# Management of *Spoladea recurvalis* (Lepidoptera: Crambidae) on amaranths using biopesticides

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## DEDICATION

To my dad, Miller Eshikhoto, son, Craig McMayabi and mum Christine Jepkoech, for your love, support and encouragement.

## ABSTRACT

Amaranths are African indigenous vegetables (AIVs) that are gaining popularity in several countries around the world due to their nutritional, medicinal and economic values. Insect pests are however a major challenge to optimum production of amaranths. The lepidopteran defoliator moth commonly known as Hawaiian beet webworm/amaranth leaf-webber, *Spoladea recurvalis* has been reported to be a major pest in amaranth fields, with the potential of causing complete defoliation under severe outbreaks. The most common management practice for *S. recurvalis* is the use of synthetic insecticides. However, resource-poor farmers cannot afford synthetic insecticides, application of insecticides poses health and environmental risks and indiscriminate use may lead to the development of insecticide resistance. Entomopathogenic fungi (EPF) and *Bacillus thuringiesis* (*Bt*) microbial pesticides have been suggested as the most promising alternatives to synthetic insecticides for management of various pests. This study, therefore evaluated the potential of fungus- and *Bt*-based products for the management of *S. recurvalis.* Twenty-four EPF isolates from three genera (14 *Metarhizium anisopliae,* 9 *Beauveria bassiana* and 1 *lsaria fumosorosea*) were screened in the laboratory to assess their pathogenicity against second instar larvae of *S. recurvalis.* Only *M. anisopliae* ICIPE 30 provided moderate control, causing 58.3% larval mortality. Eleven isolates tested against adult *S. recurvalis, viz.* 8 *M. anisopliae*, 2 *B. bassiana* and 1 *l. fumosorosea*, were pathogenic. *Metarhizium anisopliae* ICIPE 30 and *B. bassiana* ICIPE 725 caused the highest mortality of 92% and 83%, respectively. *Metarhizium anisopliae* ICIPE 30 had the shortest $LT_{50}$ value of 4.8 days. *Bacillus thuringiensis* Subsp. *kurstaki* product Halt® caused between 40 and 50% mortality of *S. recurvalis* larvae. A consecutive application of *M. anisopliae* ICIPE 30 and *Bt* did not cause a significant increase in larval mortality compared to separate applications of both products.

Compatibility of *M. anisopliae* ICIPE 30, the most effective fungal isolate against adult *S. recurvalis* and an attractant, Phenylacetaldehyde (PAA) was investigated under laboratory and field conditions. PAA completely inhibited germination of the conidia when put together in a desiccator in the

laboratory. Effects of spatial separation of conidia and PAA in an autodissemination device were investigated in the field, and results showed that conidial germination was minimized by placing PAA at 5 and 10 cm cm from *M. anisopliae* ICIPE 30 conidia. Horizontal transmission of *M. anisopliae* ICIPE 30 between freshly emerged moths inoculated with fungal conidia (‘‘donors”) and untreated freshly emerged moths (“recipients”) maintained together for 24 hours was investigated in laboratory tests. Infected moths were able to transmit the infection to untreated moths resulting in 76.9% mortality with a $LT_{50}$ value of 6.9 days. To improve the efficacy of *B. thuringiensis* Halt® against *S. recurvalis* larvae, 13 chemical additives (7 inorganic salts, 3 nitrogenous compounds, 2 protein solubilizing agents and 1 organic acid) were investigated. Boric acid was found to be the most effective additive and enhanced the potency of *Bt* by 2.9-fold. Boric acid and *Bt* could therefore be integrated in *S. recurvalis* IPM to reduce the use of synthetic insecticides in amaranth production systems.

**Key words:** African indigenous vegetables, entomopathogenic fungus, phenylacetaldehyde, Halt®, chemical additives, autodissemination, chemical insecticides, biopesticides.

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## PREFACE

This thesis follows the article format style as prescribed by the North-West University. Therefore, articles appear in published format, while manuscripts are adjusted according to the instructions to authors of internationally accredited, scientific journals. As an additional requirement by the North-West University, Table A details the contributions of authors for each article/manuscript and provides permission for use as part of this thesis.

The following Chapters were included in this work:

Chapter 1 – Introduction **(NWU Harvard, Reference Style of the Faculty of Law and APA,** published **by the Library Services of the NWU)**

Chapter 2 – Literature review **(NWU Harvard, Reference Style of the Faculty of Law and APA, published by the Library Services of the NWU)**

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Chapter 5 – Article 3 (prepared): **Journal of Microbial Pathogenesis (Elsevier)**

Chapter 6 General discussion, conclusions and recommendations **(NWU Harvard, Reference Style of the Faculty of Law and APA, published by the Library Services of the NWU)**

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**Table A:** Contributions of authors and consent for use

| Author | Article | Contribution | Consent |
|---|---|---|---|
| SO Miller | 1, 2 and 3 | Principal investigator, conducted experiments, acquisition of data, analysis and interpretation of data and wrote the manuscripts. | |
| MJ Du Plessis | 1, 2 and 3 | As promotor, supervised the design and execution of the study. Provided intellectual input on data analyses and writing of articles and thesis. | |
| KS Akutse | 1, 2 and 3 | As co-promoter, supervised the design and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis. | |
| KKM Fiaboe | 1,2 and 3 | Provided intellectual input on data analyses and writing of articles. Secured funding for the project. | |
| S Ekesi | 1, 2 and 3 | As Co-promoter, supervised the design and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis. Secured funding for the project. | |

# CHAPTER 1

# General introduction

## 1.1 Introduction

Food and nutritional security are achieved when people are able to grow or buy enough safe food to meet their daily needs for an active and healthy life (Food and Agriculture Organization *et al.*, 2013). In many African countries these conditions are threatened and malnutrition is rampant (Food and Agriculture Organization and International Fund for Argricultural Development, 2014, Food and Agriculture Organization *et al.*, 2015b). Fruits and vegetables are the richest natural sources of micronutrients. However, in developing countries, daily fruit and vegetable consumption is between 20-50% only (Ruel *et al.*, 2005, Siegel *et al.*, 2014).

In Sub-Saharan Africa (SSA), almost 33% of the population (200 million people) are undernourished (Food and Agriculture Organization *et al.*, 2014, Food and Agriculture Organization *et al.*, 2015a). In Kenya, 35% of children under five years are stunted, 16% are underweight and 7% are undernourished. In addition, 25% of women in their reproductive years (15-49 years old) have also been reported to be either overweight or obese (Kenya National Bureau of Statistics, 2015). Malnutrition and food insecurity present a challenge and an opportunity for the utilization of African indigenous vegetables (AIVs) such as amaranths. These vegetables can provide essential vitamins and minerals that are lacking in the diets, thus improving the health and livelihoods of the rural as well as the underprivileged urban poor groups (Schippers, 2000, Abukutsa-Onyango *et al.*, 2010, Alegbejo, 2013, Ojiewo *et al.*, 2013, Kaaya *et al.*, 2017).

The demand for amaranth vegetables has increased rapidly in both the rural and domestic urban markets in recent years (Abukutsa-Onyango, 2002). It is high in nutritional and medicinal values, since it is rich in vitamins A, B and C, calcium, iron, potassium, ascorbic acid and also provides an alternative source of vegetable protein (Grubben and Denton, 2004, Mlakar *et al.*, 2010, Ojiewo *et*

*al.*, 2013, Achigan-Dako *et al.*, 2014, Mbwambo *et al.*, 2015, Kaaya *et al.*, 2017). Amaranth seeds also contain a high content of Lysine, Arginine and Histidine that are good dietary supplements for the treatment of child malnutrition (Emire and Arega, 2012, Muriuki *et al.*, 2014). Amaranth grains are used to produce unsaturated oil, high in linoleic acid, which is safe for consumption by individuals that are at high risk of chronic non-communicable diseases such as coronary heart disease and diabetes (Martirosyan *et al.*, 2007, Ebert *et al.*, 2011, Alegbejo, 2013, Muriuki *et al.*, 2014, Kaaya *et al.*, 2017). Linoleic acid is also needed by children as they utilise essential fatty acids for proper growth and development (Kaaya *et al.*, 2017).

Despite the high potential value of amaranth in human nutrition and health, its production is hampered by a complex of insect pests of which lepidopteran defoliators are ranked as the most destructive (James *et al.*, 2010, Ebert *et al.*, 2011). The Hawaiian webworm, *Spoladea recurvalis* (Fabricius) (Lepidoptera: Crambidae) is the most important lepidopteran species that affects amaranth production (James *et al.*, 2010, Kahuthia-Gathu, 2011, Aderolu *et al.*, 2013, Mureithi *et al.*, 2017, Othim *et al.*, 2018). Infestations by *S. recuvalis* larvae significantly affects productivity and quality through direct feeding, contamination with their faecal matter, and webbing of leaves (James *et al.*, 2010, Kahuthia-Gathu, 2011). High larval infestation levels may result in 100% yield losses if no control measures are taken (Kahuthia-Gathu, 2011, Aderolu *et al.*, 2013).

### 1.2. Problem statement and justification

Chemical control with insecticides is the most common management practice against vegetable pests including *S. recurvalis* despite their toxicity and hazardous effects on humans and the environment (Clarke-Harris *et al.*, 2004, Badenes-Perez and Shelton, 2006, Ngowi *et al.*, 2007, James *et al.*, 2010, Aderolu *et al.*, 2013). In addition, excessive use of synthetic insecticides provide selection pressure for resistance development and eliminates potential natural enemies of the target pest (Clarke-Harris and Fleischer, 2003). Increasing concerns from consumers and retailers of agricultural produce about the adverse effects of chemical insecticides, and the restrictions to

lucrative markets due to maximum residue level (MRL) has fostered an effort to find alternative methods for management of insect pests that are ecologically sound and sustainable (Ravensberg, 2011, Ravensberg, 2015). Biological control using entomopathogenic fungi (EPF), parasitoids, attractants and *Bacillus thuringiesis* (*Bt*) are among the key potential alternatives that are being developed at the International Centre of Insect Physiology and Ecology (*icipe*) to manage amaranth pests under the African Indigenous Vegetables-IPM project.

The aim of this study was therefore to evaluate potent fungal and *Bt* biopesticides to integrate a final candidate product with the commercially available moth attractant Phenylacetaldehyde (PAA) for the management *S. recurvalis*.

### 1.3 Objectives

#### 1.3.1 General objective

The general objective of this study was to evaluate the efficacy of candidate fungal and *Bt*-based biopesticides and to integrate the most potent product with a commercially available moth attractant, Phenylacetaldehyde (PAA) for the management *S. recurvalis* on amaranth.

#### 1.3.2 Specific objectives

1. Evaluate the effects of selected EPF isolates and one commercial *B. thuringiensis* Subsp. *kurstaki*-based product, Halt®,on immature and adult stages of *S. recurvalis.*
2. Evaluate the possibility of horizontal transmission of *M. anisopliae* ICIPE 30 (most potent fungal isolate) between infected and non-infected *S. recurvalis* adults and to test its compatibility with the PAA attractant.
3. Assess the role of various low-cost chemical additives in enhancing the efficacy of selected *Bt*-based products under laboratory conditions.

### 1.3.3 Research Hypotheses

1. Fungal and *Bt*-based biopesticides are pathogenic to *S. recurvalis.*
2. Horizontal transmission of *M. anisopliae* ICIPE 30 occurs between *S. recurvalis* moths and Phenylacetaldehyde (PAA) and *M. anisopliae* ICIPE 30 are compatible.
3. Low-cost chemical additives enhance the efficacy of selected *Bt* products under laboratory conditions.

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# CHAPTER 2

## Literature review

### 2.1 Biology of *Spoladea recurvalis*

The life cycle of *Spoladea recurvalis* (Fabricius) (Crambidae), the Hawaiian beet webworm is approximately three to four weeks, the egg stage takes three to four days, the larval stage, 12–15 days and the pupal stage, 8-11 days (Bhattacherjee and Ramdas Menon, 1964, Pande, 1972). The average longevity of female is 6 days and 3.5 days in males (Pande, 1972, James *et al.*, 2010).The moths emerge usually in the early hours of the morning and copulation takes place one to two days after emergence. Males search for females in nuptial flights and after some time, the male rests near the female in a tail to tail position (Pande, 1972). As soon as mating starts both sexes cease movement and remain motionless until the act of pairing is completed (Pande, 1972). Copulation lasts for 10 to 15 minutes and oviposition commences one to two days after mating. Before oviposition, the female becomes restless and flies actively, settles on the under surface of a leaf, touches the surface with the tip of her abdomen and lays the eggs (Pande, 1972). The oviposition period varies from two to four days and up to 200 eggs are laid.

The eggs of *S. recurvalis* are tiny, scale-like, shiny, translucent yellow sacs, measuring 0.6 x 0.5 mm. Eggs are laid singly or in batches on the undersides of leaves (Pande, 1972, James *et al.*, 2010).

*Spoladea recurvalis* larvae pass through five distinct instars (four moultings) before they reach a pre-pupal stage (Pande, 1972). The newly-hatched first instar larvae are creamy white in colour but become greenish in their second, third and fourth instars with a transparent epidermis, two longitudinal white bands along the body and a dark band in the middle of the white stripes (James *et al.*, 2010, Mc Dougall *et al.*, 2013). During the fifth instar, the larvae changes to bright pink in colour (Pande, 1972, James *et al.*, 2010). The head is brown and possesses six ocelli (Pande, 1972).

First instar larvae are 2-2.5 mm long and the subsequent instars, 4.3, 6.5, 8.2 and 12.5 mm, for the second, third, fourth and fifth instars, respectively (Pande, 1972).

Last instar larva webs the leaf around itself using silken threads before it becomes a pre-pupae for 12 to 24 hours, followed by pupation inside tubular cocoons, which are about 8-12mm long (Pande, 1972). The freshly-formed pupa is light brown in colour and turns reddish brown when mature (Pande, 1972). The adult is a dark brown moth with two white translucent bands on the forewings and one on the hind wings. These bands form a continuous arch pattern when the wings are spread (Bhattacherjee and Ramdas Menon, 1964). The moths are 9 - 10 mm long with a wing span of 20 - 21 mm (Pande, 1972, Centre for Agriculture and Bioscience International, 2016). The tip of the abdomen of the male is pointed while in the female, it is broad and dilated (Pande, 1972). The moths are nocturnal, and they are found under the shady parts of the host plants during the day.

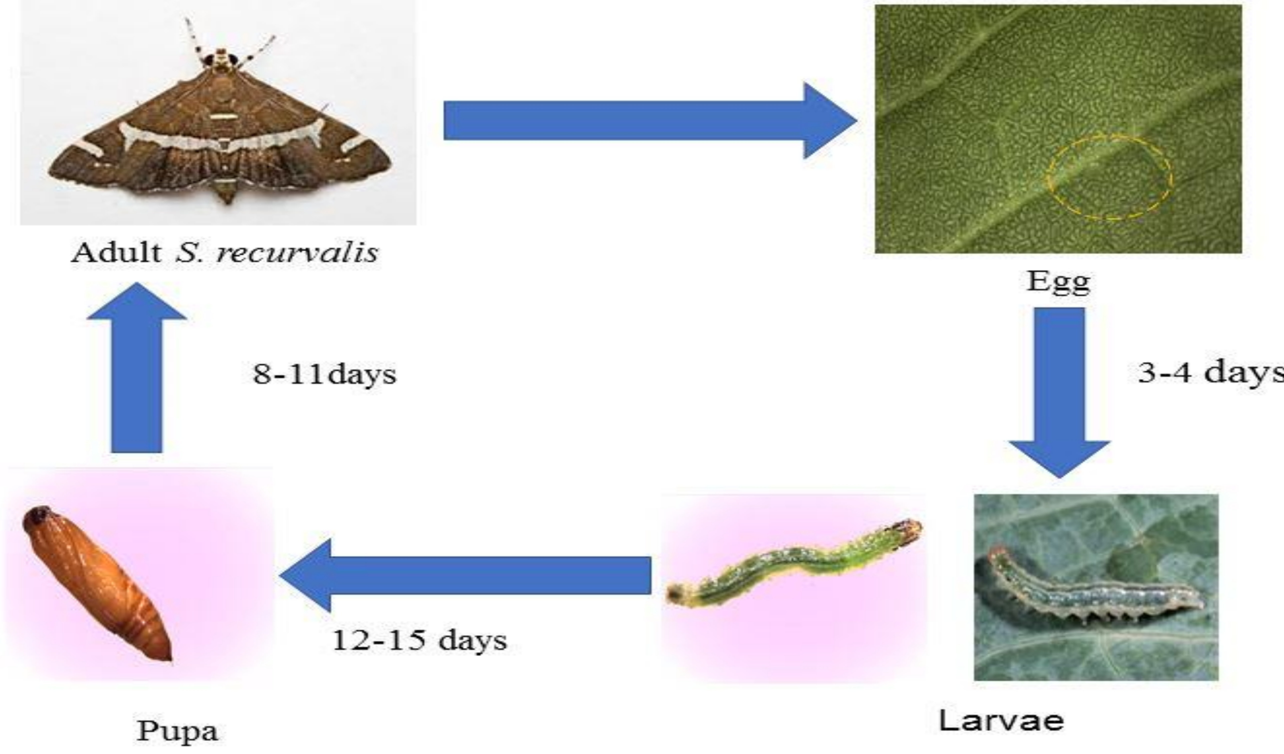


**Figure 2. 1:** Life cycle of *Spoladea recurvalis*

Photos: S.O Miller

## 2.2 Geographical distribution of *Spoladea recurvalis*

*Spoladea recurvalis* is found throughout the world but it is mainly reported in the tropical and subtropical regions of Asia, Africa, Australia, New Zealand and the Hawaiin Islands (Miyahara, 1990, Aswal *et al.*, 2005, Jež *et al.*, 2015, Centre for Agriculture and Bioscience International, 2016). *Spoladea recurvalis* has no hibernation stage and does not tolerate cold temperatures (Yamada and Koshihara, 1976, Miyahara, 1991, Shirai, 2006).

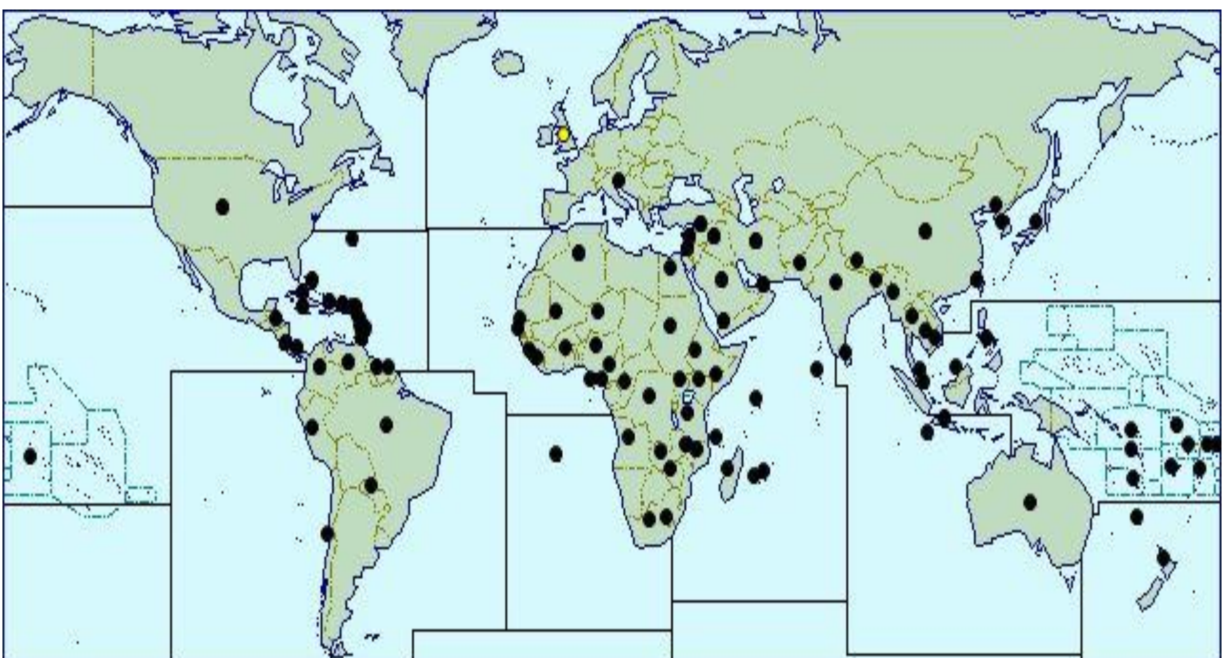

**Figure 2.2:** Geographical distribution of *Spoladea recurvalis*

Source: (Centre for Agriculture and Bioscience International, 1991)

## 2.3 Host range of *Spoladea recurvalis*

The main host plant of *S. recurvalis* is amaranth (Centre for Agriculture and Bioscience International, 2016). The pest also feeds on the leaves of other vegetable crops such as beans, beet

roots, cucurbits, eggplant aubergine, spinach, sweet potato and various weed plants (James *et al.*, 2010, Hsu and Srinivasan, 2012, Centre for Agriculture and Bioscience International, 2016).

### 2.4 Damage and economic importance of *Spoladea recurvalis*

Early instar larvae of *S. recurvalis* feed on the lower surface of leaves (Mc Dougall *et al.*, 2013). Late instars voraciously feed on the cuticle, upper epidermis and palisade layer of the leaves and curl the leaves with silvery webs forming protective leaf shelters inside which they feed (Bhattacherjee and Ramdas Menon, 1964, James *et al.*, 2010). Under high infestation, larvae can skeletonise the entire foliage of amaranth and leave only the main leaf veins intact. Yield loss of up to 100% caused by *S. recurvalis* on amaranths has been reported in Kenya (Kahuthia-Gathu, 2011). *Spoladea recurvalis* also contaminates leaves with their frass (James *et al.*, 2010). Leaf damage caused by *S. recurvalis* larvae reduces the quality of vegetable crops, making them less marketable and causes economic loss (James *et al.*, 2010, Kahuthia-Gathu, 2011).

**Figure 2.3:** Damage caused by *Spoladea recurvalis* larvae on amaranth.

Photo: S.O Miller

### 2.5 Management strategies for *Spoladea recurvalis*

#### 2.5.1 Chemical control

Organochlorides, organophosphates, pyrethroids and carbamates including Lambda-cyhalothrin, Dimethoate, Endosulfan, Abamectin, Chlorpyriphos, Spinosad and Carbaryl are widely used across the world and in Kenya in the management of agricultural pests including those of amaranth such as *S. recurvalis* and other leaf webbers (Clarke-Harris and Fleischer, 2003, Losenge, 2005, Ngowi *et al.*, 2007, Mc Leod, 2008, Arivudainambi *et al.*, 2010, Aderolu *et al.*, 2013). The majority of amaranth growers rely on insecticides applied on a calendar basis, usually every 7- 8 days (James *et al.*, 2010, Aderolu *et al.*, 2013). Although chemical control reduces the pest population effectively, indiscriminate use of synthetic insecticides impacts negatively on human health and the environment including non-target beneficial organisms (Dinham, 2003, London *et al.*, 2005). Fresh leafy amaranth may be consumed as raw salad with minimal cooking. It is therefore important to develop safe pest control options to protect consumers from poisoning due to pesticide residues on amaranth leaves (Fan *et al.*, 2013).

#### 2.5.2 Cultural control

Various cultural practices have been recommended for the control of *S. recurvalis*. Removal of weeds such as horse purslane (*Trianthema portulacastrum*) and cultivating amaranth away from crops such as maize , sugar beet, soybean, eggplant, spinach and sweet potato which serve as alternate hosts for *S. recurvalis* is recommended to minimize its population densities in amaranth crop fields (James *et al.*, 2010). Timely removal of *S. recurvalis* leaf shelters halts the pest's spread between amaranth plants (James *et al.*, 2010). Degri *et al.* (2014) recommended that amaranth farmers should plant pest resistant varieties and apply good agricultural practices, for example the correct crop spacing, for pest management and good crop performance. Intercropping and crop rotation of amaranth with African garden eggplant, lettuce, cabbage and *Vernonia* crops have

shown to reduce the damage of lepidopteran larvae on amaranth leaves (Wesonga *et al.*, 2002, James *et al.*, 2010).

### 2.5.3 Use of resistant amaranth varieties

Host plant resistance (HPR) is considered as the most effective, economical and reliable strategy in pest management. Othim *et al*. (2018) recently evaluated 35 Amaranth accessions for the expression of their antixenotic and antibiotic traits against *S. recurvalis* and found accession VI036227 to be highly resistant against the pest, exhibiting exemplary antibiosis by causing 100% larval mortality under laboratory tests. The accession is thus highly recommended for cultivation by amaranth farmers.

### 2.5.4 Botanical insecticides

Neem tree leaf and seed extracts are mostly used by farmers in West African countries and also in Kenya, against a number of lepidopteran pests on a number of vegetable crops, e.g. the African garden eggplant, amaranth, aubergine and cabbage (Wesonga *et al.*, 2002, Okunlola *et al.*, 2008, Aderolu *et al.*, 2013, Kagali, 2014). The use of modified neem leaf extracts decreased *S. recurvalis* larvae and the number of damaged amaranthus leaves per plant by 30% and 41% respectively in Nigeria (Aderolu *et al.*, 2013). Application of *Cleistanthus collinus* (Roxb.) Benth extracts also reduced *S. recurvalis* larval numbers on amaranth crops (Arivudainambi *et al.*, 2010).

### 2.5.5 Semiochemicals

Semiochemicals are chemical signals produced by one organism that causes a behavioural change in an individual of the same or a different species. Semiochemicals are used by insects for intra- and interspecies communication (Hölldobler, 1995, Blum, 1996). Due to the increasing public concern about the use of toxic insecticides for control of pests, semiochemicals are now being intergrated in IPM programs to monitor and manage insect pests by mass trapping, lure-and-kill systems and mating disruption (Ridgway *et al.*, 1990, Bjostad *et al.*, 1993, Reddy *et al.*, 2009, El-Sayed *et al.*,

2009). These naturally occurring molecules are not toxic to insects, environmentally friendly and more species specific than most conventional insecticides (Van Naters and Carlson, 2006, Witzgall *et al.*, 2010). Phenylacetaldehyde (PAA) is a floral odorant which has been reported to attract both sexes of various lepidopterans (Maini and Burgio, 1990, Meagher, 2002, Landolt *et al.*, 2011). According to studies carried out by Landolt *et al*. (2011) and Maini and Burgio (1990), PAA has shown to effectively control moths of *S. recurvalis* and can significantly contribute to environmentally friendly management of this pest in the Americas The integration of PAA with entomopathogenic fungi to suppress *S. recurvalis* in amaranth production has not been investigated.

### 2.5.6 Parasitoids

Both egg and larval parasitoids attack *S. recurvalis* (James *et al.*, 2010, Centre for Agriculture and Bioscience International, 2016). The egg parasitoids include *Trichogramma dendrolimi* (Hymenoptera: Trichogrammatidae) and the larval parasitoids include *Apanteles delhiensis, Apanteles hemara, Apanteles opacus* (Hymenoptera: Braconidae); *Campoletis chlorideae, Campoletis flavicincta,* (Hymenoptera: Ichneumonidae); *Cardiochiles fulvus, Cardiochiles hymeniae* (Hymenoptera: Braconidae); *Phanerotoma hendecasisella* (Hymenoptera: Braconidae) and *Prosopodopsis* spp. (Diptera: Tachinidae) (Narayanan *et al.*, 1957, James *et al.*, 2010). High levels of *S. recurvalis* parasitism of >90% by *Apanteles hemara* Nixon were reported under laboratory conditions (Othim *et al.*, 2017). Bhattacherjee and Ramdas- Menon (1964) reported field parasitism of 11.46% by *Apanteles delhiensis* Mues and Subba-Rao (Hymenoptera: Braconidae) on *S. recurvalis* in India, while Narayanan *et al.* (1957) reported up to 62% parasitism on *S. recurvalis* by *Apantele*s sp. in India

### 2.5.7 Entomopathogenic fungi

Entomopathogenic fungi constitute the largest single group of entomopathogens with over 750

species in approximately 90 genera. Many of the EPF genera used for control purposes belong either to the class Entomophthorales in the Zygomycota or class Hyphomycetes in the Deuteromycota (Butt and Goettel, 2000, Lacey *et al.*, 2001, Shah and Pell, 2003). Entomopathogenic fungi are promising control agents because they do not need to be ingested and can invade their hosts directly through the exoskeleton or cuticle. Entomopathogenic fungi can therefore infect non-feeding stages such as eggs and pupae (Chandler *et al.*, 2000). These fungi can be mass-produced (Roberts and Hajek, 1992), they are host specific, and can be found under different ecological conditions (Ferron, 1978). *Metarhizium anisopliae* and *Beauveria bassiana* belonging to the class Hyphomycetes have been reported to have a wide host range and are the most widely used entomopathogenic fungi for biological control of agricultural pests (de Faria and Wraight, 2007). Several mycopesticides have already been developed, formulated and commercialized in several countries for control of economically important pests and insects (Douthwaite, 2001, de Faria and Wraight, 2007). Biopesticides based on *M. anisopliae* isolates have been developed by the International Centre of Insect Physiology and Ecology (*icipe*) in collaboration with Real IPM Company (Kenya) Ltd. These commercial products include *Metarhizium anisopliae* 69 (Campaign®) to control thrips, weevils, whiteflies and mealy bugs; and *Metarhizium* 78 (Achieve®) for the control of spider mites (www.realipm.com). A number of *B. bassiana* and *M. anisopliae* isolates from the *icipe* Arthropod Germplasm have also been reported to be pathogenic to various insect pests such as *Brevicoryne* brassicae Linnaeus (Hemiptera: Aphididae), *Lipaphis pseudobrassicae* (Kaltenbach) (Hemiptera: Aphididae) and *Aphis gossypii* (Glover) (Hemiptera: Aphididae) (Bayissa *et al.*, 2017), *Megalurothrips sjostedti* (Trybom) (Ekesi *et al.*, 1998), *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) (Niassy *et al.*, 2012), *Maruca vitrata* (Fabricius) (Lepidoptera: Crambidae) (Tumuhaise *et al.*, 2015), *Liriomyza huidobrensis* (Blanchard) (Migiro *et al.*, 2010), *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae) (Wekesa *et al.*, 2005), *Cylas puncticollis* (Coleoptera: Curculionidae) (Ondiaka *et al.*, 2008) and *Ceratitis capitata* (Wiedemann), *Ceratitis rosa* var. *fasciventris* Karsch. and *Ceratitis*

*cosyra* (Walker) (Dimbi *et al.*, 2003). However, no EPF isolates have been screened for pathogenicity against *S. recurvalis.*

**2.5.7.1 Mode of action of entomopathogenic fungi**

Entomopathogenic fungi have a unique mode of infection; they reach the haemocoel through the cuticle (Ferron, 1978, Vega *et al.*, 2012, Ortiz-Urquiza and Keyhani, 2013). Asexually produced fungal spores or conidia are responsible for infection and are dispersed throughout the environment in which the insect hosts are present. The process of infection starts by the spore which makes contact with the cuticle of the host. The conidium thus adheres to the cuticle or secretes adhesive mucus that aids the conidia to attach to the cuticle (Samšiňáková *et al.*, 1971, Charnley, 1984, Boucias and Pendland, 1991, Holder and Keyhani, 2005). The virulence of an EPF is recognized by adhesion to an insect body; thus, failure of a pathogen to adhere to the cuticle is considered a feature of avirulent isolates. The conidium then germinates into a germ tube or an appressorium. Penetration of the hyphae through the cuticle is either by infection pegs produced from the underside of appressoria or by direct entry of germ tubes (Hajek and St. Leger, 1994). This process involves both mechanical and enzymatic activities. A range of cuticle degrading enzymes including lipases, proteases and chitinases are produced during penetration for hydrolyzing the cuticle of the insect (Butt *et al.*, 1990, Xiao *et al.*, 2012). The germ tube then reaches the hemocoel and encounters the cellular defence reactions of the host. Host defences include a phenoloxidase system which deposits oxidized phenols (melanin) and protease inhibitors in the cuticle, and which may restrict pathogen enzyme activity (Moore and Prior, 1993). Within the haemocoel, the main cellular defence against the fungus appears to be nodule formation, with haemocytes trapping fragments of the fungus (Charnley, 1992). However, an EPF can overcome the defence system of the host insect by producing mycotoxins (Evans, 1989). These include destruxins and desmethyl-destruxin, which have insecticidal activities on the host (Vey *et al.*, 2001). Inside the insect haemocoel, the fungus switches from filamentous hyphal growth to yeast-like hyphal bodies that circulate in the

hemolymph and multiply by budding (Boucias and Pendland, 1982). Later the fungus switches back to a filamentous phase and invades internal tissues and organs, eventually causing death (Prasertphon and Tanada, 1968, Mohamed *et al.*, 1978). The death of the insect marks the end of the parasitic phase of the fungus. After host death, the fungus proceeds to grow saprophytically and spread through virtually all tissues of the insect. Competition between the fungus and the intestinal bacterial flora occurs. Cadavers are usually transformed into mummies resistant to bacterial decay apparently because of antibiotics produced by the fungus. The fungus later erupts through the cuticle and externally the mycelia grow to cover all parts of the host and subsequently form infective spores under appropriate environmental conditions (Boucias and Pendland, 1982, Glare *et al.*, 1986, Lund and Hajek, 2005). The external hyphae produce conidia that ripen and are released into the environment and the spores are later dispersed by wind, rain, and even insects and mites (Hemmati *et al.*, 2001, Roy *et al.*, 2001).

#### 2.5.7.2 Factors affecting the efficacy of fungi as biological control agents

The pathogenicity of an EPF is determined by a variety of factors, including the physiology of the host (e.g. defence mechanisms), physiology of the fungus and the environment (Hajek and St. Leger, 1994, Lacey and Goettel, 1995, Mc Coy *et al.*, 1988).

##### 2.5.7.2.1 Environmental factors

***Temperature***

Temperature is one of the principal factors that affect the rate of conidial germination, growth, sporulation and survival of EPF in the host (Roberts and Campbell, 1977, Tanada and Fuxa, 1987, Scanlan *et al.*, 2001). The optimum temperature required for germination, growth, sporulation and virulence for most EPF has been reported to be between 20 $^{o}$C and 25 $^{o}$C but infection and disease can occur at temperatures ranging between 15 and 30 $^{o}$C. Above 30 $^{o}$C, the vegetative growth of most taxa is inhibited and growth usually ceases at ~37 $^{o}$C (Fargues *et al.*, 1992, Fargues *et al.*, 1997, Ekesi *et al.*, 1999, Dimbi *et al.*, 2004, Bayissa *et al.*, 2017).

Temperature affects EPF as well as insect host processes (Jaronski, 2010), which together interact to determine the degree of susceptibility and disease development. High temperatures accelerate insect development, and reduces the time between moults, which can subsequently reduce the prevalence of infection due to loss of inocula on exuviae (Inglis *et al.*, 2012, Ortiz-Urquiza and Keyhani, 2013). The time of inoculation prior to ecdysis, and the length of the inter-moult period are important factors that may significantly influence susceptibility of the host insect to infection by EPF (Navon and Ascher, 2000). Moulting may remove the penetrating fungus prior to colonization of the insect, if it occurs shortly after inoculation (Vey and Fargues, 1977, Navon and Ascher, 2000). There is considerable variability in temperature tolerances among the fungal entomopathogens, even among isolates of the same species (Rath *et al.*, 1995, Li and Feng, 2009). There are isolates with some degree of cold tolerance, some grows at 8 $^{o}$C (Amritha De Croos and Bidochka, 1999) while others can tolerate high temperatures (Fargues *et al.*, 1992, Ekesi *et al.*, 1999, Dimbi *et al.*, 2004). Thermotolerance of fungal strains is therefore an important factor to be evaluated during development of potent fungal-based mycoinsecticide products since EPF vary in thermo-tolerance.

***Relative humidity (RH)***

High humidity is essential for spores of EPF to geminate, penetrate the cuticle and to sporulate on cadavers (Roberts and Campbell, 1977, Inglis *et al.*, 2001). However not all EPF require high humidity for germination, some EPF can cause infection at as low as 13% RH (Ramoska, 1984, Fargues *et al.*, 1997). Low humidity was reported to be beneficial for control of the lesser grain borer, *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bruchidae), with *B. bassiana* (Lord, 2005). High moisture is also required for conidiogenesis on cadavers that have died from mycosis (Fargues and Luz, 1998).

***Solar radiation***

Natural sunlight is the most destructive environmental factor affecting the persistence of

entomopathogens in the field, particularly the UV-B spectrum in the range of 285–315 nm (Ignoffo, 1992, Moore and Prior, 1993, Braga *et al.*, 2001, Jaronski, 2010). Sunlight may directly damage the DNA of EPF or indirectly through production of highly reactive radicals (e.g. peroxides, hydroxyls, singlet oxygen) by near-ultraviolet irradiation (UV), which also inactivates and reduces the field persistence of entomopathogens (Ignoffo, 1992). The microhabitat in which fungi are deployed influences their persistence, in that survival of conidia deposited on substrates exposed to direct solar radiation is substantially reduced relative to propagules in protected locations, such as within plant canopies (Inglis *et al.*, 1993, Jaronski, 2010). Negative effects of solar radiation on persistence of EPF can be mitigated by using UV-protectants (Inglis *et al.*, 1995, Brooks *et al.*, 2004, Jackson *et al.*, 2010) .

#### 2.5.7.2.2 Biotic factors

***Pathogen population***

Pathogen population properties such as pathogen density, virulence, host specificity, infectivity, persistence, and the capacity to disperse are key factors affecting the ability of entomopathogens to produce epizootics (Inglis *et al.*, 2001, Lacey and Kaya, 2007, Vega *et al.*, 2012). An epizootic is defined as an outbreak of a disease in which there is an unusually large number of cases (Fuxa and Tanada, 1987, Onstad *et al.*, 2006).

Some EPF have restricted host ranges, for example, *Aschersonia aleyrodes* infects only scale insects, and whiteflies, while other fungal species have a wide host range, with individual isolates being more specific to target pests (Inglis *et al.*, 2001). Entomopathogens such as *M. anisopliae* and *B. bassiana* are well characterized in respect to pathogenicity to several insects, and they have been used as agents for the biological control of agricultural pests worldwide (Butt and Goettel, 2000, Vega *et al.*, 2012).

A pathogen with a high density and widespread spatial distribution in the field is well suited to cause an epizootic. Susceptible hosts encountering a high pathogen density are more likely to come

into contact with the pathogen and become infected than those in an environment with a low pathogen density and limited spatial distribution (Shapiro-Ilan *et al.*, 2012). Propagule densities must therefore be sufficiently high, especially in a field setting, to ensure a high probability that an insect will come into contact with an adequate number of propagules to exceed the inoculum threshold (Inglis *et al.*, 2001). Heavy insect population infestations should therefore be managed with inundation of high levels of the pathogen in order to initiate an epizootic. Fungal epizootics generally occur at high host population densities, thus increasing the probability of contact between the pathogen and the hosts as well as between uninfected and infected hosts (Benz, 1987, Onstad and Carruthers, 1990).

The ability of a pathogen to cycle and disperse is an important factor in the development of these epizootics (Inglis *et al.*, 2001). The ability of an entomopathogenic Hyphomycetes species to persist in an environment is another important attribute of a successful biological control agent (BCA). For propagules that exhibit good persistence, there will be a higher probability of an insect coming into contact with sufficient propagules to cause disease.

The median lethal dose of a pathogen needed to kill 50% of the tested insects ($LD_{50}$) and length of time from infection to 50% death of the host (median lethal time or $LT_{50}$) are typical measures of virulence (Tanada and Kaya, 1993, Shapiro-Ilan *et al.*, 2005). The lower the $LD_{50}$, the fewer propagules are necessary to cause mortality.

A more virulent pathogen requires fewer infective propagules to cause disease and takes a shorter time to kill its host. The production of toxins and secondary metabolites by entomopathogens reduces the $LD_{50}$ and $LT_{50}$ values, for example EPF that produces destruxins results to increased mortalities in infected insects (Schrank and Vainstein, 2010).

***The insect host***

A complex array of physiological and morphological factors influence the susceptibility of insect pests to EPF, examples of these factors include host population density, behaviour, age, nutrition,

genetics and exposure to injuries caused by mechanical, chemical or non-microbial agents (Inglis *et al.*, 2001).

The developmental stage of an insect plays an important role in the efficacy of EPF. Not all stages in an insect's life cycle are equally susceptible to infection (Hajek and St. Leger, 1994, Inglis *et al.*, 2001). For example, young larvae of the European corn-borer (*Ostrinia nubilalis*) (Lepidoptera: Pyralidae) are more susceptible to *B. bassiana* than older larvae (Feng *et al.*, 1985). Adult western flower thrips (*Frankliniella occidentalis*) (Thysanoptera: Thripidae) were found to be more susceptible to *Verticillium lecanii* than larvae (Vestergaard *et al.*, 1995). The more susceptible a host is to infection, the lower the pathogen dose necessary and subsequently the easier it is for infections to spread from one host to another (Shapiro-Ilan *et al.*, 2012). Since the penetration of the integument is the usual route of invasion by pathogenic fungi, the moulting stage in insects plays an important role in insect resistance to fungal infection (Vey and Fargues, 1977).

The behaviour of insects such as grooming, nest cleaning, secretion of antibiotics, avoidance, removal of infected individuals and colony relocation can influence epizootic development (Roy *et al.*, 2006). Grooming among termites and other social insects can result in the increased spread of a pathogen within a colony (Siebeneicher *et al.*, 1992, Oi and Pereira, 1993) or conversely serve as an effective means of actively removing pathogen propagules attached to the cuticle. For example, the termite *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) is highly resistant to entomopathogenic Hyphomycetes (e.g. *B. bassiana*), not because of any endogenous defence mechanisms, but as a result of complex social behaviours, including the removal of infected individuals from the colony (Boucias *et al.*, 1996). Termites reared in groups physically remove up to 80% of *M. anisopliae* conidia from their nest-mates and eliminate the conidia through the alimentary tract, while individually reared termites do not reduce their surface contamination (Yanagawa and Shimizu, 2007).

Insect nutrition is also a crucial factor regulating the susceptibility of insects to entomopathogens.

Inadequate nutrition often leads to increased susceptibility to entomopathogens, and the utilization of resistant plant genotypes to induce nutritional stress can substantially enhance the efficacy of entomopathogens (Inglis *et al.*, 2001). Diet can also decrease the susceptibility of insect pests to entomopathogenic Hyphomycetes. For example, Ekesi *et al*. (2000) found that thrips (*M. sjostedti*) were less susceptible to *M. anisopliae* on certain cowpea cultivars because of plant-derived fungistatic compounds. The concentration of secondary metabolites in plants is higher in young leaves than in older leaves, but older leaves contain fewer nutrients (i.e. nitrogen and water) (Feeny, 1992). Declining nutrient and water content in the mature foliage of perennial plants reduces the growth rates of lepidopteran larvae compared with those of closely related species feeding on younger leaves or on the foliage of herbaceous plants (Krischik and Denno, 1983). High protein concentrations in an insect's diet can counterbalance the toxic effect of secondary metabolites, such as alkaloids (Costa and Gaugler, 1989).

**2.5.7.3. Enhancing the efficacy of entomopathogenic fungus for use in microbial control**

The best performing EPF should be selected by screening existing species and strains that possess superior desired traits such as virulence and environmental tolerance, in the laboratory first, followed by field verification (Shapiro-Ilan *et al.*, 2012). An entomopathogen that shows high virulence under controlled laboratory conditions, could fail to suppress the target pest in the field due to various biotic or abiotic factors that render the organism incompatible (Hu and Leger, 2002, Bruck, 2005, Shapiro-Ilan *et al.*, 2012). In most countries, especially in SSA, the cost of commercial development and registration of EPF are extremely high (Jaronski, 2010). The screening processes should therefore generally include strains and species that are already commercially registered and available. In this way, production costs are reduced and extension of labels can be recommended if an already commercialized fungal strain is found to be pathogenic to the pest.

The virulence of a selected EPF candidate strain can also be maintained through regular passing of the pathogen through a susceptible host (Daoust and Roberts, 1982, Butt and Goettel, 2000).

Entomopathogens may also be combined with chemical agents to enhance microbial control efficacy; for example, a number of UV protectants have been used to shield EPF against UV radiation (Jackson *et al.*, 2010). The use of a clay solar blocker and UV absorbing optical brightener (Tinopal) increases the field persistence of *B. bassiana* conidia on grass leaves exposed to sunlight (Inglis *et al.*, 1995).

The efficacy of EPF can also be enhanced by combining it with other biological control agents; for example, a synergistic interaction between *B. bassiana* and a *B. thuringiensis tenebrionis*-based biopesticide applied against field populations of Colorado potato beetle larvae was reported by Wraight and Ramos (2005).

Efficacy may also be enhanced when an entomopathogen is combined with physical agents. For example, a synergist effect of diatomaceous earth combined with *B. bassiana* has been observed for control of coleopteran stored grain pests (Lord, 2001, Akbar *et al.*, 2004, Athanassiou and Steenberg, 2007).

The mode of applying EPF in the field has an effect on its efficacy. The most commonly used method is application of inundative sprays (Fargues *et al.*, 1996, Inglis *et al.*, 2000, Jaronski, 2010). This method, however, has several shortcomings including the use of high volumes of inoculum and short persistence in the field due to breakdown by solar radiation. As a result, repeated applications are needed that are too expensive (Fargues *et al.*, 1996, Inglis *et al.*, 2000, Jaronski, 2010). Due to these challenges, a novel application technique referred to as autodissemination or autoinoculation was developed for a number of insect pest species (Maniania, 2002, Dimbi *et al.*, 2003, Migiro *et al.*, 2010, Niassy *et al.*, 2012). It consists of an autoinoculator to which insect pests

are attracted and inoculated with a pathogen before returning to the environment to disseminate the pathogen to conspecifics (Maniania, 2002, Dimbi *et al.*, 2003, Migiro *et al.*, 2010, Niassy *et al.*, 2012).

## 2.6 *Bacillus thuringiensis (Bt)*

*Bacillus thuringiensis* (*Bt*) was first isolated from diseased larvae of the silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae), in Japan in 1901 by Ishiwata (Ishiwata, 1901). In 1911, a German biologist E. Berliner isolated a related strain of this bacterium from diseased Mediterranean flour moths, *Ephestia kuehniella* (Zell) (Lepidoptera: Pyralidae), that were found in stored grain in Thuringia (formerly East Germany) and named it *Bacillus thuringiensis* Berliner in 1915 (Berliner, 1915).

*Bacillus thuringiensis* (*Bt*) is a gram-positive, rod-shaped, spore-forming bacterium that is commonly found in natural soils and in insect-rich environments such as sericulture farms, insect rearing facilities, grain dust from flour mills, and grain storage facilities (Martin and Travers, 1989, Bernhard *et al.*, 1997, Chaufaux *et al.*, 1997, Park *et al.*, 1998, Berry *et al.*, 2002)

The life cycle of *Bt* is characterized by two phases which include vegetative cell division and a sporulation cycle. During the vegetative cell cycle, a rod-shaped vegetative cell multiplies by cell division but forms spores (endospores) within a sporangium when nutrients are depleted or when the environment becomes adverse. During spore formation *Bt* produces various insecticidal crystal proteins (ICPs) called endotoxins that are toxic to a selective range of insect orders, namely Lepidoptera, Diptera and Coleoptera (Burges, 1998, Schnepf *et al.*, 1998, Chapple *et al.*, 2000). The production of ICPs by *Bt* is a unique feature that distinguishes it from other related *Bacillus* species (Angus, 1956, Höfte and Whiteley, 1989).

*Bacillus thuringiensis*-based formulations are the most widely used microbial insecticides for control of insect pests in agriculture. (Goldberg and Margalit, 1977, Bravo *et al.*, 2013).

Insecticidal *Bt* genes have been incorporated into several major crops for example maize, cotton and tobacco for insect control (Qaim and Zilberman, 2003, Kleter *et al.*, 2007).

### 2.6.1 Mode of action of *Bacillus thuringiensis* (*Bt*)

*Bacillus thuringiensis* must be ingested by the target insect species to be effective. After ingestion, the endotoxins dissolve in the intestinal tract due to the high alkaline pH of the insect gut to form solubilized inactive protoxins (Gringorten *et al.*, 1992, Bravo *et al.*, 2005). The protoxins are then activated into active toxins by gut proteases (Knowles, 1994). The active toxin consists of three distinct domains: C-terminal, middle and the N-terminal domain (Höfte and Whiteley, 1989, Li *et al.*, 1991, Grochulski *et al.*, 1995). The C-terminal and middle domains of the toxin bind to specific receptors on the brush border membrane of the midgut epithelium columnar cells (de Maagd *et al.*, 2001, Bravo *et al.*, 2005) before inserting an N-terminal domain into the membrane. Toxin insertion leads to the formation of lytic pores in microvilli of apical membranes (Dean *et al.*, 1996, Schnepf *et al.*, 1998). The pore formation causes osmotic shock since the regulation of the trans-membrane electric potential is disturbed and this results in cell lysis and disruption of the midgut epithelium. Destruction of the midgut wall allows the haemolymph and gut contents to mix, which results in favourable conditions for germination of the *Bt* vegetative cells that proliferate in the haemocoel causing septicaemia that contributes to the mortality of the insect larva (de Maagd *et al.*, 2001, Bravo *et al.*, 2005).

### 2.6.2 *Bacillus thuringiesis* (*Bt*) formulations

Environmental conditions in the field adversely affect the insecticidal activity of *B. thuringiensis* (Mc Guire and Shasha, 1990, Mc Guire *et al.*, 1994, Hall and Barry, 1995, Behle *et al.*, 1997b). To overcome these limitations, commercially based *B. thuringiensis* biopesticides are normally

formulated with adjuvants or additives in order to make them more stable microbial agents during distribution and storage, improve their safety when handling and applying them, protect the parasporal crystals from adverse environmental factors and enhance their effectiveness on the susceptible insect pests in the field (Tamez-Guerra *et al.*, 1996, Brar *et al.*, 2006).

Commercially based *B. thuringiesis* formulations are classified either as dry solids (dusts, granules, powders and briquettes) or liquids (aqueous, oil suspensions or combination of both oil and water suspensions) (Burges, 1998, Brar *et al.*, 2006, Burges, 2012, Couch, 2014).

#### 2.6.2.1 Solid formulations

***Dusts***

Dusts are formulated by the sorption of an active ingredient into a finely ground, solid inert such as talc, clay, or chalk (Brar *et al.*, 2006). Dusts are applied as dry spot treatments to seeds or foliage and provide excellent coverage, but the small particle size that allows for this advantage also creates an inhalation and drift hazard (Naizhen, 2003). Two formulations of *B. thuringiensis* (wettable powder and dust) to control the larvae of *Lobesia botrana* (Lepidoptera: Tortricidae) were tested in Greece and dusts provided maximum kill compared to the wettable powder (Ifoulis and Savopoulou-Soultani, 2004). *Bt* dusts have also been widely used in the control of corn borer larvae (*Ostrinia nubilalis*); however, their use was restricted owing to adverse health impacts (respiratory system complications ) on the end user (Lynch *et al.*, 1980).

***Granules***

Granules are formulated by using carriers such as clay minerals, starch polymers, dry fertilizers and ground plant residues (Green, 2000). There are different types of granules commonly used in *Bt* formulations namely wheat meal granules (Navon *et al.*, 1997), corn meal baits and granules formed with gelatinized cornstarch or flour (Dunkle and Shasha, 1988, Tamez-Guerra *et al.*, 1996, Shasha and McGuire, 1999), gluten (Behle *et al.*, 1997a), cottonseed flour and sugars (Ridgway *et al.*, 1996), gelatin or acacia gum (Maldonado *et al.*, 2002), sodium alginate and paraffin (Sulaiman

*et al.*, 1990) and diatomaceous earth (Quimby Jr *et al.*, 2002). *Bt* granules (150–210 μm) with wheat meal used both as a carrier and a feeding stimulant, against *Earias insulana* (Lepidoptera: Noctuidae) on cotton, withstand UV radiation, dew and undesirable interactions with the phylloplane (Navon *et al.*, 1997). The larger particle size of granules, relative to dusts, minimizes the potential for drift and also has a reduced inhalation hazard (Brar *et al.*, 2006).

***Wettable powders***

To achieve a desired potency formulation, wettable powders consist of 50–80% technical powder, 15–45% filler, 1–10% dispersant and 3–5% surfactant by weight (Brar *et al.*, 2006). Fillers, dispersants and surfactants are added to assist in wetting the powder and to disperse it throughout the spray tank (Brar *et al.*, 2006). Wettable powders are the most commonly used dried formulations of biopesticides, because of their longer shelf life, good miscibility with water and ease of application as sprays with conventional equipment (Charles *et al.*, 2000).

#### 2.6.2.3 Liquid formulations

***Liquid flowables (Suspension concentrates)***

Liquid flowables consist of a suspension of particulates in liquids, with 10–40% microorganism, 1–3% suspender ingredient, 1–5% dispersant, 3–8% surfactant and 35–65% carrier liquid which can either be oil or water (Brar *et al.*, 2006, Burges, 2012). The products are prevented from settling due to reversible agglomeration by dispersants, while surfactants act as wetting agents and spreaders (Burges and Jones, 1998). Oil-based *Bt* formulations have advantages over water-based formulations. For example, oil-based formulations increase the ability of the biologically active agent to adhere to and disperse over hydrophobic surfaces (Bateman *et al.*, 1993, Bateman and Alves, 2000). They are also compatible with ultra-low volume (ULV) application, this ensures minimum dose usage rates of *Bt* for a maximum coverage the target site (Inglis *et al.*, 2002, Burges, 2012).

Oil-based *Bt* products have a longer shelf life than water-based because the oil formulations droplets do not evaporate during application (Cibulsky *et al.*, 1993, Charles *et al.*, 2000).

***Microencapsulates***

Microencapsulates are liquid suspensions containing microbial propagules e.g *Bt*, encapsulated in a coating (capsule) made of gelatin, starch, cellulose and other polymers (Barnes and Edwards, 1989, John *et al.*, 2011). Encapsulations are recent advances in bioinsecticidal formulations and provide protection from extreme environmental conditions and enhance residual stability due to the slow release of formulations (Brar *et al.*, 2006). *Bacillus thuringiensis* encapsulated in starch granules has been reported to have a higher insecticidal activity against *O. nubilalis* than that of *B. thuringiensis* in an unencapsulated commercial formulation (Mc Guire *et al.*, 1994). Microencapsulation of *B. thuringiensis* in a nixtamalized corn flour-based formulation were shown to control *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae) populations better than unformulated powder as well as the chemical insecticide carbaryl in the field (Tamez-Guerra *et al.*, 1999). Fine, encapsulated products can be sprayed in any volume as the pathogen is held tightly to additives causing less wastage (Shasha and McGuire, 1991).

### 2.6.3 Factors affecting *Bacillus thuringiensis* efficacy

#### 2.6.3.1 Sunlight / UV radiation

Sunlight rapidly degrades residual insecticidal activity of *B. thuringiensi*s (Frye *et al.*, 1973, Griego and Spence, 1978, Behle *et al.*, 1997b). *Bacillus thuringiensis* spores and their endotoxins are highly sensitive to the UV radiation spectra: UV-B (280–310 nm) and UV-A (320–400 nm) (Myasnik *et al.*, 2001).

#### 2.6.3.2 Rainfall

Rain physically removes spores and crystals from the target site, reducing residual insecticidal activity of a *B. thuringiensis* application (Mc Guire *et al.*, 1994, Hall and Barry, 1995, Behle *et al.*, 1997b). Recent advanced formulations improved on the rainfastness of *Bt* products; for example experimental cornstarch based formulations showed increased activity following rainfall, possibly because of the ability of cornstarch to stick onto crop foliage (Mc Guire and Shasha, 1992). Similar results were reported regarding maize flour microcapsules (Tamez-Guerra *et al.*, 2000).

#### 2.6.3.3 pH

*Bt* activity has been shown in many studies to be stable above pH 3 and below 11 (Nishiitsutsuji-Uwo *et al.*, 1977, Jones and Burges, 1998). The sensitivity of *Bt* to extreme pHs increases with increased duration of exposure. Similarly, as temperature increases, *Bt* sensitivity to extreme pHs also increases resulting in a challenge in areas with highly acidic water. Wheat gluten based formulations have been developed that could sustain the entire pH range between 3–11 (Behle *et al.*, 1997a). Effects of pH are mostly encountered while loading the spray mixtures into containers (aluminum or iron) which could get corroded and affect the effective field application of the biopesticides adversely, but it can be addressed by using buffering agents (Burges, 1998).

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## CHAPTER 3 ARTICLE 1

**Effects of entomopathogenic fungi and *Bacillus thuringiensis*-based biopesticides on *Spoladea recurvalis* (Lepidoptera: Crambidae)**

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# Effects of Entomopathogenic fungi and *Bacillus thuringiensis*-based biopesticides on *Spoladea recurvalis* (Lepidoptera: Crambidae)

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## Abstract

*Spoladea recurvalis* (Fabricius) is one of the most devastating pests of amaranths causing severe yield losses of 60%–100% to the crop. Unfortunately use of chemical pesticides is the most common control strategy that vegetable farmers rely on to control the pest. However, it is not effective and harmful to environmental and human health. Aiming to provide more environmentally friendly alternatives, this study evaluated the effects of various entomopathogenic fungal isolates and commercial based *Bacillus thuringiensis* Subsp. *kurstaki* product Halt®, on the pest. Twenty-four entomopathogenic fungal (EPF) isolates from three genera (14 *Metarhizium anisopliae*, 9 *Beauveria bassiana* and 1 *Isaria fumosorosea*) were screened in the laboratory to assess their pathogenicity against second instar larvae of *S. recurvalis*. Only *M. anisopliae* ICIPE 30 reached a moderate threshold, causing 58.3% larval mortality. All the 11 isolates (8 *M. anisopliae*, 2 *B. bassiana* and 1 *I. fumosorosea*) tested against adult *S. recurvalis* were pathogenic, with *M. anisopliae* ICIPE 30 and *B. bassiana* ICIPE 725 causing the highest mortality of 92% and 83%, respectively. *Metarhizium anisopliae* ICIPE 30 had the shortest $LT_{50}$ value of 4.8 days. *Bacillus thuringiensis* Subsp. *kurstaki* product Halt® caused <50% mortality on *S. recurvalis* larvae. A consecutive application of *M. anisopliae* ICIPE 30 and *Bt* did not cause a significant increase in larval mortality compared to separate applications of both products. Results of this study suggest that *M. anisopliae* ICIPE 30 was the most potent candidate and could be used in an autodissemination approach for management of adult *S. recurvalis*.

**KEYWORDS**
African indigenous vegetables, autodissemination, chemical insecticides, Halt®, ICIPE 30, pathogenicity

## 1 | INTRODUCTION

Amaranths are among the three most important African indigenous vegetables (AIVs) consumed in Sub Saharan Africa (SSA) (Abukutsa-Onyango, 2002; Ebert, Wu, & Wang, 2011). They are highly nutritious (Mbwambo, Abukutsa-Onyango, Dinssa, & Ojiewo, 2015; Muriuki, Sila, & Onyango, 2014; Muyonga, Nabakabya, Nakimbugwe, & Masinde, 2008; Ojiewo, Tenkouano, Hughes, & Keatinge, 2013) and drought tolerant with a short maturity period (DAFF, 2010). The sale of amaranth produce in rural, peri-urban and urban markets provides an important source of income for smallholder farmers across SSA (Abukutsa-Onyango, 2002; Onyango, 2010; Shackleton, Pasquini, & Drescher, 2009). Amaranth production has therefore excellent potential for mitigating the effects of climate change because of its drought tolerant nature, generation of income and by improving food and nutritional security in SSA.

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Despite all these advantages, production of amaranths has not kept pace with the high demand, mainly due to insect pests that attack the crop, particularly lepidopteran defoliators which affect the yield and marketability (James et al., 2010; Robert N. Kagali, Kioko, Osiemo, Muya, & Wachera, 2013; Mureithi et al., 2015; Schippers, 2000; Sithanantham, Olubayo, Reddy, & Nyarko, 1997). Among the lepidopteran species that infest amaranths, the Hawaiian webworm, *Spoladea recurvalis* (Fabricius) (Lepidoptera: Crambidae) has been reported as the major amaranth pest which can cause 60%–100% yield loss (Aderolu, Omooloye, & Okelana, 2013; James et al., 2010; Robert N. Kagali et al., 2013; Mureithi et al., 2015). *Spoladea recurvalis* larvae web distinctive leaf shelters from where they skeletonize the foliage, leaving only the main leaf veins intact. This makes the management of the pest difficult, especially the late instar larvae that are protected inside the leaf web shelters (James et al., 2010).

Unfortunately, due to the rising importance and high demand of amaranth, lepidopteran larval infestations on the crop are controlled with widespread application of synthetic chemical insecticides in an effort to increase crop yield (James et al., 2010; Robert Nesta Kagali, 2014; Ngowi, Mbise, Ijani, London, & Ajayi, 2007). However, excessive and indiscriminate use of these insecticides not only increases the cost of production, but also results in detrimental effects on humans, the environment and often disrupts the activity of natural enemies (Dinham, 2003; Jeyanthi & Kombairaju, 2005; Macharia, 2015). Resistance of *S. recurvalis* to chemical insecticides has been reported elsewhere in the world (Clarke-Harris & Fleischer, 2003), and a similar scenario is likely to develop in Africa. There is therefore a strong need to look for alternative strategies that are safe, environmentally friendly and cost-effective to control this pest.

Due to the short maturity period of amaranths and the fact that amaranth leafy vegetables are consumed fresh with minimal cooking, the pest complex on this crop must be managed with care. Biopesticides are the most appropriate option with no accumulation of residues and no requirements for a pre-harvest interval (PHI) (Copping & Menn, 2000; Gupta & Dikshit, 2010; Gwynn & Maniania, 2010). Currently, biological control using entomopathogenic fungi (EPF) and *Bacillus thuringiensis* (*Bt*)-based biopesticides offer a sound alternative management strategy against many insect pests either as stand-alone control agents or as a component of integrated pest management programmes (Bravo, Gill, & Soberón, 2007; Chandler et al., 2011; Gill, Cowles, & Pietrantonio, 1992; Inglis, Goettel, Butt, & Strasser, 2001; Shah & Pell, 2003). Many commercial formulations of *B. thuringiensis* Subsp. *kurstaki* and *aizawai* are available for use in management of several vegetable pests (Kabaluk, Svircev, Goettel, & Woo, 2010). For instance, *B thuringiensis* biopesticides have been used to control lepidopteran pests as an alternative for synthetic chemical pesticides. The larvicidal effects of *Bt*-based biopesticides have been demonstrated through the susceptibility bioassays of second instar larvae of *M. vitrata* to the insecticidal crystal proteins (ICPs) from *B. thuringiensis*, namely Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca and Cry2Aa assessed in Taiwan, showed that the toxin Cry1Ab was the most potent toxin ($LC_{50}$ = 0.207 ppm), followed by Cry1Ca, Cry1Aa, Cry2Aa and Cry1Ac, with $LC_{50}$s of 0.477, 0.812, 1.058 and 1.666 ppm, respectively (Srinivasan, 2008). In addition, *Metarhizium*-based fungal biopesticides *M. anisopliae* ICIPE 69 (Campaign®), *M. anisopliae* ICIPE 78 (Achieve®) and *M. anisopliae* ICIPE 62 (Met 62®) developed by the International Centre of Insect Physiology and Ecology (*icipe*), formulated, commercialized and marketed by Real IPM Kenya are also available against major vegetable arthropod pests (http://www.realipm.com/). However, little has so far been performed to exploit such management options for *S. recurvalis* control. Therefore, the objectives of this study were to screen and evaluate the effects of selected entomopathogenic fungal isolates and one commercial *B. thuringiensis* Subsp. *kurstaki*-based product, Halt® on immature and adult *S. recurvalis*, as well as to assess the interactions of both biocontrol candidates for the suppression of the pest population.

## 2 | MATERIALS AND METHODS

### 2.1 | Insects

A colony of *S. recurvalis* was established using adults and larvae which were collected from Transmara, Narok county (0°35′32.892″N 3°0′49.14″E) and Yatta, Machakos County (01°08.295′S 037°25.892′E) on amaranth crops during a field survey in May and June 2014. The moths were kept in ventilated, sleeved Perspex cages (40 × 40 × 45 cm) and were fed with a 10% honey solution soaked in balls of cotton wool. Potted amaranth plants were placed in the cages for oviposition. The plants were removed after 24 hr and transferred to separate wooden cages (50 × 50 × 60 cm) ventilated with netting material at the sides and on the top until the eggs hatched. Leaves with larvae were removed from these plants, 3 days after the larvae hatched and placed into plastic containers (15 × 7 × 5 cm) lined with paper towel to absorb excess moisture and fine netting infused lid for ventilation. The larvae were supplied daily with fresh amaranth leaves as food until they pupated. The pupae were collected from the plastic containers using a fine camel hair brush and placed inside a clean plastic container for adult emergence. Prior to conducting an experiment, newly emerged adults were released into a separate cage (40 × 40 × 45 cm) with potted amaranth plants for oviposition as described above, and the plants were replaced every 24 hr to ensure a population homogenous in age. Field collected populations of *S. recurvalis* were introduced into the laboratory colony after every 3 months to enhance genetic vigour. The colony was maintained in a rearing room at 25 ± 2°C, 50%–70% RH and 12:12 L: D photoperiod at the Animal Rearing and Quarantine Unit (ARQU) of *icipe*.

### 2.2 | Fungal isolates

Fungal isolates used in this study were obtained from the *icipe* Arthropod Germplasm Centre. The origin of the fungal isolates, their host source and year of isolation are summarized in Table 1. The isolates were cultured on Sabouraud dextrose agar (SDA) except for *Beauveria* isolates which were cultured on potato dextrose agar (PDA) and maintained at 25 ± 2°C in darkness. Conidia were

**TABLE 1** Identity of the selected fungal isolates tested against *S. recurvalis* and their percentage germination on SDA and PDA 18 hr post-inoculation at 26 ± 2°C

| Fungal species | Isolate | Source | Locality (Country) | Year of isolation | % Germination ± SE |
|---|---|---|---|---|---|
| *Metarhizium anisopliae* | ICIPE 69 | Soil | Matete (DRC) | 1990 | 92.0 ± 3.1ab |
| | ICIPE 78 | *Temnoschoitanigroplagiata* | Ungoe (Kenya) | 1990 | 97.1 ± 2.0ab |
| | ICIPE 62 | Soil | Matete (DRC) | 1990 | 95.5 ± 1.3ab |
| | ICIPE 7 | *Rhipicephalusappendiculatus* | Rusinga Island (Kenya) | 1996 | 91.5 ± 2.2b |
| | ICIPE 30 | *Busseolafusca* | Kendubay (Kenya) | 1989 | 97.0 ± 0.9ab |
| | ICIPE 18 | Soil | Mbita (Kenya) | 1989 | 96.2 ± 1.7ab |
| | ICIPE 20 | Soil | Migori (Kenya) | 1989 | 99.0 ± 0.7ab |
| | ICIPE 40 | Soil | Kitui (Kenya) | 1990 | 96.3 ± 0.9ab |
| | ICIPE 74 | Soil | Mtwapa (Kenya) | 1990 | 94.1 ± 1.0ab |
| | ICIPE 48 | Earwing | Homabay(Kenya) | 1989 | 98.8 ± 0.7ab |
| | ICIPE 63 | Soil | Matete (DRC) | 1990 | 97.9 ± 0.8ab |
| | ICIPE 727 | Caterpillar | Kenya | 2014 | 99.3 ± 0.5a |
| | ICIPE 41 | Soil | Lemba (DRC) | 1990 | 98.5 ± 0.9ab |
| | ICIPE 723 | Soil | Naivasha (Kenya) | 2015 | 96.8 ± 1.2ab |
| *Beauveria bassiana* | ICIPE 725 | Soil | Diani (Kenya) | 2008 | 92.2 ± 1.5ab |
| | ICIPE 644 | Soil | Mauritius | 2007 | 94.0 ± 2.4ab |
| | ICIPE 676 | Soil | Muhaka (Kenya) | 2008 | 96.0 ± 1.3ab |
| | ICIPE 10 | Soil | Mbita (Kenya) | 2002 | 93.5 ± 1.0ab |
| | ICIPE 273 | Soil | Mbita (Kenya) | 2006 | 99.1 ± 0.7ab |
| | ICIPE 279 | Coleopteran larvae | Kericho(Kenya) | 2005 | 98.3 ± 0.6ab |
| | ICIPE 281 | Soil | Mauritius | 2005 | 96.1 ± 1.7ab |
| | ICIPE 726 | Caterpillar | Kenya | 2014 | 96.2 ± 0.8ab |
| | ICIPE 35 | Coffee berry borer | Kenya | 2009 | 93.3 ± 1.7ab |
| *Isaria fumosorosea* | ICIPE 682 | Soil | Maasai Mara (Kenya) | 2015 | 98.3 ± 0.5ab |

Means within a column followed by the same letter are not significantly different by Student–Newman–Keuls (SNK) test ($p < .05$).

harvested from 2-to 3-week old sporulated cultures and suspended in 10 ml distilled water with 0.05% Triton X-100 in universal bottles containing glass beads (φ = 3 mm). The suspension was vortexed for 5 min at 100 rpm to break the conidial clumps and ensure a homogeneous suspension. Conidial concentrations were quantified using a haemocytometer under a light microscope. The conidial suspensions were adjusted to $1 \times 10^8$ conidia/ml through dilution prior to bioassays. For viability tests, a concentration of $3 \times 10^6$ conidia/ml was prepared and 0.1 ml of the suspension was evenly spread on a SDA or PDA plate and three sterile microscope cover slips were placed randomly on the surface of each inoculated plate. The plates were sealed with Parafilm and incubated under complete darkness at 25 ± 2°C. Conidia germination was assessed after 18 hr by counting 100 randomly selected conidia beneath each coverslip under a light microscope. Conidia were considered to have germinated when the length of the germ tube was at least twice the diameter of the conidium (Goettel & Inglis, 1997; Inglis, Enkerli, & Goettel, 2012). Four replicate plates were used per isolate, and viability of each isolate was determined, where 90%–99% of conidia germinated (Table 1).

For pathogenicity screening bioassays against moths, conidia of *B. bassiana*, *M. anisopliae* and *I. fumosorosea* were mass produced using grain rice on a long-rice substrate in Milner bags (60 cm long by 35 cm wide). Rice was autoclaved for 1 hr at 121°C and inoculated with a 3-day-old culture of blastopores (Jenkins, Heviefo, Langewald, Cherry, & Lomer, 1998). The inoculated bags were then incubated for 21 days at 20–26°C and 40%–70% relative humidity (RH). The rice substrate containing fungal spores was then allowed to dry for 5 days at room temperature. Conidia were harvested by sifting the substrate through a 295-μm mesh sieve and then stored in plastic bags in a refrigerator (4–6°C) until use.

#### 2.2.1 | *Bacillus thuringiensis* (Bt)

Halt® 5 WP, a commercially available biological insecticide based on *Bacillus thuringiensis*, *serovar kurstaki* H3a, 3b, 3c, formulated as wettable powder (WP) with 32,000 I.U potency, obtained from Osho Chemical Industries LTD (Nairobi, Kenya), was used in this study. The wettable powder (0.05 g) was mixed with 100 ml

distilled water containing 0.05% Triton X-100 in a sterilized conical flask. An aqueous suspension was diluted to the respective dose of concentrations to be tested and stirred for 3 min with a magnetic stirrer.

### 2.3 | Pathogenicity of EPF isolates against second instar larvae of *Spoladea recurvalis*

Twenty-four isolates of entomopathogenic fungi from three genera (14 *M. anisopliae*, 9 *B. bassiana* and 1 *I. fumosorosea*) were screened against second instar larvae of *S. recurvalis*. Two fresh amaranth leaves of similar size and age, approximately 2–3 weeks old, were placed in a Petri dish (90 mm in diameter). For each treatment, twenty, second instar *S. recurvalis* larvae obtained from the rearing colony, were transferred onto the leaves in each Petri dish, using a carmel hair brush. Each Petri dish represented a replicate and repeated three times per isolate. Insects were allowed to settle on the amaranth leaves and were then sprayed with 10 ml of $1 \times 10^8$ conidia/ml suspension using Burgerjon's spray tower (Burgerjon 1956). The control groups were sprayed with sterilized distilled water containing 0.05% Triton X-100. After treatment, leaves containing insects were transferred to clear plastic dishes (110 × 60 mm) lined with moist filter paper and incubated at 25 ± 2°C. The insects were supplied with fresh amaranth leaves (surface-sterilized and untreated) daily as food. Larval mortality was recorded daily for 7 days. All the treatments were arranged in a completely randomized design with each treatment replicated three times as well as the controls. For the mycosis test, the dead insects were surface sterilized with 70% alcohol and then rinsed thrice in distilled water. They were kept separately in Petri dishes lined with sterile moistened filter paper to record fungal outgrowth and verify if mortality could be attributed to the respective fungal isolates they were treated with.

### 2.4 | Screening of EPF isolates against adults of *Spoladea recurvalis*

Eleven EPF from the above tested isolates (8 *M. anisopliae*, 2 *B. bassiana* and 1 *I. fumosorosea*) were screened for their virulence against *S. recurvalis* moths. For each fungal isolate (treatment), 10, 1-day-old virgin *S. recurvalis* male and female moths were contaminated with EPF using velvet-coated plastic jars (150 × 80 mm) following the procedure described by Migiro, Maniania, Chabi-Olaye, and Vandenberg (2010). The devices were contaminated with 1 g of dry conidia. Moths were introduced into the device for 3 min to pick up spores. Control insects were exposed to fungus-free velvet plastic jars. After 3 min of exposure, contaminated insects were transferred into clean ventilated Perspex cages (300 × 300 × 300 mm) and provided with 10% sucrose solution as food. Adult mortality was recorded daily for 7 days. Mycosis was assessed as described above, and mortality due to fungal infection was confirmed by the presence of hyphae and conidia on the surface of the cadaver. All the treatments were arranged in a completely randomized design with each treatment replicated three times as well as the controls.

### 2.5 | Efficacy of *Bacillus thuringiensis* against second instar *Spoladea recurvalis* larvae

The *Bt* suspension was prepared by mixing 100 mg of commercial *Bt* Subsp. *kurstaki* product, Halt® 5 WP into 100 ml distilled water containing 0.05% Triton X-100. Five serial concentrations were prepared based on the recommended label rates of this product at 0.5 g/L application against caterpillars of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) and *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae), *viz*. 100, 50, 25, 12.5 and 6.25 mg/100 ml, respectively. Application of the respective *Bt* concentrations was performed, and larvae were maintained as described above for evaluation of the pathogenicity of EPF isolates. Larval mortality was recorded daily for 7 days.

### 2.6 | Interactions between *Metarhizium anisopliae* isolate ICIPE 30 and *Bacillus thuringiensis* Subsp. *kurstaki* (*Bt*) for control of second instar *Spoladea recurvalis* larvae

This experiment was conducted with three treatments: (i) *M. anisopliae* isolate ICIPE 30+ *B. thuringiensis* Subsp. *kurstaki*, (ii) *M. anisopliae* isolate ICIPE 30 alone and (iii) *B. thuringiensis* Subsp. *kurstaki* alone. For combined treatment, ten millilitre of *M. anisopliae* isolate ICIPE 30 fungal suspension at the concentration of $1 \times 10^8$ conidia/ml, followed by *B. thuringiensis* Subsp. *kurstaki* were applied consecutively to 20-s instar *S. recurvalis* larvae. The *Bt* application was according to the recommended application rate of *Bt* for lepidopterans (50 mg/100 ml). For each individual treatment with *M. anisopliae* isolate ICIPE 30 and *B. thuringiensis* Subsp. *Kurstaki*, the insects were treated using the same application or spray procedures as described earlier. The control groups were sprayed with sterilized distilled water containing 0.05% Triton X-100. Larval mortality was recorded daily for 7 days. The treatments were arranged in a completely randomized design with three replicates per treatment.

### 2.7 | Data analysis

Mortality data were corrected using Abbott's formula (Abbott, 1925) to correct natural mortality before being subjected to one-way analyses of variance (ANOVA). Whenever treatments were found to be significantly different ($p < .05$), means were separated using the Student–Newman–Keuls (SNK) test. Lethal time to 50% mortality ($LT_{50}$) values was estimated for the EPF isolates that caused greater than 50% mortality of the larvae and moths. Time-mortality data were analysed by means of generalized linear model (GLM) using the function "dose.p" from the MASS library, to generate $LT_{50}$ estimates, along with slopes of the regression curves. GLM analysis was run for each replication, and the resultant $LT_{50}$ values and their respective slopes were subjected to ANOVA to generate

**TABLE 2** Effects of EPF on the second instar larvae of *Spoladea recurvalis* at 7 days post-treatment

| Fungal species | Fungal isolate | % Mortality ± SE |
|---|---|---|
| *M. anisopliae* | ICIPE 18 | 6.7 ± 1.7ef |
| | ICIPE 30 | 58.3 ± 3.3a |
| | ICIPE 69 | 10.0 ± 2.9ef |
| | ICIPE 7 | 16.7 ± 1.7cde |
| | ICIPE 62 | 13.3 ± 3.3def |
| | ICIPE 78 | 21.7 ± 1.7bcd |
| *B. bassiana* | ICIPE 644 | 26.7 ± 4.4bc |
| | ICIPE 725 | 30.0 ± 2.9b |
| *I. fumosorosea* | ICIPE 682 | 21.7 ± 1.7bcd |
| | Control | 3.3 ± 1.7f |

Means within a column followed by the same letters are not significantly different by Student–Newman–Keuls (SNK) test ($p < .05$).

means. Means were separated using the Student–Newman–Keuls (SNK) test at $p < .05$. All data analyses were performed using R (version 3.2.5) statistical software packages (R Development Core Team, 2016).

## 3 | RESULTS

### 3.1 | Pathogenicity of EPF isolates against second instar larvae of *Spoladea recurvalis*

Viability tests showed that conidia germination of the different fungal isolates used in this study exceeded 90% after 18-hr incubation at 25 ± 2°C (Table 1). Of the 24 fungal isolates screened at the concentration of $1 \times 10^8$ conidia/ml against *S. recurvalis* larvae, only nine isolates caused larval mortalities in *S. recurvalis* (Table 2). The 15 isolates that caused no larval mortalities are 8 *M. anisopliae* (ICIPE 20, 40, 74, 48, 63, 727, 41 and 723) and 7 *B. bassiana* (ICIPE 676, 10, 273, 279, 281, 726 and 35). *Metarhizium anisopliae* isolate ICIPE 30 was the only isolate that caused moderate mortality of 58.3%, which was significantly higher than mortalities caused by any of the other eight isolates as well as the control ($F = 34.21$, $df = 9, 20$, $p < .0001$; Table 2). The lethal time to 50% mortality ($LT_{50}$) caused by *M. anisopliae* isolate ICIPE 30 was 5.6 days. The other eight isolates caused mortalities below 50% and their $LT_{50}$ values were therefore not computed.

### 3.2 | Screening of EPF isolates against adults of *Spoladea recurvalis*

All eleven fungal isolates screened were pathogenic to the moths, but the mortality varied significantly between the fungal isolates ($F = 9.46$, $df = 10, 22$, $p < .0001$). *Metarhizium anisopliae* isolate ICIPE 30 and *B. bassiana* isolate ICIPE 725 caused the highest mortalities of 92% and 83%, respectively, 7 days after treatment (Table 3). The lethal time required to achieve 50% mortality ($LT_{50}$) was calculated for isolates that caused >50% mortality 7 days post-treatment. The shortest $LT_{50}$ of 4.8 days was recorded for *M. anisopliae* isolate ICIPE 30 (Table 3).

**TABLE 3** Pathogenicity of entomopathogenic fungal isolates against *Spoladea recurvalis* moths and their $LT_{50}$ values 7 days post-treatment

| Fungal species | Isolate | % Mortality± SE | $LT_{50}$ (days) (95% FL) |
|---|---|---|---|
| *Metarhizium anisopliae* | ICIPE 69 | 74 ± 7.5bc | 6.2 (6.2–6.3) |
| | ICIPE 78 | 64 ± 4.7bc | 5.9 (5.8–6.0) |
| | ICIPE 62 | 56 ± 10.6bcd | 7.1 (6.9–7.2) |
| | ICIPE 7 | 68 ± 4.0bc | 6.3 (6.2–6.3) |
| | ICIPE 30 | 92 ± 4.0a | 4.8 (4.7–4.8) |
| | ICIPE 18 | 67 ± 8.3bc | 6.6 (6.4–6.7) |
| | ICIPE 20 | 58 ± 4.3bcd | 6.8 (6.7–6.9) |
| | ICIPE 74 | 42 ± 8.3cde | - |
| *Beauveria bassiana* | ICIPE 725 | 83 ± 4.3ab | 5.4 (5.3–5.4) |
| | ICIPE 644 | 27 ± 4.7e | - |
| *Isaria fumosoroseus* | ICIPE 682 | 30 ± 4.3de | - |

Means within a column followed by the same letters are not significantly different by Student–Newman–Keuls (SNK) test ($p < .05$). LT50 (in days); FL represents fiducially limit at 95%. $LT_{50}$ values for ICIPE 74, 644 and 682 were not computed as they only caused mortality <50% in *S. recurvalis*.

### 3.3 | Efficacy of *Bacillus thuringiensis* against second instar *Spoladea recurvalis* larvae

All five serial diluted concentrations which varied from 6.25 to 100 mg/ml tested against the second instar larvae of *S. recurvalis* caused <50% mortalities, 7 days post-treatment. Although there were significant differences in the mortalities caused by the respective *Bt* concentrations applied ($F = 33.13$, $df = 4{,}100$, $p < .0001$), none controlled 50% of the larvae and it was therefore not possible to calculate a $LC_{50}$ value for the tested concentrations (Figure 1).

### 3.4 | Interactions between *Metarhizium anisopliae* isolate ICIPE 30 and *Bacillus thuringiensis kurstaki* (*Bt*)

There was no significant difference in *S. recurvalis* larval mortality between the consecutive application of *M. anisopliae* isolate ICIPE 30 and *B. thuringiensis* Subsp. *kurstaki* (*Bt*) as a treatment (56.9%), compared to the mortality recorded for individual treatments of each biocontrol agent (51.0% for ICIPE 30 and 35.5% for *Bt*) separately ($F = 3.7$, $df = 2{,}6$, $p = .0892$).

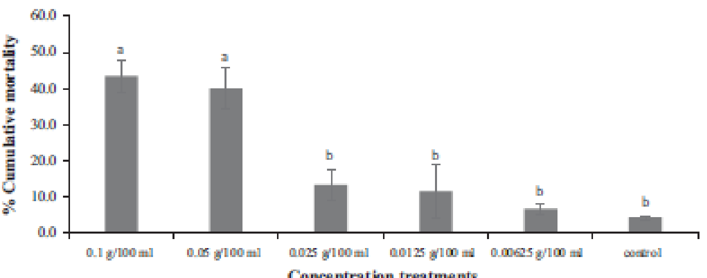


**FIGURE 1** Pathogenicity of *Bt* against second instar larvae of *Spoladea recurvalis* at five different concentrations. Bars denote means ± standard errors ($p < .05$ and those indicated by the same letter do not differ significantly (Student–Newman–Keuls (SNK) test)

## 4 | DISCUSSION

This study focused on developing efficient entomopathogenic fungal and *Bt*-based biopesticides for the management of *S. recurvalis*, a devastating pest of amaranth.

The EPF bioassays results from screening of 24 fungal isolates against the second instar *S. recurvalis* larvae indicated that only *M. anisopliae* ICIPE 30 caused moderate larval mortality of 58.3%. All the other 23 isolates recorded mortalities below 50%. Although *Metarhizium anisopliae* ICIPE 30 has been reported to be highly virulent against the other lepidopteran larvae such as the second instar larvae of the cereal stem borers, *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) and against fifth and sixth instar larvae of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) causing 96.2% larval mortality in both species (Inglis et al., 2001; Maniania, 1992), our findings showed that *S. recurvalis* is moderately susceptible to the same fungal isolate. In addition, these results are opposite to Tumuhaise et al. (2015) who demonstrated that the legume pod borer, *Maruca vitrata* (Fabricius) (Lepidoptera: Crambidae), was highly susceptible to *M. anisopliae* ICIPE 18 that caused 91% larval mortality, *S. recurvalis* larvae were less susceptible to ICIPE 18 with only 6.7% mortality recorded. The mode of action of EPF involves conidial attachment to the host surface followed by germination and penetration of the host cuticle through a combination of enzymatic activity and mechanical force (Arruda, Lübeck, Schrank, & Vainstein, 2005; Inglis et al., 2012; Quintela & McCoy, 1998). However, some insects have evolved a number of mechanisms to keep pathogens at bay that include (a) production of cuticular antimicrobial lipids, proteins and metabolites (Greenfield, Lord, Dudley, & Butt, 2014; Pedrini et al., 2015); (b) shedding of the cuticle during development; (c) behavioural–environmental adaptations such as induced fever, burrowing and grooming (Oi & Pereira, 1993; Siebeneicher, Bradleigh, & Kenerley, 1992).The low pathogenicity of the selected EPF isolates to *S. recurvalis* larvae could be due to cuticular or behavioural environmental adaptations that warrant further research to elucidate the exact reasons for this low pathogenicity reported in the present study.

This study also evaluated and documented for the first time the efficacy of a commercially formulated *Bt kurstaki* product, Halt® 5 WP against the second instar larvae of *S. recurvalis* under laboratory conditions, where low mortality of 40% was recorded following the recommended concentration for lepidopterans. Similarly when a high concentration was applied on the larvae (as the recommended dose is not for *S. recurvalis*), low mortality of 43.3% was observed. *Bt* mode of action is through ingestion of toxin crystal proteins (Bravo et al., 2007, 2013; Endo & Nishiitsutsuji-Uwo, 1980). We therefore hypothesed that the low mortalities caused by *Bt* could be possibly due to development of resistance by *S. recurvalis* larvae to the *Bt* toxins. This hypothesis therefore needs to be verified further by means of chemical and/or molecular techniques investigation. In contrast to our findings on low efficacy of *Bt* to *S.recurvalis* larvae, many studies have reported susceptibilities of lepidopteran larvae to *Bt* toxins (González-Cabrera, Mollá, Montón, & Urbaneja, 2011; Srinivasan & Yun-Che, 2008). For instance, evaluation of three commercial formulates based on *B. thuringiensis* against the first, second and third instar larvae of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in the laboratory, greenhouse and open-field experiments demonstrated that *B. thuringiensis* effectively controls *T. absoluta* (González-Cabrera et al., 2011). In the same line, Diamondback moth (DBM), *P. xylostella*, cabbage head caterpillar, *Crocidolomia binotalis* (Zeller) (Lepidoptera: Pyralidae) and cabbage webworm, *Hellula undalis* (Fabricius) (Lepidoptera: Pyralidae) were reported to be highly susceptible to four *Bt* δ-endotoxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca) and a commercial formulation based on *Bt* Subsp. *aizawai* (Xentari®) (Srinivasan & Yun-Che, 2008), while only 43.3% *S. recurvalis* larval mortality was recorded at high concentration of *B. thuringiensis* Subsp. *kurstaki* product Halt®. *Plutella xylostella* was equally susceptible to the toxins Cry1Ac, Cry1Aa and Cry1Ca with $LC_{50}$s of 25.7, 28.5 and 30.3 ppm, respectively; *Crocidolomia binotalis* was highly susceptible to all the Cry1A toxins, *viz.* Cry1Ac, Cry1Ab and Cry1Aa with $LC_{50}$ values of 3.6, 3.7 and 4.0 ppm; the toxin Cry1Ca was the most potent ($LC_{50}$ = 13.2 ppm) against *Hellula undalis* and *Bt* Subsp. *aizawai* was highly toxic to *H. undalis* ($LC_{50}$ = 14.8 ppm), and less toxic to *C. binotalis* or *P. xylostella* (Srinivasan & Yun-Che, 2008).

Results from combined treatments of *M. anisopliae* ICIPE 30 and *Bt* Halt® against second instar larvae of *S. recurvalis* showed no significant difference in the mortalities after combining the two entomopathogens compared to when the entomopathogens were applied individually. While many studies have shown significant synergistic or additive mortality effects to their target pests after

combination and application of both *Bt* and EPF based biopesticides (Lewis, Berry, Obrycki, & Bing, 1996; Mantzoukas, Milonas, Kontodimas, & Angelopoulos, 2013; Wakil et al., 2013; Wraight & Ramos, 2005), our findings showed the opposite for *S. recurvalis* in this study. The low mortalities obtained after combining the two entomopathogens might be a result of cuticular barriers by the larvae to EPF as its mode of infection is penetration through the external cuticle, their or possible resistance to *Bt* toxins. However, further studies are required to elucidate this phenomenon of *S. recurvalis* larvae towards the two biocontrol agents.

Adult *S. recurvalis* were, however, found highly susceptible to all eleven fungal isolates screened against them. The virulence of the evaluated isolates varied in terms of mortality as well as lethal time values. *Metarhizium anisopliae* ICIPE 30 and *B. bassiana* ICIPE 725 were highly effective and outperformed the other nine fungal isolates with 92% and 83% mortalities, respectively. *Spoladea recurvalis* pest is reported for the first to be susceptible to *B. bassiana* ICIPE 725 although a wide range of insects have already been reported to be susceptible to *M. anisopliae* ICIPE 30, namely the cabbage aphid, *Brevicoryne brassicae* (Linnaeus) (Hemiptera: Aphididae), turnip aphid *Lipaphis pseudobrassicae* (Davis) (Hemiptera: Aphididae) and the melon/cotton aphid, *Aphis gossypii* (Glover) (Hemiptera: Aphididae) (Bayissa et al., 2017), legume flower thrips, *Megalurothrips sjostedti* (Trybom) (Thysanoptera: Thripidae) (Ekesi, Maniania, Onu, & Löhr, 1998) and termite *Macrotermes michaelseni* (Sjostedt) (Isoptera: Termitidae) (Gitonga, 1996). Therefore, the diverse host range of *M. anisopliae* ICIPE 30 makes it a suitable candidate to be developed as a fungal biopesticide against a broad range of insect pests, and especially against *S. recurvalis* adults where the extension of labels upon registration and commercialization could benefit many users especially small scale farmers. The ability of EPF to infect their hosts through the cuticle offers a better alternative management strategy, because they do not need to be ingested like bacteria, viruses and protozoa (Chandler et al., 2011). The major challenge for using dry EPF conidia to control moths could be their application in the field. However, recently, autodissemination contamination devices were developed to infect fruit flies (Dimbi, Maniania, Lux, Ekesi, & Mueke, 2003), tsetse flies (Maniania, 1998, 2002) and thrips (Niassy, Maniania, Subramanian, Gitonga, & Ekesi, 2012). These have been tested in the field with success (Ekesi, Dimbi, & Maniania, 2007; Maniania, Ekesi, Odulaja, Okech, & Nadel, 2006; Mfuti et al., 2016). This novel cost-effective autodissemination strategy could consequently also be exploited or adapted for *S. recurvalis* population suppression. The moths can therefore be used as vectors to transmit the inoculum among conspecifics in the environment after they have been attracted and have acquired the pathogen (Vega, Meyling, Luangsa-ard, & Blackwell, 2012). An effective autodissemination strategy requires an autoinoculation trap baited with powerful attractants. The entomopathogens should also be compatible with the attractants and further research is therefore warranted to test and identify powerful attractants of adult *S. recurvalis*. An autodissemination trap for *S. recurvalis* moths should also be developed and evaluated using the potent *M. anisopliae* ICIPE 30 isolate. In order to efficiently combine EPF with an attractant in an autodissemination device, further studies to assess the compatibility of *M. anisopliae* ICIPE 30 with *S. recurvalis* attractants are also needed to optimize the mass trapping or dissemination of the pathogen. Management of the adult stage of *S. recurvalis* will offer an excellent opportunity to trap and kill the pest before it could lay eggs and eventually develop into larvae which are the damaging stage to crops.

Our finding showed that entomopathogenic fungi can cause infections in all life stage but not all host stages in an insect's life cycle are equally susceptible to pathogen infection. Pupal stages are often being reported to be the most resistant stage and adults as the most susceptible (Butt & Goettel, 2000; Hajek & St Leger, 1994). This has been clearly demonstrated in the present study where the adults of *S. recurvalis* were highly susceptible to EPF while susceptibility of the larval stage was insignificant and only *M. anisopliae* ICIPE 30 caused more than 50% mortality. The results of this study have shown that *M. anisopliae* ICIPE 30 is a promising alternative for synthetic chemical insecticides in the management of adult *S. recurvalis*. Further studies aiming to identify powerful attractants of adult *S. recurvalis* that are compatible with *M. anisopliae* ICIPE 30 are warranted for establishment of an attract and kill strategy through autodissemination techniques. To the best of our knowledge, the present study is the first laboratory-based report on the susceptibility of *S. recurvalis* to entomopathogenic fungi and *Bt* and their potential in amaranth lepidopteran management.

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## AUTHOR CONTRIBUTION

All authors conceived and designed the research. SO conducted experiments and analysed data. KKMF and SE provided the fungal isolates, reagents materials, analytical tools and the facilities. SO, HP, KSA, KKMF and SE wrote the manuscript. KKMF and SE secured funding. All authors read and approved the manuscript.

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CHAPTER 4: ARTICLE 2

# Horizontal transmission of *Metarhizium anisopliae* between *Spoladea recurvalis* (Lepidoptera: Crambidae) adults and compatibility of the fungus with the attractant phenylacetaldehyde

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**Abstract**

The compatibility of the entomopathogenic fungus *Metarhizium anisopliae* ICIPE 30 which was proved to be pathogenic to adult *Spoladea recurvalis*, and phenylacetaldehyde (PAA) floral attractant for lepidopteran moths, was investigated under laboratory and field conditions through spatial and temporal separations. Horizontal transmission of *M. anisopliae* ICIPE 30 between adult *S. recurvalis* and the number of conidia acquired by a single moth from the autoinoculation device were also determined under laboratory conditions. Freshly emerged moths were inoculated with fungal conidia (‘‘donors”) and maintained together with an equal number of untreated freshly emerged moths (“recipients”) in the laboratory. Transmission of the infection to untreated moths resulted to 76.9% mortality with an $LT_{50}$ value of 6.9 days. The overall mean number of conidia acquired by a single moth was significantly higher immediately after exposure (0 hours) ($14.3 \pm 2.5 \times 10^5$) than what it retained at 24, 48, and 72 hours after inoculation (F = 10.26, Df = 3,8, P = 0.003), though a single moth still retained $4.6 \pm 0.9 \times 10^5$ conidia 72 hours post inoculation. Laboratory results showed that PAA completely inhibited the germination of the conidia 8 days post exposure, while the conidial viability was not affected in the control treatment without PAA. Under field conditions, the inhibitory effects of PAA on conidial germination was minimized by placing it at a distance of 5 to 10 cm from *M. anisopliae* isolate ICIPE 30 conidia. There was no significant difference between conidial germination in the control treatment and in treatments where PAA was placed at 5 cm and 10 cm away from *M. anisopliae* isolate ICIPE 30. Germination was low for the conidia that was directly exposed to PAA in the the autoinnoculation device. PAA is therefore compatible with *M. anisopliae* ICIPE 30 for use in integrated management of *S. recurvalis*, if spatially separated by at least 5 cm from the fungus. This fungus strain can therefore be combined in autocontamination devices for the control of *S. recurvalis.*

**Keywords:** Amaranths, synthetic insecticides, entomopathogenic fungus, lure and kill, autoinoculation device, biopesticide

## 4.1 Highlights

- Adult *Spoladea recurvalis* moth acquired and retained conidia for up to 72 hours
- Infected moths (‘‘donors”) horizontally infected untreated moths (“recipients”)
- Phenylacetaldehyde completely inhibited conidia germination in laboratory tests
- Negative effect of PAA on conidial persistence was reduced by distance separation of 5 and 10 cm

## 4.1 Introduction

The beet webworm*, Spoladea recurvalis* (Fabricius) (Lepidoptera: Crambidae) is one of the most damaging insect pests of vegetable crops in the tropical and subtropical regions of the world (Centre for Agriculture and Bioscience International, 2016). Its main host plant is amaranth (James *et al.*, 2010, Othim *et al.*, 2018). Severe larval infestation can cause up to 100% yield loss if not effectively controlled (Kahuthia-Gathu, 2011). Amaranth farmers rely heavily on insecticides to control the pest (Ngowi *et al.*, 2007, James *et al.*, 2010). However, an integrated pest management (IPM) approach that is ecologically sound and sustainable is needed because control by means of synthetic insecticide application is not only inefficient, but also has deleterious effects on the environment and human health, while leading to development of insecticide resistance in *S. recurvalis* and eliminating natural enemies (Clarke-Harris and Fleischer, 2003, Dinham, 2003, Jeyanthi and Kombairaju, 2005, Manyilizu *et al.*, 2017).

Microbiological treatments with entomopathogenic fungi offer an alternative to the use of chemical insecticides (Inglis *et al.*, 2001). A recent study on the pathogenicity of entomopathogenic fungi

(EPF) to *S. recurvalis,* identified *M. anisopliae* ICIPE 30 isolate to be more virulent to adults than to the larval stage (Opisa *et al.*, 2018). Since entomopathogenic fungi infect their host through the cuticle, they hold greater potential as biocontrol agents for moths than other entomopathogens such as bacteria and viruses, which must be ingested to be effective. Management of the adult stage of *S. recurvalis* would therefore offer an excellent opportunity to trap and kill the pest and to reduce the moth population numbers before it could lay eggs and eventually develop into larvae which are the damaging stage to amaranth crops. However, the high mobility of flying *S. recurvalis* moths could pose a challenge for the use of dry conidia or suspension for management of this pest. The effectiveness of application and persistence of EPF in the field has been improved through the development of an autodissemination management approach ("attract and infect"). This approach involves the attraction of the moths to a semiochemical-baited inoculation device where they are infected with the pathogen and on returning to the environment, they can disseminate the pathogen among the insect population (Vega *et al.*, 2007). Autodissemination of EPF provides an additional advantages over the use of chemical insecticides, where (1) attractant traps ensure that only the targeted pest is attracted to the pheromone or other attractants in the traps and therefore minimizes the effects of the fungus on other non-target organisms and the environment; (2) small quantities of fungus (and pheromone) are required, thus minimizing treatment costs; and (3) the trap is designed to protect the fungus from the harmful effects of UV and to provide suitable humid conditions to promote the activity of the fungus. Contamination devices were developed to infect fruit flies (Dimbi *et al.*, 2003), tsetse flies (Maniania, 1998, Maniania, 2002) and thrips (Niassy *et al.*, 2012), and have been tested in the field with success (Maniania *et al.*, 2006, Ekesi *et al.*, 2007, Mfuti *et al.*, 2016b). The ability of an insect pest to acquire conidia from an autoinoculation device and horizontally transfer it to other members of the population is a very important factor to be considered in designing an autoinoculation device for successful EPF microbial control. This study investigated whether fungus-treated moths can transfer conidia to fungus-free moths during mating

or physical contact and evaluated the quantity of conidia a moth could acquire and retain from the autoinoculation device after inoculation in the laboratory.

Phenylacetaldehyde (PAA), a floral odorant has been reported to attract adults of both sexes of various lepidopteran species including *S. recurvalis* (Maini and Burgio, 1990, Meagher, 2002, Landolt *et al.*, 2011b) and can thus be combined with EPF, *M. anisopliae* ICIPE 30 in a lure and infect strategy for *S. recurvalis*. However, a successful autodissemination system depends on the virulence and persistence of the EPF, the efficacy of the autoinoculation device in attracting the target insect and transmitting the inoculum, and most importantly the compatibility of the attractants with EPF as demonstrated in previous research studies (Vega *et al.*, 1995, Klein and Lacey, 1999, Maniania *et al.*, 2006, Lyons *et al.*, 2012, Nana *et al.*, 2012, Mfuti *et al.*, 2016a, Mfuti *et al.*, 2016b, Mfuti *et al.*, 2017).

This study thus evaluated the effects of PAA on conidial viability and persistence of *M. anisopliae* ICIPE 30, and tested whether the two can be used together in a semiochemical-baited trap for control of *S. recurvalis* for improvement of the horizontal pathogen transmission.

## 4.2 Materials and methods

### *Study site*

The study was conducted in the laboratory and under field conditions at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville Campus in Nairobi, Kenya (1.221S, 36.896E; 1616 m above sea level). The mean annual temperature is between 15 and 24 °C and an average of 1,000 mm bimodal annual rainfall (Kenya Meteorological Department, 2015).

### *Semiochemical*

Phenylacetaldehyde (PAA) (Sigma-Aldrich Chemie GmbH) used in this study was purchased from Kobian, Kenya Limited.

***Fungal culture***

*Metarhizium anisopliae* isolate ICIPE 30, isolated from a *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) larva in 1989 was used in this study. It was obtained from the Arthropod Germplasm Centre of *icipe*, and its pathogenicity and virulence against *S. recurvalis* has been previously reported (Opisa *et al.*, 2018). The fungus was cultured on Sabouraud dextrose agar (SDA) in Petri dishes (90 mm diameter) at 25 ± 2 °C and incubated in darkness following the methodology described by Maniania (1992). Conidia were harvested from 2 to 3-week old sporulated cultures and suspended in 10 ml distilled water with 0.05% Triton X-100 in universal bottles containing two glass beads (ϕ = 3 mm). The bottles were vortexed to produce a homogeneous conidial suspension. The spore concentration was determined using an improved Neubauer hemacytometer. Viability tests were carried out using the technique described by Goettel and Inglis (1997). Conidial suspension (0.1 ml) titrated to $3 \times 10^6$ conidia $ml^{-1}$ was spread-plated on Petri dishes (90 mm diameter) containing SDA. The suspension was spread evenly on the plate and three sterile microscope cover slips were placed randomly on each plate. Plates were incubated at 25 ± 2 °C and percentage germination was determined by counting 100 spores for each plate after 18 to 24 h under a light microscope (Leica DMLB) at ×400 magnification. Lactophenol cotton blue was added to terminate germination and also stain the spores for ease of counting. Conidia were considered to have germinated when the length of the germ tube was at least twice the diameter of the conidium (Goettel and Inglis, 1997, Inglis *et al.*, 2012).

For the autodissemination experiments, conidia of *M. anisopliae* ICIPE 30 were mass-produced on a long-rice substrate in Milner bags (60 cm long by 35 cm wide) following the technique described by Maniania (1998). Rice was autoclaved for 1 h at 121 °C and inoculated with a three-day-old culture of blastopores (Jenkins *et al.*, 1998). The inoculated bags were then incubated for 21 days at 20 - 26 °C and 40 - 70% relative humidity (RH). The rice substrate containing fungal spores was

then allowed to dry for 5 days at room temperature. Conidia were harvested by sifting the substrate through a 295 μm mesh sieve and then stored in plastic bags in a refrigerator (4 - 6 $^{o}$C) until use.

### 4.2.1 Conidial acquisition and retention by a single moth after inoculation from an autoinoculation device

To determine the quantity,of *M. anisopliae* ICIPE 30 that moths retain after inoculation moths were inoculated according to the technique described by Opisa *et al*. (2018). Twenty 1-day-old virgin *S. recurvalis* moths were contaminated with ICIPE 30 dry conidia using velvet-coated plastic jars (150 × 80 mm). The devices were contaminated with 2 g of dry conidia. Moths were introduced into the device for 5 min to acquire spores and thereafter transferred into clean ventilated Perspex cages (300 × 300 × 300 mm). Immediately after exposure, 5 moths were placed individually in a universal bottle containing 1 ml of 0.05% Triton X-100. The universal bottles were then vortexed to dislodge conidia from the insect's body and quantify spore rentention. The number of conidia acquired by each moth was estimated using a haemocytometer (Dimbi *et al.*, 2013). The number of conidia retained by moths was monitored after 0, 24, 48 and 72 hours.

### 4.2.2 Horizontal transmission of *Metarhizium anisopliae* ICIPE 30 between *Spoladea recurvalis* adults

Five freshly emerged moths regardless of sex were treated with 1 g of dry conidia of *M. anisopliae* ICIPE 30 for 5 minutes as previously described above and introduced into clean ventilated Perspex cages (300 × 300 × 300 mm). Twenty four hours later, the treated moths (served as ‘donors’) were placed with five untreated moths (served as ‘recepients’) in Plexiglas for 24 h to allow for horizontal transmission of *M. anisopliae* conidia through contact. The recipients moths were stained with nontoxic poster colours (ACRON®, Pidilite Industries Ltd., Regent Chambers, Mumbai, India) on the white band of their wing to allow them to be distinguished from inoculated

donors. The moths were separated after 24 hours into two groups of donors and recipients. Both groups were supplied with 10% honey solution as food source and mortality was monitored for 10 days. The same experimental protocol was used for the control, except that donor moths were not exposed to the fungal treatment. Each combination of five donors and five recipients was considered a single replicate per treatment. There were three replicates per treatment. Dead moths were surface sterilized with 1% sodium hypochlorite followed by three rinses with sterile distilled water, placed on sterile moist filter paper in sterile petri dishes that were then sealed with parafilm and kept at room temperature to be inspected for development of mycosis on the cadavers.

### 4.2.3 Effect of temporal separation of PAA and *Metarhizium anisopliae* ICIPE 30 on conidial viability

To assess the viability of the conidia in a compatibility test, the methodology described by Mfuti et al. (2016a) was used. The conidial suspension was prepared as described earlier and titrated to $1\times10^7$ conidia $ml^{-1}$. The spores were retained on a nitrocellulose filter membrane (diameter 47 mm, pore size 0.45 µm, Sigma Chemicals) by pouring 10 ml suspension through a filter holder unit (MFS) under an aspirator vacuum (Maniania, 1994). The nitrocellulose filter membranes were dried for 30 min in a laminar flow cabinet. Five nitrocellulose filter membranes were transferred to a single glass desiccator (2.5 L) for exposure to the PAA attractant. A cotton wick was soaked in 4 ml PAA (Landolt *et al.*, 2011a) and placed in the desiccator to allow for volatile diffusion. Fungus-treated nitrocellulose membranes were exposed to PAA attractant for 1, 2, 3, 6 and 8 days respectively. After these exposure times, one fungus-treated nitrocellulose filter membrane containing conidia was removed from the desiccator without replacement, and transferred into 10 ml sterile distilled water containing 0.05% Triton X-100 and vortexed for 3 min to dislodge the conidia. Suspension (0.1 ml) titrated to $3 \times 10^6$ conidia $ml^{-1}$ was spread-plated on SDA plates. Plates were incubated at $25 \pm 2$°C, L12:D12 photoperiod and examined after 18 to 24 h for conidial germination. Germination of conidia was determined as described above following the various

exposure time. The same procedure, but without PAA attractant was applied for the control treatment. Each of the five nitrocelloluse filter membranes served as treatments at each exposure time for fungal viability. The fungal viability treatments were completely randomized and the experiment was replicated three times, with each glass desiccator containing five nitrocellulose filter membranes serving as a replication.

### 4.2.4 Effects of spatial separation of PAA on thepersistence of *Metarhizium anisopliae* ICIPE 30 conidia in the field

The experiments were intended to validate the compatibility of PAA and *M. anisopliae* ICIPE 30 conidia placed at different positions in an autoinoculation device. The autoinoculation device and the procedure for the inoculation device used in the present study were as described by Niassy *et al.* (2012). A Lynfield trap (11 cm diameter × 10 cm height) was perforated with six entry/exit holes (2 × 3 cm) near the top and another set of six holes near thebottom of the trap at alternate positions. A piece of velvet cloth (8 × 8.5 cm) was wrapped around a smaller inner cylindrical bottle (5.2 cm diameter × 6 cm height) that was then hung in the trap. Approximately 2 - 3 g of dry conidia were spread evenly on the velvet cloth of the autoinoculation device. Five cotton wicks were individually imbibed into 4 ml of PAA (Landolt *et al.*, 2011a) and placed at five different positions in the trap following the methodology and design developed by Mfuti *et al.* (2016b). These positions were as follows: (i) direct exposure of the cotton wick imbibed in PAA to fungal conidia in the device (direct), (ii) a small container (5.2 cm diameter × 6 cm height) containing the PAA fixed just below the autoinoculation device (0 cm), (iii) at 5 cm and (iv) 10 cm below the device, respectively as well as a control treatment in which a device containing the fungal conidia, but with no PAA added. The autoinoculation devices were hung at a height of 70 cm. The distance of separation of PAA from the fungal conidia served as treatments i.e. direct, 0 cm, 5 cm, 10 cm and control, respectively. Each of the five treatments were randomly deployed at three sites that served as

replicates. The sites were separated by a distance of at least 10 m to avoid interferences between adjacent sites.

### 4.2.5 Conidial viability and persistence assessment

Persistence of *M. anisopliae* conidia in the autoinoculation devices was studied over two weeks from 1-12 September 2017. A moist cotton bud was used to collect samples of conidia from the autoinoculation devices from each of the five treatments (Niassy *et al.*, 2012) at day 3, 6, 9 and 12 post-treatment from all the three replicated sites for each sampling day (Niassy *et al.*, 2012, Mfuti *et al.*, 2016b). The end of the cotton bud was cut, suspended in 10 mL of 0.05% (w/v) Triton X-100 and vortexed for 1 min to dislodge conidia. A sample of 100 µL was spread plated on SDA plates and incubated for 16 h at 25±2 °C and L12:D12 photoperiod. Germination of conidia was determined as described earlier.

### 4.2.6 Data analysis

Data on conidial germination of *M. anisopliae* ICIPE 30 were subjected to generalized linear model (GLM) using binomial regression analysis (Warton and Hui, 2011), with time and site as factors. Analysis of deviance was used to test the effects of the position of PAA in the autodissemination device on the viability or persistence of *M. anisopliae* ICIPE 30. Means were separated using Tukey's HSD test. Count data on conidial retention were checked for normality using Shapiro–Wilk test before analysis. After the normality test, analysis of variance (ANOVA) for the data on number of spores acquired by a single moth from the autoinoculation device based on the different exposure durations. Means were compared using the Student–Newman–Keuls (SNK) test. Percentage moth mortality data were corrected for natural mortality using Abbott's formula (Abbott, 1925) before being subjected to ANOVA. Lethal time to 50% mortality ($LT_{50}$) values were analyzed by Generalized Linear Model (GLM), using the function 'dose.p' from the MASS

library. All data analyses were performed using R (version 3.2.5) statistical software packages (R Core Team, 2016).

## 4.3 Results

### 4.3.1 Rate of conidia acquisition and retention by a single moth from an autoinoculation device

The average number of spores acquired by a single moth was significantly higher immediately after exposure (0 hour) ($14.3 \pm 2.5\times 10^5$) than at 24, 48, and 72 hours after inoculation ($F = 10.26$, $Df = 3,8$, $P = 0.003$). However, there were no significant differences in the number of spores retained by a single moth at 24 , 48, and 72 hours post-treatment (Table 4.1).

### 4.3.2 Horizontal transmission of *Metarhizium anisopliae* ICIPE 30 between *Spoladea recurvalis* adults

Both “donor” and “recipient” moths acquired and died due to fungal infection, resulting in $92.3 \pm 7.7$ and $76.9 \pm 13.3$% mortality in the two groups respectively. There was however, no significant difference in mortalities between the donor and recipient moths ($F = 0.99$, $Df = 1,4$, $P = 0.374$). The lethal time to 50% mortality (LT50), 6.9 days, was similar in both the “donors” and “recipients”. All dead moths developed 100% mycosis due to *M. anisopliae* ICIPE 30 infection.

**Table 4.1**: Conidia retention by *Spoladea recurvalis* moths after aquisition over time

| **Exposure duration (hours)** | **Number of conidia/ moths ± SE** |
|---|---|
| 0 | (14.3 ± 2.5) x $10^5$a |
| 24 | (7.9 ± 1.8) x $10^5$b |
| 48 | (5.9 ± 1.1) x $10^5$b |
| 72 | (4.6 ± 0.9) x $10^5$b |

Means (± SE) followed by the same lower case letters within the column are not significantly different according to Student–Newman–Keuls (SNK) test at $P < 0.05$.

### 4.3.3 Effects of temporal separation of PAA and *Metarhizium anisopliae* ICIPE 30 on conidial viability of in the laboratory

No conidia germinated after an exposure period of 1, 2, 3, 6 and 8 days to PAA Although the conidia germination was completely inhibited in all the PAA treatments, high conidial viability was recorded in the control treatment after the 8 days experimental period (85.2 ± 0.5%). However, conidial germination in the control treatment differed significantly over time ($\chi^2$ = 60.83; Df = 4; P = 0.0024 ; Figure 4.1). Conidia persistence over time or percentage germination in the control treatment was significantly higher for 1 day (97.6 ± 0.3%) and 2 days (94.1 ± 0.3%) than at 8 day (85.2 ± 0.5%) post exposure (Figure 4.1). Conidial persistence was however not significantly different from day 1 to day 6 (Figure 4.1).

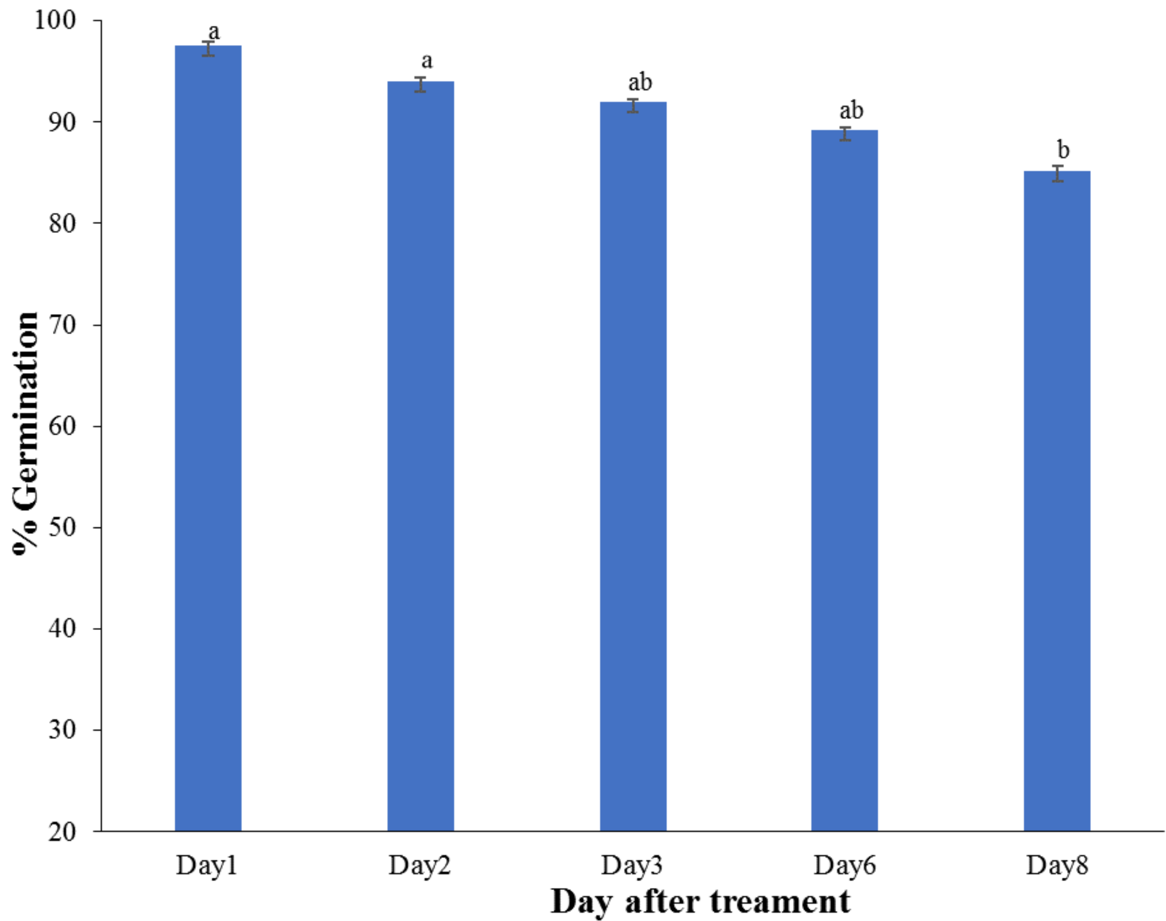


Figure 4. 1: Conidial germination or persistence of *Metarhiziium anisopliae* isolate ICIPE 30 in the control treatment over time in the laboratory. Bars denote means ± standard errors and means followed by the same letter(s) are not significantly different by GLM, Tukey's HSD test, $P < 0.05$.

#### 4.3.4 Effect of spatial separation of PAA on persistence of *Metarhizium anisopliae* ICIPE 30 conidia in the field

The separation distances of PAA and *M. anisopliae* ICIPE 30 conidia had a significant effect on viability of conidia ($\chi^2 = 180.8$; Df = 4; P < 0.0001; Table 4.2). The direct exposure of cotton wick imbibed in PAA to fungal conidia in the device (direct) caused significantly lower germination rates than when the PAA container was fixed just below the autoinoculation device (0 cm). Germination rate at 0 cm were significantly lower compared to separation by 5 cm, 10 cm and in

the control treatment (Table 4.3). There was, however, no significant difference in conidial viability when PAA was placed at 5 and 10 cm away from the autoinoculation device, with conidial germination of 50.1 ± 2.2%, and 54.1 ± 2.2%, respectively (Figure.4.2). Furthermore, the conidial viability in these two PAA treatments (5 and 10 cm) did also not differ significantly from the conidial viability in the control (55.1 ± 2.2%). Exposure duration had a significant effect on conidial germination which decreased over 12 days of exposure in all the treatments ($\chi^2$ =393.7; Df = 3; P < 0.0001) (Table 4.2).

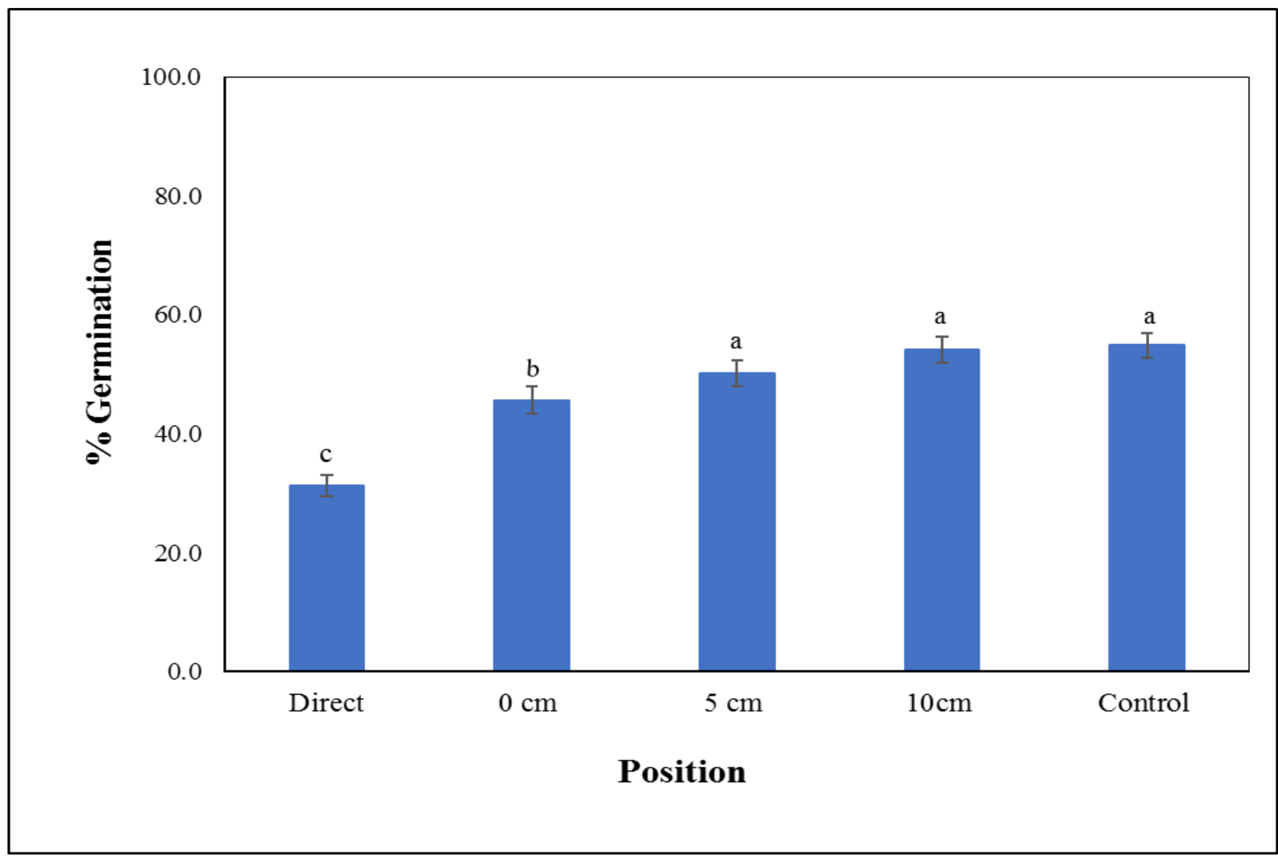


**Figure 4.2**: Effect of spatial separation of PAA on persistence of *Metarhizium anisopliae* isolate ICIPE 30 conidia in the field. Bars denote means ± standard errors. Means followed by the same letter are not significantly different by GLM, Tukey's HSD test, *P* < 0.05. Interaction between the distance of separation (treatments) and sites was not statistically significant different ($\chi^2$ =125.4; Df = 8; P = 2578) (Table 4.2).

**Table 4.2**: Results from the generalized linear model with exposure duration in the field, distance of separation, and their effects on conidial germination of *M. anisopliae* ICIPE 30

| Parameter | Df | Deviance | Resid. df | Resid. Dev ($\chi^2$) | P value |
|---|---|---|---|---|---|
| Site | 2 | 17.11 | 237 | 729.58 | 0.0001 |
| Day | 3 | 335.88 | 234 | 393.7 | < 0.0001 |
| Distance of separation | 4 | 212.9 | 230 | 180.81 | < 0.0001 |
| Site*Day | 6 | 45.35 | 224 | 135.46 | <0.0001 |
| Site* Distance of separation | 8 | 10.1 | 216 | 125.35 | 0.2578 |
| Day* Distance of separation | 12 | 8.71 | 204 | 116.65 | 0.7278 |
| Site* Day* Distance of separation | 24 | 14.85 | 180 | 101.8 | 0.9250 |

Df, degrees of freedom; Resid. df, residual degrees of freedom; Resid. Dev, residual deviance.

Viability of conidia decreased significantly over time, with the highest viability at 3 days and the lowest at 12 days exposure duration (Table 4.3). In the latter case, the lowest germination of 14.6 ± 3.0% was recorded in the treatment that had PAA directly exposed to *M. anisopliae* ICIPE 30 and the highest germination of 35.2 ± 4.5%, in the control treatment (Table 4.3).

**Table 4.3**: Effect of spatial separation of PAA on the persistence of conidia of *Metarhizium anisopliae* isolate ICIPE 30 in autoinoculation devices over time

| Separation distance | Exposure duration (days) | | | | % Mean* |
|---|---|---|---|---|---|
| | 3 | 6 | 9 | 12 | |
| Control | 67.2 ± 4.3 | 61.7 ± 3.0 | 55.5 ± 2.7 | 35.2 ± 4.5 | 54.9 ± 2.1 a |
| Direct | 43.0 ± 2.6 | 36.1 ± 4.0 | 31.4 ± 2.7 | 14.6 ± 3.0 | 31.3 ± 1.8 c |
| 0 cm | 58.7 ± 5.1 | 52.6 ± 4.1 | 48.9 ± 3.1 | 22.4 ± 3.6 | 45.7 ± 2.3 b |
| 5 cm | 63.2 ± 3.1 | 57.2 ± 3.8 | 50.6 ± 3.9 | 29.2 ± 5.3 | 50.0 ± 2.2 a |
| 10 cm | 66.6 ± 2.8 | 61.2 ± 6.8 | 55.4 ± 3.8 | 33.4 ± 4.1 | 54.1 ± 2.2 a |
| % Mean** | 59.7 ± 1.5A | 53.7 ±1.7B | 48.3 ± 1.4C | 27 ± 1.4D | |

* Means (± SE) followed by the same lower case letters within the column are not significantly different according to Tukey's HSD test. ** Means (± SE) followed by the same upper case letters within the row are not significantly different according to GLM, Tukey's HSD test, $P < 0.05$.

### 4.4 Discussion

Semiochemical attractants play a significant role in enhancing effectiveness of fungal-based biopesticides in arthropod pest management approaches (Nchu *et al.*, 2009, Nana *et al.*, 2012, Mfuti *et al.*, 2016b, Mfuti *et al.*, 2017). Phenylacetaldehyde attractant has been used as a monitoring tool of many lepidopteran pests (Maini and Burgio, 1990, Landolt *et al.*, 2011a); however its intergration with entomopathogenic fungi in suppressing pest populations has not been explored. Studies on compatibility of EPF with attractants are needed before their integration in an autodissemination pest management strategy. The use of incompatible attractants may inhibit the efficacy of EPF in a pest control strategy.

Opisa *et al*. (2018) recently demonstrated the pathogenicity and virulence of *M. anisopliae* ICIPE 30 against adult *S. recurvalis* (See Chapter 3). Compatibility of PAA and EPF ICIPE 30 in managing *S. recurvalis* will be advantageous since PAA attracts both sexes of the pest which may offer quick and double suppression of the pest population (Tóth *et al.*, 2009, Landolt *et al.*, 2011a, Landolt *et al.*, 2011b). Use of PAA in an autodissemination device will attract the moths, but it needs to be coupled with horizontal transmission of fungal conidia from treated to healthy moths within the moth population. Mortality in the donor group (92.3%) was not significantly different from to mortality of the recipient group (76.9%), indicating that the transmitted conidia still have the same potential in suppressing the pest population through the autodissemination strategy. Horizontal transmission of EPF from inoculated individuals to healthy ones can occur through direct contamination by passive transfer from inoculated adults (Dimbi *et al.*, 2013, Maniania *et al.*, 2013), indirect contamination by conidia deposited in the crop by inoculated adults (Pell *et al.*, 1993), and secondary transmission of conidia from the sporulating mycosed cadavers of diseased individuals which died within the crop (Furlong and Pell, 2001). The death and mycosis of untreated *S. recurvalis* (recipients) moths when they were placed together with treated moths (donors), demonstrated that horizontal transmission of *M. anisopliae* ICIPE 30from infected moths to healthy moths occurred either through mating or physical contact between the moths. Horizontal transmission of fungal conidia from treated to healthy individuals has also been demonstrated in other lepidopterans, such as the stem borer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) (Maniania *et al.*, 2011) and the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Furlong and Pell, 2001). Results from the present study have shown the potential of *M. anidopliae* ICIPE 30 for horizontal transmission in a *S. recurvalis* population under laboratory conditions, and further studies are warranted to test and validate its efficacy under field conditions. The similar $LT_{50}$ value of 6.9 days reported for both donors and recipients showed the ability of a single moth to still retain conidia for 72 hours, which is a fundamental attribute in an

autodissemination management approach since such a delay in the killing of the pest will ensure that the moths continue dispersing the inoculum in the population before death.

Compatibility tests carried out in the laboratory indicated that PAA completely inhibited conidial germination of *M. anisopliae* ICIPE 30 after exposure to the semiochemical in desiccators for 1 – 8 days. Jay and Rivers (1984) reported similar results of antifungal effects of PAA when used as a food flavouring compound. Incompatibility between insect attractants and EPF has also been reported previously; for example, Lurem-TR an attractant for thrips inhibited the germination of *M. anisopliae* ICIPE 69 conidia in autoinoculation devices (Niassy *et al.*, 2012). Similarly, the tick attraction-aggregation-attachment pheromone (AAAP) was also reported to inhibit radial growth and spore production for *M. anisopliae* ICIPE 7 that was found efficient for tick management (Nana *et al.*, 2012). The results of this study showed that conidia of *M. anisopliae* ICIPE 30, which was found pathogenic to *S. recurvalis,* can therefore not be used in direct combination with PAA inside an autoinoculation device. The autoinoculation device should therefore be modified as a lure and infect device that will not negatively affect conidial viability and persistence of *M. anisopliae* ICIPE 30 for control of *S. recurvalis*.

Conidial viability in the autoinoculation devices under field conditions that had conidia separated from PAA was high, compared to the autoinoculation device that had the PAA directly exposed to *M. anisopliae* ICIPE 30 conidia. The negative effect of PAA was therefore reduced with distance of separation (5 and 10 cm) from *M. anisopliae* ICIPE 30 conidia. Similar results were also reported by Mfuti *et al*. (2016b) where the fungal persistence of *M. anisopliae* isolate ICIPE 69 was increased with distance of separation of Lurem-TR (0-20 cm) in the development of a thrips management strategy.

Persistence of *M. anisopliae* ICIPE 30 conidia applied in the autoinoculation device under field conditions over 12 days was low, with only 35% conidial germination after 12 days of exposure. A similar result of 41% conidial germination was reported by Mfuti *et al*. (2016b) for *M. anisopliae* ICIPE 69 in the autoinoculation device after 15 days of field exposure. However, Maniania *et al*.

(2002) reported 60% and 43% conidia viability of *M. anisopliae* ICIPE 30 in a contamination device placed in the sun and shade, respectively for 31 days. Despite the loss of conidial viability to 43% the level of fungal infection remained unchanged for 31 days at > 90% mortality in tsetseflies. However, compared to 85% fungal viability of the control treatment under laboratory conditions in the present study, the low persistence of fungal propagules reported in the field suggests that abiotic factors especially the solar ultraviolet (UV) radiation (UV-A and UV-B) (Jaronski, 2010) may be the main factor detrimental to the fungus in the trap. Formulations of dry conidia with adjuvants to absorb or to block solar radiation and thereby protect fungi from UV radiation (Inglis *et al.*, 1995) before use in the trap are recommended. This should increase the persistence of the fungal conidia and consequently decrease the application frequency.

### 4.5 Conclusion

This study has revealed that PAA and *M. anisopliae* ICIPE 30 conidia can be used together in an autoinoculation device, but the two should be spatially separated by 5 to 10 cm. PAA and *M. anisopliae* ICIPE 30 could therefore be considered for use ina "lure and kill" management strategy against *S. recurvalis.* However, additional field studies need to be conducted with an autoinoculation device and PAA separated as from 5 cm during *S. recurvalis* infestation outbreaks to assess the efficacy of the autodissemination delivery system in suppressing the pest population. There is also a need to screen for other lures for *S. recurvalis* and to test their compatibility with *M. anisopliae* ICIPE 30 to to boost up with PAA in suppressing pest populations.

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## CHAPTER 5 ARTICLE 3

**Role of chemical additives in enhancing the efficacy of *Bacillus thuringiensis* subsp. *kurstaki* for biological control of *Spoladea recurvalis* (Lepidoptera: Crambidae) on amaranths**

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**Abstract**

*Spoladea recurvalis* (Fabricius) (Lepidoptera: Crambidae) larvae can cause 100% foliage loss on amaranths during severe outbreaks. *Bacillus thuringiensis* Subsp. *kurstaki* product Halt® is a biologically safe biopesticide recommended for the management of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) and *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae). *Spoladea recurvalis* is not very susceptible to this product. Thirteen chemical additives (7 inorganic salts, 3 nitrogenous compounds, 2 protein solubilizing agents and 1 organic acid) to improve on the efficacy of *Bt* spray for control of *S. recurvalis*, were evaluated in laboratory bioassays against second instar larvae. Among the 7 inorganic salts, boric acid was the only additive that caused more than 50% larval mortality. Two nitrogenous compounds, peptone and sodium nitrate caused 54 and 51% larval mortalities, however, this efficacy was not significantly different from the 40% mortality caused by *Bt* applied without any additive. Among the protein solubilizing agents, urea increased larval mortality from 40% to 51 % although the increase was not significant from a *Bt* spray application only. Citric acid, the only organic acid evaluated at the concentration of 0.05% in combination with *Bt,* had no significant effect on the efficacy of *Bt* spray against *S. recurvalis* larvae. Overall, among the additives evaluated, the efficacy of *Bt* spray was most enhanced by boric acid and could be further evaluated under field conditions for validation, and integration into an IPM strategy for *S. recurvalis* management.

Key words: Boric acid, chemical additives, synergistic interaction, *Bt* spray, insecticides, lepidopteran pests

## 5.1 Highlights

- *Spoladea recurvalis* larvae are less susceptible to Halt®, a *Bacillus thuringiensis* Subsp. *kurstaki*-based biopesticides
- Peptone, sodium nitrate and urea caused >50% mortality though not significantly different from *Bt* application on its own
- Among the 13 chemical additives evaluated, only boric acid enhanced the efficacy of *Bt*

## 5.1 Introduction

Amaranths, *Amaranthus* spp. (Caryophyllales: Amaranthaceae), are valuable vegetable and grain crops that are grown in many countries in the world (Borsch, 1998, Dinssa *et al.*, 2016). Vegetable amaranths are good sources of minerals and vitamins, levels of which are higher than in popular non-indigenous vegetables like cabbage or tomatoes (Abukutsa-Onyango, 2003, Ebert *et al.*, 2011, Muriuki *et al.*, 2014). The protein and fibre contents in grain amaranths are also higher than in other cereal grains (Breene, 1991, Mburu *et al.*, 2012, Venskutonis and Kraujalis, 2013). Amaranth oil is highly nutritious, containing a high amount of squalene (Bressani *et al.*, 1992), which has been shown to reduce cholesterol levels in humans (Berger *et al.*, 2003, Martirosyan *et al.*, 2007, Kaaya *et al.*, 2017). Amaranth plants are able to grow in poor soils with moderate salinity levels (Liu and Stützel, 2002), and also exhibit C4 photosynthesis that enables them to have high photosynthetic efficiency under a wide range of temperatures, moisture gradients and water-stress environments (Ueno, 2001, Tsutsumi *et al.*, 2017).

In Africa (James *et al.*, 2010, Aderolu *et al.*, 2013, Kagali *et al.*, 2013, Mureithi *et al.*, 2015), Asia (Sharma and Ramamurthy, 2009) and the Americas (Clarke-Harris *et al.*, 2004, García *et al.*, 2011, Niveyro *et al.*, 2013), quality and production of amaranths are curtailed by severe infestations of insect pests. Among these insects pests, lepidopteran defoliators, especially the leafwebber, *Spoladea recurvalis* (Fabricius) (Lepidoptera: Crambidae) are considered the major and most damaging pest of amaranth. *Spoladea recurvalis* is economically important, causing yield losses of 60-100% (James *et al.*, 2010, Kagali, 2014, Mureithi *et al.*, 2017, Othim *et al.*, 2018).

Most amaranth farmers rely heavily on the use of chemical insecticides to control amaranth pests (Clarke-Harris and Fleischer, 2003, Losenge, 2005, Ngowi *et al.*, 2007, Mc Leod, 2008, Arivudainambi *et al.*, 2010, Aderolu *et al.*, 2013). However, with the short cropping cycle of amaranth, pesticide residues may remain on plant products following chemical control of *S. recurvalis.* This means of control is therefore not advisable (Fan *et al*., 2013). Adding to the health and environmental risks, is the price of chemical insecticides which makes them often not affordable to the resource constrained small-scale farmers in Kenya (M'Ribu, 2003, Dinham, 2003, Mbugua *et al.*, 2011). Insecticide resistance has also been reported in *S. recurvalis* in Jamaica (Clarke-Harris and Fleischer, 2003). *Bacillus thuringiensis* based biopesticides are widely used as an alternative to chemical insecticides in suppressing many lepidopteran pests because of their safety to humans, beneficial insects and other non-target organisms (Salama *et al.*, 1990, Ela *et al.*, 1993, Baum *et al.*, 1999, Navon, 2000, Nexter *et al.*, 2002, Chattopadhyay *et al.*, 2004, Roh *et al.*, 2007).

In a study conducted by Opisa *et al*. (2018) on the efficacy of entomopathogenic fungi (EPF) and *Bt* based biopesticides for the control of *S. recurvalis*, low efficacy of the commercial *Bt* Subsp. *kurstaki*-based product, Halt® was reported. There are several studies that aimed at increasing the

efficacy of *Bt* sprays by incorporating chemical additives (Govindarajan *et al.*, 1976, Salama *et al.*, 1984, Salama *et al.*, 1985, Salama *et al.*, 1986, Salama *et al.*, 1989, Salama *et al.*, 1990, El-Moursy *et al.*, 1992, Salama *et al.*, 1992, Gibson *et al.*, 1995, Morris, 1995, Sabbour *et al.*, 2012, Zhang *et al.*, 2013, Vimala Devi and Vineela, 2015). Addition of Ethylenediamine tetraacetate (EDTA) to *Bt spray* against Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), greasy cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) and diamondback moth, provided synergistic effects and improved efficacy of the biopesticide against these pests (Salama *et al.*, 1989, El-Moursy *et al.*, 1992, Liu and Tabashnik, 1997). Determining a *Bt*-additive-mixture that could enhance the efficacy of *Bt* sprays for the management of *S. recurvalis* would also reduce the costs of field applications of the biopesticide, as well as limiting negative human and environmental impacts.

The aim of this study was therefore to determine if the efficacy of *B. thuringiensis* Subsp. *kurstaki* spray applications for control of *S. recurvalis* larvae could be enhanced when combined with chemical additives.

## 5.2 Materials and methods

### Insects

Second instar larvae of *S. recurvalis* were used in all the experiments and were obtained from the Animal Rearing and Quarantine Unit at *icipe.* The colony was maintained in a rearing room at 25 ± 2 °C, 50–70% RH, and 12L:12D photoperiod as described in Chapter 3 (see section 3.2).

**Chemical additives**

The four groups of chemical additives used in this study were inorganic salts (sodium carbonate, potassium carbonate, calcium carbonate, zinc sulphate, calcium chloride, magnesium sulphate and boric acid), nitrogenous compounds (peptone, tryptone and sodium nitrate), protein solubilizing agents (urea and ethylene diamine tetracetate (EDTA)) and organic acids (citric acid). These additives were obtained from Sigma-Aldrich® Chemical company (St. Louis MO 63178, USA).

***Bacillus thuringiensis* (*Bt*)**

Halt® 5 WP, a commercially available biological insecticide based on *Bacillus thuringiensis, serovar kurstaki* H3a, 3b, 3c, formulated as a wettable powder (WP) with 32,000 I.U potency, was used in this study. Detailed methodology is described in Chapter 3 (see section 3.2).

**5.2.1 Effects of additives on second instar *Spoladea recurvalis* larvae**

Each of the additives was evaluated separately to determine their effect on second instar *S. recurvalis* larvae. Two fresh amaranth leaves of similar size and age, approximately 2–3 weeks old were placed in a Petri dish (90 mm in diameter). Ten milliliters of each additive at a concentration of 0.05 % were applied to the leaves in the Petri dishes using a Burgerjon's spray tower (Burgerjon, 1956). The control groups were sprayed with sterilized distilled water containing 0.05% Triton X-100. After treatment, the leaves were air-dried in a sterile laminar airflow hood for approximately 5 minutes to remove excessive moisture or solution. The leaves were then transferred into transparent plastic Petri dishes (110 × 60 mm) lined with moist filter paper. A group of 20 second instar *S. recurvalis* larvae were transferred onto the treated amaranth leaves, using a soft camel hair brush. Each Petri dish represented a replicate. There were three replicates per additive. The insects were fed on treated leaves ad libitum for the first 24 hours after treatment. Thereafter, insects were supplied with fresh amaranth leaves (surface-sterilized and untreated) as food and renewed daily.

Larval mortality was recorded daily for 7 days. All the treatments, including the control were arranged in a completely randomized design.

### 5.2.2 *Bacillus thuringiensis* (*Bt*) and chemical additives bioassays

All additives evaluated (see section 5.2.1) were combined with the *Bt* spray in the interaction bioassays. A similar experiment as described above was conducted to evaluate possible interaction effects of the respective chemical additives added to *Bt.* A mixture of 5 ml of 0.05% additive and 5 ml Halt® 5 WP (*Bacillus thuringiensis* Subsp. *kurstaki*) were applied as treatments. Larval mortality was recorded daily for 7 days.

### 5.2.3 Statistical analyses

Data on mortalities were corrected for natural mortality using Abbott's formula (1925) followed by a normality test (Shapiro and Wilk, 1965), before being subjected to analyses of variance (ANOVA). Whenever treatments were found to be significantly different ($p < 0.05$), means were separated using the Student–Newman–Keuls (SNK) post hoc test. For the organic acid group with citric acid only, the Welch's t-test was used to compare the combined treatment of citric acid and *Bt* with the *Bt*-only treatment. All data analyses were performed using R (version 3.2.5) statistical software packages (R Core Team, 2016).

## 5.3 Results

### 5.3.1 Effects of additives on second instar *Spoladea recurvalis* larvae

There were significant differences in larval mortality caused by the respective additives applied to *S. recurvalis* larvae ($F = 2.71$, $df = 12,29$; $P < 0.02$). The larval mortalities caused by the additives ranged from 1.7 to 20% (Table 5.1).

**Table 5.1 Effects of additives on second instar *S. recurvalis* larvae**

| Group | Additive | % Mortality± SE |
|---|---|---|
| Inorganic salts | Magnesium sulphate | 15.0 ± 2.9ab |
| | Potassium carbonate | 5.0 ± 2.9b |
| | Calcium carbonate | 3.3 ± 1.7b |
| | Zinc sulphate | 11.7 ± 1.7ab |
| | Calcium chloride | 6.7 ± 4.4ab |
| | Boric acid | 20.0 ± 2.9a |
| | Sodium carbonate | 10.0 ± 2.9ab |
| Nitrogenous compounds | Peptone | 8.3 ± 3.3ab |
| | Sodium nitrate | 1.7 ± 1.7b |
| | Tryptone | 10.0 ± 5.8ab |
| Protein solubilizing agents | Urea | 11.7 ± 3.3ab |
| | EDTA | 10.0 ± 2.9ab |
| Organic salts | Citric acid | 6.7± 1.7ab |

Means within the column followed by the same letter (s) are not significantly different at $P < 0.05$ (Student–Newman–Keuls (SNK) test)

### 5.3.2 Efficacy of *Bacillus thuringiensis* (*Bt*) and chemical additives against *S. recurvalis* larvae

**Inorganic Salts**

There were significant differences in larval mortality caused by the respective inorganic salts + *Bt*-mixtures and the *Bt* spray without additives ($F = 5.47$, $df = 7,16$; $P < 0.002$). Except for calcium chloride, the efficacy of all mixtures was higher (Table 5.2) compared to application of the inorganic salts only (Table 5.1 Mortality caused by the calcium chloride + Bt spray mixture was significantly lower than the mortality caused by mixtures containing magnesium sulphate, calcium carbonate and boric acid as well as the Bt spray application. The only *Bt* - inorganic salt mixture that provided a significantly higher mortality rate than *Bt* spray applied without any additive, was the *Bt*-boric acid mixture, with 60 % larval mortality compared to 40% when only *Bt* spray was applied (Table 5.2).

**Table 5.2**: Efficacy of *Bt* only and inorganic salts applied in mixture against *Spoladea recurvalis* larvae at 0.05% 7 days after treatment

| Treatment | % Mortality ± SE |
|---|---|
| Bt + Magnesium sulphate | 31.9 ± 10.6b |
| Bt + Potassium carbonate | 25.0 ± 7.6bc |
| Bt + Zinc sulphate | 9.9 ± 9.9bc |
| Bt + Calcium carbonate | 33.3 ± 10.9b |
| Bt + Sodium carbonate | 18.3 ± 1.7bc |
| Bt + Calcium chloride | 3.6 ± 3.6c |
| Bt + Boric acid | 60.0± 5.8a |
| Bt Only | 40 ± 5.8b |

Means within a column followed by the same letter(s) are not significantly at P < 0.05 (Student–Newman–Keuls (SNK) test)

**Nitrogenous compounds**

In combination with *Bt* spray, 0.05% peptone and sodium nitrate caused 54 and 51 % larval mortality respectively, compared to 40 % caused by *Bt* without any additives There was, however, no significant difference in efficacy between the three nitrogenous compounds separately combination with *Bt* as well as *Bt* applied without additives (i.e. *Bt* only) (F = 0.97, df = 3,8; P = 0.45).

**Table 5.3**:Efficacy of *Bt* only and nitrogenous compounds applied in mixtures against *S. recurvalis* larvae 7 days after treatment

| Treatment | % Mortality ± SE * |
|---|---|
| Bt + Peptone | 54.4 ± 7.5 |
| Bt + Sodium nitrate | 51.4 ± 9.4 |
| Bt + Tryptone | 42.0 ± 5.1 |
| *Bt* only | 40.0 ± 5.8 |

* No significant difference between treatments

**Protein solubilizing agents**

There was no significant difference between efficacy of *Bt*-only spray and that of *Bt* + urea mixture, as well as *Bt* + EDTA mixture (F = 1.73, df = 2,6; P = 0.26), although the additive effect of urea-*Bt* mixture increased the mortality of *S. recurvalis* larvae from 40% (*Bt* only) to 51% (urea-*Bt* mixture) (Table 5.3).

**Table 5.4**:Efficacy of *Bt* only and protein solubilizing agents applied in mixtures against *S. recurvalis larvae* after 7 days of treatment

| Treatment | % Mortality ± SE * |
|---|---|
| Bt + Urea | 51.1 ± 11.2 |
| Bt + EDTA | 30 ± 5.8 |
| *Bt*-only | 40 ± 5.8 |

* No significant difference between treatments

**Organic acids**

The efficacy of control of *S. recurvalis* larvae with the *Bt* + citric acid mixture was significantly lower than the application of *Bt* alone (t = 4.72, df = 2.33, P= 0.03)

## 5.4 Discussion

Chemical additives were evaluated to determine their synergetic role in enhancing the efficacy of *Bacillus thuringiensis* Subsp. *kurstaki* (product Halt®) against *S. recurvalis* larvae. Among the seven inorganic salts (sodium carbonate, potassium carbonate, calcium carbonate, zinc sulphate, calcium chloride, magnesium sulphate and boric acid) tested, only boric acid increased the potency of *Bt*. from 40% larval mortality caused by *Bt* alone to 60% from the Bt spray mixture with boric acid The potency-enhancing effect of boric acid with *Bt* has also been reported against tobacco cutworm *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) (Govindarajan *et al.*, 1976), gypsy moth larvae *Lymantria dispar* (Linnaeus) (Lepidoptera: Lymantriidae) (Charles and Wallis, 1964),

bertha armyworm larvae *Mamestra configurata* Walker (Lepidoptera: Noctuidae) (Morris, 1995) and African bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Sabbour *et al.*, 2012, Vimala Devi and Vineela, 2015). Boric acid is used as a stomach poison and contact insecticide (Cisneros *et al.*, 2002, Zurek *et al.*, 2003, Xue and Barnard, 2003). It is assumed that boric acid destroys the protective layer of the peritrophic membrane and lining of the gut, thereby facilitating the diffusion of *B. thuringiensis* toxin to the insect midgut epithelium and hence the enhancement effects of Boric acid on *Bt* (Govindarajan *et al.*, 1976, Metcalf *et al.*, 1993). The other six inorganic salts tested were ineffective in enhancing the potency of *Bt* and caused mortalities lower than when *Bt* was applied alone. This is in contrast to the findings of Zhang *et al.* (2013) who reported enhancement of *B. thuringiensis*. Subsp. *kurstaki* against Diamondback moth, *P. xylostella* by these salts. In addition, (El-Moursy *et al.*, 1992) also reported that Zinc sulphate (0.25%) and calcium carbonate (0.25%) increased the activity and potency of *B. thuringiensis* var. *kurstaki* (Dipel 2X) against *P. interpunctella* larvae by 2.7 and 2.5-fold, respectively. In a field trial on soybean plants, potassium carbonate was reported to enhance and significantly increase the effect of *B. thuringiensis* var. *kurstaki* HD-1 (Dipel 2x) against the larvae of cotton leafworm, *Spodoptera littoralis* (Boisduval) Lepidoptera: Noctuidae) (Salama *et al.*, 1990). Magnesium sulphate (0.05%), potassium carbonate (0.05%), sodium carbonate (0.05%) and zinc sulphate (0.05%) in combination with *B. thuringiensis* Subsp. *kurstaki* increased toxicity of the pathogen against *M. configurata* larvae by 3.5, 2.2, 1.8 and 2.2 fold, respectively (Morris, 1995). Similarly, Salama *et al.* (1985) demonstrated that calcium carbonate (0.25 %) and zinc sulphate (0.05%) compounds potentiated the efficacy of *B. thuringiensis* var. *entomocidus* against *S. littoralis* by 13 and 24 fold respectively, while *S. recurvalis* larvae were not very susceptible to these combinations. These salts are alkaline which provides a conducive midgut environment for *Bt* crystals to solubilise after ingestion (Nickerson, 1980, Knowles, 1994). Failure to potentiate *Bt* against *S. recurvalis* larvae might be due to differences in the strain of *Bt* used in this study and the diverse nature of the

lepidopteran insect species, as well as their related gut flora or microbiota. The efficacy of control of *S. recurvalis* larvae with Bt spray was not enhanced with the addition of either of the two-protein solubilizing agents, *viz*. urea or ethylene diamine tetracetate (EDTA). The more than 50% mortality of *S. recurvalis* larvae caused by urea may be linked to the ability of urea to denature proteins (Pace, 1986, Bennion and Daggett, 2003) by reducing the disulphide bonds of the protein molecules to sulphydryl groups, thus increasing the dissolution of the δ-endotoxin in the insect gut (Nickerson, 1980, Zhang *et al*., 2013). Similar antagonistic effects of EDTA when combined with *Bt* were reported by Salama *et al*. (1985) against the cotton leafworm *S. littoralis*.

Citric acid did not enhance the efficacy of *Bt* when applied as a mixture against *S. recurvalis* at a 0.05% concentration. This is in agreement with the results reported by Salama *et al*. (1989) for control of *A. ipsilon.* Similarly, no enhancing effect of Bt spray by the addition of any of four organic acids, *viz*. calcium acetate, lauric acid, sodium thioglycolate or malic acid against fourth instar *M. configurata* larvae, was reported by Morris *et al*. (1995). Maleic acid and citric acid were however, found to enhance the activity of *Bt* against Diamondback moth, *P. xylostella* (Zhang *et al.*, 2013). El-Moursy *et al.* (1992) also reported that succinic, malic and formic acids increased the potency of *Bt* against *P. interpunctella.* The differences observed in the synergistic effects of organic acids combined with *Bt* indicate that further investigations using biochemical and molecular techniques are needed. Since citric acid, the only organic acid tested in combination with *Bt* in the current study, was ineffective in increasing the efficacy of control, more organic acids to be combined with *Bt* should be tested.

None of the three nitrogenous compounds evaluated in bioassays, *viz.* peptone, tryptone and sodium nitrate was singly effective in controlling S. *recurvalis* larvae. When added together with Bt spray, no synergisms in these mixtures were found.

Wigglesworth (1977) ascribed the synergistic effects of nitrogenous compounds and *Bt* in control of lepidopteran larvae, to alteration in the physiology of haemolymph caused by leakage through the midgut cells of the *Bt* treated larvae.

## 5.5 Conclusion

Results from this laboratory study indicated that only boric acid has the potential to enhance the activity of the *B. thuringiensis* Subsp. *kurstaki* product Halt® against *S. recurvalis* larvae and could significantly contribute to the management of the pest. However, further field experiments using this key potent chemical additive in combinations with the *Bt* product against *S. recurvalis* larvae is warranted for validation.

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# CHAPTER 6

## General discussion, conclusions and recommendations

### 6.1 General discussion

There has been an increased interest in the use of microbial control agents to manage pests in vegetable cropping systems because of consumer demand for products that are free of insecticide residues (Chandler *et al.*, 2008). Furthermore, several vegetable pests have developed resistance to one or more pesticides (Mc Caffery, 1998, Pérez *et al.*, 2000, Roush and Tabashnik, 2012) leaving growers to search for alternative management strategies that are safe, environmentally friendly and sustainable. Amaranth is a short duration vegetable crop and managing its most important pest, *S. recurvalis* using chemical insecticides is highly discouraged because of the accumulated pesticide residues that are toxic to consumers. This study was therefore aimed to evaluate the potential of entomopathogenic fungal and *Bt*-based biopesticides, and to integrate the fungal isolate identified as potent, with a commercially available moth attractant (PAA) for the management of *S. recurvalis* on amaranths through an "attract and kill" strategy. Chemical additives were also explored in boosting the efficacy of *B. thuringiensis* Subsp. *kurstaki* product Halt® against *S. recurvalis* larvae, the most damaging life stage of this pest.

The study identified *M. anisopliae* ICIPE 30 as the most potent EPF against *S. recurvalis,* with the adult stage being more susceptible to the pathogen, than the larval stage. *Metarhizium anisopliae* ICIPE 30 has also been reported to be potent against other tropical pests (Maniania, 1992, Gitonga, 1996, Ekesi *et al.*, 1998, Bayissa *et al.*, 2017). This broad host range of ICIPE 30 is a very important attribute in the process of development and commercialization of fungal-based biopesticides. It would not only save on production costs, but also on application costs since one mycopesticide could be used in the management of many insect pests. The outcomes of this study showed that *M. anisopliae* ICIPE 30 is a potential candidate for biopesticide development that could

be integrated in the management of *S. recurvalis* targeting the adult stage through an autodissemination approach.

After illustrating the efficacy of the *M. anisopliae* ICIPE 30 fungal biopesticide against adult *S. recurvalis*, one of the crucial factors to consider for integration of the biopesticide into an IPM program for this pest, is the compatibility of the fungus with available attractants and semiochemicals. Studies on the compatibility of Phenylacetaldehyde (PAA), a floral attractant for adult *S. recurvalis,* and *M. anisopliae* ICIPE 30 were therefore done in the laboratory and field. The aim was to exploit it together in a "lure and infect" strategy for suppression of the pest population. In the laboratory, PAA completely inhibited the germination of *M. anisopliae* ICIPE 30 conidia, which implies that the two cannot be used when in direct contact. A field study with *M. anisopliae* ICIPE 30 and PAA separated at different distances in an autodissemination trap was done to find the optimal spatial separation distance to overcome this incompatibility constraint. Results showed that the inhibitory effect of PAA on conidial germination was minimized by increasing the distance between PAA from *M. anisopliae* isolate ICIPE 30 conidia. The fungus was still very viable and pathogenic to the pest at a distance of 5 to 10 cm from the attractant. These results have confirmed that spatial separation of fungal conidia from PAA in an autodissemination trap can be adopted to improve on autoinoculation efficacy. Autodissemination of *M. anisopliae* isolate ICIPE 30 can therefore be integrated in a *S. recurvalis* management program. An autodissemination device uses small amounts of conidia as compared to inundative applications and is therefore a more economical management strategy. Frequent applications as with inundative applications, is also not necessary and can therefore be recommended to amaranth farmers in SSA. Horizontal transmission of *M. anisopliae* ICIPE 30 from infected to healthy moths was also illustrated in this study under laboratory conditions. Based on this result, the use of the autodissemination technique for the management of *S. recurvalis* can be feasible in amaranth production. Moths that became infected in the autodissemination device will disseminate conidia during mating or other behaviours that lead to physical contact, and this has the potential to suppress the pest population and consequently

decrease plant damage. Mfuti *et al*. (2016) described the autodissemination device used in this study. It is made from locally available materials. It will therefore be affordable to small scale farmers once they adopt this control technique. The use of the autodissemination device will also prolong the persistence of *M. anisopliae* in the field by shielding the fungus from the detrimental effects of solar radiation.

A combined application of EPF and *Bt* may offer more effective control for pests (Thomas *et al.*, 2003, Wraight and Ramos, 2005, Ma *et al.*, 2008, Mantzoukas *et al.*, 2013). In the present study, this hypothesis was investigated for control of *S. recurvalis* compared to the use of each pathogen separately. No difference in efficacy of control of *S. recurvalis* larvae was found when the two entomopathogens were applied simultaneously compared to separate applications. The cause of low susceptibility of *S. recurvalis* larvae to *Bt* and EPF was not determined. However, it may be due to the larvae developing some tolerance and adaptation mechanisms to avoid the entomopathogens (Greenfield *et al.*, 2014, Pedrini *et al.*, 2015), but this phenomenon needs to be further investigated. It is also vital to search for ways to improve the efficacy of *Bt* against *S. recurvalis* larvae. Chemical additives have been reported to improve the efficacy of *Bt.* The efficacy of control by combined treatments of chemical additives and *Bt* on *S. recurvalis* mortality showed only boric acid to significantly improve the efficacy of *Bt*. Therefore, Boric acid, which is locally available in Kenya can be combined with *Bt* to control *S. recurvalis* on amaranth. This product may contribute to reduce excessive use of chemical insecticides by farmers.

## 6.2 Conclusions

Of the twenty-four entomopathogenic fungal isolates and one commercial based *Bacillus thuringiensis* Subsp. *kurstaki* product Halt® screened in the laboratory against second instar larvae of *S. recurvalis*, only *M. anisopliae* ICIPE 30 reached a moderate threshold, causing 58.3% larval mortality.

Based on this result, this study concludes that *S. recurvalis* larvae are moderately susceptible to EPF and *Bt- based* biopesticides. Consecutive application of *M. anisopliae* ICIPE 30 and *Bt* did not cause a significant increase in larval mortality compared to separate applications of each product. Further studies are therefore warranted to elucidate the exact reasons for low pathogenicity caused by the two biocontrol agents against the larval stage of *S. recurvalis*.

On the other hand, adult *S. recurvalis* were, highly susceptible to all the 11 screened EPF isolates screened against them with *M. anisopliae* ICIPE 30 and *B. bassiana* ICIPE 725 being the most potent. Compatibility bioassays under laboratory conditions revealed that direct contact between *M. anisopliae* ICIPE 30 and PAA completely inhibits the germination of the fungus. Spatial separation of PAA and *M. anisopliae* ICIPE 30 was therefore investigated in an autodissemination device for use against *S. recurvalis* moths under field conditions. As a result, the negative effect of PAA on conidial viability and persistence of ICIPE 30 was reduced at separation distances of 5 and 10 cm. Horizontal transmission of EPF was demonstrated in this study between infected ('donors') to uninfected moths ('recipients') resulting in high mortality in both the 'donors' and 'recipients' moths under laboratory conditions.

Among the thirteen chemical additives evaluated to improve the potency of *Bt* spray only boric acid enhanced its efficacy.

### 6.3 Recommendations

1. In the process of developing a sustainable *M. anisopliae* ICIPE 30 microbial biopesticides, it is highly recommended to study its effects on non-target insects including natural enemies before it could be adopted for *S. recurvalis* management.
2. Development of novel formulations of this potent fungal-based biopesticide is warranted to improve on its efficacy and application technologies.
3. Since farmers are still using synthetic chemical insecticides, compatibility of the ICIPE 30-based biopesticide with these insecticides should be investigated.
4. Training of farmers and agricultural extension officers on the benefits associated with using *M. anisopliae* ICIPE 30 as well as a combination of *Bt* and Boric acid in the management of *S. recurvalis* in amaranth vegetable production systems is recommended.
5. Moths infested with conidia from the autodissemination devices are likely to lose some of the conidia during flight, which might reduce the rate of horizontal transmission. It is therefore necessary to test fungus formulations that are electrostatically charged or lipophilically attractive that may improve the adherence of conidia to the host cuticle.
6. Additional lures (including pheromones) for *S. recurvalis* should be tested with PAA as a reference, and compatibility of the promising lures with ICIPE 30 be determined.

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