depends on the host finding mechanism and the ability of the pest to move. Polyculture or intercropping tomatoes with other crops or non-crops avoids the physical movement of pests (Smith and McSorley, 2000). *Tuta absoluta* eggs deposited onto the tomato plant can be reduced by intercropping with sainfoin (Zarei *et al.*, 2019). The intercrop between tomatoes and sainfoin increases the diversity index of predator species. Some of the predators recorded in the intercrops are; *Nabis punctatus costa, Macrolophud pygmaeus* (Rambur), *Deraeocoris punctulatus* (Fallen), *Dicyphus* sp., *Nabis pseudoferus* Remane, and *Geocoris punctla* (Khafagy, 2015). According to the same authors, intercropped tomatoes have a higher yield than the monocropped. Some herbs such as geranium have proven to be a good intercrop with tomatoes. The effect of inter-planting geranium and tomatoes is reduction in the number of Tuta larvae on leaflets, on fruits and the total number of mines on tomatoes (Khafagy, 2015).

Nitrogen and water management significantly affects the development of different stages of *Tuta absoluta*. Variation in nitrogen and water levels available to a tomato plant affects the pupa's survival rate, development rate, and weight. An increase in the amount of nitrogen available to a plant causes a decrease in survival rate of all developmental stages to the adult stage. The interaction between water and nitrogen level significantly affects the weight of *Tuta absoluta* pupae. Drought conditions and low nitrogen availability causes reduced pupal weight (Jin. *et al.*, 2014). High phosphorus levels interacting with nitrogen increase the developmental time at all stages of *Tuta absoluta* development (Blazhevski *et al.*, 2018). Hence, varying nitrogen and water can be a management technique to control *Tuta absoluta*.

2.6.3 Biological control methods

Several parasitoids attacking *Tuta absoluta* have been reported. In Egypt two hymenopteran larval parasitoids, *Diglyphus sp*. (Eulophidae) and *Elasmus sp* (Scelionidae) and an egg parasitoid *Telenomus sp*. (Scelionidae) have been recorded (Rashwan, 2016). *Diglyphus* sp is an ectoparasitoid of *Tuta absoluta* larvae. It has also been recognized in Spain, Algeria, and Greece. *Elasmus spp* is a parasitoid that attacks the larvae and pupa of *Tuta absoluta.* The parasitoids have been recorded in Spain and Italy (Mahdi, 2011). *Telenomus sp* is a parasitoid that attacks the eggs of *Tuta absoluta*. The parasitoid has been recorded in Egypt and Iraq. Some predators like *Nesidiocoris tenuis* have been documented attacking *Tuta absoluta* eggs. The predator is also registered in Spain, France, Algeria, and Iran (Rashwan, 2016). Batalla-Carrera *et al.* (2010) reported that nematodes found in the families like Steinernematidae and Heterorhabditidae like; *Steinernema feltiae* and *Heterorhabditis bacteriophora* were lethal against the later stage of *Tuta absoluta* larvae (Van Damme *et al*., 2016). According to the study, EPNs can kill all the stages of the development of *Tuta absoluta* (Arthurs *et al.*, 2004). The EPNs can also penetrate the galleries of the tomato mines to get the *Tuta absoluta*. Under field conditions, the J1s can find the *Tuta absoluta* larvae in the mines and kill them (Gözel and Kasap, 2015; Gözel and Gözel, 2020). The EPNs can kill up to 50% of the adult *Tuta absoluta* when applied at the rate of 50 J1s/cm^3 (Kamali and Koppenhöfer, 2017).

Nesidiocoris tenuis is a predator that feeds on *Tuta absoluta* eggs (Ferracini *et al.,* 2019) and larvae, especially the first instar larvae (Öztemiz, 2013). The female consumes more *Tuta absoluta* eggs than the male (Urbaneja *et al.,* 2009). Calvo *et al*. (2012) reported that the pre-plant release of *Nesidiocoris tenuis* can reduce the populations of *Tuta absoluta* in greenhouses.

2.6.4 Chemicals used for controlling *Tuta absoluta*

Under laboratory conditions, chlorantraniliprole was effective in killing 93% *Tuta absoluta* larvae (Deleva and Harizanova, 2014). Under field conditions, Chlorantraniliprole showed a positive impact against *Tuta absoluta* in two (2) hours after application (Cherif *et al.*, 2018). Braham *et al.* (2012) demonstrated that spirotetramat has 47.5% efficiency against *Tuta absoluta* larvae under laboratory conditions while Gacemi and Guenaoui (2012) confirmed that emamectin-Benzoate was efficacious on *Tuta absoluta* larvae. According to Peris *et al*. (2018) *Tuta absoluta* larvae can develop resistance to chemical pesticides hence studies to evaluate *Metarhizium anisopliae* as a potential alternative to manage the pest or as a component of IPM.

2.6.5 Botanical pesticides used against *Tuta absoluta*

According to Kona *et al.* (2014), application of neem extracts onto the eggs of *Tuta absoluta* can cause up to 26% egg mortality, while combined application of neem and Jatropha on the larvae of *Tuta absoluta* causes 100% mortality. Brito *et al*. (2015) also showed that ethanolic leaf extract from *Piper amalago* var. *medium, P. glabratum,* and *P. mikaninum* had activity against the larvae of *T. absoluta*. The same authors have reported that the extracts also cause prolonged development for the different life stages of the pest (Brito *et al.,* 2015). Some oils such as clove, eugenol, and isoeugenol cause a reduction in the hatching of *Tuta absoluta* larvae with approximate effectiveness of 100% (Moawad *et al.,* 2013). The eugenol oil, clove oil, and lavender oil have a repellency effect towards the first instar of *T. absolute* larvae, with eugenol oil achieving 82% efficacy (Goudarzvand and Abbasipour, 2017). *Tuta absoluta* adults are susceptible to the fumes released from *Citrus aurantium* essential oil and pure limonene (Zarrad *et al.,* 2017).

2.6.6 Semiochemicals used to manage *Tuta absoluta*

Some of the semiochemicals produced by insects include pheromones. The pheromones are used for luring male adult moths into traps. The traps can be either sticky or with water that has detergent to break the surface tension. Ettaib *et al.* (2016) demonstrated that pheromone traps capture more *Tuta absoluta* moths than other forms of trap. Pheromone traps are also used to monitor the population of *Tuta absoluta* in the greenhouse or open field (Balzan and Moonen, 2012).

2.6.7 Integrated pest management strategy for *Tuta absoluta*

A good IPM program contains the following aspects: host plant resistance, microbial, biological control, entomopathogenic nematodes, botanicals, semiochemicals, synthetic pesticides, cultural methods, sterile insect technique, and insecticide resistance management. Low-risk substances and biological control agents are emphasized in the management program. A judicious application of target-specific pesticides is typically done (Tarusikirwa *et al.,* 2020).

2.7 Mode of action of *Metarhizium anisopliae*

Adhesion to the pest is a crucial stage in the pathogenesis process of *Metarhizium anisopliae* (Leão *et al.,* 2015). Host structure surface and the chemical composition of the cuticle of the target organism affect the adhesion of *Metarhizium anisopliae* onto the host. Based on the host's signals, *M. anisopliae* secretes host-specific proteins. *Metarhizium anisopliae* contains genes that encode proteins that degrade the cuticle to enable penetration (Wang *et al.*, 2012). Proteins that help in cuticle degeneration are proteases, including chymotrypsin, elastase, trypsin, subtilisins, and carboxypeptidases (Santi *et al.*, 2010). These traits make the fungus very selective (Lord and Howard, 2004). Ment *et al*. (2010) showed that exposure time of the host to the conidia of *Metarhizium anisopliae* influences the adhesion to the cuticle of the pest. The longer the period of exposure, the more conidia retention. After a successful landing, the conidia germinate by developing a swelling called appressorium that helps it hold onto the host. The appressorium then creates penetration pegs used to invade the host (Leger *et al.*, 1989). Infection pathway of *Metarhizium anisopliae* has been demonstrated in figure 2.3.

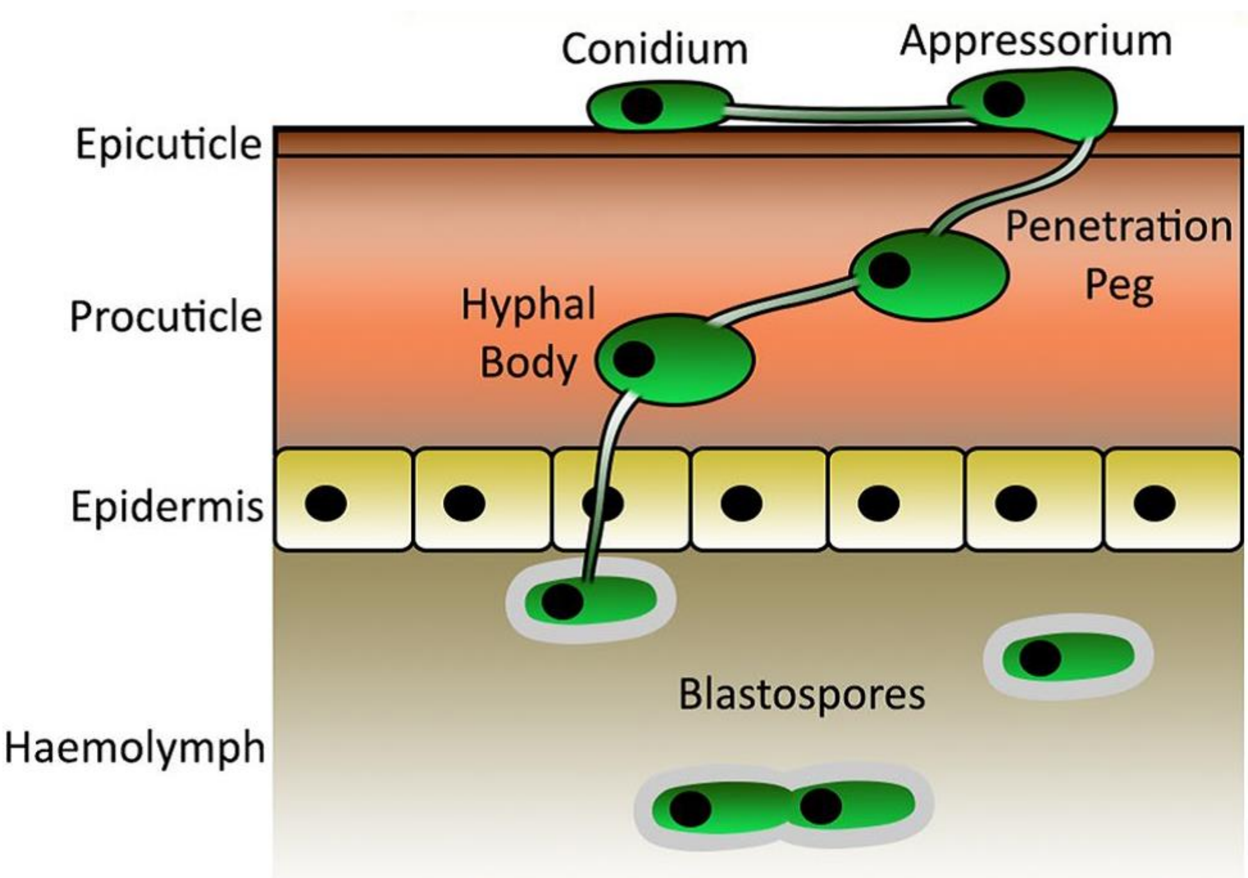

Fig 2. 3: Host infection pathway of Metarhizium spp (Lovett and Leger, 2015)

2.8 Metarhizium anisopliae in the management of other pests affecting tomatoes

Aphids *(Aphis gossypii)* are sucking insects that directly affect tomatoes by sucking the plant sap and indirectly by vectoring virus diseases or attracting the black sooty mold that interferes with photosynthesis (Tripathi, 2018). Aphid (*A. gossypii*) populations can be managed using *M. anisopliae,* with maximum population reduction observed after 21 days (Jugno *et al.*, 2018). More than 330 species of Liriomyza have been described but *Liriomyza trifoli* attacks explicitly tomatoes. *Metarhizium anisopliae* has been described as an entomopathogen that can potentially manage *Liriomyza trifoli* (Liu *et al.,* 2009). Wekesa *et al.* (2006) examined the effect of *Metarhizium anisopliae* on the fecundity and egg fertility of spider mites *Tetranychus evansi* under screen house conditions. There was a significant reduction in hatchability of the spider mite eggs caused by *Metarhizium anisopliae*. Investigation revealed that the eggs and adult stage are the most susceptible to *Metarhizium anisopliae* isolate ICIPE 78 (Wekesa *et al.*, 2006). The adult female mites also showed reduced oviposition rate due to the effect of *Metarhizium anisopliae*. According to a study done by Maniania *et al.* (2016) the entomopathogen, *M. anisopliae* had significant potential in managing the spider mite, *Tetranchus evansi,* infesting tomato under field and screen house conditions. Bugeme *et al.* (2015) demonstrated that *M. anisopliae* isolate, ICIPE 78, effectively controlled the two-spotted spider mite, *Tetranychus urticae*, infesting tomatoes and beans. The study showed that applying an aqueous and slurry formulation of *M. anisopliae* and abamectin significantly reduced the population of *Tetranychus urticae* (Bugeme *et al.*, 2015).

Several *Metarhizium anisopliae* isolates have been tested for managing *Tetranychus urticae,* and the results are promising (Mugisho and Markus, 2009; Chandler *et al.,* 2004). Strains of *Metarhizium anisopliae* virulent to *Helicoverpa armigera* have been isolated from *Annona squamosal* (Pathan and Deshpande, 2019). Kumar and Chowdhry (2004) also confirmed virulence of several *Metarhizium anisopliae* strains against *Helicoverpa armigera* under greenhouse conditions. Similarly, Vvestergaard et al. (1995) evaluated several isolates of *Metarhizium anisopliae* and found that they caused more than 94% mortality against the western flower thrips *Frankliniella occidentalis* within seven days.

2.9 Other *Metarhizium* species and their uses in agriculture

Metarhizium anisopliae var. *acridum,* also called *Metarhizium acridum,* is an entomopathogenic pathogen virulent to Acrididae. Central American Locust *Schistocerca piceifrons* Walker has been proven susceptible to *Metarhizium anisopliae* var. *acridum*. The conidia have been shown to cause up to 97 % mortality of adult pests (Hernández-Velázquez *et al.,* 2003). According to *Tokarev et al.* (2011) the nymph of the migratory locust (*Locusta migratoria*) is susceptible to combined infection of *Metarhizium acridum* and *Paranosema locustae*. The findings of Hunter *et al.* (2016) demonstrated that *Metarhizium acridum* is effective when used against the Italian locust *Calliptamus italicus* (L) (Orthoptera: Acrididae) and that within 14 days *Metarhizium acridum*, application, up to 67 %, population reduction can be recorded.

An investigation on the efficacy of *Metarhizium acridum* and neem seed oil mixture against the tree locust (*Anacridium melanorhodon*) (Orthoptera: Acrididae) showed that the combination can cause up to 92% mortality of the tree locust (Haroon *et al.,* 2011). *Metarhizium majus* has efficacy against the Scarabaeidae family of beetles. Depending on the conidia density of the spores, a mortality rate of up to 92% has been reported (Velavan *et al.*, 2017). *Metarhizium majus* has caused 100% death of *Oryctes rhinoceros* (L) larvae under laboratory conditions (Oetari *et al.*, 2020).

CHAPTER THREE
THE EFFECTS OF REGULAR APLLICATION AND LEAF MORPHOLOGY ON *METARHIZIUM ANISOPLIAE* CONIDIA RETENTION

ABSTRACT

Tomato (*Solanum lycopersicum*) is a high-value vegetable crop. In Kenya, tomatoes contribute to the national GDP as a horticultural crop. Tomato production is negatively affected by pest and diseases. *Metarhizium anisopliae* is an entomopathogenic fungus applied to tomato plants to control pests such as *Tuta absoluta.* This experiment aimed to determine the effect of tomato varieties on the regular application of *Metarhizium anisopliae* conidia. Five tomato varieties commonly grown in Kenya were evaluated during the study: Eden, Rio Grande, M82, Moneymaker, and Cal-J. A hand-held sprayer was used to inoculate the tomato leaves with *Metarhizium anisopliae* (4.9 x 10^9 cfu /ml) on the lower and upper sides of the leaves. The leaves were sampled once per week and incubated on Potato Dextrose Agar. The *Metarhizium anisopliae* colonies on the leaves were counted after 48hrs. Weekly averages of the number of colonies formed on leaves were determined, and the Pearson correlation between the numbers of colonies on leaves and leaf area. During the eight weeks of sampling, Rio grande variety had the largest leaf area in both seasons. This area was not significantly different from the leaf area of M82, Eden, Cal J, and Moneymaker. Riogrande variety significantly ($p<0.05$) retained more *M. anisopliae* conidia than the other varieties at 221.7 cfu and 238.7 cfu in in season one and two, respectively.

A positive correlation between leaf area and colony number(0.823** and 0.820**) was recorded indicating that the leaf area influences the number of conidia retained on the leaf.

3.1 Introduction

Tomato (*Solanum lycopersicum*) is both a commercial and food crop (Geoffrey *et al.*, 2014). It is many nutritional and health benefits. The fruits are reported to contain phytochemicals such as carotenoids and polyphenols. These phytochemicals reduce the likelihood of contracting diseases such as cancer (Arah *et al.,* 2015).

Two types of tomatoes exist, the determinate and the indeterminate. The determinate tomatoes can grow upright, while the indeterminate require support to grow upright. Determinate tomato types are primarily grown in the field, while the in-determinate type are grown in the greenhouse. Within the determinate kind of tomato, there are several varieties, including; Eden, Monyalla, Cal J, Tanzanite, and Onyx. The indeterminate varieties include Keno, Moset , Nemonneta, and Anna F1 (Mwangi *et al.*, 2020).

Metarhizium genus contains a wide range of entomopathogenic fungi. *Metarhizium anisopliae* (Metchnikoff) contains various lineages that are pathological to insect pests and has the potential for controlling many pests that are of economic importance in the agricultural sector (Nishi and Sato, 2017). *Metarhizium anisopliae* attacks its host by first attaching to the cuticle. After adhering, it penetrates the host using a penetration peg. This is followed by colonization of the haemocoel by the mycelium. After killing the host, the green mycelium then emerges from the cadaver (Lovett and Leger, 2015). The application of *Metarhizium anisopliae* conidia can be made through soil drenching around the crown region of the plant to control soil-borne pests (Greenfield *et al.,* 2016).

It can also be applied by spraying on leaves to manage foliar and other aerial problems (Hong *et al.,* 2017).

3.2 Materials and methods

3.2. 1 Site description

This study was conducted at the University of Nairobi field station and the plant pathology laboratory at the Faculty of Agriculture.

3.2.2 Tomato varieties used in the experiments

Five tomato varieties were grown in pots under greenhouse conditions. The varieties evaluated were obtained from the Kenyan market based on their availability during the study period. The varieties used in the experiment were Eden, Rio grande, M82, Money maker, and Cal-j. Each plant variety represented a treatment. Each treatment contained ten plants replicated three times in a complete randomized design.

3.2.3 Inoculation of the leaves with *Metarhizium anisopliae* conidia

Metarhizium anisopliae colonies were cultured in the laboratory on PDA media for use in the experiment. All the treatments were inoculated with *Metarhizium anisopliae* using a handheld sprayer (500 ml). The treatments were inoculated with a standard conidia density of 4.9×10^9 cfu/ml on the lower and upper sides of the leaves following the procedure of Batta **(**2013). The inoculation was done eight times at an interval of seven days. The first spray was done three weeks after transplanting.

3.2.4 Leaf sampling and incubation

From each treatment, leaflets were sampled from the top, middle and bottom plant canopy at seven days interval. The leaflets were carried to the laboratory in a cool box sterilized using 70% ethanol.

In the laboratory, each leaf was handled under aseptic conditions before plating on Potato Dextrose Agar. Every single leaflet obtained from the compound leaves was spread on solid Potato Dextrose Agar and incubated at 24^{o}C for 48 hrs following Last (1955) procedure with modifications.

3.2.5 Leaf area determination

Leaf area determination was done by combined use of a scanning device with a resolution of 1080×2340 pixels. A modification of the Michal and Kinga method (Stawarczyk and Stawarczyk, 2015) was used to develop a scale for the images. A millimeter ruler was placed alongside each leaf before taking digital photos. The ruler was calibrated to show the distance covering 10 mm, under the scanner. The scanned images were then saved using the JPEG format. The analysis of the image was done in Image-J. After setting the scale, the type of measurement was selected to be the area. Before obtaining the site, the image was processed for color threshold, specifically, brightness, and color intensity. After implementing the analysis, the generated data were recorded in a table of summary exported to Microsoft excel. Surface area was measured in millimetres squared (Stawarczyk and Stawarczyk, 2015).

3.2.6 Determination of the number of *Metarhizium anisopliae* colonies

A modification of the method used by Last (1955) to determine the fungal colonies formed on plant leaves was used in the experiment. The total number of colonies established on the lower side of each leaf that were plated on PDA were counted using a colony counter. The leaves were divided into four sections to make counting easy.

3.2.7 Data analysis

The weekly means and ANOVA of the number of colonies formed on each leaf were determined using SPSS and GenStat 15th Edition. A comparison for the difference in the determined means was made using Turkey's test in the GenStat statistical software version 2009. The leaf area and colony number date were plotted on a scatter plot to determine the linearity. Correlation analysis was done to determine the relationship between leaf area and the number of colonies. Pearson correlation analysis was then carried out to assess the association of leaf area and the number of colonies formed.

3.3 Results

3.3.1 Effect of tomato varieties and on the growth characteristics of *Metarhizium anisopliae*

In the first season there were no significant difference in the leaf area of all tomato varieties during the sampling period. Riogrande had the largest leaf area while Money maker had the lowest leaf area throughout the sampling period (Table 3.1).

Table 3. 1: Mean tomato leaf area of five selected tomato varieties season one

	WEEKS							
Tomato	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Moneymaker	763.1a	886.5a	1012a	1189a	1384a	1562a	1663a	1773a
Cal J	775.7a	901.7a	1029a	1208a	1401a	1581a	1679a	1793a
Eden	784.8a	912.9a	1036a	1216a	1405a	1598a	1698a	1804a
M82	797.6a	931.9a	1050a	1228a	1413a	1607a	1710a	1820a
Riogrande	822.7a	950.1a	1074a	1248a	1440a	1623a	1735a	1856a
CV	4.50	8.80	6.30	5.50	5.50	4.40	4.30	4.50
LSD	64.01	146.70	120.10	120.80	140.00	128.40	131.50	146.90
P	0.35	0.88	0.83	0.85	0.93	0.85	0.88	0.84

Means with similar letters in the same column are not significantly different ($P<0.05$).

All the tomato varieties retained viable conidia of *Metarhizium anisopliae* on their leaves. In season one, there were significant ($p<0.001$) differences in number of colonies retained by all tomato varieties between the first and fifth week of sampling. During the eight weeks of observation, the *M. anisopliae* conidia retained by Eden and M82 varieties varied with M82 retaining higher colony forming units except in the 8th week. *Metarhizium anisopliae* conidium retained by Riogrande was significantly (p<0.001) higher than that of from M82 except in week 6, 7 and 8. Rio Grande had the highest number of *M. anisopliae* conidia retained on the leaf compared to other varieties in the experiment. Money maker variety had the lowest number of *M. anisopliae* conidium which was significantly (p<0.001) lower than that recorded in the rest of the varieties, throught out the 8 weeks of observation (Table 3.2).

Table 3. 2: Mean number of colony forming units retained on tomato leaves of five selected tomato varieties season one

	WEEKS							
Tomato	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Moneymaker	31.67 a	49.67 a	59.3 a	67.7 a	77.3 a	122.3 a	142.3 a	158.0 a
Cal J	55 b	74.7 b	94.3 b	104.3 b	109.3 b	133.3 ab	165.3 b	187.0 b
Eden	63.67 c	90.7 c	104.3 c	121.3 c	123.3 c	144.3 b	178.3 b	195.7 bc
M82	80 d	115.7 d	131.3 d	152.3 d	159.3 d	179.3 c	211.7 c	214.7 cd
Rio grande	95.33 e	133.7 e	150.3 e	174.3 e	175.3 e	193.3 c	220 c	221.7 d
CV	4.10	6.10	1.40	1.60	3.40	4.60	4.40	4.20
LSD	4.81	10.34	2.78	3.52	7.76	12.86	14.79	14.89
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Means with similar letters in the same column are not significantly different (P<0.05).

A strong positive correlation was observed of the colony number formed on the leaf and the leaf area (**0.823****). The leaf area and the varieties had a weak positive correlation (**0.060****). There was a strong positive correlation of the colony number formed on the leaf and the number of sampling weeks (**0.803****). The Pearson correlation findings are displayed in Table 3.3

Table 3. 3: Pearson Correlation matrix of the tomato leaf area, *Metarhizium anisopliae* colony forming unit number, sampling period and tomato variety for season one

		Weeks	Tomato variety	leaf area	colony number
Weeks	Pearson Correlation	1	.000	.982**	.803**
	Sig. (2-tailed)		1.000	.000	.000
Tomato varierty	Pearson Correlation	.000	1	.060	.563**
	Sig. (2-tailed)	1.000		.514	.000
leaf area	Pearson Correlation	.982**	.060	1	.823**
	Sig. (2-tailed)	.000	.514		.000
colony number	Pearson Correlation	.803**	.563**	.823**	1
	Sig. (2-tailed)	.000	.000	.000	

**. Correlation is significant at the 0.01 level (2-tailed). Pearson correlation coefficient r value |<0.3| are considered negligible, |0.3≤ r <0.5| is deemed to be weak, and |0.5≤ r <0.7| is moderate, while |0.7≤ r <0.9| is strong.

The findings in the second season of the study had a similar pattern to the one observed in the first season. No significant differences in leaf area of all tomato varieties were recorded during the sampling period. Riogrande still had the largest leaf area recorded during the entire experiment duration while Money maker variety had the least leaf area. Money maker had the lowest leaf area throughout the sampling period (Table 3.4).

Table 3. 4: **Mean tomato leaf area of five selected tomato varieties in season two**

	WEEKS							
Tomato	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Moneymaker	760.7a	875.4a	994a	1179a	1379a	1577a	1680a	1786a
Cal J	779.9a	904.6a	1035a	1214a	1407a	1588a	1682a	1800a
Eden	791.9a	915.8a	1042a	1222a	1412a	1604a	1705a	1807a
M82	802.1a	939.2a	1056a	1234a	1420a	1612a	1713a	1822a
Riogrande	828.9a	958.9a	1062a	1252a	1446a	1632a	1740a	1844a
CV	7.40	7.80	7.00	5.60	4.40	4.00	4.60	3.60
LSD	107.10	130.80	132.40	123.20	112.50	117.60	141.90	119.40

Means with similar letters in the same column are not significantly different (P<0.05)

All tomato varieties retained viable conidia of *Metarhizium anisopliae* on their leaves. In season two, there were significant (p<0.05) differences in the number of colonies retained by all the tomato varieties between the first and fourth week of sampling. The *M. anisopliae* conidia retained by Rio grande, Eden and Cal J varieties were significantly (p<0.05) different throughout the eight weeks of observation. *Metarhizium anisopliae* conidia retained by Riogrande was the highest followed by M82 and Eden varieties in the second and third place, respectively. Moneymaker had the least number of *Metarhizium anisopliae* conidia per leaf (P<0.05). These results are presented in Table 3.5.

The findings in the second season showed a similar pattern to that observed in the first season. A strong positive correlation was observed between the colony number formed on the leaf and the leaf area (**0.820****). The leaf area and the varieties had a weak positive correlation (**0.062****). There was a strong positive correlation between the colony forming units retained on the leaf and the number of sampling weeks (**0.798****). The Pearson correlation results are displayed in Table 3.6.

Table 3. 5: **Mean colony numbers retained on tomato leaves of five selected tomato varieties season two**

	WEEKS							
Tomato	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Moneymaker	33.7 a	54.7 a	58.7a	74.3a	86.3a	126.3a	142.3a	154.7a
Cal J	53.7 b	78.7 b	97.3b	113.3b	116.3b	143.0ab	176.3b	195.7b
Eden	66.7 c	92.7 b	106.3c	127.0c	122.3b	152.0b	188.0b	196.3b
M82	86.7 d	122.7c	142.3d	159.0d	165.3c	190.7c	212.0c	223.7c
Rio grande	99.7 e	134.7c	159.3e	180.3e	183.3d	204.0c	229.7d	238.7c
CV	2.20	5.90	1.90	2.10	3.20	4.40	2.70	3.70
LSD	2.78	10.34	3.96	5.015	7.96	13.01	9.45	13.63

Means with similar letters in the same column are not significantly different (P<0.05).

Table 3. 6: **Pearson Correlation matrix of the tomato leaf area, *Metarhizium anisopliae* colony number, sampling period and tomato variety for season two**

		Weeks	Tomato variety	leaf area	colony number
Weeks	Pearson Correlation	1	.000	.982**	.798**
	Sig. (2-tailed)		1.000	.000	.000
Tomato varierty	Pearson Correlation	.000	1	.062	.574**
	Sig. (2-tailed)	1.000		.503	.000
leaf area	Pearson Correlation	.982**	.062	1	.820**
	Sig. (2-tailed)	.000	.503		.000
colony number	Pearson Correlation	.798**	.574**	.820**	1
	Sig. (2-tailed)	.000	.000	.000	

**. Correlation is significant at the 0.01 level (2-tailed). Pearson correlation coefficient r value |<0.3| is considered negligible, |0.3≤ r <0.5| is deemed to be weak, and |0.5≤ r <0.7| is moderate, while |0.7≤ r <0.9| is strong.

3.4 Discussion

There was an increase in the leaf area during the assessment period of eight weeks. The number of *Metarhizium anisopliae* colonies on the leaf plated on Potato Dextrose Agar increased from week one to week eight. The Pearson correlation confirms a positive relationship of leaf area and the number of *Metarhizium anisopliae* colonies on the leaf. Pearson correlation also demonstrates a strong positive relationship of the sampling period and number of *Metarhizium anisopliae* colonies. These findings are similar to the ones of Guinossi *et al.* (2012) who reported, that the conidia of *Metarhizium anisopliae* can be retained on plant leaves. A similar study by Bamisile *et al*. (2020) on Citrus limon leaves showed that endophytic activities of *M. anisopliae* were observed in plant tissues after inoculation.

This study showed that all the tested tomato varieties retained *Metarhizium anisopliae* conidia. *Metarhizium anisopliae* conidia germinate to form a germ tube that penetrates plant tissues leading to more retention through systemic colonization (Batta, 2013). Elena *et al.* (2011) reported high endophytic activities in tomato leaves after inoculation with *Metarhizium anisopliae* conidia. The increase in the number of colonies resulted from the repeated application of *Metarhizium anisopliae*. An alternative evaluation should be done to determine whether tomato leaves can support the multiplication of *Metarhizium anisopliae* conidia by using tomato leaf extracts to culture the fungi (Inyang *et al.*, 1999).

3.5 Conclusions

The tomato varieties Eden, Rio grande, M82, Money maker and Cal-j retained *Metarhizium anisopliae* conidia applied on the leaves at seven days intervals. The conidia on the leaves germinated when placed on PDA media. The tomato leaf area influences the number of conidia retained by the leaf where an increase in leaf area causes an increase in the number of conidia retained. Repeated application causes cumulative retention.

CHAPTER FOUR

EFFECTS OF SELLECTED ADJUVANTS, NONYLPHENOL ETHOXYLATE 15%, TWEEN 80 AND LIQUID SOAP ON INCUBATED *Metarhizium anisopliae*

ABSTRACT

The objective was to test the effect of three adjuvants, Nonylphenol ethoxylate 15%, Tween 80, and liquid soap on radial growth of *Metarhizium anisopliae* ICIPE 69 and ICIPE 78. Each adjuvant was diluted into three concentrations, 0.5ml of adjuvant in l Liter of sterile distilled water, 1.0ml of adjuvant in l Liter of clean filtered water, and 3.0 ml of adjuvant in l Liter of sterile distilled water compared with control. Five (5) millimetre diameter *Metarhizium anisopliae* mycelial discs obtained from pure cultures grown in the laboratory were soaked in the adjuvants for 30 minutes before plating on Potato Dextrose Agar. The isolate ICIPE 69 showed the following findings at the end of the study, a 35.8mm diameter was recorded in the Nonylphenol ethoxylate 15% experiment, while the control had 16.5mm. An increase in diameter to 33.4mm was recorded in Tween 80 experiments, while the control had 17.5mm. There was no change in diameter in the liquid soap experiment where the diameter of the mycelial discs remained at 5mm. Similar results were recorded in experiments with ICIPE 78 isolate where the mycelial disc diameter increased to 29.3mm in Nonylphenol ethoxylate 15% experiment and 32.9mm in Tween 80 while the controls recorded 19.1mm and 16.2mm, respectively. Based on these findings, Nonylphenol ethoxylate 15% and Tween 80 do not affect ICIPE 69 and ICIPE 78 isolates and the adjuvants can be used to formulate *Metarhizium anisopliae* before application.

4.1 Introduction

Adjuvants are substances that cause modification of other compounds without directly causing any effect on the attributes. They are mainly added to pesticides to increase the efficacy of the active ingredients (Nobels *et al.,* 2011). Classification of adjuvants is done by; effect they cause in each step of spray application, the function of the adjuvant, and their chemical class (Hazen, 2000). The first category is divided into utility modifiers, spray modifier adjuvants, or activator adjuvants. The classification due to function can further be subdivided based on uses such as stabilization, defoaming and antifoaming, buffering, wetting, spreading and sticking (Duke and Powles, 2008).

Environmental factors like temperature, moisture, UV light, and pH affect germination and growth of *Metarhizium anisopliae* (Zimmermann, 1982). High temperatures cause delayed conidia germination of the *M. anisopliae* (Zimmermann, 1982). Thermal death point of *M. anisopliae* is related to the moisture level of the conidia (Zimmermann, 1982). The higher the moisture level, the higher the thermal death point because of this relationship (Zimmermann, 1982). The optimum temperature for *M.* is 25^{0}C; any further increase in temperature beyond this causes a decline in the rate of change (Ekesi *et al.,* 1999). According to a study conducted by Athanassiou *et al.* (2017), temperature can influence the virulence of *M. anisopliae* against some pests. According to the same authors, *M. anisopliae* became more virulent to the larvae of *Ephestia kauhniella* when the temperature increased to 30^{0}C. Some conidia of *Metarhizium anisopliae* are more tolerant to UV radiation, while for others, the radiation causes inhibited growth (Zhao *et al*., 2016). Ultraviolet – A (UV-A), UV-B radiation, and sunlight heat are the main environmental factors which reduce the efficacy of entomopathogens in the field (Rangel *et al.,* 2005). Exposure to UV-B radiation for 24 hours reduces germination of *M. anisopliae* by up to 95% (Rangel *et al.,* 2005). The pH level in the environment influences the enzymatic expression of *Metarhizium anisopliae* during host penetration (Leger *et al.,* 1998). At pH 3 to 4, high amounts of proteolytic and chitinolytic enzymes are released by the *Metarhizium anisopliae*. These enzymes cause the degradation of the host's cuticle (Leger *et al.,* 1998).

Moisture is essential for conidial germination, but exposure to very high moisture content hinders the growth of *Metarhizium anisopliae* (Hallsworth and Magan, 1999). On the other hand, when the environmental temperatures are beyond the optimum requirement, the virulence of *Metarhizium anisopliae* against a host like *African Tephritid* fruit flies reduces with each level of temperature increase (Dimbi *et al.,* 2004).

Oil-based formulations do not cause any adverse effects on *Metarhizium anisopliae* conidial germination (Alves *et al.,* 2002). When refined, paraffinic oils are mixed, they give very high conidia germination. Equally, when the shells and Ondina oils (refined paraffinic oils) are applied individually, they also yield high conidia germination after 24h of incubation (Alves *et al.,* 2002). When peanut oil, Tween 80 (Polyoxyethylene Sorbitan Monooleate), or Agral are used during formulation, conidial germination of up to 99% can be obtained after 24h and 48h (Alves *et al.,* 2002).

Several types of oils used as adjuvants show compatibility with *Metarhizium anisopliae* (Ummidi and Padmaja, 2014). These oil formulations are almond oil and gingerly at 1, 2, and 3%. Mustard oil, sunflower, castor, and coconut oil are compatible with *Metarhizium anisopliae* at a low concentration of 1%. Any higher concentration becomes toxic (Ummidi and Padmaja, 2014). Neem oil, in particular, increases the virulence and persistence of *Metarhizium anisopliae* under field and laboratory conditions (Gomes et al., 2015).

Polyoxyethylene Sorbitan Monooleate (Tween 80) is a non-ionic surfactant; other non-ionic surfactants are Tween 20 and Tween 40 (Mohajeri and Noudeh, 2012). On the other hand, soap is classified as an ionic surfactant and others like bile salts (Gloxhuber, 1974). Nonylphenol ethoxylate(NPnEO) is a non-ionic surfactant with a wide range of use (Maguire, 1999). Several studies have been carried out to determine effects of Tween 80 and NPnEO on characteristics of *Metarhizium anisopliae*. None have specifically focused on impact of the concentrations on radial growth of the fungi (Langdon *et al.*, 2012).

4.2 Materials and methods

4.2.1 Treatment description

This study was conducted on two commercial *Metarhizium anisopliae* strains (ICIPE 69 and ICIPE 78). For each strain, the effect of the three adjuvants, Nonylphenol ethoxylate 15%, Tween 80 and liquid soap on radial growth was assessed. The investigation of the three adjuvants was done concurrently. In each experiment, mycelial discs of 5 mm diameter were obtained from pure culture of *Metarhizium anisopliae*. The discs were cut using a cork borer. Each experiment had four treatments. The cultures were soaked for 30 minutes before plating onto PDA media. T1; contained 0.5 mL of the adjuvant in 1000 mL of distilled sterile water. T2; contained 1.0 mL of the adjuvant in 1000 mL of distilled sterile water. T3; contained 3.0 mL of the adjuvant in 1000 mL of distilled sterile water.T4; was the control experiment containing *Metarhizium anisopliae* only. The procedure follows that of Chandler et al. (2016) with modifications. *Metarhizium anisopliae* discs of 5.0 mm were excavated from pure cultures using a cork borer. After soaking, the fungal disks were placed on PDA before incubating at 270C. The diameter covered by the fungus (the radial growth) was measured with a millimeter ruler every 24h for ten days. The bases of the plate were divided into four quarters which allowed the calculation of an average diameter even when the growth was irregular. Three petri plates were used in each treatment, with each plate acting as a replicate. The experiment design was CRD, and the procedure follows that of Gabiatti *et al*. (2006) with modifications.

4.2.2 Morphological identification of *Metarhizium anisopliae* cultures and data collection

The characteristics of the different strains of *Metarhizium anisopliae* growing on PDA were evaluated following Fernandes *et al*. (2010) method. During the 21 days of the experiment, the growth parameters were observed regularly for the following factors; colony size and conidial mass color. The mycelial color, shape, and color of conidia were honored at the end of the experiment using a light microscope (Ayele *et al*., 2020).

4.2.3 Data analysis

Data analysis for the effects of Nonylphenol ethoxylate 15%, Tween 80 and liquid soap on the radial growth of *Metarhizium anisopliae* was done according to procedure. Mean diameters of the cultures were calculated using ANOVA, and the significant differences between the means were compared using a Fisher's protected LSD at a probability level of 0.05. The difference between the growth rates was also compared (Raypuriya and Bhowmick, 2019).

4.3 Results

4.3.1 Pure culture growth

On culturing *Metarhizium anisopliae* characteristics of ICIPE 78 and ICIPE 69 were observed. Figure 4.1 shows the attributes of ICIPE 78 and ICIPE 69 when germinated on PDA media.

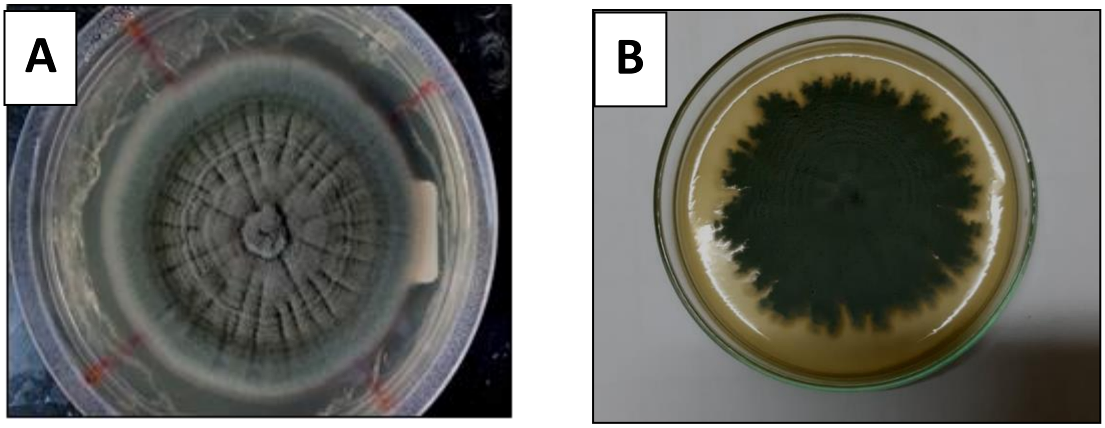

Fig 4. 1: Image (a) pure culture of *Metarhizium anisopliae* strain ICIPE 78 image (b) *Metarhizium anisopliae* strain ICIPE 69

4.3.2 Effect of adjuvants on *Metarhizium anisopliae* ICIPE 69 radial growth

Radial growth significantly ($p<0.05$) increased with time for the 10 weeks of observation compared to control. The 3ml concentration had the highest mean radial growth/diameter and was significantly different ($p<0.05$) from the control and the rest of the concentrations except in the 9th week where it compared to that of 1ml concentration. The control had the least radial growth followed by 0.5ml concentration (Table 4.1).

The diameter of *Metarhizium anisopliae* (ICIPE 69) in all the treatments enlarged during the study period, but at varying rates. The treatment containing 3.0 (Nonylphenol ethoxylate 15%) mL per Liter of water had the fastest radial growth with the highest diameter (35.2 mm) recorded in week 10 of the measurement period. According to table 7, the longest diameter recorded in the treatment containing 1.0 (Nonylphenol ethoxylate 15%) mL per Liter of water was 32.7mm, while the treatment containing 0.5 (Nonylphenol ethoxylate 15%) mL per Liter of water had 28.4mm as the longest diameter recorded. Control treatment containing water only had the lowest radial growth compared to other treatments. Although the rate of increase in the diameter varied depending on the treatment, the mean treatment of 0.5 (Nonylphenol ethoxylate 15%) mL per Liter of water (lowest concentration) was not significantly different from that of treatment 1 ml(Nonylphenol ethoxylate 15%) per Liter of water (medium concentration). The treatment means of 0.5 (Nonylphenol ethoxylate 15%) mL per Liter of water was however significantly different from other treatments as recorded in Table 4.1.

Table 4. 1: Mean radial growth of *Metarhizium anisopliae* ICIPE 69 (mm) as affected by Nonylphenol ethoxylate 15% in experiment one

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml NPnEO per Liter of water	5.0a	9.3ab	11.6a	14.4a	16.7b	18.5b	20.7b	22.3b	25.5b	28.4b
1.0 ml NPnEO per Liter of water	5.0a	13.3b	17.4b	16.1c	19.3c	21.3c	23.7c	26.8c	29.27c	32.7c
3.0 ml NPnEO per Liter of water	5.0a	11.1ab	17.4b	19.9d	22.6d	24.3d	27.8d	29.6d	31.3c	35.2d
Water	5.0a	6.3a	7.6a	8.7a	9.5a	10.4a	12.7a	13.5a	14.3a	16.5a
CV	**	18.40	13.90	3.40	4.10	4.00	3.50	2.40	3.70	1.40
LSD	**	3.46	3.53	0.93	1.31	1.40	1.42	1.06	1.74	0.72

Means with similar letters in the same column are not significantly different ($P<0.05$).

In second season of experiment, the diameter increased over time and 3ml with longest mean diameter was significantly ($p<0.05$) higher than control. Two lower concentrations significantly differed ($p<0.05$) from control from the third week of evaluation. Control had the smallest diameter followed closely by the means recorded in 0.5ml concentration (Table 4.2). During the same period, the diameter of *Metarhizium anisopliae* in all the treatments enlarged, although at different rates. The treatment containing 3.0 ml (Nonylphenol ethoxylate 15%) per Liter of water had the fastest radial growth with the longest diameter (35.8mm) recorded on the tenth week of sampling. The longest diameter recorded in the treatment containing 1.0 ml (Nonylphenol ethoxylate 15%)

per Liter of water was 32.5mm and that of 0.5 ml (Nonylphenol ethoxylate 15%) per Liter of water had 28.3 as the longest diameter recorded. The control treatment with distilled water only, had the lowest radial growth compared to all the other treatments. The mean diameters recorded in 0.5 ml (Nonylphenol ethoxylate 15%) per Liter of water were significantly ($p<0.05$) higher than those of control and the other treatments (Table 4.2).

Table 4. 2: Mean radial growth of *Metarhizium anisopliae* ICIPE 69 (mm) as affected by Nonylphenol ethoxylate 15% in experiment two (repeat)

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml NPnEO per Liter of water	5.0a	9.0b	11.8b	14.6b	16.5b	18.4b	20.3b	22.6b	25.6b	28.3b
1.0 ml NPnEO per Liter of water	5.0a	13.6c	15.33c	16.4c	19.7c	21.4c	23.5c	26.6c	28.3c	32.5c
3.0 ml NPnEO per Liter of water	5.0a	14.6c	17.4d	19.3d	22.5d	24.6d	27.3d	29.4d	31.3d	35.8d
Water	5.0a	7a	8.6a	9.5a	10.5a	12.3a	15.7a	16.5a	17.3a	19.6a
CV	**	18.4	4.7	2.7	3.8	2.2	2.7	1.2	1.5	1.6
LSD	**	1.02	1.19	0.77	1.24	0.79	1.09	0.52	0.74	0.89

Means with similar letters in the same column are not significantly different ($P<0.05$).

Diameter of *Metarhizium anisopliae* cultures increased over time in first assay and the growth significantly differed ($p<0.05$) from control. The concentration 3.0 mL (Tween 80) per Liter of water had the longest diameter recorded on the 10th week of data collection followed by the diameter of 1.0 mL (Tween 80) per Liter of water and the two differed significantly ($p<0.05$) from one another and from of 0.5 mL (Tween 80) per Liter of water. Control with water only had least diameter recorded at 18.0mm (Table 4.3).

Table 4. 3: Mean radial growth of *Metarhizium anisopliae* ICIPE 69 (mm) as affected by Tween 80 in experiment one

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml Tween 80 per Liter of water	5.0a	11.0b	11.0b	13.0b	16.0b	17.0b	19.0b	21.0b	24.0b	27.0b
1.0 ml Tween 80 per Liter of water	5.0a	13.0c	15.0c	16.0c	19.0c	20.0c	22.0c	25.0c	26.7c	31.0c
3.0 ml Tween 80 per Liter of water	5.0a	14.0c	17.0d	19.0d	22.0d	24.0d	26.0d	28.0d	30.0d	34.0d
Water	5.0a	8.0a	9.0a	10.0a	11.0a	12.0a	14.0a	15.0a	16.0a	18.0a
CV	**	3.6	5.4	3.2	3.5	3.7	2.3	2.8	2.8	2.4
LSD	**	0.79	1.33	0.87	1.12	1.26	0.87	1.16	1.29	1.22
P	**	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001

Means with similar letters in the same column are not significantly different (P<0.05).

Second season of this experiment, the diameter of *Metarhizium anisopliae* cultures increased over the assessment period and the growth was significantly different (p<0.05) from control. Concentration 3.0 mL (Tween 80) per Liter of water had the longest diameter recorded on the 10th week of data collection followed by the mean diameter of 1.0 mL (Tween 80) per Liter of water. Three different concentrations, 0.5 ml, 1ml and 3.0 mL (Tween 80) per Liter of water significantly (p<0.05) differed during assessment period (Table 4.4).

Table 4. 4: Mean radial growth of *Metarhizium anisopliae* ICIPE 69 (mm) as affected by Tween 80 in run two

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml Tween 80 per Liter of water	5.0a	10.6ab	10.9b	12.5b	15.5b	16.3b	18.6b	20.4b	23.5b	26.5b
1.0 ml Tween 80 per Liter of water	5.0a	12.3b	14.2c	15.8c	18.6c	19.2c	21.7c	24.9c	26.4c	27.87bc
3.0 ml Tween 80 per Liter of water	5.0a	13.5b	13.3c	18.4d	21.7d	23.6d	25.8d	27.7d	29.6d	33.4c
Water	5.0a	7.2a	8.7a	9.5a	10.3a	11.7a	13.8a	14.5a	15.4a	17.5a
CV	**	12.5	6.2	3.3	3.5	5.5	2.6	1.8	3.0	8.5
LSD	**	2.564	1.37	0.87	1.1	1.83	0.99	0.76	1.36	4.21
P	**	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Means with similar letters in the same column are not significantly different (P<0.05).

Liquid soap significantly ($p<0.05$) stopped the growth of Metarhizium *anisopliae*. The diameter of *Metarhizium anisopliae* in 0.5 mL (liquid soap) per Liter of water, 1.0 mL(liquid soap) per Liter of water, and 3.0 mL(liquid soap) per Liter of water was constant at 5.0mm throughout the period of the study while that in water only continued to increase reaching 15.8 mm by the 10th week of assessment (Table 4.5).

Table 4. 5: Mean radial growth of *Metarhizium anisopliae* ICIPE 69 in mm as affected by liquid soap in experiment one

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
1.0 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
3.0 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
Water	5.0a	7.0b	7.9b	8.5b	9.9b	11.0b	12.1b	13.4b	14.7b	15.8b
CV	**	1.8	1.7	6.0	1.6	2.3	2.2	4.2	2.7	3.9
LSD	**	0.19	0.19	0.66	0.19	0.28	0.28	0.56	0.38	0.56
P	**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Means with similar letters in the same column are not significantly different ($P<0.05$).

Similar observations were made during the repeat experiment (second run). Liquid soap significantly ($p<0.05$) stopped growth of Metarhizium *anisopliae*. Diameter of *Metarhizium anisopliae* in 0.5 mL per Liter of water, 1.0 mL per Liter of water and 3.0 mL liquid soap per Liter of water was constant at 5.0mm throughout the period of the study while that in water only, continued to increase reaching 15.6 mm by the 10th week of assessment (Table 4.6).

Table 4. 6: Mean radial growth of *Metarhizium anisopliae* ICIPE 69 in mm as affected by liquid soap in repeat experiment (run two)

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
1.0 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
3.0 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
Water	5.0a	7.4b	8.3b	9.2b	10.6b	11.8b	12.1b	13.5b	14.5b	15.6b
CV	**	2.70	3.40	4.10	10.20	5.20	4.40	2.80	6.10	3.30
LSD	**	0.28	0.38	0.47	1.22	0.66	0.56	0.38	0.85	0.47

Means with similar letters in the same column are not significantly different ($P<0.05$).

4.3.3 Effect of adjuvants on *Metarhizium anisopliae* ICIPE 78 radial growth

The diameter of *Metarhizium anisopliae* in all the treatments increased over time during the study period. Nonylphenol ethoxylate 15% significantly ($p<0.05$) increased *Metarhizium anisopliae* ICIPE 78 radial growth compared to control. The treatment containing 3.0 mL per Liter of water had the fastest radial growth with the longest diameter (24.5mm) recorded during the assessment period followed by the treatment containing 1.0 mL per Liter of water and lastly that with 0.5 mL per Liter of water. The control treatment containing water only had the least radial growth compared to all the other treatments. Although the increase in diameter varied depending on the treatment, the treatment means of 0.5 mL per Liter of water did not differ from that of treatment with 1 mL per Liter of water. The two were significantly ($p<0.05$) higher than the mean diameter recorded in control where water only was used (Table 4.7).

Table 4. 7: Mean radial growth of *Metarhizium anisopliae* ICIPE 78 in mm as affected by Nonylphenol ethoxylate 15% in experiment one

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml NPnEO per Liter of water	5.0a	9.6b	11.7b	12.6b	14.7b	15.8ab	15.3a	17.6b	19.5b	20.5b
1.0 ml NPnEO per Liter of water	5.0a	10.5b	12.2bc	13.3b	15.7bc	16.4b	17.5b	18.4b	20.5b	21.3b
3.0 ml NPnEO per Liter of water	5.0a	12.4c	13.4c	14.7c	16.4c	16.4b	20.3c	21.4c	23.6c	24.5c
Water	5.0a	7.2a	8.6a	10.3a	12.3a	13.5a	14.4a	15.4a	17.5a	18.2a
CV	**	5.40	4.40	2.60	3.20	6.80	4.20	1.90	2.10	3.60
LSD	**	1.01	0.95	0.63	0.88	1.98	1.33	0.65	0.79	1.42

Means with similar letters in the same column are not significantly different ($P<0.05$).

Similar observations were made in the repeat experiment. The diameter of *Metarhizium anisopliae* in all the treatments increased over time and Nonylphenol ethoxylate 15% significantly ($p<0.05$) increased *Metarhizium anisopliae* ICIPE 78 radial growth compared to control. The treatment containing 3.0 mL per Liter of water had the fastest radial growth with the longest diameter recorded during the assessment period followed by the treatment containing 1.0 mL per Liter of water and lastly that with 0.5 mL per Liter of water. The control treatment containing water only had the least radial growth compared to all treatments. Although the increase in diameter varied depending on the treatment, the treatment means of 0.5 mL per Liter of water did not differ from that of treatment with 1 mL per Liter of water except in week 7 and 10. The two were significantly higher than mean diameter recorded in control where water only was used (Table 4.8).

Table 4. 8:Mean radial growth of *Metarhizium anisopliae* ICIPE 78 (mm) as affected by Nonylphenol ethoxylate 15% in experiment two (repeat run)

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml NPnEO per Liter of water	5.0a	9.6b	11.6b	12.4b	14.8b	15.5b	15.4b	18.4b	19.4b	21.2a
1.0 ml NPnEO per Liter of water	5.0a	10.5b	12.1b	13.4bc	15.4b	16.6bc	17.6c	18.8b	20.7b	24.9b
3.0 ml NPnEO per Liter of water	5.0a	12.5c	13.3c	14.4c	16.6b	17.7c	20.5d	21.6c	23.3c	29.3c
Water	5.0a	7.2a	8.7a	10.5a	12.5a	13.5a	14.4a	15.8a	17.5a	19.1a
CV	**	4.80	3.80	5.00	4.70	3.90	1.50	4.30	2.60	4.80
LSD	**	0.90	0.82	1.18	1.32	1.18	0.49	1.52	0.98	2.13

Means with similar letters in the same column are not significantly different (P<0.05).

Diameter of *Metarhizium anisopliae* in all the treatments increased over time during the study period. Tween 80 significantly (p<0.05) increased *Metarhizium anisopliae* ICIPE 78 radial growth compared to control. The treatment containing 3.0 mL per Liter of water had the fastest radial growth with the longest diameter (32.9mm) recorded during the assessment period followed by the treatment containing 1.0 mL per Liter of water and lastly that with 0.5 mL per Liter of water. The control treatment containing water only had the least radial growth compared to all the other treatments. The increase in diameter varied depending on the treatment and the treatment means of the concentrations significantly (p<0.05) differed among themselves and water. The two were significantly higher (p<0.05) than mean diameter recorded in control where water only was used (Table 4.9).

Table 4. 9: Mean radial growth of *Metarhizium anisopliae* ICIPE 78 in mm as affected by Tween 80 in experiment one

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml Tween 80 per Liter of water	5.0	9.0b	10.8b	12.6b	15.4b	16.5b	17.7b	18.7b	22.2b	23.9b
1.0 ml Tween 80 per Liter of water	5.0	10.4c	13.1c	15.1c	18.6c	18.9c	20.7c	21.8c	26.3c	28.8c
3.0 ml Tween 80 per Liter of water	5.0	12.3d	15.4d	18.2d	20.9d	23.2d	24.7d	27.1d	27.5d	32.9d
Water	5.0	7.2a	7.6a	9.7a	10.4a	11.5a	13.1a	13.5a	14.1a	16.2a
CV	**	4.60	4.00	4.00	3.60	3.70	2.80	4.20	3.80	2.50
LSD	**	0.84	0.89	1.04	1.10	1.22	1.00	1.59	1.61	1.21

Means with similar letters in the same column are not significantly different ($P<0.05$).

Similar observations were made in the repeat experiment. The diameter of *Metarhizium anisopliae* in all treatments increased over time and Tween 80 significantly increased ($p<0.05$) *Metarhizium anisopliae* ICIPE 78 radial growth compared to control. The treatment containing 3.0 mL per Liter of water had the fastest radial growth with the longest diameter recorded during the assessment period. This was followed by the treatment containing 1.0 mL per Liter of water and lastly that with 0.5 mL per Liter of water. The control treatment containing water only had the least radial growth compared to all treatments. Although the increase in diameter varied depending on the treatment, the treatment means of the three test concentrations significantly ($p<0.05$) differed. All three were significantly higher than the mean diameter recorded in control where water only was used (Table 4.10).

Table 4. 10: Mean radial growth of *Metarhizium anisopliae* ICIPE 78 in mm as affected by Tween 80 in run two (repeat experiment)

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml Tween 80 per Liter of water	5.0	9.5b	9.8b	11.3b	14.5b	15.3b	17.7b	19.7b	22.7b	25.4b
1.0 ml Tween 80 per Liter of water	5.0	11.3c	13.2c	14.8c	17.6c	18.2c	20.8c	23.7c	25.3c	29.2c
3.0 ml Tween 80 per Liter of water	5.0	12.5c	15.4d	17.4d	20.7d	22.5d	24.7d	26.9d	28.5d	32.5d
Water	5.0	6.2a	7.7a	8.4a	9.5a	10.5a	12.8a	13.3a	14.4a	16.6a
CV	**	4.8	4.2	3.2	2.9	2.7	2.5	2.2	2.0	2.9
LSD	**	0.89	0.92	0.78	0.85	0.83	0.91	0.87	0.87	1.42

Means with similar letters in the same column are not significantly different $P < 0.05$.

Liquid soap significantly ($p<0.05$) stopped the growth of *Metarhizium anisopliae* ICIPE 78. Diameter of *Metarhizium anisopliae* in 0.5 mL per Liter of water, 1.0 mL per Liter of water and 3.0 mL liquid soap per Liter of water was constant at 5.0mm throughout the period of the study while that in water only, continued to increase reaching 13.4 mm by the 10^{th} week of assessment (Table 4.11).

Similar observations were made during the repeat experiment (second run). Liquid soap significantly ($p<0.05$) stopped the growth of Metarhizium *anisopliae* ICIPE 78. The diameter of *Metarhizium anisopliae* in 0.5 mL per Liter of water, 1.0 mL per Liter of water and 3.0 mL liquid soap per Liter of water was constant at 5.0mm throughout the period of the study while that in water only. continued to increase reaching 12.3 mm by the 10^{th} week of assessment (Table 4.12).

Table 4. 11: Mean radial growth of *Metarhizium anisopliae* ICIPE 78 in mm as affected by liquid soap in experiment one

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
1.0 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
3.0 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
Water	5.0a	6.3b	7.1b	8.3b	9.2b	10.5b	11.3b	11.9b	12.6b	13.4b
CV	**	1.9	4.5	4.3	1.7	6.3	1.5	1.5	5.8	4.2
LSD	**	0.19	0.47	0.47	0.19	0.75	0.19	0.19	0.75	0.56

Means with similar letters in the same column are not significantly different. (P<0.05).

Table 4. 12: Means of the radial growth of *Metarhizium anisopliae* ICIPE 78 in mm as affected by liquid soap in run two

	SAMPLING DURATION IN WEEKS									
TREATMENT	1	2	3	4	5	6	7	8	9	10
0.5 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
1.0 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
3.0 ml soap per Liter of water	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
Water	5.0a	6.1b	6.9b	7.4b	7.9b	8.5b	9.3b	10.8b	11.7b	12.3b
CV	**	1.9	2.7	4.5	2.6	1.7	1.6	0.8	3.7	2.9
LSD	**	0.2	0.3	0.5	0.3	0.2	0.2	0.1	0.5	0.4

Means with similar letters in the same column are not significantly different (P<0.05).

4.4 Discussion

Adjuvants are used to increase the efficacy of the active ingredient of pesticides. Studies have been carried out to determine compatibility of *Metarhizium anisopliae* strains and adjuvants used in Kenya. Two strains of *Metarhizium anisopliae* (ICIPE 78 and ICIPE 69) were used for the laboratory experiment. The leading drawbacks of deploying entomopathogenic fungi for pest and vector control is their short life span under field environment (Gomes, 2016). Various formulations have been developed to increase the persistence of these fungi in field environment (Campos *et al.,* 2016).This study demonstrates that Nonylphenol ethoxylate 15% and Tween 80 positively affected the vegetative growth of both ICIPE 78 and ICIPE 69 strain of *Metarhizium anisopliae*. The study also revealed that the liquid soap hinders the vegetative growth of both ICIPE 69 and ICIPE 78. This study demonstrates that the adjuvants tested about the two strains of *Metarhizium anisopliae* (ICIPE 69 and ICIPE 78) increased growth with increase in concentration. On the flip side, there are shortcomings of the study. Only the vegetative growth of the fungi was evaluated, unlike a similar survey conducted by Polar *et al.*(2005) that evaluated other factors like spore germination and spore concentration. The increase in diameter observed in the organosilicon experiment is attributed to fungal nutrition. The NPnEO surfactant is easily broken down into Nonylphenol (NP) compounds with little persistence in the environment (De Weert *et al.,* 2011). These NP derivatives of NPnEO can be managed by using several types of micro-organisms found in the background (Corvini *et al.,* 2006). *Metarhizium robetsii* species utilize NP from the environment (Rózalska *et al.,* 2013). *Metarhizium anisopliae* also degrades (NP) from the environment (Nowak *et al.,* 2019). The other species of Metarhizium that can also utilize the NP are *Metarhizium majus, Metarhizium guizhouense, Metarhizium lepidiotae* and *Metarhizium globosum* (Nowak *et al.,* 2019).

The results observed in the Tween 80 experiment confirm that *Metarhizium anisopliae* is compatible with Tween 80 and that it can support growth of the fungus as an adjuvant. These findings are similar with those reported with Alves *et al.* (2002)**.** Carolino *et al.* (2014) demonstrates Tween 80 increases the persistence of *Metarhizium anisopliae.* However, further studies need to be done to confirm the effect of Tween 80 on the germination and concentration of *Metarhizium anisopliae* spores (Wu *et al.*, 2010). The soap used in this experiment showed antiseptic properties because no radial increase was observed in all the treatments containing the soap. A different investigation has reported compatibility between *Metarhizium anisopliae* and neem soap (Raypuriya and Bhowmick, 2019). Other types of soap can be evaluated for compatibility with *Metarhizium anisopliae*.

4.5 Conclusions

Results show that the growth characteristics of *Metarhizium anisopliae* are maintained with formulation with Nonylphenol ethoxylate 15% or Tween 80. Ten weeks after cultures are done for *Metarhizium anisopliae* ICIPE 69 and ICIPE 78, growth was robust. Therefore, these adjuvants can be used during the formulation of *Metarhizium anisopliae* for application as a spray on plants.

CHAPTER FIVE

EFFECT OF *METARHIZIUM ANISOPLIAE* IN THE MANAGEMENT OF TOMATO LEAF MINER, *TUTA ABSOLUTA*

ABSTRACT

Tuta absoluta is an invasive tomato pest. The pest infests tomatoes and other Solanaceae plants. It causes direct tomato yield loss of up to 100% in areas where the pest has been established. The pest develops resistance to synthetic pesticides as a result of continuous appliction of chemical pesticides. *Metarhizium anisopliae* is an entomopathogenic fungus that is an alternative in managing arthropod pests. The experiment was conducted to determine the potential of *Metarhizium anisopliae* in managing *Tuta absoluta*. The laboratory assay was conducted using *Tuta absoluta* larvae extracted from tomato fruits. The larvae were treated with *Metarhizium anisopliae* conidia of density, 1.2×10^2 cfu/ml, 1.2×10^3 cfu/ml, 1.2×10^4 cfu/ml and 1.2×10^6 cfu/ml. Data was recorded every 12hrs from the incubated treated larvae for 84 hrs. In the greenhouse an experiment of five treatments; i) *Metarhizium anisopliae* (6.0×10^3 cfu/ml) formulated with Nonylphenol ethoxylate, ii) *Metarhizium anisopliae* (6.0×10^3 cfu/ml), iii) Nonylphenol ethoxylate alone, iv) Indoxacarb 85g/L together with Emmamectin benzoate 15g/L and control was conducted and repeated. Similarly, a field experiment was conducted also with five treatments which were i) *Metarhizium anisopliae* (6.0×10^3 cfu/ml) formulated with Nonylphenol ethoxylate, ii) *Metarhizium anisopliae* (6.0×10^3 cfu/ml), iii) Nonylphenol ethoxylate alone, iv) Indoxacarb 85g/L together with Emmamectin benzoate 15g/L and control was undertaken. The findings show that *Metarhizium anisopliae* significantly ($p<0.05$) caused mortality of *Tuta* larvae and that within 36 hrs of treatment, 1.2×10^6 cfu/ml concentration had achieved 100% mortality. The greenhouse experiment showed that *Metarhizium anisopliae* &

NPnEO's treatment means were lowest. This was not significantly different from the results for *Metarhizium anisopliae* (6.0×10^3 cfu/ml). The outputs of the field experiment showed that the treatment means for Indoxacarb 85g/L and Emmamectin Benzoate 15g/L were the lowest. This was not significantly different from the results for *Metarhizium anisopliae* (6.0×10^3 cfu/ml) formulated with Nonylphenol ethoxylate. The adjuvants can be used to facilitate the distribution of conidia on the plant and furthermore they enhance growth of the fungus on the pests.

5.1 Introduction

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is a tomato leaf miner that was first reported in Kenya in 2013 (Mansour *et al*., 2018). It is an invasive pest whose origin is the South American region. The pest affects tomato production that is done in greenhouses and field environments. *Tuta absoluta* is also reported to affect other Solanaceae crops like potatoes. The adult *Tuta absoluta* female has the potential of laying aprotxymately 250 to 300 eggs (Doğanlar and Yİğİt, 2011). The insect pest undergoes three developmental stages. It has a complete life cycle of aproximately 24 days at 25°C to 38 days at 19°C. The pest has a significant economic effect in places where it is established. Kenya is estimated to be losing between 59.8 million USD and 66.5 million USD annually due to the pest (Venkatramanan *et al*., 2019).

In areas where *Tuta absoluta* has been introduced and established, like in Kirinyaga, Kenya, farmers prefer using primarily synthetic pesticides to manage *Tuta absoluta* pest (Mwangi *et al.*, 2015). This trend is influenced by the fact that they do not have an explicit knowledge of the available alternatives that can be used in Integrated Pest Management IPM (Ochilo *et al.*, 2019). In some places, resistance by *Tuta absoluta* to pesticides that were previously effective has been reported. Lietti *et al.* (2005) demonstrated that *Tuta absoluta* population in Argentina had developed resistance to Deltamethrin. The Italian population of *Tuta absoluta* has shown resistance to chlorantraniliprole and flubendiamide (Roditakis *et al*, 2015).

Emphasis should be placed on IPM rather than the exclusive use of pesticides to reduce the pest's rate of developing resistance to synthetic pesticides. The use of entomopathogenic fungi like *Metarhizium anisopliae* as a biological control agent has proven effective under different conditions (Tadele and Emana, 2017). The infected larvae show symptoms of mycosis within 15 days after treatment. Green sporulation and mycelium can be seen covering the integument of the cadaver (Contreras *et al*., 2014). *Metarhizium anisopliae* is an economical and environmentally friendly method of managing *Tuta absoluta*. *Metarhizium anisopliae* does not affect non-target organisms, and it is compatible with other pest management methods in an IPM system.

5.2 Materials and methods

5.2.1 Selection of the study site

The study was conducted at the University of Nairobi plant pathology laboratory at the Faculty of Agriculture.

5.2.2 Entomopathogenic fungi conidia production

Metarhizium anisopliae fungus was isolated from a commercial product in the Kenyan market containing *Metarhizium anisopliae* strain ICIPE 69. The fungus was multiplied for use in the experiment by plating and sub culturing the fungal colonies obtained from the commercial product on Potato Dextrose Agar (PDA) media to get pure colonies. The cultures were incubated at 25 ± 1^{o}C for 21 days. The conidia were harvested from the Potato Dextrose Agar (PDA) after 21 days by scraping using a sterile surgical blade. The quantification of the conidia in the *Metarhizium* *anisopliae* powder was done by dissolving 0.5 g of the dry conidia powder in 10 ml sterile distilled water containing 0.01% Tween 80. The conidia density of the suspension was determined using a hemocytometer.

5.2.3 *Tuta absoluta* larvae

The second and third instar larvae of *Tuta absoluta* were obtained from infested fruits that were obtained from a greenhouse at the University of Nairobi Field Station (Upper Kabete campus).

5.2.4 Laboratory bioassays of *Metarhizium anisopliae*

The larvae were extracted from the infested tomato fruits using a sterile surgical blade. The live nymphs were immediately placed on a Petri dish lined with moist filter paper. Ten live larvae of the second and third instar were placed on each plate. Five cm diameter Petri dishes lined with a moistened filter paper were used in the lethal concentration (LC_{50}) study. Larvae from the first treatment were inoculated with 2 ml of Metarhizium anisopliae at 1.2 × 102 cfu/ml. The larvae in treatment two were inoculated with 2 ml of *Metarhizium anisopliae* at 1.2×10^3 cfu/ml. The larvae in treatment three were inoculated with 2 ml of *Metarhizium anisopliae* at 1.2×10^4 cfu/ml. The larvae in treatment four were inoculated with 2 ml of *Metarhizium anisopliae* at 1.2×10^6 cfu/ml. The larvae in treatment five were inoculated with 2 ml of water only. The inoculation was done by using a sterile micropipette. The treated larvae were incubated at 24 ± 2^o C. The number of dead larvae was counted and collected within 84 hrs. The collected cadavers were incubated at 24^oC to determine the cause of death. Each dead corpse showed external mycelia growth on the surface, confirming the cause of death. The bioassay process was carried out using a completely randomized design, with the five treatments being replicated three times.

5.2.5 Experimental site description for field and greenhouse experiments

The experimental site was an open field located at the University of Nairobi, Faculty of Agriculture field station. The elevation of the area is approximately 1890m above sea level. It is classified under the Agro-ecological zones, which extend from the eastern slopes of the Nyandarua (Aberdare) Range to the isohyets. Farmers in Kabete can obtain yield with additional irrigation because it is found under the Marginal Coffee Zone. First rain typically starts from mid to end of March. The second rainy season generally begins in mid-October. The soils of this region are moderate to highly fertile (Jaetzold and Schmidt, 1983). In addition, a greenhouse experiment was undertaken in the same location.

5.2.6 Tomato seedlings establishment for greenhouse and field experiment

The tomato plants were established in a nursery bed. The nursery was sited at a place that has no history of planting Solanaceae plants like potatoes in the recent past. After one month in the seedbed, the seedlings were uprooted for transplanting. Plastic pots filled with three kilograms of soil were used to plant the greenhouse's tomato plants. The pots were measuring six miters width by 16miters length. The seedlings had at least four to five leaves; the roots had some soil at transplanting. Phosphate fertilizer was applied after transplanting to enhance root development. Ammonium nitrate and calcium fertilizer was applied to improve leaf formation. A high phosphorus fertilizer was applied at the flowering stage: N-P-K (5-10-5). Irrigation was done in the greenhouse by using a drip irrigation system. Poles and wires were put in place to support the tomato plant during growth. The plants were regularly pruned, to prevent the formation of a humid microclimate, hence reducing the relative humidity within the canopy. Older leaves were pruned after the plant had flowered correctly. This helped the plant to flower well and produced more fruits. The Cal J variety was used because it is susceptible to *Tuta absoluta* and is popular with farmers. *Tuta absoluta* was introduced 21 days after transplanting. The natural infestation of *Tuta absoluta* into the pest was also allowed (Geofrey *et al.*, 2014).

5.2.7 Experimental treatment description for greenhouse experiment

The experiments were conducted to evaluate the effectiveness of *Metarhizium anisopliae* in the management of *Tuta absoluta* under greenhouse conditions. The effect of the combined application of *Metarhizium anisopliae* with Nonylphenol ethoxylate 15% in the management of *Tuta absoluta* was also evaluated. These experiments were compared to Indoxacarb 85g/L and Emmamectin Benzoate 15g/L as a positive standard. Treatment one consisted of *Metarhizium anisopliae* (6.0 x10^3 cfu/ml) and Nonylphenol ethoxylate 15% (1ml/L). Treatment two had *Metarhizium anisopliae* (6.0 $\times 10^3$ cfu/ml) alone. Treatment three had Nonylphenol ethoxylate 15% (1ml/L) only. Treatment four contained Indoxacarb 85g/L and Emmamectin Benzoate 15g/L (0.25ml/L) the positive standard. While treatment five was the control, where only distilled water was applied. The experiment was laid down in a complete randomized design, with each treatment being replicated three times in the greenhouse. Data collected was the damage level caused by the pest, the number of larvae in four leaves, and the yield of damaged and marketable fruits.

5.2.8 Experimental treatment description for the field experiment

The experiments were conducted to evaluate the effectiveness of *Metarhizium anisopliae* in the management of *Tuta absoluta* under field conditions. The effect of the combined application of *Metarhizium anisopliae* with Nonylphenol ethoxylate 15% in the management of *Tuta absoluta* was also evaluated. These experiments were compared to Indoxacarb 85g/L and Emmamectin Benzoate 15g/L. Treatment one contained *Metarhizium anisopliae* (6.0 x10^5 cfu/ml) and

Nonylphenol ethoxylate 15% (1ml/L). Treatment two consisted of *Metarhizium anisopliae* (6.0 x10^5 cfu/ml) alone while treatment three had Nonylphenol ethoxylate 15% (1ml/L) only. Treatment four contained Indoxacarb 85g/L and Emmamectin Benzoate 15g/L (0.25ml/L). While treatment five was the control, with no treatment measures applied to manage *Tuta absoluta*. The experiment was laid down in a randomized complete block design, with each treatment being replicated three times. The data collected was the damage level caused by the pest, the number of larvae in four leaves, and the yield of damaged and marketable fruits.

5.2.9 Data analysis

After inoculation, larval mortality was evaluated for 84 hours at 12 hours. The percent cumulative mortality was calculated by finding the difference between dead and live larvae divided by the original number of larvae multiplied by one hundred.

$$\text{Cumulative mortality} = \frac{\text{number of live larvae} - \text{number of dead larvae}}{\text{10 (starting population)}} \times 100.$$

The cumulative mortality data were assessed for normality using Genstat statistical software 15th edition. The mortality means were analyzed using one-way ANOVA and Turkey's test was used to compare the means and determine the differences in the means for each treatment.

The greenhouse and field experiment data were analyzed using one way ANOVA. The data for leaf damage, number of larvae in four leaves, and damage on fruits were first transformed using ARCSINE($\sqrt{(X}/100$) (Tiago *et al.,* 2011). The transformed data were processed through the analysis of variance (ANOVA) to determine the effects of the treatment. Turkey's test was done to compare treatment means. The data analysis was performed by with the help of Genstat-PC v14.1, 14th Edition.

5.3 Results

5.3.1 Effect of *Metarhizium anisopliae* entomopathogenic fungi (EPF) on the management of tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) under laboratory conditions

The EPF, *Metarhizium anisopliae* significantly ($p<0.05$) caused mortality to Tuta larvae**.** In the first experiment, a conidia density of 1.2×10^6 cfu/ml achieved 100% larval mortality within 36 hours of the sampling period. A conidia density of 1.2×10^4 cfu/ml recorded 100% mortality within 48 hrs and at the third position was 1.2×10^3 cfu/ml, recorded 100% mortality within 84 hrs of assessment. The highest mortality reported in conidia density 1.2×10^2 cfu/ml during the sampling period was 66.7%, and where *Metarhizium anisopliae* was not applied had 50% mortality within the assessment period. Significant differences in the achieved kill between the tested concentrations of *Metarhizium anisopliae* were observed from 12th hour where 1.2×10^6 cfu/ml conidia density, consistently achieved the highest mortality till 100% was achieved. The treatment with the firstest kill effect is conidia density 1.2×10^6 cfu/ml with 100% mortality of *Tuta absoluta* larvae at 36 hrs after application. There were no differences in the mortality caused by 1.2×10^3 cfu/ml, 1.2×10^4 cfu/ml, and 1.2×10^6 cfu/ml 72 hours after treatment application. Close to half of the larvae in the control experiment died naturally after 84 hours (Table 5.1).

Table 5. 1: Mean percentage cumulative mortality of *Tuta absoluta* larvae inoculated with *Metarhizium anisopliae* under laboratory conditions experiment one

	SAMPLING TIME (HRS)						
CONCENTRATION	12	24	36	48	60	72	84
0	6.7a	6.7a	16.7a	26.7a	40.0a	50.0a	50.0a
1.2×10^2 cfu/ml	10.0a	30.0b	30.0b	40.0b	50.0b	56.7b	66.7b
1.2×10^3 cfu/ml	16.7b	26.7b	56.7c	76.7c	93.4c	96.7c	100.0c
1.2×10^4 cfu/ml	20.0b	40.0c	86.7d	100.0d	100.0d	100.0c	100.0c
1.2×10^6 cfu/ml	40.0c	70.0d	100.0e	100.0d	100.0d	100.0c	100.0c
CV%	11.7	17.5	4.9	4.4	4.0	3.1	1.2
LSD	3.99	11.04	5.22	5.55	5.59	4.52	1.86
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Means with similar letters in the same column are not significantly different ($P<0.05$).

The results of the second run were similar to those of the first run experiment. The EPF, *Metarhizium anisopliae* significantly ($p<0.05$) caused mortality to Tuta larvae**.** In the first experiment, a conidia density of 1.2×10^6 cfu/ml achieved 100% larval mortality within 36 hours of the sampling period. A conidia density of 1.2×10^4 cfu/ml recorded 100% mortality within 48 hrs and at the third position was 1.2×10^3 cfu/ml, recorded 100% mortality within 84 hrs of assessment. The highest mortality reported in conidia density 1.2×10^2 cfu/ml during the sampling period was 66.7%, and where *Metarhizium anisopliae* was not applied 50% mortality had taken place during the assessment period. Significant differences in the achieved kill between the tested concentrations of *Metarhizium anisopliae* were observed from 12th hour where 1.2×10^6 cfu/ml conidia density, consistently achieved the highest mortality till 100% was achieved. The treatment with the firstest kill effect is conidia density 1.2×10^6 cfu/ml with 100% mortality of *Tuta absoluta* larvae at 36 hrs after application. There were no differences in the mortality caused by 1.2×10^3 cfu/ml, 1.2×10^4 cfu/ml, and 1.2×10^6 cfu/ml 72 hours after treatment application. Close to half of the larvae in the control experiment died naturally after 84 hours (Table 5.2).

Table 5. 2: Mean percentage cumulative mortality of *Tuta absoluta* larvae inoculated with *Metarhizium anisopliae* under laboratory conditions run two

	SAMPLING TIME (HRS)						
CONCENTRATION	12	24	36	48	60	72	84
0	6.7a	13.4a	26.7a	26.7a	36.7a	46.7a	50.0a
1.2×10^2	10.0ab	30.0bc	33.4a	43.4b	50.0b	56.7b	66.7b
1.2×10^3	13.4b	26.7b	50.0b	76.7c	93.4c	96.7c	100.0c
1.2×10^4	16.7bc	40.0d	83.4c	100.0d	100.0d	100.0c	100.0c
1.2×10^6	40.0d	73.4e	100.0d	100.0d	100.0d	100.0c	100.0c
CV%	19.4	8.0	6.7	3.6	2.8	3.5	8.7
LSD	6.12	5.42	7.18	4.49	4.15	5.21	13.14

Means with similar letters in the same column are not significantly different ($P<0.05$).

5.3.2 Effect of *Metarhizium anisopliae* as a biological control agent in the management of *Tuta absoluta* under greenhouse conditions

Tuta absoluta larvae made entry and exit holes on tomatoes fruits. Sometimes the pest enters and only forms puncture marks on the tomato fruits. The larvae destroyed the tissue between the cuticles developing transluscent windows. The windows on the leaves sometimes had dark frass. Figure 5.1 shows the symptoms of *Tuta absoluta* larvae damage on tomato fruits and leaves.

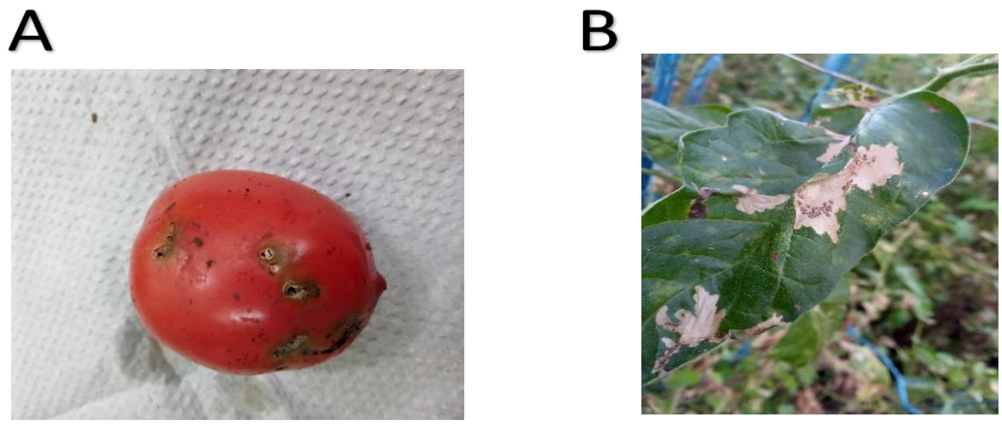

Fig 5. 1: Photo A; Tomato fruit damaged by *Tuta absoluta* larvae. Photo B; Tomato leaf damaged by *Tuta absoluta*.

Treatments significantly differed with control only in the 8th week of observation. All the treatments recorded *Tuta absoluta* populations below those in the control treatment. The control experiment consistently recorded the highest number of larvae. The treatment containing *Metarhizium anisopliae* & NPnEO, *Metarhizium anisopliae,* NPnEO, and Indoxacarb 85g/L & Emmamectin Benzoate 15g/L did not differ in the Tuta larval population recorded. Also, the treatments containing *Metarhizium anisopliae* & NPnEO and those containing *Metarhizium anisopliae* alone did not differ. The treatment containing *Metarhizium anisopliae* & NPnEO had the lowest mean population of Tuta larvae recorded (Table 5.3).

Table 5. 3: Mean number of *Tuta absoluta* larvae affected by *Metarhizium anisopliae* Nonylphenol ethoxylate under greenhouse conditions (Season one)

	SAMPLING DURATION IN WEEKS							
Treatment	1	2	3	4	5	6	7	8
Metarhizium anisopliae & NPnEO	1.0a	1.7a	2.0a	1.3a	1.3a	1.7a	2.7a	1.3a
Metarhizium anisopliae	1.3a	2.0a	2.3a	1.7a	1.7a	2.0a	2.3a	1.7a
NPnEO	1.7a	2.3a	2.7a	2.3a	2.3a	2.3a	2.7a	2.0ab
Indoxacarb 85g/L & Emamectin Benzoate 15g/L	1.7a	2.3a	2.7a	2.3a	2.3a	2.3a	3.0a	2.3b
Control (Water)	2.7b	3.3ab	3.3a	3.0b	2.7a	3.3a	3.3a	3.0bc
CV%	55.9	35	46.6	43.6	41.4	48.2	18.4	21.6
LSD	1.69	1.49	2.20	1.69	1.56	2.05	0.94	0.81

Means with similar letters in the same column are not significantly different ($p<0.05$).

Metarhizium anisopliae significantly ($p< 0.05$) reduced *Tuta absoluta* populations from the 4th week of assessment after treatment application. *Metarhizium anisopliae* & NPnEO had the least *Tuta absoluta* larvae population but comparable to Emamectin benzoate with Indoxacarb, *Metarhizium anisopliae* alone followed closely. All treatments recorded *Tuta absoluta* numbers below those recorded in control in the second season. The treatments containing *Metarhizium anisopliae* & NPnEO and *Metarhizium anisopliae* alone had no differences. Similarly, the treatment containing NPnEO and Indoxacarb 85g/L & Emmamectin Benzoate 15g/L were not different. Like the first season, the treatment containing *Metarhizium anisopliae* & NPnEO had the lowest mean population of Tuta larvae while control recorded the highest number of *Tuta absoluta* larvae consistently throughout the assessment period (Table 5.4.)

Table 5. 4: Mean number of *Tuta absoluta* larvae affected by *Metarhizium anisopliae* Nonylphenol ethoxylate under greenhouse environments (Season two)

	SAMPLING DURATION IN WEEKS							
TREATMENT	1	2	3	4	5	6	7	8
Metarhizium anisopliae & NPnEO	1.0a	2.0a	2.0a	1.3a	1.7a	1.3a	2.3ab	1.0a
Metarhizium anisopliae	1.7a	1.7a	2.7a	1.7a	2.0ab	2.3abc	2.7b	1.7ab
NPnEO	2.0a	2.0a	2.7a	2.7ab	2.7bc	2.3ab	3.0bc	1.7ab
Indoxacarb 85g/L & Emmamectin Benzoate 15g/L	2.0a	2.0a	2.0a	1.7a	1.3a	1.7ab	1.7a	2.3abc
Control (Water)	2.3a	3.4b	3.4a	3.3bc	3.3bc	3.3bc	3.7c	3.7c
CV%	40.6	38.9	30.6	27.1	23.5	33.2	19.4	39.5
LSD	1.33	1.56	1.41	1.05	0.94	1.33	0.94	1.49

Means with similar letters in the same column are not significantly different *($p<0.05$).*

The most damage of tomato fruit was recorded in control (90.24%) which was significantly ($p<0.05$) higher than damage in the treated tomatoes. The NPnEO treatment recorded percentage damage of 60%. The lowest amount of damage was recorded in treatment with Indoxacarb 85g/L & Emmamectin Benzoate 15g/L which was not different from the second lowest that was recorded in *Metarhizium anisopliae* & NPnEO (Table 5.5). The highest total yield and the consequent undamaged fruits was recorded in treatment with Indoxacarb 85g/L & Emmamectin Benzoate 15g/L followed in the second place by the treatment with *Metarhizium anisopliae* & NPnEO. The two were significantly ($p<0.05$) different from each other and the rest of the treatments *Metarhizium anisopliae* alone and NPnEO alone (Table 5.5).

Table 5. 5: Mean weight of the greenhouse tomato fruits damaged and not damaged by *Tuta absoluta* (Season one)

AVERAGE FRUIT WEIGHT (TONES PER HA)				
Treatment	Damaged	Not damaged	Total	% Damaged
Metarhizium anisopliae & NPnEO	3.80ab	7.60d	11.40d	33.30ab
Metarhizium anisopliae	4.30cd	4.90c	9.20c	46.73c
NPnEO	5.10e	3.40b	8.50b	60.00d
Indoxacarb 85g/L & Emmamectin Benzoate 15g/L	4.10abc	9.10e	13.20e	31.05a
Control (Water)	3.70a	0.40a	4.10a	90.24e
LSD	0.37	0.37	0.29	2.28
%CV	4.9	4.0	1.7	2.4

Means with similar letters in the same column are not significantly different ($p<0.05$). Percent damaged tomato fruits= (Damaged fruit weight/total fruit weight) *100. (/ is the division sign, while * is the sign of multiplication).

Similar results were observed in the second season. In the second season, the most damage of tomato fruit was recorded in control (86.4%) which was significantly ($p<0.05$) higher than damage in the treated tomatoes. The NPnEO treatment recorded percentage damage of 62.5%. The lowest amount of damage was recorded in treatment with Indoxacarb 85g/L & Emmamectin Benzoate 15g/L which was not different from the second lowest that was recorded in *Metarhizium anisopliae* & NPnEO (Table 5.6). The highest total yield and the consequent undamaged fruits was recorded in treatment with Indoxacarb 85g/L & Emmamectin Benzoate 15g/L followed in the second place by the treatment with *Metarhizium anisopliae* & NPnEO. The two were significantly ($p<0.05$)

different from each other and the rest of the treatments *Metarhizium anisopliae* alone and NPnEO alone (Table 5.6).

Table 5. 6: Mean weight of the greenhouse tomato fruits damaged and not damaged by *Tuta absoluta* (Season two)

AVERAGE FRUIT WEIGHT (TONES PER HA)				
Treatment	Damaged	Not damaged	Total	% Damaged
Metarhizium anisopliae & NPnEO	4.3b	8.8d	13.1d	32.8ab
Metarhizium anisopliae	4.7bc	5.1c	9.8c	48.0c
NPnEO	5.5d	3.3b	8.8b	62.5d
Indoxacarb 85g/L & Emmamectin Benzoate 15g/L	4.4b	9.3de	13.7e	32.1a
Control (Water)	3.8a	0.6a	4.4a	86.4e
LSD	0.4	0.4	0.3	2.1
%CV	4.5	3.8	1.6	2.2

Means with similar letters in the same column are not significantly different ($p<0.05$). Percent damaged tomato fruits= (Damaged fruit weight/total fruit weight) *100. (/ is the division sign, while * is the sign of multiplication).

5.3.3 Effect of *Metarhizium anisopliae* as a biological control agent in the management of *Tuta absoluta* under field conditions

The findings show that all the treatments that were evaluated namely; *Metarhizium anisopliae* and NPnEO, *Metarhizium anisopliae*, NPnEO and Indoxacarb 85g/L & Emmamectin Benzoate 15g/L varied in effect against Tuta populations had slightly lower populations compared to control. Control treatment recorded the highest mean of *Tuta absoluta* throughout the study period. Treatment containing NPnEO recorded the second-highest mean. The treatments did not differ in effect but Indoxacarb 85g/L & Emmamectin Benzoate 15g/L and *Metarhizium anisopliae* & NPnEO treated tomatoes had slightly lower mean populations. The treatment containing

Metarhizium anisopliae & NPnEO was not significantly different (P<0.05) from treatment containing *Metarhizium anisopliae*. Furthermore, the treatment containing *Metarhizium anisopliae alone* was not different from NPnEO and control (Table 5.7).

Table 5. 7: Mean number of *Tuta absoluta* larvae affected by *Metarhizium anisopliae* Nonylphenol ethoxylate and Indoxacarb combined with Emamectin Benzoate under field conditions

	SAMPLING DURATION IN WEEKS							
TREATMENT	1	2	3	4	5	6	7	8
Metarhizium anisopliae & NPnEO	1.3abc	1.7ab	1.3ab	1.0ab	2.0a	2.0ab	2.3ab	2.0ab
Metarhizium anisopliae	1.3abc	2.0ab	1.7abc	1.7bc	1.7a	2.3abc	2.7bc	2.3ab
NPnEO	2.3c	2.3ab	2.0abc	2.0c	2.0a	2.7abc	3.0c	2.7bc
Indoxacarb 85g/L & Emmamectin Benzoate 15g/L	1.0a	1.3a	1.0a	0.7a	1.7a	1.7a	2.0a	1.7a
Control (Water)	2.3c	3.0b	2.7c	3.0d	3.4a	3.3c	3.7d	3.3c
CV%	33.8	49.2	40.1	26.8	45.3	27.4	13.4	17.8
LSD	1.06	1.91	1.31	0.84	1.82	1.24	0.69	0.81

Means with similar letters in the same column are not significantly different (P<0.05).

Treatments significantly (p<0.05) increased yield compared to control. The yield data recorded in the field showed that highest yield was achieved in Indoxacarb with Emmamectin but it was not different from that of *Metarhizium anisopliae* & NPnEO. The consequent amount of damage from the two treatments was lowest and second lowest, respectively with no significant differences. The highest amount of damage on the tomato fruits occurred in the control plots and the consequent yield was significantly (p<0.05) lower than for treated tomatoes. The amount of damage observed in *Metarhizium anisopliae* & NPnEO was not different from that observed in *Metarhizium* *anisopliae* only and the standard, Indoxacarb 85g/L & Emmamectin Benzoate 15g/L treatment plots (Table 5.8).

Table 5. 8: Mean weight of field tomato fruits damaged and not damaged by *Tuta absoluta*

AVERAGE FRUIT WEIGHT (TONES PER HA)				
Treatment	Damaged	Not damaged	Total	% Damaged
Metarhizium anisopliae & NPnEO	3.8ab	8.6c	12.4c	30.7a
Metarhizium anisopliae	3.2a	5.9b	9.1b	34.8a
NPnEO	5.1cd	4.4b	9.5b	54.2b
Indoxacarb 85g/L & Emmamectin Benzoate 15g/L	4.1bc	9.1c	13.2c	31.0a
Control (Water)	3.7ab	1.4a	5.1a	74.0c
LSD	0.7	1.1	1.4	13.9
%CV	9.2	9.5	7.5	11.7

Means with similar letters in the same column are not significantly different ($p<0.05$). Percent damaged tomato fruits= (Damaged fruit weight/total fruit weight) *100. (/ is the division sign, while * is the sign of multiplication).

5.4 Discussion

5.4.1 Potential of *Metarhizium anisopliae* in the management of tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) under laboratory conditions

Tuta absoluta is infested tomatoes in the experiment. The developmental stages that it undergoes are egg, larvae, pupa and adult moth. The larva is the stage that causes damage while feeding (Mansour *et al.*, 2018). The larvae caused mines in the leaves by feeding on the mesophyll found between the cuticles. The larvae also caused mines in the tomato fruits. These findings are similar to what was reported by Sanda *et al.* (2018). *Metarhizium anisopliae* is an entomopathogenic fungus that has shown high potential for managing *Tuta absoluta* (Ayele *et al.,* 2020). Four different concentrations of *Metarhizium anisopliae* were evaluated. All the concentrations of *Metarhizium anisopliae* caused *Tuta absoluta* mortality. The concentration 1.2×10^6 cfu/ml of

Metarhizium anisopliae caused 100% mortality of *Tuta absoluta* larvae within 36 hours of application.

The incubated cadavers of *Tutu absoluta* showed external germination of green mycelium. This observation and microscopic evaluation confirmed that *Metarhizium anisopliae* was responsible for the death of the larvae. The process through which *Metarhizium anisopliae* infects and kills the host is initiated with adhesion of the conidia onto its host. The exogenous sources of carbon and nitrogen initiate germination of the conidia followed with appresorium formation. During penetration of the larvae, proteins are released to digest the procuticle in order to allow colonization of the host's haemolymph. After *Metarhizium anisopliae* has killed its host, the mycelium extrudes the host and covers the surface in a green mat of mycelium (Aw and Hue, 2017). According to Contreras *et al.* (2014) the effect of *Metarhizium anisopliae* was evaluated on different population of *Tuta absoluta*. A confirmatory test similar to the one described by Gabarty *et al. (2*014) was conducted to ascertain that the larvae that died because of being infected by *Metarhizium anisopliae*. The larvae that died due to infection by *Metarhizium anisopliae* were covered by a mat of green mycelium after incubation similar to what is described by Contrerase *et al.* (2014).

5.4.2 Effect of *Metarhizium anisopliae* as a biological control agent in the management of *Tuta absoluta* within greenhouse and field conditions

Metarhizium anisopliae combined with NPnEO is efficacious in the management of *Tuta absoluta* wihin greenhouse and field environment. A combination of *Metarhizium anisopliae* and NPnEO results in a significantly low *Tuta absoluta* damage on the leaves compared with control and the effect was comparable to standard pesticide emmamectin& indoxacarb. Indoxacarb 85g/L & Emmamectin Benzoate 15g/L and *Metarhizium anisopliae* and NPnEO prevented damage caused on leaves and fruits when compared to control. One limitation of this study is that a laboratory experiment was not conducted to determine effect of NPnEO on larvae of *Tuta absoluta*. Due to this, effect of NPnEO on damage level and population of *Tuta absoluta* larvae reported in treatment containing only NPnEO cannot be directly attributed to the organosilicon. The major strength to this study comparing with experiments that evaluates combined effect of *Metarhizium anisopliae* and the organosilicon adjuvant NPnEO. The finding is consistent with that of Buragohain *et al*. (2021) who reported the efficacy of *Metarhizium anisopliae* against *Tuta absoluta* larvae. Similar findings were also recorded by Michaelides *et al*. (2018) that Indoxacarb and emmamectin are efficacious in management of *Tuta absoluta* population. It compares the effectiveness of different formulations of *Metarhizium anisopliae* to formulate a technique that can deliver the spores without affecting fungal virulence. *Metarhizium anisopliae* and the organosilicon adjuvant NPnEO was as effective as the synthetic product Indoxacarb 85g/L combined with Emmamectin Benzoate 15g/L. Similar experiments only consider the effectiveness of *Metarhizium anisopliae* in the field without adjuvants (Alikhani *et al.*, 2019).

When *Metarhizium anisopliae* is deposited on to the surface of its host, the conidia germinate and penetrates through the cuticle of the host. *Metarhizium anisopliae* then forms hyphal bodies that colonize the hemolymph. The host is killed through depletion of nutrients. After killing the host green mycelium of *Metarhizium anisopliae* emerges from the cuticle and covers the surface of the cadaver (Lovett and Leger, 2015). Emamectin causes paralysis of lepidopteran larvae after ingestion. Emamectin interferes with neuromuscular processes through activation of the chlorine channel causing permanent relaxation of muscles that leads to death and the process is irreversible (Berxolli and Shahini, 2018). Indoxacarb causes inhibition of production of digestive enzymes like amylase, trehalase and invertase in *Tuta absoluta*. These consequently affect the presses of cuticle formation and molting of target pest (Taha and Al-Hadek, 2016).

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General discussion

Tomato (*Solanum lycopersicum* L) is a vegetative crop of high economic and nutrative value in the world (Seisuka and Neelima, 2014) and *Tuta absoluta* is an invasive pest that attacks tomatoes by feeding on the mesophyll of leaves and fruits (Sanda *et al.*, 2018). The larvae mines in leaves and fruits while feeding (Mansour *et al.,* 2018). This study showed that tomato leaves morphology affects the retention and distribution of *Metarhizium anisopliae* conidia. The area of the tomato leaves increased as the crop also developed. Similarly, the *Metarhizium anisopliae* conidia retained on the leaves was increasing. Plant leaf area and the rate of growth influences the retention of the *Metarhizium anisopliae* conidia (Guinossi *et al*, 2012). A study conducted by Inyang *et al.* (1999) showed that plant leaf area influences the distribution of the fungal conidia.

The study showed that Nonylphenol ethoxylate 15% and Tween 80 positively enhanced the growth of *Metarhizium anisopliae* ICIPE 69 and ICIPE 78. While liquid soap negatively affected (stopped) the growth of *Metarhizium anisopliae* conidia. NPnEO has nutritive components that enhance the growth of *Metarhizium anisopliae* because it breaks down to Nonylphenol compounds (NP) (De Weert *et al,* 2011). *Metarhizium anisopliae* utilizes the (NP) components in the environment for nutrients. The experiment also demonstrated that *Metarhizium anisopliae* is compatible with both Nonylphenol ethoxylate 15% and Tween 80 for formulation, results that agree with those of Alves *et a.,* (2002). The soap evaluated in this study was not compatible with *Metarhizium anisopliae* conidia which could possibly be explained by the ions present in it making it an antiseptic. A different study using neem soap demonstrated its compatibility with *Metarhizium anisopliae* for formulation during spraying (Raypuriya and Bhowmick 2019).

The laboratory bio assay showed that conidia density of 1.2 x 10^3 cfu/ml to 1.2 x 10^6 cfu/ml induces 100% death of the larvae. Whereas the lower concentrations take longer up to 48hours to achieve 100% kill, 1.2 x 10^6 cfu/ml achieved the same within 36 hours of application. Germination of green mycelium on the cadavers confirmed that they died as a result of *Metarhizium anisopliae* infection (Gabarty *et al,* 2014). *Metarhizium anisopliae* conidia attach on to the larvae surface, and germination is initiated stimulated by exogenous sources of carbon and nitrogen followed by appressorium formation (Aw and Hue, 2017). The findings in the field and greenhouse experiment agree with what Buragohain *et al.*, (2021) reported that *Tuta absoluta* larvae can be managed by using *Metarhizium anisopliae.*

6.2 Conclusions

The tomato varieties Eden, Rio grande, M82, Money maker and Cal-j retained *Metarhizium anisopliae* conidia applied on the leaves at seven days intervals. The conidia on the leaves germinated when placed on PDA media. The tomato leaf area affected the number of conidia retained by the leaf where an increase in leaf area caused an increase in the number of conidia retained.

Growth characteristics of *Metarhizium anisopliae* are not affected by formulation using Nonylphenol ethoxylate 15% or Tween 80. Ten weeks after cultures were done for *Metarhizium anisopliae* ICIPE 69 and ICIPE 78, growth was robust. Therefore, these adjuvants can be used during the formulation of *Metarhizium anisopliae* for application as a spray on plants. The adjuvants facilitated distribution and sticking of the conidia onto the plant surface.

The assay in the laboratory demonstrated that *Metarhizium anisopliae* is able to infect Tuta larvae killing them between 36-48 hours depending on the concentrations. *Metarhizium anisopliae* is efficacious in managing *Tuta absoluta* larvae under greenhouse and open field conditions and that *Metarhizium anisopliae* conidia formulated with Nonylphenol ethoxylate 15% will manage the population of *Tuta absoluta* under greenhouse and field conditions when applied at 7 days interval reducing populations infesting the crop. The resultant effect is increased yields and low fruit damage.

6.3 Recommendations

- Establish role and mode of action of Tween 80 in supporting growth of *Metarhizium anisopliae* conidia.
- Establish the effect of adjuants on growth characteristics of *Metarhizium anisopliae*.
- Nonylphenol ethoxylate 15% and Tween 80 can be used as a formulant during the application of *Metarhizium anisopliae* conidia as foliar spray.

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