

**EFFICACY OF ENDOPHYTIC FUNGI IN THE
MANAGEMENT OF *Spoladea recurvalis*
(LEPIDOPTERA: CRAMBIDAE) ON AMARANTH
(*Amaranthus dubius*)**

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Efficacy of Endophytic Fungi in the Management of *Spoladea recurvalis* (Lepidoptera: Crambidae) on Amaranth (*Amaranthus dubius*)

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**A Thesis Submitted in Partial Fulfilment of the Requirements for
the Degree of Master of Science in Plant Health Science and
Management of the Jomo Kenyatta University of
Agriculture and Technology**

DECLARATION

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

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DEDICATION

This work is dedicated to my dearest parents and my sister for their endless prayers, love, encouragement and support that made it easier to walk through this journey.

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ACRONYMS AND ABBREVIATIONS

AIVs	African Indigenous Vegetables
APU	Arthropod Pathology Unity
ARQU	Animal Rearing Quarantine Unit
ASR	Acquired Systemic Resistance
CRD	Completely Randomized Design
EPFs	Entomopathogenic Fungus
FAO	Food
GC-MS	Gas Chromatography Mass Spectrometry
HPR	Host Plant Resistance
ICIPE	International Centre of Insect Physiology and Ecology
IPM	Integrated Pest Management
NMDS	Non-metric multidimensional scaling
PAA	Phenylacetaldehyde
PCA	Principal Component Analysis
PDA	Potato Dextrose Agar
RCBD	Randomized Complete Block Design
ROS	Reactive Oxygen Species
SDA	Sabouraud Dextrose Agar

TAVs	Traditional African Vegetables
UK	United Kingdom
VOCs	Volatile Organic Compounds
WHO	World Health Organization

ABSTRACT

Amaranth is a Traditional African Vegetable (TAV) that in recent years has gained global popularity due to its exceptional nutritional, medicinal and economic attributes. However, its production is significantly limited by insect pest infestations, mainly lepidopteran defoliators. *Spoladea recurvalis*, commonly known as Hawaiian beet webworm has been reported as the major pest that feeds voraciously on the leaves leading to substantial yield losses of up to 100% under severe pest infestation. Chemical control remains a widely used method for the management of *S. recurvalis*. The use of fungal endophytes has emerged as a promising, sustainable and eco-friendly alternative to chemical pesticides in the management of insect pests. Therefore, this study evaluated the efficacy of selected 15 fungal isolates to endophytically colonize *Amaranthus dubius* host plant. Only two fungal isolates *Hypocrea lixii* F3ST1 and *Trichoderma asperellum* M2RT4 were found to be endophytic to *A. dubius* with varied colonization rates among them. The results revealed that both endophytes successfully colonized over 90% of all the examined plant tissues (root, stem and leaves). The two fungal isolates were further evaluated for their endophytic persistence, effect on host plant growth promotion and insecticidal activity against *S. recurvalis* life cycle and F1 progeny. Also, the behavioral and chemical mechanism through which the presence of the most potent endophytes *H. lixii* F3ST1 and *T. asperellum* M2RT4 within amaranth plants influence *S. recurvalis* host selection and herbivory were investigated. Both endophytes persisted in the host plant throughout the four-week assessment period exhibiting varying colonization rates influenced by significant interaction between time, fungal isolates and plant parts. Although *T. asperellum* M2RT4 outperformed *H. lixii* F3ST1 in the weekly assessment on plant growth promotion, the two endophytes significantly improved plant height, the number of leaves, leaf length and leaf width. *Trichoderma asperellum* M2RT4 significantly enhanced dry shoot biomass when compared with *H. lixii* F3ST1 and the control. Further, both endophytes significantly reduced number of egg masses laid, egg hatchability, increased larval mortality, reduce pupae formation, adult emergence and reduced survival/longevity of F1 progeny. *Amaranthus dubius* plants endophytically colonized by *H. lixii* F3ST1 significantly recorded the lowest number of egg masses (3.75 ± 1.11) resulting in 5% and 49.8% reductions, followed by *T. asperellum* M2RT4 (36.75 ± 3.77) compared to (73.25 ± 5.14) in the control. Upon egg hatching, *H. lixii* F3ST1 endophytically colonized plants significantly recorded the lowest number of larvae (13.75 ± 1.75) followed by *T. asperellum* M2RT4 (94.5 ± 11.66) compared to 196.5 ± 22.14 in the control. There was no significant difference in larval mortality by *H. lixii* F3ST1 endophytically colonized ($40 \pm 0.82\%$) and *T. asperellum* M2RT4 plants ($42 \pm 0.74\%$) compared to ($2.91 \pm 0.29\%$) in the control. Fewer pupae were obtained in *H. lixii* F3ST1 (3.75 ± 1.75) followed *T. asperellum* M2RT4 (44.5 ± 1.94) compared to 76.25 ± 1.43 in the control. Besides, adult emergence of *S. recurvalis* varied significantly between the fungal isolates with the lowest number of moths emerging from *H. lixii* F3ST1 endophytically colonized plants (3.25 ± 0.25) followed by *T. asperellum* M2RT4 (38.5 ± 1.19) compared to the control (66.5 ± 2.25). Furthermore, the two endophytes *H. lixii* F3ST1 and *T. asperellum* M2RT4 significantly reduced the survival of *S. recurvalis* F1 progenies. The behavioral bioassay using the Y-tube olfactometer revealed that *S. recurvalis* showed a preference for non-colonized amaranth plants over

those colonized by both endophytes. Nevertheless, the findings showed that *S. recurvalis* moths were not attracted to either infested non-colonized or infested endophyte-colonized amaranth plants. Gas chromatography-mass spectrometry (GC-MS) analysis of volatiles emitted from non-inoculated, inoculated, non-inoculated infested, and inoculated infested amaranth plants colonized by *H. lixii* F3ST1 and *T. asperellum* M2RT4 revealed the presence of 80 and 74 volatile organic compounds (VOCs), respectively. Similarity percentage analysis highlighted the major compounds contributing to the differences observed among treatments. In *H. lixii* F3ST1-colonized plants, key discriminating VOCs included (Z)-3-Hexenyl acetate, pentadecane, α -Cedrene, o-Xylene, and (E)- β -Ocimene while for *T. asperellum* M2RT4-colonized plants, predominant differentiating compounds were Germacrene D, (Z)-3-hexenol, 1,3-Dimethylbenzene, Tetradecane, and 2,4,4-Trimethyl-1-pentene. The distinct VOC profiles linked to their colonization and treatment status suggest that these endophytes may influence plant defense signaling pathways, further contributing to their biocontrol efficacy for the management of *S. recurvalis*. Hence, providing insights for their integration into environmentally friendly pest management strategies in amaranth cropping systems. However, further studies are warranted to assess the effects of the key identified synthetic compounds on the pest and validate the results under field conditions.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background of the Study

Food security exists when all people, at all times, have physical, social, and economic access to sufficient, safe, and nutritious food that meets their dietary needs and food preferences (FAO, 2008). The number of people experiencing hunger globally is increasing, with approximately 924 million people facing severe levels of food insecurity in 2021 (Burki, 2022). Childhood malnutrition is evidently a major global issue, particularly in Africa (Black et al., 2013). In Sub-Saharan Africa, children suffer from Vitamin A deficiency, while women suffer from iron deficiency (Stevens et al., 2015). Children who suffer from acute malnutrition have a higher risk of serious sickness that can lead to slower growth, death, impaired learning and development (Brown et al., 2020; Medialdea et al., 2021; Mullen, 2021; Headey & Ruel, 2022). In Kenya, high levels of under-nutrition, especially stunting, have been persistent for decades (Masibo & Makoka, 2012). Kimani-Murage et al. (2015) reported that among the 3335 children sampled in Nairobi, Kenya, 46% were stunted, 11% underweight, 2.5% wasted and 9% overweight/obese. Additionally, among the 5190 adults sampled in the study, 43% and 37% of their mothers were overweight and obese, respectively, while 9% were underweight and 22% overweight (Kimani-Murage et al., 2015). Citaristi (2022) reported that approximately one-third of all children in Kenya exhibit delayed physical development due to insufficient micronutrient intake.

Traditional African Vegetables have been widely used for subsistence farming in East Africa for several years (Krause et al., 2019). In the last decade, rural-urban relocation and a change of consumer choice have given rise to the demand for commercially marketed TAVs because of their high nutritional and medicinal values (Cernansky, 2015). The nutritional potential of African indigenous vegetables has the capability to significantly alleviate food insecurity and malnourishment (Imathiu, 2021). This can be achieved by including TAVs in the household diet to promote good health and reduce micronutrient deficiencies (van Rensburg et al., 2007). In Kenya, Tanzania and Uganda, amaranth (*Amaranthus* spp.) is ranked among the top four TAVs because of

the economic, medicinal and nutritional value of both seeds and leaves (Mkpuma et al.,2026). Among TAVs, amaranth has been categorized as a prospective vegetable with a promising nutritional, medicinal and economic value (Jimoh et al., 2018). Amaranth belongs to the family Amaranthaceae, which originated from South America (Ogwu, 2020). Within the genus, *Amaranthus* L. consists of 60-70 species (Xu & Sun, 2001; Ebert et al., 2011).

Amaranth crop is rich in nutrients such as vitamins A and C, iron, calcium, zinc, and magnesium, protein, dietary fiber, lipids and unsaturated fatty acids, bioactive compounds, e.g., phytosterols, squalene, saponins, and polyphenols, which are essential for the development of a healthy body (Mbhenyane, 2017; Kambabazi et al., 2021). Nyonje et al. (2022) reported that amaranth helps in boosting blood and milk production in breastfeeding mothers, increases eye vision, eradicates childhood marasmus, cleanses the body and relieves constipation. *Amaranthus* contains no cholesterol, hence, it can be a healthy dietary choice for people who are observing a strict diet with the goal of weight loss, thereby ensuring a healthy living (Ramdwar et al., 2017). Due to its high lysine content, *Amaranthus* is a useful supplement for diets rich in cereals and tubers. It is used to treat gastrointestinal problems as well as aid in the recovery of new and nursing mothers (Coelho et al.,2018). According to Martirosyan et al. (2007), amaranth helps in boosting the body's immunity, reduce blood pressure and increasing antioxidant levels (Martirosyan et al., 2007). The grains are high in nutrients and commonly used to feed children and the elderly to boost their immunity (Amicarelli & Camaggio, 2012).

Despite all these benefits of amaranth, its production is constrained by insect pest infestation, which attacks mostly the roots, stems, leaves and seeds (Othim et al., 2018). Studies conducted by Agbodzavu (2019). *Amaranthus* defoliator, *Spoladea recurvalis* Fabricius (Lepidoptera: Crambidae), commonly known as Hawaiian beet webworm or amaranth leaf-webber, is the major lepidopteran pest of amaranth across Africa. *Spoladea recurvalis* can cause a 50-70% reduction in amaranth yield (Azandémè-Hounmalon et al.,2023) . In Kenya, Kahuthia-Gathu (2011) reported that *S. recurvalis* can cause up to 100% yield loss during extreme outbreaks. According to Aderolu et al. (2013), the third instar larvae are the most ravenous and destructive stage that feeds

on succulent amaranth leaves. The larvae skeletonize the foliage of amaranth by wrapping and rolling the leaves into shelters where they feed frequently, resulting in the entire foliage being covered in frass (James et al., 2010).

Management of *S. recurvalis* has been primarily the indiscriminate and massive use of chemical pesticides, which are not only ineffective but hazardous to humans and the environment (Ahouangninou et al., 2012). However, the potential of endophytic fungi to control *S. recurvalis* on amaranth has not been systematically evaluated.

1.2 Problem Statement

Spoladea recurvalis is the major amaranth pest that poses a significant threat to amaranth production (Azandémè-Hounmalon et al., 2023). This pest causes extensive damage by feeding on foliage, resulting in reduced photosynthetic capacity, stunted growth, and yield losses of up to 50–70% ((Azandémè-Hounmalon et al.,2023). In Kenya, severe infestations of *S. recurvalis* can lead to complete crop failure, with yield losses reaching 100% (Kahuthia-Gathu, 2011). Such losses threaten the livelihoods of smallholder farmers and compromise household food and nutritional security by reducing the availability of this nutrient-rich vegetable.

Current management strategies rely predominantly on chemical pesticides. However, their frequent and indiscriminate use is often ineffective and can lead to pesticide residues on vegetables, environmental contamination, adverse effects on human health, pest resistance, and the destruction of beneficial organisms such as parasitoids, predators, and pollinators (Sankoh et al., 2016). Consequently, increasing attention has been directed toward biological control agents as environmentally sustainable alternatives. Among these, endophytic fungi microorganisms that inhabit plant tissues without causing disease have shown considerable potential because of their ability to promote plant growth, produce insecticidal metabolites, and induce systemic plant defense responses (Bamisile et al., 2018).

Despite these promising attributes, there is limited knowledge regarding which endophytic fungal isolates effectively colonize amaranth, their ability to suppress *S. recurvalis* populations, their effects on the pest's life-history traits, and the underlying

mechanisms responsible for pest suppression, including plant growth promotion, induced resistance, and changes in plant volatile emissions. This lack of information has constrained the development of endophyte-based management strategies for *S. recurvalis* in amaranth. Therefore, this study aimed to assess the effectiveness of endophytic fungi in controlling *S. recurvalis* on amaranth by identifying fungal isolates with biocontrol potential, evaluating their effects on plant growth promotion, assessing their impact on the life-history parameters of *S. recurvalis*, and investigating their mechanisms of action.

1.3 Justification

With the increased awareness on the importance and high demand of leafy amaranth, *S. recurvalis* being a serious pest causes considerable yield losses, threatening food security and the income for smallholder farmers who rely on amaranth production. Excessive application of chemical pesticides to control vegetable pests, despite their short-term effectiveness, have increasingly become a concern due to their environmental toxicity, high economic cost, promotes pest resistance, and have detrimental impacts on human health and non-target beneficial organisms. Biopesticides, particularly fungal-based biopesticides, are a promising new alternative with high biodegradability and specificity, low likelihood of resistance development, suitability for incorporation into integrated pest management practices, and practically no known health risks (La Spada et al., 2020).

Endophytic fungi have emerged as promising biological control agents due to their ability to colonize plant tissues internally, produce bioactive metabolites, and enhance plant defense mechanisms against herbivorous insects (Vega, 2008). Therefore, this study focused on the promising potential of endophytic fungi to offer a long-term, environmentally sustainable solution for managing *S. recurvalis* and elucidating their mechanisms of action. The research aimed at contributing to the advancement of integrated pest management (IPM) strategies. This would reduce dependence on chemical pesticides, enhance environmental health, and improve the productivity and safety of amaranth cultivation systems.

1.4 Research Objectives

1.4.1 General Objective

To assess the efficacy of endophytic fungi in the management of *S. recurvalis*.

1.4.2 Specific Objectives

1. To investigate the endophytic colonization and insecticidal activity of the fungal endophytes in colonized amaranth plants to *S. recurvalis*
2. To assess the behavioral and reproductive responses of female *S. recurvalis* to endophytically inoculated amaranth plants
3. To evaluate plant growth promotion of the endophytically colonized amaranth plants and quantify shoot biomass

1.5 Research Hypotheses

- i. Endophytic colonization does not confer systemic resistance against *S. recurvalis*
- ii. Female *S. recurvalis* do not show any behavioral and reproductive responses towards endophytically colonized amaranth plants
- iii. Endophytes do not stimulate or promote the growth of amaranth plants after their successful colonization

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and Taxonomy of Amaranth Species

Central America is considered the center of origin for *Amaranthus*, with evidence of its cultivation dating back to around 6700 BC (Mposi, 1999). The genus *Amaranthus* includes approximately 60 to 70 species, with around 40 species native to the Americas. Of these, 17 are cultivated as vegetable amaranths for their edible leaves, while three are recognized as grain amaranths valued for their edible seeds (Ebert et al., 2011). The remaining species are primarily used as ornamentals or are considered weedy species.

2.2 Amaranth Production in Kenya

In Kenya, Krause et al. (2019), Ochieng et al. (2019) and Nyonje et al. (2022) reported that Kisii, Nyamira, Vihiga, Bomet, Kisumu, Kiambu, Bungoma, Kakamega, Kajiado, Kilifi, and Nakuru are the leading areas for amaranth production. The most common species of amaranth grown as vegetables are *Amaranthus dubius*, *A. blitum* and *A. hybridus*, commonly known as red amaranth. Additionally, *A. cruentus* and *A. hypochondriacus* are grown for both grain and vegetable (Muthoni & Nyamongo, 2010; Muriuki et al., 2014). Amaranth is often slighted as “poor man’s food” or “famine food” (Weinberger & Msuya, 2004; Mbhenyane et al., 2005) but its demand by the consumer is rising in response to increased awareness of its nutritional and medicinal importance, as well as a source of income for small-scale farmers (Ouma, 2004). Studies conducted by Oniang’o et al. (2006), reveals that major supermarkets in Nairobi and other cities sell amaranth. Nevertheless, Cernansky (2015) demonstrated that amaranth is being sold in various restaurants in Nairobi and other urban areas.

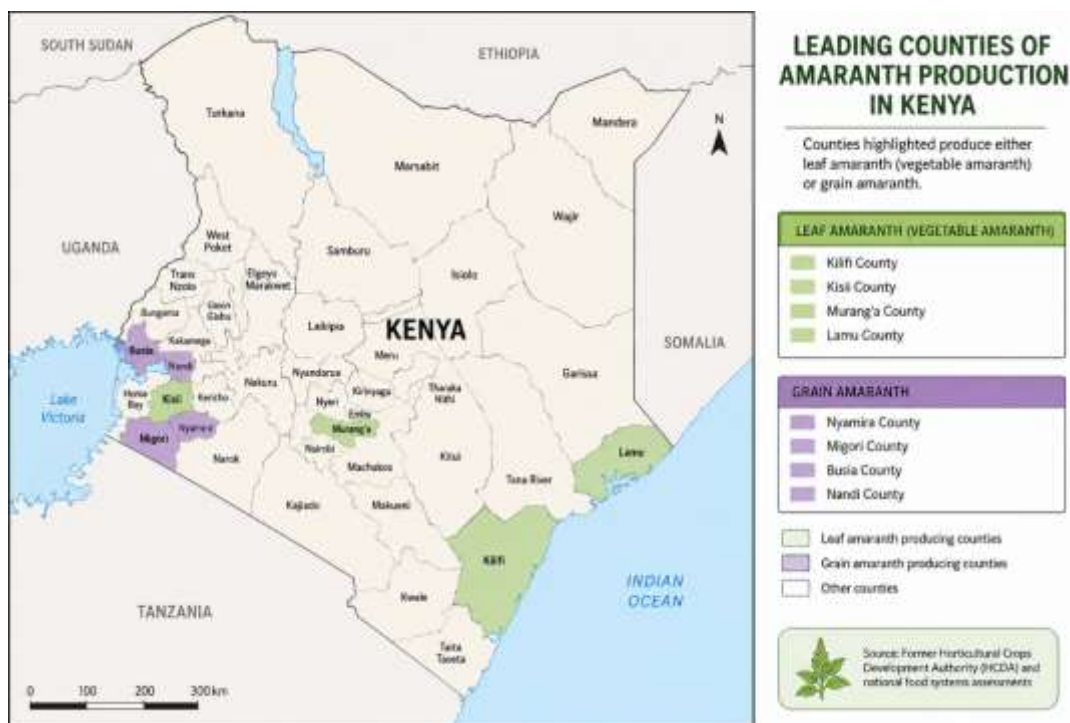


Figure 2.1: A map Showing Leading Amaranth Counties in Kenya

Source: (Agriculture and Food Authority, Horticultural Crops Directorate [HCD], 2014)

2.3 Nutritional, Medicinal and Economic Uses of Amaranth

Amaranths have excellent nutritional value because they contain high amounts of essential micronutrients such as β -carotene, iron, calcium, vitamin C and folic acid (Anuradha et al., 2023). For instance, vegetable amaranth such as *A. dubius* are a rich source of proteins, minerals like calcium, iron and phosphorus, vitamins A, C and K, riboflavin, niacin, vitamin B6 and folate, which help in combating and avoiding malnutrition (Emokaro et al., 2007; Makobo et al., 2010; Ebert et al., 2011).

Alabi et al. (2005) stated that a cup of amaranth leaves, which are cooked, boiled, and drained, contains 90 % vitamin C, 73 % vitamin A, 28 % calcium and 17 % iron. The amino acid content of amaranth seeds is close to the FAO and WHO recommendations for a healthy human diet (Gambus et al., 2002; Schoenlechner et al., 2010). Amaranth

seeds are beneficial as a dietary supplement for treating childhood malnutrition due to their high lysine, arginine, and histidine content (Gimplinger et al., 2008; Aragón-García et al., 2011). Amaranth leaves are a good source of antioxidant compounds such as phenolic acids, flavonoids and carotenoids (López-Mejía et al., 2014). These antioxidants act as a natural defense against diseases like cardiovascular, cancer, cataracts, atherosclerosis, retinopathy, arthritis, emphysema, and neurodegenerative diseases (Sarker & Oba, 2019). Amaranth has been used to treat diseases such as diabetes, urinary failure, cardiovascular complications, gynaecological disorders, pulmonary problems, jaundice and other infections (Caselato-Sousa & Amaya-Farfan, 2012). Amaranth is rich in bioactive compounds capable of inhibiting the proliferation of cancer cells (Venskutonis & Kraujalis, 2013).

In Kenya, Mkpuma et.al. (2026) reported that TAVs offer a significant opportunity for the resource poor people in western Kenya to earn a living because their production is easy to grow and can be done with little capital investment. Hence, low input TAVs are the only cash resource at the disposal of the women for the welfare of their families. Thus, their improved consumption and utilization is the most direct, low-cost way for children, lactating mothers and urban and rural poor to improve their nutritional, health and income status.



Figure 2.2: (a) *Amaranthus dubius* and (b) *Amaranthus cruentus*

Source: (Agbodzavu, 2019)

2.4 Key Insect Pests of Amaranth

The production of amaranth is limited by insect pests such as lepidopteran defoliators and amaranth stem weevils that are causing damage to amaranth crops worldwide (Othim et al., 2018). However, the leaf-webber *S. recurvalis* has been pointed out as a major pest (Othim et al., 2018; Agbodzavu, 2019). The caterpillar weaves the leaf into a distinctive leaf shelter, ferociously feeds on green matter and pupates within the rolled leaf. A severe infestation causes the leaves to quickly dry out and become completely skeletonized (Navarajan, 2007; James et al., 2010).

2.5 Biology of *Spoladea recurvalis*

The life cycle of *S. recurvalis* is approximately 3-4 weeks; the egg stage takes 3-4 days, the larval stage 12–15 days, and the pupal stage 8-11 days (Agbodzavu et al., 2020; Pande, 1972, Figure 2.2). It consists of the egg, six larval instars, a pre-pupa and a pupa prior to adult emergence (Aderolu et al., 2019). The adult is a dark brownish 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 (Agbodzavu et al., 2020). Mature adult male measures 10.25 mm long and 2.83 mm wide with a slender body, while the mature female measures 11.97 mm long and 3.27 mm wide with a robust and compact physique (Omoloye & Oyedokun, 2018). The average life span of males and females is 3.5 and 6 days respectively (James et al., 2010). Mating occurs within one day after emergence, with the male and female remaining in coition facing opposite directions (Omoloye & Oyedokun, 2018). Oviposition begins one or two days after mating and lasts between five - six days, and each female lays between 200 and 400 eggs (SM El-Gendi et al., 2006; Aderolu et al., 2019). *Spoladea recurvalis* eggs are oval in shape, scale-like, shiny, translucent yellow sacs deposited singly or in batches on the lower side of the leaves. The eggs measure 0.6 mm × 0.5 mm (Aderolu et al., 2019). The first instar larvae hatches after three days of egg deposition (Figure 2.2). The trunk is uniformly whitish, featuring a dark spot at the anterior end of the body. As the larvae feeds progressively, it turns into second instar larvae which is light green in appearance, the green color slowly turns to pale green as the larvae advance towards the third instar. It retains the pale green color at the fourth instar and changes

to milky-white at the fifth instar (Figure 2.2). As the sixth instar gets closer to the pupa stage, it becomes reddish and constricted. The pre-pupa develops into a light brown. One day old pupa is white brown, but it becomes dark brown and tanned as days go by (Omoloye & Oyedokun, 2018).



Figure 2.3: Life Cycle of *Spoladea recurvalis*, showing the Different Stages

Source: (Opisa, 2019)

2.6 Geographic Distribution and Host Range of *Spoladea recurvalis*

Spoladea recurvalis has been identified as a migratory species in Europe (Shirai, 2006). It is widely distributed worldwide, including the tropics and subtropics regions of Central America, the Caribbean, South America, Europe, Africa, North America and Asia (Aswal et al., 2005; Jež et al., 2015; Husain et al., 2020).

In addition to amaranth, *S. recurvalis* larvae feed on the leaves of beans, cucurbits, beetroot, sweet potato, and eggplant, causing severe damage to these host plants (Kim et al., 2014; Jeyasankar & Gokilamani, 2016).

2.7 Damage of *Spoladea recurvalis* on Amaranth

The early instar larvae of *S. recurvalis* only consume the leaves beneath the epidermis skeletonizing the tissues, and occasionally spin webs of silk under which they remain protected (Jeyasankar & Gokilamani, 2016). After the second instar, they skeletonise the foliage, leaving only the main veins intact, roll the leaves into distinctive leaf shelters, form webbing on leaves and leave frass on the leaves (Navarajan, 2007; James et al., 2010). A severe attack on amaranths causes the leaves to quickly dry up and become completely skeletonized (Navarajan, 2007; James et al., 2010). In Kenya, yield loss of up to 100% by *S. recurvalis* larvae has been reported (Kahuthia-Gathu, 2011). Leaf damage caused by the larvae reduces its quality and quantity, making it lose its commercial value (Kahuthia-Gathu, 2011). The ravenous feeding habit by the larvae leaves the fecal pellets, which significantly affect the quality and the consumer's preference by defoliating the leaves, hence making it unmarketable (Aderolu et al., 2013).

2.8 Management of *Spoladea recurvalis*

2.8.1 Chemical Control

Synthetic pesticides such as organochlorides, organophosphates, pyrethroids, carbamates, endosulfan, abamectin, chlorpyrifos, spinosad and carbaryl are primarily relied by farmers in the management of agricultural pests, including the key insect pest of amaranth *S. recurvalis* and other leaf webbers (Losenge, 2005; Arivudainambi et al., 2010; Aderolu et al., 2013). Over-reliance and indiscriminate use of chemical pesticides pose a risk to human and environmental health, resistance to the target pest due to their persistence in the soil and bioaccumulation and moreover, causes environmental pollution (Losenge, 2005). Additionally, when chemical pesticides are used improperly, they lead to the presence of pesticide residues in amaranth leaves, which might cause harm to the consumers (Awasthi, 2001; Fan et al., 2013). Most

contact insecticides have a broad spectrum, affecting target and non-target organisms; hence, the non-judicious use is disruptive to naturally occurring biological control agents like natural enemies and predators (Fernandes et al., 2010). As a result, reduced activity of biological agents usually leads to pest resurgence and eruption of secondary pests (Fernandes et al., 2010).

2.8.2 *Bacillus thuringiensis* (Bt)

Bacillus thuringiensis (Bt) has been successfully used as a biopesticide in the management of lepidopteran pests (Leena et al., 2005). All lepidopteran larvae are susceptible to high doses of *Bt kurstaki* (Fifi et al., 2016). Aswal & Bisht (2012) reported that there was a reduction of 85% *S. recurvalis* larvae after treatment with *Bt*. Under laboratory conditions El Hussein (2019) reported 100% larval mortality of *S. recurvalis* when fed on a diet mixed with different concentrations of *Bt kurstaki* (Dipel 2X). While Opisa et al. (2018) under laboratory conditions indicated between 40-50% *S. recurvalis* larval mortality after treatment with *Bt kurstaki* product Halt®.

2.8.3 Use of Natural Enemies

The use of natural enemies including predators and parasitoids in the management of *S. recurvalis* is one of the crucial components of integrated pest management (IPM). They attack various life stages of the pest. For instance, successful incidents in the use of parasitoids in regulating *S. recurvalis* populations have been reported. Egg parasitoids, e.g., *Trichogramma* species (Hymenoptera: Trichogrammatidae); larval parasitoids, including *Apanteles* sp., *Cardiochiles* sp., and *Phanerotoma* sp., *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae), *Campoletis* sp., *Venturia infesta* Cresson (Hymenoptera: Ichneumonidae), and *Prosopodopsis* sp. (Diptera: Tachinidae) have been successfully reared on *S. recurvalis* (Agbodzavu, 2019). Narayanan et al. (1957) reported up to 60% parasitism by *Apanteles* sp on *S. recurvalis*. Parasitism rate of more than 90% by *A. hemara* on *S. recurvalis* under laboratory conditions has also been reported (Othim et al. 2017).

2.8.4 Botanical Insecticides

Botanical insecticides are pest control agents that are derived from plant materials and they are used to manage agricultural pests (Ngegba et al., 2022). The use of neem extracts showed a wide range of effects including antifeedancy, repellency, growth inhibition, oviposition, insecticidal, and larvicidal activity when used on lepidopterans. Besides, it showed no toxicity on non-target organisms (Niroumand et al., 2016). This is in agreement with (Morimoto et al., 1999) who reported that plant extracts exhibit similar antifeedant properties against lepidopteran pests. For example, herbal-based compounds, e.g., neem derived from *Azadirachta indica*, have been reported to cause 52.70% antifeedant activity on *S. recurvalis* larvae while feeding on amaranth (Jeyasankar & Gokilamani, 2016). In Nigeria, the application of neem extracts decreased *S. recurvalis* larvae and the number of damaged leaves per plant by 30% and 41%, respectively (Aderolu et al., 2013). According to Aderolu et al. (2013), the active ingredients in that specific plant hinder's larvae from feeding. Arivudainambi et al. (2010) reported that *Cleistanthus collinus* (Roxb.) extract upon application resulted in low numbers of *S. recurvalis* larvae feeding on amaranth crops.

2.8.5 Use of Host Plant Resistance

Host plant resistance (HPR) refers to the intentional use of resistant crop cultivars to reduce the negative impacts of pests or diseases on crop production systems (Stout, 2014). The method has been fully adopted as a pest management tactic in most of the vegetable production systems (Eigenbrode & Trumble, 1994). Recent studies by Othim et al. (2018), who in his work assessed a total of 35 amaranth successions for their antixenotic and antibiosis properties against *S. recurvalis* reported accession V1036227 demonstrated high resistance against the pest by impairing larval development. In addition, accession V1036227 exhibited exemplary antibiosis by causing 100% larval mortality under laboratory bioassays (Othim, et al., 2018).

2.8.6 Cultural Control

These includes sanitation, destroying the pest's alternate habitats and hosts, tillage, crop rotation, adjustment of planting date, plant diversity, use of fertilizer and harvest

time (Schellhorn et al., 2000). Numerous cultural practices have been proposed for the management of *S. recurvalis*. Some of them are weeding and planting amaranth at a distance from maize, sugar beet, soybean, eggplant, spinach, and sweet potato, which serve as alternative hosts (James et al., 2010). Timely removal of *S. recurvalis* leaf shelters is suggested in order to stop the pests from spreading between amaranth plants (James et al., 2010). Maloth (2023) reported that amaranth producers should plant pest-resistant cultivars and use sound agricultural methods to ensure pest management and optimal crop performance. Also, amaranths grown as per the recommended intra-spacing and weeding measures could be safer, healthier and pest-free (Sokoto & Johnbosco, 2017). Cultural practices like adequate spacing between plants and frequent weed control were effective in reducing *S. recurvalis* infestation and leaf damage thus improving amaranth growth and yield (Maloth.,2023).

2.8.7 Use of Semiochemicals

Semiochemicals are compounds produced by one organism that elicit a behavioural or physiological response in members of the same or different species. They affect the behavior of the insect pest through insect-insect and plant-insect interactions (El-Ghany, 2019). Host-plant volatiles are used to locate food, mates and oviposition sites for insect herbivores (Witzgall et al., 2010). Phenylacetaldehyde (PAA) a flower volatile and attractant, has been reported to be a strong attractant for Lepidoptera, Coleoptera, Diptera and Hymenoptera (El-Sayed et al., 2008; Guédot et al., 2008). Landolt et al. (2011) showed that PAA was effective in controlling *S. recurvalis*. Recent studies by Opisa et al. (2019) demonstrated that PAA could be used together with *M. anisopliae* ICIPE 30 conidia in an autoinoculation device to control *S. recurvalis* under field conditions. This could be achieved by spatially separating PAA and *M. anisopliae* ICIPE 30 by five to 10 cm in order to effectively reduce the pest populations (Opisa et al., 2019). On the contrary, Othim et al. (2018) & Agbodzavu (2019) in their work in Tanzania and Kenya respectively concluded that PAA was ineffective in the management of *S. recurvalis*.

2.8.8 Use of Endophytic Fungus

Endophytic fungi refers to microorganisms that primarily live within plant tissues throughout the entire or partial life cycle by establishing a mutually beneficial symbiotic relationship without harming the host plant (Patchett & Newman, 2021). Moreover, recent research has shown that several species of entomopathogenic fungi can also establish themselves as endophytes within plant tissues (Paradza et al., 2021). This endophytic colonization offers additional ecological benefits, including enhanced tolerance to environmental stress, stimulation of plant growth, and improved defense against biotic threats such as insect herbivores and plant pathogens (Shikano et al., 2017). These endophytes can stimulate the production of key phytohormones and secondary metabolites involved in plant growth (Proietti et al., 2023). Consequently, the activation of these plant defense mechanisms can impact insect performance by reducing their survival, growth, feeding efficiency, and reproductive success (Jaber & Ownley, 2018; Jensen et al., 2020). Screening of endophytic fungi against some plant pathogens has been recently intensified due to their effectiveness as a source of biocontrol agents. For example, *Cochliobolus lunatus* has been successfully used as an entomopathogenic fungus in the management of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) by causing 60% larval mortality (Saad et al., 2019). Additionally, Hussein et al. (2013) reported 90% mortality by *S. littoralis* larvae after treatment with *Paecilomyces fumosoroseus* under laboratory conditions. Through seeds inoculation, *Trichoderma asperellum* M2RT4, *Hypocrea lixii* F3ST1 and *Beauveria bassiana* ICIPE 706 negatively affected the life history parameters of *Tuta absoluta* (Lepidoptera: Gelechiidae) (Agbessenou et al. 2020). Opisa et al. (2018) reported that *Metarhizium anisopliae* ICIPE 30 and *B. bassiana* ICIPE 725 caused mortality of 92% and 83% respectively on *S. recurvalis* larvae. However, no endophytic fungi have been screened through seeds inoculation for pathogenicity against *S. recurvalis*.

CHAPTER THREE

FRIENDS OR BETRAYERS? ENDOPHYTIC COLONIZATION OF AMARANTHUS DUBIUS AND ITS EFFECTS ON THE REPRODUCTIVE TRAITS OF SPOLADEA RECURVALIS (*Lepidoptera: crambidae*)

The work in this chapter covers the activities of objective three and has been published in ‘Arthropod Plant Interactions Journal’. The authorship is as shown below:

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Abstract

Amaranth is a highly nutritious and medicinal crop with great potential for food security. However, its productivity is severely constrained by insect pests, particularly *Spoladea recurvalis*. Farmers’ reliance on chemical insecticides for its control poses high risks to human and environmental health. This study therefore explored the potential of fungal endophytes as a sustainable alternative approach in managing *S. recurvalis*. We screened 15 fungal isolates belonging to six different genera (*Trichoderma*, *Hypocrea*, *Beauveria*, *Fusarium*, *Bionectria* and *Metarhizium*) to assess their potential to endophytically colonize *Amaranthus dubius* through seed inoculation and determined the systemic induced resistance potential of the endophytes in controlling *S. recurvalis*. *Hypocrea lixii* F3ST1 and *Trichoderma asperellum* M2RT4 successfully colonized all the host seedling parts. *Hypocrea lixii* F3ST1 and *T. asperellum* M2RT4 were further investigated for their endophytic persistence, effect on plant growth, pathogenicity to *S. recurvalis*, and longevity of F1 progenies. Both endophytes remained persistent for the four-week assessment period with varied colonization rates. The two endophytes significantly improved leaf number, leaf width, leaf length, plant height, and shoot dry weight. Additionally, *H. lixii* F3ST1 and *T. asperellum* M2RT4 significantly affected the life-history parameters of *S. recurvalis*,

resulting in reductions in egg masses (5% and 49.8%, respectively; $p < 0.001$), egg hatchability (7% and 48%, respectively; $p < 0.001$), increased larval mortality ($> 35\%$), decreased number of pupae (4.9% and 58.4%, respectively; $p < 0.001$), reduced adult emergence (4.9% and 57.9%, respectively; $p < 0.001$), and lower survival of F1 progeny. Our findings suggest that *H. lixii* F3ST1 and *T. asperellum* M2RT4 may enhance amaranth growth and reduce *S. recurvalis* infestation under controlled conditions. However, further field evaluations are required to validate these effects under practical farming systems.

3.1 Introduction

The African continent is rich in traditional vegetable species, with amaranth being among the most important and widely consumed leafy vegetables across the continent (Kansiime et al., 2018). It is highly valued for its exceptional nutritional profile, providing essential micronutrients such as β -carotene, iron, calcium, vitamin C, and folic acid. In addition, amaranth is a rich source of antioxidants, including flavonoids, carotenoids, and phenolic compounds, that mitigate oxidative stress and offer protection against diseases such as atherosclerosis, cancer, cardiovascular disorders, diabetes, stroke, and rheumatoid arthritis (Venskutonis and Kraujalis, 2013).

Amaranth production has recently increased from a subsistence to a commercial crop (Cernansky 2015). However, plant parts. Nevertheless, the major insect pest species causing significant losses to amaranth belong to the orders Lepidoptera- *S. recurvalis* (F.), *Spodoptera littoralis* (Boisduval) (cotton leafworm), Coleoptera- *Hypolixus* sp. (F.) (amaranth stem weevils), Diptera- *Liriomyza huidobrensis* (Blanchard) (pea leaf miner), and Hemiptera- *Myzus persicae* (Sulzer) (green peach aphid) (Othim et al. 2018). In addition, *S. recurvalis* is reported as a major pest in amaranth fields with the potential to cause complete defoliation under extreme outbreaks (Agbodzavu 2019).

Spoladea recurvalis is a highly destructive pest of leafy vegetables, particularly on *Amaranthus* species (Husain et al., 2020). The larvae feed voraciously on the underside of leaves, consuming the epidermis, skeletonizing tissues, and occasionally spinning light webs in which they rest (Jeyasankar and Gokilamani, 2016). This feeding damage severely reduces leaf quality, rendering the crop unmarketable and causing substantial

economic losses (Aderolu et al., 2019). In Kenya, yield losses of up to 100% have been reported (Kahuthia-Gathu et al., 2011). Farmers commonly rely on indiscriminate use of synthetic insecticides for controlling the pest (Lucas et al., 2015), a practice that poses significant risks to human health, the environment, and biodiversity. Moreover, excessive use of chemical insecticides increases production costs, accelerates pest resistance, and eliminates natural enemies, often leading to pest resurgence (Sharma & Singhvi, 2017). High pesticide residues on export crops also create trade barriers due to non-compliance with international residue limits (Bailey et al., 2010).

Among non-chemical management approaches, biological control, particularly using entomopathogenic fungi, offers an effective and environmentally friendly alternative (Fanning et al., 2018). These fungi can be incorporated into Integrated Pest Management (IPM) programs, especially for vegetable crops (Akutse et al., 2020). Some entomopathogenic fungi could also establish endophytically within plant tissues without harming the host (Tejesvi and Pirttilä, 2018), offering advantages such as protection from adverse environmental conditions and improved persistence against abiotic factors (Chaudhary et al., 2022). Endophytic fungi enhance plant defense responses (Kambrekar, 2016), promote growth (Agbessenou et al., 2020; Paradza et al., 2021; Kinyungu et al., 2023), and facilitate nutrient uptake (White et al., 2019). Given the limitations of synthetic insecticides and the need for sustainable management strategies against *S. recurvalis*, this study aimed to (i) evaluate the ability of some selected fungal isolates to establish endophytically within amaranth tissues, (ii) assess the persistence of these fungal endophytes over time in the host plant, (iii) determine the effects of endophytic fungal colonization on amaranth growth performance, and (iv) examine the impact of endophytically colonized amaranth on the life-history parameters of *S. recurvalis*. Specifically, it was hypothesized that (1) the selected fungal isolates would not establish endophytically in amaranth tissues, (2) the endophytes would not persist within the plant over time, (3) endophytic colonization has no effect on amaranth growth performance, and (4) endophytic colonization of amaranth plants by the selected fungal isolates has no effect on the life-history parameters of *S. recurvalis*.

3.2 Materials and Methods

3.2.1 Experimental Site

The experiments were carried out in the Arthropod Pathology Unit (APU) laboratory and in a screen house at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville Campus, Nairobi, Kenya (1.2219° S, 36.8967° E; 1616 m above sea level).

3.2.2 Insect Rearing

A colony of *S. recurvalis* was established from adults and larvae collected on amaranth during a field survey in Naivasha (N 0° 43' 0.01" E 36° 26' 9.28") and Mwea, (S 0° 36' 31.3" S 037° 22' 29.7" E) in September 2022. The selection of Naivasha and Mwea was based on the availability of experimental facilities, collaborating farmers, and the feasibility of conducting the field trials. The moths were maintained in ventilated, sleeved Perspex cages (40 × 40 × 45 cm) and fed *ad libitum* with a 10% honey solution dipped in cotton wool balls to serve as a food source (Othim et al., 2017). Five weeks old potted amaranth plants were later introduced in the cages for oviposition. After 24 h exposure, the plants were removed and transferred to separate cages (50 × 50 × 60 cm), ventilated with netting material at the sides until the eggs hatched. Foliage containing larvae were removed from these plants three days after hatching and placed into plastic lunch boxes (15 × 7 × 5 cm) lined with tissue paper to absorb excess moisture and fine netting infused lid for ventilation. The larvae were fed daily with fresh amaranth leaves until they pupated. With the aid of a fine camel hair brush, pupae were collected from the lunch boxes and placed inside sleeved Perspex cages (40 × 40 × 45 cm) for adult emergence. Field collected populations of *S. recurvalis* were infused into the laboratory colony every three months in order to enhance outbreeding (Agbessenou et al., 2020; Akutse et al., 2020). The colony was maintained in a rearing room at 25 ± 2 °C, 50%–70% RH and 12:12 L:D photoperiod respectively, at the Animal Rearing and Quarantine Unit (ARQU) of *icipe*.

3.2.3 Fungal Cultures and Viability Test

In this study, 15 fungal isolates belonging to six different genera (3 *Trichoderma*, 1 *Fusarium*, 1 *Bionectria*, 7 *Beauveria*, 1 *Hypocrea* 2 *Metarhizium*) were selected, cultured and screened for their ability to colonize amaranth plant through seed inoculation (Table 3.1). The fungal isolates were obtained from the International Centre of Insect Physiology and Ecology (*icipe*)’s repository of pathogens maintained at the Arthropod Germplasm Unit (APU), and subcultured on Potato Dextrose Agar (PDA) (OXOID CM0139, Oxoid Ltd., Basingstoke, United Kingdom), exclusive of *Metarhizium anisopliae* isolates that were subcultured on Sabouraud Dextrose Agar (SDA) (OXOID CM0041, Oxoid Ltd., Basingstoke, UK), and maintained at 25 ± 2 °C in complete darkness. The selection of *H. lixii* F3ST1 and *T. asperellum* M2RT4 was based on previous studies that demonstrated their successful establishment as endophytes, their ability to enhance plant defense responses, and their effectiveness against *T. absoluta* on tomatoes (Agbessenou et al., 2020) and Fall army worm on maize (Kinyungu et al., 2023). However, their efficacy against *Spoladea recurvalis* had not been investigated, representing the knowledge gap addressed by this study

Table 3.1. Identity of Fungal Isolates That Were Used in This Study

Fungal species	Isolate	Source	Location	Year of isolation
<i>Trichoderma asperellum</i>	M2RT4	Maize and Sorghum	Nakuru (Kenya)	2009
<i>T. atroviride</i>	F2S21	Onion	Loitoktok (Kenya)	2012
<i>T. harzianum</i>	F2R41	Onion	Kericho (Kenya)	2009
<i>Fusarium proliferatum</i>	F2S21	Onion	Embu (Kenya)	2012
<i>Bionectria ochroleuca</i>	F3R21	Onion	Kericho (Kenya)	2008
<i>Beauveria bassiana</i>	ICIPE 279	Coleopteran larvae	Kericho (Kenya)	2005
<i>B. bassiana</i>	ICIPE 273	Soil	Suba (Kenya)	2006
<i>B. bassiana</i>	ICIPE 283	Soil	Mauritius	2005
<i>B. bassiana</i>	ICIPE 621	Soil	Kericho (Kenya)	2008
<i>B. bassiana</i>	ICIPE 676	Soil	Diani (Kenya)	2008
<i>B. bassiana</i>	ICIPE 609	Soil	Meru (Kenya)	2008
<i>B. bassiana</i>	ICIPE 706	Monocots	Kenya	2012
<i>Hypocrea lixii</i>	F3ST1	Maize and Sorghum	Kenya	2009
<i>Metarhizium anisopliae</i>	ICIPE 7	<i>Amblyomma variegatum</i>	Rusinga (Kenya)	1996
<i>M. anisopliae</i>	ICIPE 41	Soil	Lemba (DRC)	1990

Source: *icipe* germplasm

Conidia were harvested from two-week-old plates by scraping off the agar surface with a sterile spatula into 10 ml sterile distilled water containing 0.05% Triton X-100 (MERCK KGaA, Darmstadt, Germany) in a universal bottle containing 5-10 glass beads. The suspension was vortexed for 5 min at about 700 rpm to break conidial clumps and ensure a homogenous suspension (Akutse et al., 2013). Conidial concentrations were quantified using an improved Neubauer hemocytometer under a light microscope (Inglis et al., 2012). The conidial suspension was adjusted to a concentration of 1×10^8 conidia ml^{-1} through serial dilution before inoculating amaranth seeds.

The conidial viability was assessed prior to any bioassay under a microscope by inoculating 0.1 ml of the 3×10^6 conidia/ml suspension onto four fresh plates of PDA using a glass spreader. Three sterile microscope cover slips (2×2 cm) were placed on the surface of the inoculated plates. The plates were sealed with a parafilm and incubated under complete darkness at 25 ± 2 °C and examined after 16–20 h. Percentage conidial germination was determined by counting the number of germinated conidia from 100 randomly selected conidia in a selected field covered by each cover slip under a microscope at 400× magnification (Leica DM500; Microsystems, Wetzlar, Germany). (Agbessenou et al., 2020; Akutse et al., 2013; Opisa et al., 2018). Conidia were considered to have germinated when the length of the germ tube was about twice the diameter of the conidium (Goettel & Inglis, 1997). For each isolate, four replicates were used and replicated over time.

3.2.4 Amaranth Seeds Inoculation and Colonization Assessment

A local vegetable variety Dubious of amaranth seeds (*Amaranthus dubius*) used for this study was selected because it is one of the most widely cultivated leafy amaranth species in Kenya and a known host of *Spoladea recurvalis*, making it suitable for evaluating the endophytic fungi. The seeds were sourced from Simlaw Seeds Company Limited, Nairobi, Kenya, and used for all the experiments.

3.2.5 Amaranth Seed Inoculation

Prior to inoculation, *A. dubius* seeds were surface-sterilized with 70% ethanol (2 min) and 1.5% sodium hypochlorite (three min), rinsed thrice with sterile distilled water, and aseptically dried on sterile filter paper in a safety cabinet (Agbessenou et al. 2020; Akutse et al. 2013). To confirm the effectiveness of the surface sterilization procedure, tissue (sterilized seeds) imprinting and plating 0.1 ml of the last rinse water was conducted on PDA media supplemented with 100 mg/L Streptomycin and the plates were incubated at 25 ± 2 °C for 14 days (Agbessenou et al. 2020). Failure of fungal growth after incubation of the plates was an indication of the effectiveness of the sterilization procedure. The seeds were then soaked overnight for 12 h in conidial suspensions containing 1×10^8 conidia/ml. For the controls, sterilized seeds were also soaked overnight for 12 h in sterile distilled titrated water containing 0.05% Triton X-100 (MERCK KGaA, Darmstadt, Germany) (Agbessenou et al. 2020). Field soil mixed with manure at a 5:1 soil: manure ratio, autoclaved at 121 °C for 2 h, and left to cool for 72 h before sowing, was used as the planting substrate (Akutse et al. 2013; Jaber and Enkerli 2016). Ten seeds were sown in individual pots (8 cm diameter x 7 cm height) containing autoclaved planting substrate and maintained at room temperature (25 ± 2 °C, 60% RH, and 12:12 L: D photoperiod) for 4-5 weeks. After germination, seedlings were thinned to one per pot and watered twice daily (morning and evening) to maintain adequate soil moisture for seedling growth. No fertilizer was added to the planting substrate. Plants four to five weeks old were used for the various experiments

3.2.6 Endophytic Colonization Assessment

To check for the success rate of fungal endophyte colonization, four to five weeks-old seedlings were carefully uprooted and washed under running tap water, to remove any soil particles attached to the plants. A destructive sampling method was employed to evaluate the colonization of the plant tissues (leaves, stems and roots). A total of 12 plants were used per treatment. Five randomly selected leaf, stem and root sections from each plant were surface-sterilized as described above. The different plant tissues were then aseptically cut under a laminar flow hood into 1×1 cm and placed 4 cm

apart from each other on freshly prepared PDA plates supplemented with 0.05% solution of antibiotic (Streptomycin sulfate salt) to suppress bacterial contamination (Bamisile et al. 2020). Plates were then sealed with parafilm and incubated at 25 °C for 10 days. The presence of endophyte growth was determined by regularly examining the plates at two - three day intervals (Bamisile et al. 2020). Subsequently, the last rinse water was plated, and tissue imprinting was also conducted to check the effectiveness of surface sterilization of the plant materials (Inglis et al. 2012). The endophytic colonization of the different plant parts was recorded based on the morphological characteristics and microscopic examination of conidia through slides preparation in comparison with the mother plates according to Koch's postulates (Petrini and Fisher, 1986). Positive colonization was recorded only if there was the presence of the inoculated endophytes on the incubated plant materials in the plates (Muvea et al. 2014). Treatments were arranged in a completely randomized design (CRD) and the experiment was conducted with four replicates per treatment over time. Fungal endophyte colonization rate (%) of amaranth plant parts plated was calculated as shown below.

$$\text{Colonization (\%)} = \frac{\text{Number of plant tissue exhibiting fungal out growth}}{\text{Total number of plant tissue pieces plated out}} \times 100$$

3.2.7 Endophytic Persistence and Assessment of Plant Growth Parameters in Amaranth

Endophytic persistence and plant growth were assessed as described by Paradza et al. (2021). To examine the endophytic colonization persistence and the effect of the fungal isolates on plant growth promotion parameters, seed inoculation was conducted with some selected endophytes that colonized the whole plant and untreated control as described above in the colonization experiment. The plants were grown in plastic pots (8 cm diameter x 7 cm height) arranged in a completely randomized block design in a greenhouse. Weekly sampling comprised new plants from four different pots, and each pot represented a replicate (Paradza et al. 2021). After recording the growth parameters, colonization assessment was investigated for four weeks within amaranth plants over different growth stages starting at one-week post-germination. Four plants

were sampled per replicate, with four replicates per treatment, resulting in a total of 16 plants per treatment. Plant height was measured from the plant base to the tip of the uppermost leaf with a visible collar, leaf number was determined by counting fully developed leaves, while leaf length (bottom to its tip), leaf width (at the widest part of the leaf) and plant fresh and dry shoot biomass (fresh and oven-dried at 60°C for 48 h) were also recorded. Fresh and dry shoot biomass were measured only in the final week of the experiment to assess the total accumulated shoot biomass for the entire growing period (Paradza et al. 2021).

3.2.8 Pathogenicity of Endophytically Colonized Amaranth Plants on Life-History Parameters of *Spoladea recurvalis*

The best performing endophytic fungal isolates (*H. lixii* F3ST1 and *T. asperellum* M2RT4), based on their ability to colonize the root, stem, and leaf tissue of the host plant, were selected to assess their effect on the oviposition potential, egg hatchability, larval mortality, pupation, adult emergence and survival/longevity of *S. recurvalis* F1 progeny. Three-day-old, freshly emerged and mated adults (21 individuals at a sex ratio of 1:2 male: females) were exposed for 24 h to four-week-old endophytically colonized amaranth plants in Plexiglas cages (50 × 50 × 45 cm). Each cage contained four potted plants representing a treatment, maintained at 25 ± 2 °C, 60 % RH and 12:12 L: D photoperiod. The potted plants were arranged in cages in a completely randomized design, and the experiment was replicated four times over time. After 24 h post exposure, the insects were removed from the cages using a clean glass vial and introduced into clean cages (50 × 50 × 45 cm) (Akutse et al. 2013). The control moths were exposed to endophyte-free amaranth plants in all the experiments. For each treatment, a hand lens was used to count the number of egg masses laid on endophytically colonized and control (endophyte-free) plants. The eggs were maintained on the plants until they hatched. The experiment was replicated four times over time. After hatching, the larvae were allowed to feed upon their natal plants until they reached the second instar (approximately 8 -10 days post-exposure). The larvae were then counted and transferred using a fine camel brush into lunch boxes lined with a paper towel to absorb excess moisture. For each treatment, 80 larvae were randomly picked and individually placed in another set of clean lunch boxes lined with a paper

towel. The larvae were fed daily on endophytically colonized amaranth plants in the lunch boxes. On the contrary, the controls were fed with endophyte-free amaranth plants (Russo et al. 2021). Thereafter, larval mortality, pupation, adult emergence and longevity of F1 progeny were assessed and recorded. Larval mortality was scored daily by counting dead larvae. For each treatment, the number of pupae formed was recorded, and the pupae were incubated at 25 ± 2 °C. The number of adults that emerged was also determined for each of the treatments. Thirty (30) newly emerged adult moths (F1 progenies) were selected per treatment, and their mortality recorded daily until the last moth died (Akutse et al. 2013). These F1 moths were kept in a cage as described above and were provided with 10% sugar solution soaked in cotton wool balls as a source of food. The temperature of the cage was maintained at 25 ± 2 °C, 60% RH and 12:12 L:D photoperiod. To ensure that the fungal infection directly caused the moth's mortality, dead *S. recurvalis* were put on a moistened filter paper in petri dishes, followed by the mycosis test. To achieve mycosis, the dead insects were surface sterilized with 1% sodium hypochlorite followed by three rinses in sterile distilled water. The insect cadavers were placed on sterile Whatman wet filter paper in sterile petri dishes, then sealed with parafilm and kept at 25 °C. The presence of hyphae and conidia on the surface of the cadaver was attributed to mortality due to fungal infection (Agbessenou et al. 2020; Akutse et al. 2013).

3.3 Statistical Analyses

All statistical analyses were performed in R version 4.3.3 (R Development Core Team, 2024). Prior to analysis, all the data collected were subjected to normality and homogeneity test of variances using Shapiro-Wilk (Shapiro and Wilk, 1965) and Levene test respectively (at $p < 0.05$ significance level). Colonization (roots, stems, leaves) data were analyzed using generalized linear model (GLM) with binomial error distribution (link = logit). Similarly, percentage colonization data on weekly endophytic persistence were analyzed using GLM with binomial error distribution (link = logit). Besides, Wilcoxon rank sum test was performed on the percentage colonization per week in each plant part. Data collected on plant height, leaf length, leaf width, number of leaves, wet and dry shoot weight were analyzed using ANOVA, and the differences in means were separated using Tukey's honestly significant

difference (HSD) test. Count data (number of egg masses, egg hatchability, larval mortality, number of pupae and number of adults emerged) were analyzed using GLM with Poisson with log link and the means were separated using Tukey's HSD test. The goodness of fit of the data to the GLM was evaluated through residual diagnostics by assessing the ratio of residual deviance to degrees of freedom relative to the dispersion parameter. The survival probability curves were generated using the Kaplan–Meier estimator method, and log-rank test was used to compare the effect of the different endophytic fungal isolates on F1 progenies' survival using the “Survival” package (Therneau 2015).

3.4 Results

3.4.1 Endophytic Colonization of *Amaranthus dubius* by Fungal Isolates

All the isolates used in this study had conidial viability rates greater than 90% after 18h of incubation. After fourteen days of inoculation, every tested isolate was effectively recovered from the inoculated plants as fungal endophytes with varying colonization rates and plant part preferences except *B. bassiana* ICIPE 279, *M. anisopliae* isolates and *T. harzianum* KF2R41 which failed to colonize the seedling tissues. Only the isolates that successfully colonized at least one plant part are considered as endophytes in amaranth. Out of the 15 fungal isolates screened, only *H. lixii* F3ST1 and *T. asperellum* M2RT4, successfully colonized all the different parts of the host plant. However, the colonization rates were subject to the fungal species and plant parts. Colonization rates varied significantly among fungal isolates across all plant parts; roots, stems, and leaves (roots: LR $\chi^2 = 192.79$; stems and leaves: LR $\chi^2 = 1.21 \times 10^{13}$; df = 15; p < 0.001) (Figure 3.1). *Hypocrea lixii* F3ST1 exhibited colonization rate of 100% in the roots, stems, and leaves, while *T. asperellum* M2RT4 colonized 90, 100 and 100% of the roots, stems and leaves respectively. However, *F. proliferatum* NS2F51, *T. atroviride* F2S21, *B. ochroleuca* F3R21, *B. bassiana* isolates ICIPE 621, ICIPE 273, ICIPE 676, ICIPE 609, ICIPE 283 and ICIPE 706 colonized only the roots at the rates of 70, 15, 5, 35, 15, 15, 10, 5 and 5% respectively. *Trichoderma harzianum* F2R41, *B. bassiana* ICIPE 279, *M. anisopliae* isolates ICIPE 7 and ICIPE 41 failed to colonize any parts of the host plant (Fig 3.1). In addition, the

uninoculated plants (controls), the tissue imprints and the last rinsed distilled water, did not show any fungal growth; indicating the effectiveness of the surface sterilization technique used (Figure 3.1).

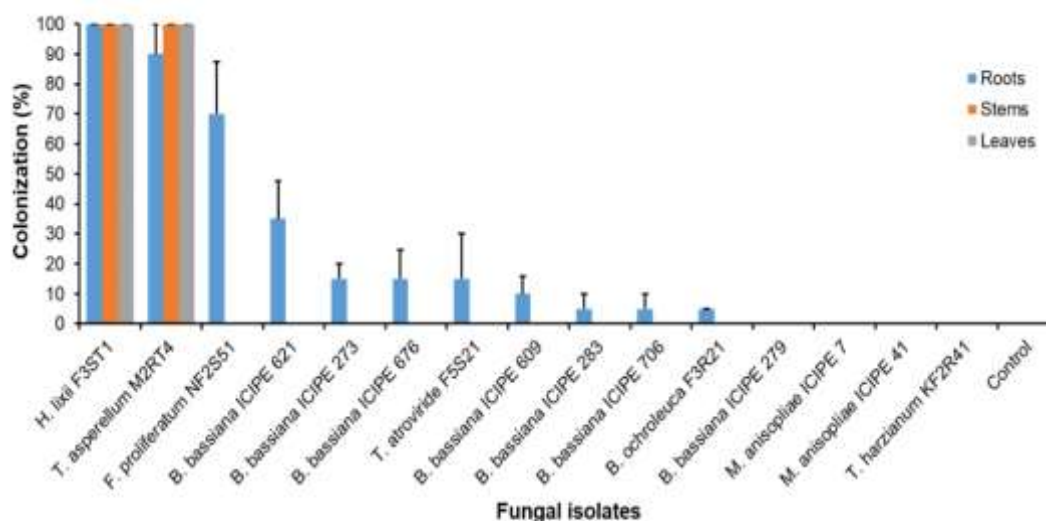


Figure 3.1: Endophytic Colonization of the Different Parts of *Amaranthus dubius* Host Plant by the Different Fungal Isolates

The bar chart represents means $\pm$ SE (standard error) at 95% CI (Tukey's HSD test, $p \leq 0.05$).

3.4.2 Endophytic Colonization Persistence for Selected Fungal Isolates

Following the successful colonization of all the host plant parts through seed inoculation, *H. lixi* F3ST1 and *T. asperellum* M2RT4 were selected as best performing isolates, and their endophytic persistence evaluated. A significant interaction occurred between time and plant parts colonization persistence ($LR \chi^2 = 61.42$, $df = 6$, $p < 0.001$), as well as between fungal isolates and time ($LR \chi^2 = 13.63$, $df = 3$, $p < 0.01$). However, no significant interaction was observed between fungal isolates and plant parts ($LR \chi^2 = 2.87$, $df = 2$, $p = 0.24$), and among fungal isolates, plant parts, and time ($LR \chi^2 = 10.80$, $df = 6$, $p = 0.09$).

Also, when considering the colonization persistence of the plant parts between treatments per week, there was no significant difference observed in the roots i.e week 1 ($w = 10$, $p = 0.453$), week 2 ($w = 12$, $p = 0.186$), week 3 ($w = 11$, $p = 0.429$) and week 4 ($w = 13$, $p = 0.163$) for both isolates (Figure 3.2a). Additionally, the colonization persistence in the stems by the two endophytes did not show any significant variations during week 1 ($w = 8$, $p = 1$), week 3 ($w = 5$, $p = 0.429$), and week 4 ($w = 9.5$, $p = 0.76$); except for week 2 where significant difference was observed between the treatments ($w = 16$, $p = 0.027$). This could suggest a transient phase in endophyte colonization, reflecting differences in early establishment efficiency and interaction with host defense responses (Figure 3.2b). However, there were significant differences in the persistence of the endophyte in the leaves during week 1 ($w = 16$, $p = 0.017$) and week 2 ($w = 11.5$, $p = 0.037$); except for week 3 ($w = 8$, $p = 1$) and week 4 ($w = 13.5$, $p = 0.122$) where no variations were observed (Fig. 3.2c).

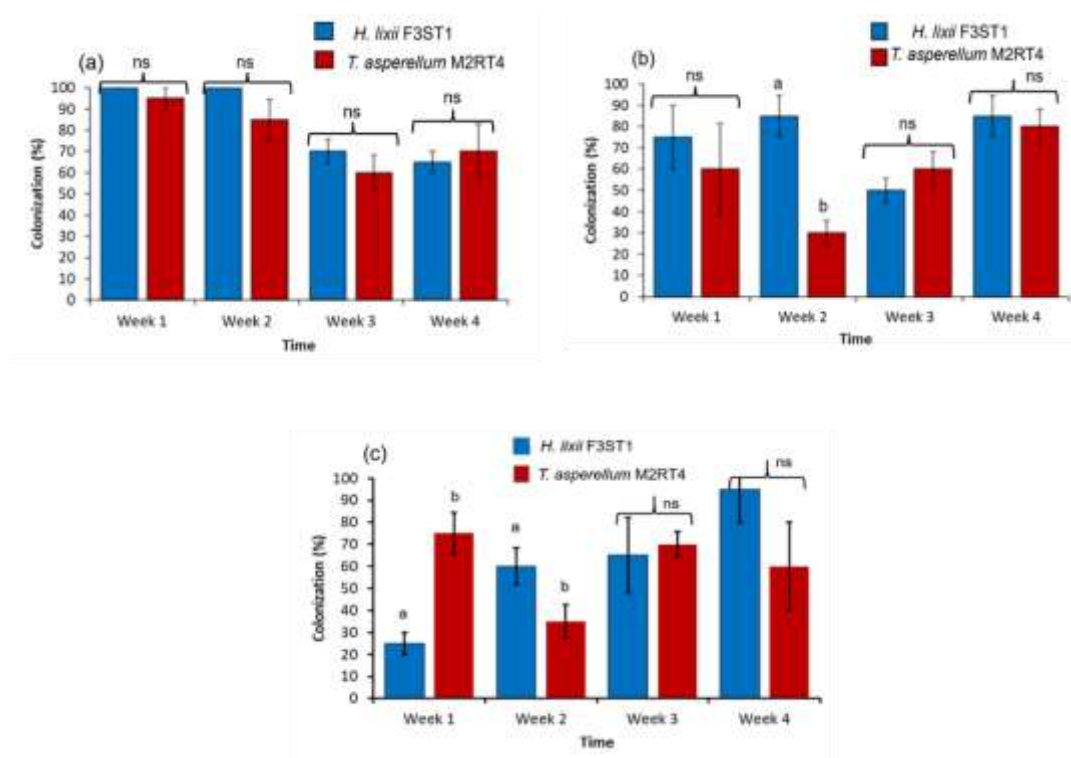


Figure 3.2: Endophytic Colonization Persistence of *Hypocrea lixii* F3ST1 and *Trichoderma asperellum* M2RT4 Four Weeks Post-Germination

Bar charts represent means $\pm$ SE at 95% Confidence Level ($p \leq 0.05$; $n = 4$), where (a) = Roots, (b) = Stems and (c) = Leaves.

3.4.3 Effect of Endophytic Colonization of the Fungal Isolates on *Amaranthus dubius* Growth Parameters

3.4.3.1 Leaf Number

Leaf number did not differ significantly among treatments during the first three weeks ($p > 0.05$), but significant differences emerged in weeks 4 and 5 ($F = 17.51$ and 10.51 , respectively, $df = 2$, $p < 0.001$), with both endophyte-treated plants differing from the control but not from each other (Table 3.2).

3.4.3.2 Plant Height

Plant height did not differ significantly between *T. asperellum* M2RT4 and *H. lixii* F3ST1 throughout the five-week evaluation, but both treatments were significantly taller than the control in weeks 1 and 2 ($p < 0.05$), and weeks 4 and 5 ($p < 0.001$), with no significant difference observed in week 3 ($p > 0.05$) (Table 3.2).

3.4.3.3 Leaf Length

Leaf length growth in *T. asperellum* M2RT4 and *H. lixii* F3ST1 inoculated plants was significantly greater than the control, with differences observed in weeks 1 and 2 ($p < 0.05$) and highly significant differences in weeks 3 to 5 ($p < 0.01$) (Table 3.2).

3.4.3.4 Leaf Width

Leaf width growth in *T. asperellum* M2RT4 and *H. lixii* F3ST1 inoculated plants was significantly higher than in the control. Differences were observed in weeks 1 and 2 ($p < 0.05$) and became highly significant from weeks 3 to 5 ($p < 0.001$) (Table 3.2).

Table 3.2. Effect of *Trichoderma asperellum* M2RT4 and *Hypocrea lixii* F3ST1 on *Amaranthus dubius* Plants Growth Parameters

Parameter	Week 1	Week 2	Week 3	Week 4	Week 5
Leaf number					
<i>T. asperellum</i>					
M2RT4	8.50 ± 0.21 a	10.1 ± 0.26 a	11.5 ± 0.4 a	24.3 ± 1.39 a	34.9 ± 2.07 a
<i>H. lixii</i> F3ST1	7.81 ± 0.12 a	9.25 ± 0.37 a	11.1 ± 0.08 a	21 ± 0.26 a	33.4 ± 0.74 a
Control	7.44 ± 0.42 a	9.19 ± 0.48 a	10.5 ± 0.4 a	17 ± 0.55 b	26.8 ± 0.7 b
F	3.84	1.88	2.19	17.51	10.51
df	2	2	2	2	2
P	0.062	0.208	0.167	0.001	0.004
Plant height (cm)					
<i>T. asperellum</i>					
M2RT4	10.95 ± 0.31 a	14.4 ± 0.66 a	18.46 ± 0.73 a	25.47 ± 0.66 a	32.24 ± 0.93 a
<i>H. lixii</i> F3ST1	10.8 ± 0.32 a	13.7 ± 0.3 ab	18.5 ± 0.7 a 16.43 ± 0.96	25.34 ± 0.40 a	32.45 ± 0.67 a
Control	9.26 ± 0.45 b	11.83 ± 0.7 b	a	21.7 ± 0.71 b	27.49 ± 0.38 b
F	6.63	5.19	2.15	12.57	16.11
df	2	2	2	2	2
p	0.017	0.032	0.173	0.002	0.001
Leaf length (cm)					
<i>T. asperellum</i>					
M2RT4	5.04 ± 0.09 a	7.12 ± 0.34 a	10.7 ± 0.28 a 10.1 ± 0.24	11.7 ± 0.25 a	13.9 ± 0.34 a
<i>H. lixii</i> F3ST1	4.9 ± 0.14 a	6.45 ± 0.01 ab	ab	10.9 ± 0.01 b	12 ± 0.21 b
Control	4.33 ± 0.17 b	5.95 ± 0.99 b	9.25 ± 0.22 b	9.75 ± 0.20 c	10.3 ± 0.22 c
F	7.55	7.43	8.96	25.22	45.08
df	2	2	2	2	2
p	0.0119	0.012	0.007	0.001	0.002
Leaf width (cm)					
<i>T. asperellum</i>					
M2RT4	3.7 ± 0.05 a	4.96 ± 0.12 a	7.73 ± 0.1 a	8.24 ± 0.09 a	9.46 ± 0.18 a
<i>H. lixii</i> F3ST1	3.46 ± 0.16 ab	4.5 ± 0.22 b	6.91 ± 0.25 b	7.58 ± 0.25 ab	8.11 ± 0.18 b
Control	3.2 ± 0.11 b	4.31 ± 0.12 c	6.64 ± 0.13 b	7.04 ± 0.13 b	7.38 ± 0.18 c
F	4.53	4.31	11.09	12.66	34
df	2	2	2	2	2
p	0.044	0.049	0.003	0.002	0.001

Means followed by the same letter within a column are not significantly different at $p \leq 0.05$ according to Tukey's honest significant difference (HSD) test.

3.4.3.5 Shoot Biomass

No significant differences were observed in fresh shoot weight among the treatments at the end of the evaluation period (week 5) ($p > 0.05$) (Table 3.3). In contrast, dry shoot weight was significantly higher in endophyte-treated plants compared to the control ($p < 0.01$) (Table 3.3).

Table 3.3. Effect of endophytic colonization of *T. asperellum* M2RT4 and *H. lixii* F3ST1 on Fresh and Dry Biomass

Fungal isolate	Fresh shoot weight (g)	Dry shoot weight (g)
<i>T. asperellum</i> M2RT4	169.39 ± 11.85 a	51.38 ± 4.14 a
<i>H. lixii</i> F3ST1	160.46 ± 7.13 a	40.44 ± 1.69 ab
Control	152.13 ± 4.17 a	32.95 ± 2.65 b
F	1.07	9.53
df	2	2
<i>p</i>	0.382	0.01

Means followed by the same letter within a column are not significantly different at $p \leq 0.05$ according to Tukey's honest significant difference (HSD) test.

3.4.4 Effect of Endophytically Colonized *Amaranthus dubius* Plants on Egg Masses and Hatchability of *Spoladea recurvalis*

The number of egg masses laid on amaranth plants differed significantly among treatments ($\chi^2 = 290.91$, $df = 2$, $p < 0.001$). Plants colonized by *H. lixii* F3ST1 had the fewest egg masses (3.75 ± 1.11), followed by those with *T. asperellum* M2RT4 (36.75 ± 3.77), while control plants recorded the highest (73.25 ± 5.14) (Figure 3.3a). In addition, egg hatchability differed significantly among treatments ($\chi^2 = 242.56$, $df = 2$, $p < 0.001$). *Hypocrea lixii* F3ST1 recorded the fewest hatched larvae (13.75 ± 1.75), followed by *T. asperellum* M2RT4 (94.5 ± 11.66), while the control had the highest (196.5 ± 22.14) (Figure 3.3b).

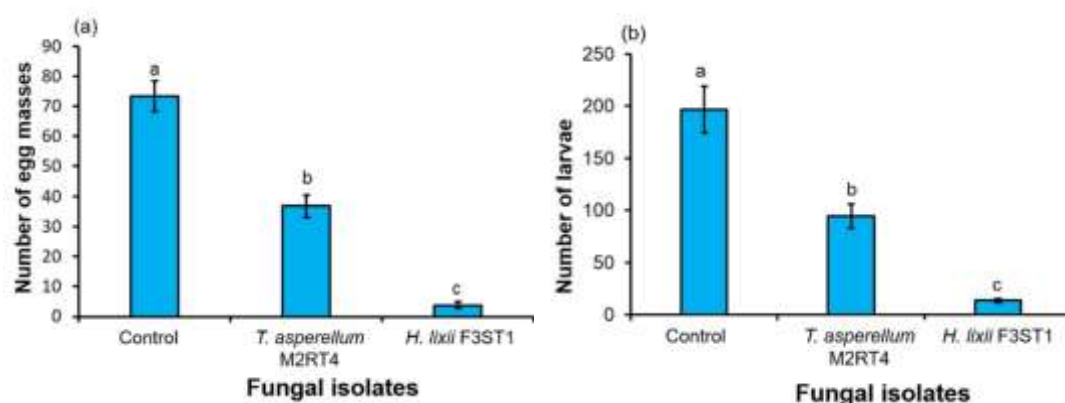


Figure 3.3: Effect of Endophytically Colonized *Amaranthus dubius* Plants on (a) Number of Egg Masses after 24 h and (b) Number of Larvae of *Spoladea recurvalis* after Three Days

Bar charts represent means $\pm$ SE at 95% Confidence Level ($p \leq 0.05$; $n = 4$).

3.4.5 Effect of Endophytically Colonized *Amaranthus dubius* Plants on Larval Mortality and Pupation of *Spoladea recurvalis*

There was a significant difference between the endophytically colonized and the control plants in regard to the mortality rates of *S. recurvalis* larvae ($\chi^2 = 175.32$, $df = 2$, $p < 0.001$). However, larval mortality did not differ significantly between *T. asperellum* M2RT4 and *H. lixii* F3ST1, which caused $42 \pm 0.74\%$ and $40 \pm 0.82\%$ mortality rates, respectively, compared to $2.91 \pm 0.29\%$ in the control (Figure 3.4a).

Similarly, the pupation of *S. recurvalis* differed significantly among treatments ($\chi^2 = 323.81$, $df = 2$, $p < 0.001$). *Hypocrea lixii* F3ST1 colonized plants produced the fewest pupae (3.75 ± 1.75), followed by *T. asperellum* M2RT4 (44.5 ± 1.94), while the control had the highest (76.25 ± 1.43) (Figure 3.4b).

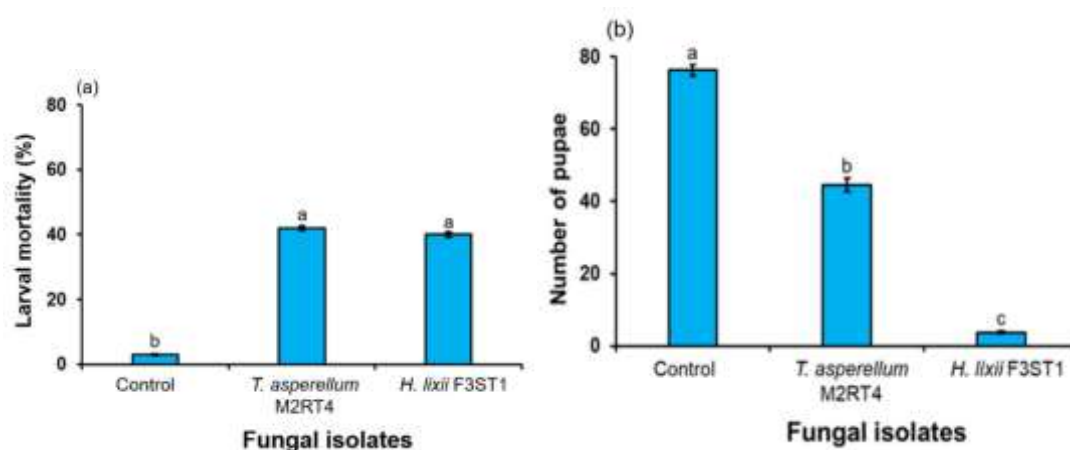


Figure 3.4: Effect of Endophytically Colonized *Amaranthus dubius* Plants on (a) Larval Mortality and (b) Number of Pupae of *Spoladea recurvalis*.

Bar charts represent means $\pm$ SE at 95% Confidence Level ($p \leq 0.05$; $n = 4$).

3.4.6 Effect of Endophytically-Colonized *Amaranthus dubius* Plants on Adult Emergence and Survival of F1 Progenies of *Spoladea recurvalis*

Adult emergence of *S. recurvalis* differed significantly among treatments ($\chi^2 = 282.63$, $df = 2$, $p < 0.001$). *Hypocrea lixii* F3ST1 had the fewest emerged adults (3.25 ± 0.25), followed by *T. asperellum* M2RT4 (38.5 ± 1.19), while the control recorded the highest emergence rate (66.5 ± 2.25) (Figure 3.5a). In addition, the survival of the first-generation (F1) progeny of *S. recurvalis* differed significantly among treatments (proximate log-rank test = 72, $df = 2$, $p < 0.001$), with progeny from endophytically colonized *A. dubius* showing lower survival rates than those from the controls (Figure 3.5b).

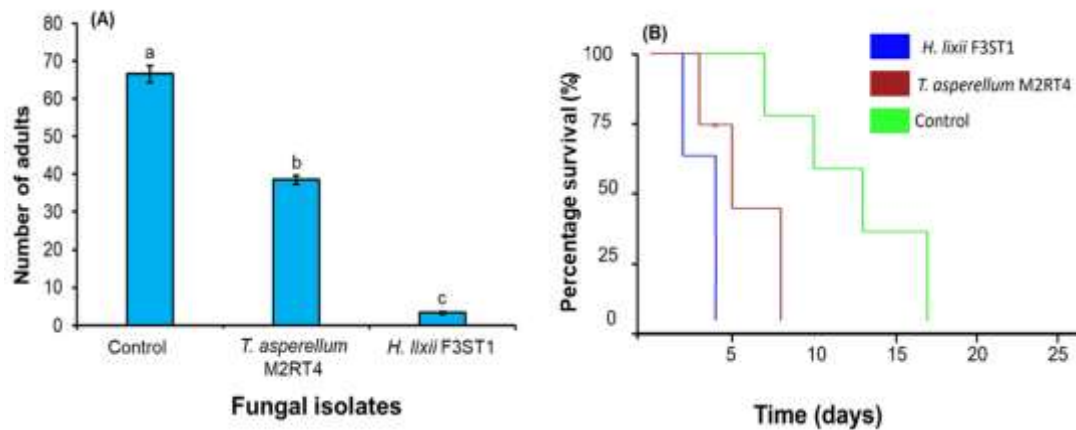


Figure 3.5: Effect of Endophytically Colonized *Amaranthus dubius* Plants on (a) Number of Adults Emergence

Bar charts represent means $\pm$ SE at 95% Confidence Level ($p \leq 0.05$; $n = 4$). (b) Kaplan-Meier survival curves for *Spoladea recurvalis* F1 progenies after emergence from *Hypocrea lixii* F3ST1 and *Trichoderma asperellum* M2RT4 endophytically colonized and control (endophyte-free) plants ($p \leq 0.05$, $n = 4$).

3.5 Discussion

The colonization of *A. dubius* by fungal endophytes varied depending on fungal strain and host tissue type. *Hypocrea lixii* F3ST1 and *T. asperellum* M2RT4 successfully colonized all the plant tissues, whereas *F. proliferatum* NF2S51, *T. atroviride* F5S2, *B. ochroleuca* F3R21, and some *B. bassiana* isolates colonized only the roots. In contrast, *B. bassiana* ICIPE 279, *T. harzianum* KF2R41, and *M. anisopliae* isolates (ICIPE 7 and ICIPE 41) failed to establish in any of the plant tissue. Similar observations of selective colonization have been reported in *Phaseolus vulgaris*, *Vicia faba* (Akutse et al., 2013), and *Solanum lycopersicum* (Qayyum et al., 2015). The limited colonization by *B. bassiana* isolates may be due to host-endophyte incompatibility or isolate-specific adaptation linked to their source, despite reports of the broad plant colonization capacity of *B. bassiana* (Behie et al., 2015; Qayyum et al., 2015; Quesada-Moraga et al., 2014). Likewise, *M. anisopliae* isolates failed to colonize *A. dubius*, consistent with previous studies indicating *M. anisopliae*

preference for rhizospheric or root tissue habitats (Greenfield et al., 2016). Successful establishment of

entomopathogenic fungi as endophytes depends on inoculation method, host, fungus compatibility, and inoculum density (Parsa et al., 2013; Russo et al., 2015; Bamisile et al., 2018).

In this study, *H. lixii* F3ST1 and *T. asperellum* M2RT4 consistently colonized all *A. dubius* tissues, confirming their strong endophytic potential as previously observed in onions (Muvea et al., 2014), tomato, nightshade (Agbessenou et al., 2020), and french bean (Paradza et al., 2021). Both isolates persisted within host tissues for up to four weeks, with colonization patterns varying by tissue type and time, but with *H. lixii* F3ST1 showing superior persistence overall. This aligns with findings by Paradza et al. (2021), who associated colonization rate and systemic spread with passive migration through connected tissues, and with Mengistu (2020), who noted that persistence depends on both fungal strain and plant tissue.

Endophytic colonization significantly enhanced *A. dubius* growth parameters. Plants inoculated with *H. lixii* F3ST1 and *T. asperellum* M2RT4 exhibited increased height, leaf number, and shoot biomass compared to the control, with *T. asperellum* M2RT4 showing the highest improvements in leaf size and dry shoot weight. Similar growth-promoting effects were also reported in maize (Kinyungu et al., 2023) and tomato (Paradza et al., 2021; Khan et al., 2015; Liao et al., 2017), which might likely be due to fungal stimulation of phytohormones such as auxins, gibberellins, and cytokinins.

Moreover, *H. lixii* F3ST1 and *T. asperellum* M2RT4 endophytically colonized plants negatively affected the life-history parameters of *S. recurvalis*. Both isolates significantly reduced oviposition, egg hatchability, larval survival, pupation, adult emergence, and longevity of F1 progeny, with *H. lixii* F3ST1 showing greater suppression. Similar pest-suppressive effects of endophytic fungi have been demonstrated against *Liriomyza huidobrensis* in beans (Akutse et al., 2013), *Thrips tabaci* in onions (Muvea et al., 2014), and *Ophiomyia phaseoli* in beans (Mutune et al., 2016). Reduced oviposition may be linked to volatile organic compounds (VOCs) emitted by endophyte-colonized plants that repel or deter insects (Holighaus & Rohlf, 2016).

2016; Agbessenou et al., 2022), while elevated larval mortality may result from toxic secondary metabolites (Agbessenou et al., 2022; Akutse et al., 2013). Although no mycosis was observed, similar findings by Schulz and Boyle (2005), Strobel (2006), and Herre et al. (2007) attribute larval mortality to endophyte-induced chemical defenses deterring feeding or exerting toxicity. Studies in maize further support this mechanism, showing reduced tunnelling by *Ostrinia nubilalis* (Cherry et al., 2004) and smaller larvae of *Sesamia calamistis* due to antibiosis or feeding deterrence caused by endophyte-derived metabolites. Likewise, *H. lixii* F3ST1 and *T. asperellum* M2RT4 significantly reduced pupation and adult longevity of *S. recurvalis* F1 progenies, consistent with findings by Ramos Aguila et al. (2022) in *Diaphorina citri* on *Citrus limon*, where insect mortality occurred without external fungal growth, implicating antibiosis and VOC-mediated effects (Garrido-Jurado et al., 2017).

However, despite the demonstrated efficacy of these endophytes in suppressing different developmental stages of the pest and promoting amaranth growth, the chemical and molecular mechanisms underlying these effects remain poorly understood. Furthermore, validation under field conditions is necessary to confirm their effectiveness, consistency, and practical applicability before large-scale implementation in amaranth production systems.

3.6 Conclusion

This study demonstrated that, among 15 screened fungal isolates, *H. lixii* F3ST1 and *T. asperellum* M2RT4 effectively colonized all tissues of *A. dubius* and persisted endophytically for up to four weeks, whereas other isolates showed limited or no colonization. Both endophytes significantly enhanced plant growth and suppressed *S. recurvalis* by reducing oviposition, egg hatchability, larval survival, pupation, and adult longevity. These findings highlight their potential as endophytic fungal agents for incorporation into Integrated Pest Management (IPM) strategies. However, despite the efficacy and ability of these endophytes in controlling various stages of the pest and promoting amaranth growth, further studies are warranted to establish the chemical and molecular defense mechanisms behind these findings and validate the

results under field conditions. Further studies are therefore required to validate their applicability before large-scale implementation in amaranth cropping systems.

CHAPTER FOUR

ASSESSING THE BEHAVIORAL AND REPRODUCTIVE RESPONSES OF FEMALE *Spoladea recurvalis* TO ENDOPHYTICALLY INOCULATED AMARANTH PLANTS

Abstract

The management of *Spoladea recurvalis*, a major pest of amaranth, poses a significant challenge in agriculture due to its destructive feeding habits that causes up to 100% yield losses under massive attack. Chemical pesticides are commonly employed to mitigate these losses. However, their use is associated with negative human and environmental impacts, as well as the development of pest resistance. In response to these challenges, the use of endophytic fungi has emerged as a promising alternative for the sustainable management of insect pests. These fungi live symbiotically within plant tissues without causing any harm/symptoms to the host plant and enhance plant resistance to herbivory attack by altering the behavior of the insect. Previously, we demonstrated that endophytic fungus *Hypocrea lixii* F3ST1 and *Trichoderma asperellum* M2RT4 inoculated amaranth plants significantly reduced oviposition, egg hatchability, larval mortality, pupation and adult emergence of *S. recurvalis*. Additionally, the survival of F1 progenies was significantly reduced. However, despite their suppressive effects, the exact underlying mechanisms through which the presence of endophytic fungus in inoculated amaranth plants affects *S. recurvalis* life history traits remain unclear. The behavioral responses of *S. recurvalis* in Y-tube olfactometer bioassays were therefore tested. Gravid *S. recurvalis* were evaluated in the olfactometer by introducing a single female into the stem of the olfactometer, allowed five minutes to acclimatize, and then given another five minutes to make a choice. A response was recorded when the insect moved at least 5 cm into one arm of the olfactometer and remained there for two or more minutes. The females of *S. recurvalis* showed a preference for non-inoculated amaranth plants over those inoculated with both endophytes. Furthermore, *S. recurvalis* females were neither attracted to non-inoculated infested plants nor to inoculated-infested amaranth plants. Gas chromatography-mass spectrometry (GC-MS) analysis of volatiles emitted from non-

inoculated, inoculated, non-inoculated infested, and inoculated infested amaranth plants colonized by *H. lixii* F3ST1 and *T. asperellum* M2RT4 revealed the presence of 80 and 74 volatile organic compounds (VOCs), respectively. Similarity percentage analysis highlighted the major compounds contributing to the differences observed among treatments. In *H. lixii* F3ST1-colonized plants, key discriminating VOCs included (Z)-3-Hexenyl acetate, pentadecane, α -Cedrene, o-Xylene, and (E)- β -Ocimene while in *T. asperellum* M2RT4-colonized plants, predominant differentiating compounds were Germacrene D, (Z)-3-hexenol, 1,3-Dimethylbenzene, Tetradecane, and 2,4,4-Trimethyl-1-pentene. The distinct VOC profiles linked to their colonization and treatment status suggest that these endophytes may influence amaranth plant defense signaling pathways, further contributing to their biocontrol efficacy for the management of *S. recurvalis*. The synthetics of these identified major compounds, were later used to assess and confirm *S. recurvalis* behavior and herbivory observed. The findings support the integration of these endophytic fungi into IPM strategies for sustainable pest management through the natural enhancement of plant chemical defenses.

4.1 Introduction

Amaranth is a highly nutritious crop whose productivity is severely constrained by the lepidopteran pest *Spoladea recurvalis*, which can cause yield losses of up to 100% under severe infestations while reducing leaf quality and marketability (Opisa et al., 2018). Management of this pest has relied heavily on synthetic insecticides, whose indiscriminate use has resulted in pesticide residues, environmental contamination, pest resistance, destruction of natural enemies, and risks to human and livestock health (Nampeera et al., 2019). Consequently, there is an urgent need for environmentally sustainable pest management alternatives.

Endophytic fungi have emerged as promising biological control agents because they colonize plant tissues without causing disease and can enhance plant resistance to herbivorous insects while minimizing environmental impacts (Akutse et al., 2013; Agbessenou et al., 2020; Agbessenou et al., 2022; Sharma et al., 2023). Beyond their direct effects on insect survival, endophytes can influence plant defense mechanisms

by activating salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) signaling pathways, which alter the production of defensive metabolites and volatile organic compounds (VOCs) (Garcia-Brugger et al., 2006).

Herbivore-induced plant volatiles (HIPVs) play a central role in mediating insect behavior. They can repel herbivores, attract natural enemies, and signal neighboring plants to prepare for herbivore attack (Turlings & Erb, 2018; Lin et al., 2017; Lin et al., 2021; Lin et al., 2022). Importantly, female moths, including lepidopteran pests, rely on these volatile cues to locate suitable host plants for feeding and oviposition (Egigu et al., 2011). Changes in VOC profiles induced by endophytic fungi may therefore alter host selection, feeding, and oviposition behavior of *S. recurvalis*, providing an indirect mechanism of pest suppression in addition to any direct pathogenic effects (Zhu et al., 2023).

The exploration of herbivore-induced plant volatiles (HIPVs) has been widely proposed as a promising strategy for plant protection and for enhancing biological control within the framework of integrated pest management (Ayelo et al., 2021). Secondary metabolites produced by fungal endophytes can function as repellent compounds, possessing properties that repel or deter other organisms, such as insect pests (Poveda (2021), These repellent compounds include volatile organic compounds such as alcohols, aldehydes, and ketones that interfere with insects' sensory mechanisms, making the environment less attractive or even driving them away (Wang et al., 2016). However, the underlying mechanisms mediating these interactions between endophytic fungi and how they prime plant defense systems in amaranth pest *S. recurvalis* management is not yet elucidated.

This study, therefore, aimed at unravelling and identifying the specific volatile organic compounds released by *H. lixii* F3ST1 and *T. asperellum* M2RT4-endophytically colonized amaranth plants in the sustainable management of *S. recurvalis* in amaranth cropping systems. The hypothesis stipulating that inoculated amaranth plants with *H. lixii* F3ST1 and *T. asperellum* M2RT4 trigger the production of phytohormones, which would affect the reproductive traits of *S. recurvalis* was tested. Specifically, (i) the behavioral responses of *S. recurvalis* females to non-inoculated, inoculated, non-

inoculated infested and inoculated infested amaranth plant volatiles using the Y-tube olfactometer were assessed; (ii) identification of the odor profile associated with non-inoculated and inoculated amaranth plants upon attack by *S. recurvalis* was also undertaken.

4.2 Materials and Methods

4.2.1 Fungal Cultures

The fungal isolates *Hypocrea lixii* F3ST1 and *Trichoderma asperellum* M2RT4 used in this study were obtained from the Arthropod Pathology Unit's Germplasm at the International Centre of Insect Physiology and Ecology (*icipe*) Nairobi, Kenya, and cultured on potato dextrose agar (PDA) (OXOID CM0139, Oxoid Ltd., Basingstoke, United Kingdom), and maintained at $25 \pm 2^\circ\text{C}$ in complete darkness. Culturing was done as described in Chapter 3, section 3.2.3.

4.2.2 Inoculation of Seeds with *Hypocrea lixii* F3ST1 and *Trichoderma asperellum* M2RT4 and Colonization Assessment

In this study, a local vegetable variety of amaranth seeds Dubious, *Amaranth dubius* was used because it is the most widely cultivated leafy vegetable species within amaranth genus. The seeds were purchased locally from Simlaw Seeds Company Ltd., Nairobi, Kenya. Seeds were surface-sterilized by soaking them in 70% ethanol for two minutes, submerged for two minutes in 1.5% sodium hypochlorite and finally rinsed three times in sterile distilled water. The seeds were then placed on sterile filter paper in a safety cabinet to allow all the residual water to evaporate (Agbessenou et al., 2020; Akutse et al., 2013). To check for effectiveness of the surface sterilization technique, 0.1 mL of the last rinse water was plated onto potato dextrose agar (PDA) and also imprinting of surface sterilized seeds onto PDA (tissue imprint) supplemented with 100 mgL^{-1} Streptomycin, followed by incubation of plates at 25°C for 14 days (Inglis et al., 2012). Seeds were then soaked overnight for 12 h in conidial suspensions titrated at 1×10^8 conidia mL^{-1} . For the control, sterilized seeds were soaked overnight for 12 h in sterile distilled titrated water containing 0.05% Triton X-100 (MERCK KGaA, Darmstadt, Germany) (Agbessenou et al., 2020). The surface sterilized *A. dubius* seeds

were then planted in plastic pots (8 cm diameter × 7.5 cm high) containing a planting substrate (mixture of 0.5 Kg of peat compost and soil at the ratio of 1:5) (Mea Ltd., Nairobi, Kenya). The substrate was sterilized in an autoclave for 2 h at 121 °C and allowed to cool for 72 h prior to sowing (Akutse et al., 2013). Five seeds were sowed per pot and maintained at room temperature ($25 \pm 2^\circ\text{C}$), 60% RH and 12:12 L:D photoperiod. Pots were transferred immediately after germination to the screen house (2.8 m length × 1.8 m width × 2.2 m height) at $25 \pm 2^\circ\text{C}$, 60% RH, and 12:12 L:D photoperiod for four – five weeks. After germination, seedlings were thinned to two per pot and watered with distilled water twice per day (morning and evening). No additional fertilizer was added to the planting substrate. Plants of four – five-week-old were used for the various experiments.

To confirm endophytic colonization before the behavioral and reproductive bioassays, amaranth plants were carefully uprooted from the pots 4–5 weeks post-inoculation and washed under running tap water to remove any soil attached to the plants. Seedlings (ca. 30 cm in height) were divided into three different sections (ca. 5 cm long): leaves, stems, and root using a sterile scalpel (Akutse et al., 2013; Agbessenou et al., 2022). Five sections each from the leaf, stem, and root of each plant were randomly selected and surface-sterilized as previously described above for seeds. The different plant parts were then aseptically cut under a laminar flow hood into 1×1 cm pieces and placed four cm apart from each other on PDA plates supplemented with a 0.05% solution of antibiotic (streptomycin sulfate salt) (Akutse et al., 2013). Plates were incubated at $25 \pm 1^\circ\text{C}$ for 10 days, during which the presence of endophyte was determined at 2-3 days interval. In addition to the last rinse water that was plated, plate imprinting was also conducted to assess the effectiveness of surface sterilization of plant materials (Inglis et al., 2012). The colonization of the different plant parts was recorded by counting the number of pieces of plant parts that showed the presence of inoculated fungal growth/mycelia according to Koch's postulates (Petrini and Fisher, 1986). Only the presence of endophyte that was inoculated was scored from each incubated plate. Fungal isolate was identified morphologically using slides which were prepared from the mother plates. Treatments (non-inoculated and inoculated plants) were arranged in a randomized complete block design (CBD) with four replicates. The success rate of

fungal endophyte colonization (%) of amaranth host plant parts was calculated as follows:

$$\text{Colonization (\%)} = \frac{\text{Number of plant tissue exhibiting fungal out growth}}{\text{Total number of plant tissue pieces plated out}} \times 100$$

4.2.3 Insect Rearing

For the experiments, insect colony was established using *S. recurvalis* adults and larvae collected from amaranth plants. The insects were obtained from a colony maintained $25 \pm 2^{\circ}\text{C}$, 50%–70% RH and 12:12 L: D light/ dark photoperiod reared on potted *A. dubius* variety plants (seeds sourced from Simlaw Seeds Company Limited., Nairobi, Kenya) in well-ventilated, sleeved Perspex cages ($40 \times 40 \times 45$ cm) at the insectary section of the Animal Rearing and Quarantine Unit (ARQU) of *icipe*. The initial colony was established from adults and larvae of *S. recurvalis* collected from infested amaranth plants in Naivasha (N $0^{\circ} 43' 0.01''$ E $36^{\circ} 26' 9.28''$) and Mwea, (S $0^{\circ} 36' 31.3''$ S $037^{\circ} 22' 29.7''$ E) in September 2022. The moths were fed on 10% honey solution ad libitum dipped in cotton wool balls to serve as a food source (Othim et al., 2017). Five weeks old potted plants were later introduced in the cages for oviposition for 24 h. After 24 h exposure, the plants were removed and transferred to separate cages ($50 \times 50 \times 60$ cm), ventilated with netting material at the sides until the eggs hatched. Foliage containing larvae were removed from these plants three days after the larvae hatched and placed into plastic boxes ($15 \times 7 \times 5$ cm) lined with tissue paper to absorb excess moisture and fine netting infused lid for ventilation. On a daily basis, the larvae were fed with fresh amaranth leaves until they pupated (Nyamamba et al., 2025). With the aid of a fine camel hairbrush, pupae were collected from the lunch boxes and placed inside sleeved Perspex cages ($40 \times 40 \times 45$ cm) for adult emergence. Field collected populations of *S. recurvalis* were infused into the laboratory every three months in order to reduce inbreeding (Akutse et al., 2020).

4.2.4 Behavioural Responses of Female *Spoladea recurvalis* to Inoculated *Amaranthus dubius*

4.2.4.1 Y-Tube Olfactometer Bioassays

The responses of females of *S. recurvalis* to volatiles of non-inoculated, inoculated, non-inoculated infested, and inoculated infested amaranth plants were tested in dual-choice assays using a Y-tube olfactometer, which had the following dimensions (internal diameter = 3 cm; stem = 15 cm; side arms = 10 cm) (Sigma Scientific, Gainesville, FL, United States) connected with Teflon tubing to the test odor sources which were headspace of amaranth plant volatiles (Agbessenou et al., 2022). The control arm was an empty oven bag (25 cm × 380 cm, Baco & BacoFoil, Wrap- Film Systems Ltd., United Kingdom).

Each arm of the olfactometer was connected to either the amaranth plant volatiles or an empty oven bag with Teflon tubing. A vacuum pump (Cole-Parmer Instrument Co., Chicago, IL, United States) was used to generate the air which was filtered by an active charcoal filter and passed through each odor source tied in an oven baked bag at a constant flow rate of 150 mL min⁻¹. Four-week-old amaranth plants were infested with 18 second instar larvae of *S. recurvalis* for 24 h. Each pot contained one plant wrapped in aluminum foil (base of the plant) to prevent contamination of volatiles emitted from the pot and soil (Adams et al., 2023; Agbessenou et al., 2022). To get rid of visual cues from the experiment area, the Y-tube olfactometer was illuminated using 20 W red fluorescent bulb (Sokame et al., 2019; Fiaboe et al., 2024). An individual gravid *S. recurvalis* female was released into the stem of the olfactometer and allowed five minutes to settle and another five minutes to make a choice. An insect was considered to have made a choice when it walked at least five (5) cm into one of the arms and stayed for two or more minutes. Each gravid *S. recurvalis* female was tested only once. Insects that failed to make a decision within five minutes were considered to be non-respondents and were thus not included in the statistical analysis. To ensure no positional bias in the olfactometer bioassay, the odor sources were interchanged after every five tests. After every ten females tested, the plants were replaced with new ones. Teepol odorless detergent (2%) (Sudi Chemicals Industries Limited, Nairobi, Kenya)

was used to clean the Y-tubes followed by rinses with sterile distilled water. The Y-tubes were dried in the laboratory in an oven overnight at 80°C before use. The experiment was conducted in a controlled laboratory setting at a temperature of 25 ± 2°C, and 50%–70% relative humidity. The responses of three-day-old mated gravid females of *S. recurvalis* toward the different odor sources are summarized in Table 4.1. Forty insects were tested per odor combination, and a total of 440 insects were used in the whole bioassay.

Table 4.1: Summary of Odor Sources and the Combinations Tested in the Y-Tube Olfactometer Assays

Combination	Odor sources
A	Air vs. air (control)
B	Air vs. non-inoculated plant
C	Air vs. non-inoculated infested plant
D	Air vs. inoculated plant
E	Air vs. inoculated infested plant
F	Non-inoculated plant vs. inoculated plant
G	Inoculated plant vs. inoculated infested plant
H	Non-inoculated plant vs. inoculated infested plant
I	Inoculated plant vs. non-inoculated infested plant
J	Non-inoculated infested plant vs inoculated infested plant
K	Non-inoculated plant vs. non-inoculated infested plant

4.2.4.2 Collection of Headspace Volatiles

Headspace plant volatiles were collected from four-week-old *A. dubius* plants as per the following treatments: (i) non-inoculated, (ii) inoculated, (iii) non-inoculated infested, (iv) inoculated infested amaranth plants and (v) an empty roasting bag (control). The plants from the screen house used for this experiment were allowed to acclimatize in the laboratory conditions for 12 h before collecting the volatiles. A sterilized roasting bag (35 × 30 cm) (Lifetime Brands Europe Ltd, Valepits Road, Birmingham), pre-conditioned in an oven at ~ 100 °C for 24 h before use, was used to cover the aerial part of each test plant and carefully tightened with a rubber band around the base of the stem to ensure airtight conditions while ensuring that the plant is not damaged. Moreover, the base of each potted plant was wrapped with aluminum

foil to prevent emissions of volatiles from the pot and soil. To ensure that the air was humid, charcoal-purified air (400 mL min^{-1}) was passed through a glass tube filled with distilled water. Volatiles were trapped (350 mL min^{-1}) from the bags through a vacuum tube connected to a Super-Q adsorbent (30 mg) (Analytical Research System, Gainesville, FL, United States) for 24 h (5:00 pm-5:00 pm the following day), targeting both day and night volatiles. The volatiles that were trapped in Super-Q were eluted with $150 \text{ }\mu\text{L}$ gas chromatography (GC) grade dichloromethane (Analytical Sigma-Aldrich, St, Louis, MO) under a gentle stream of charcoal scrubbed nitrogen gas into 1.5 mL glass vials. All the eluted samples were stored at $-80 \text{ }^{\circ}\text{C}$ until required for chemical analyses.

4.2.4.3 Analysis of Volatiles

The volatile extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) on an HP 7890A series gas chromatograph (Agilent Technologies, Wilmington, USA) coupled with an HP 5975C mass spectrometer (Agilent Technologies, Wilmington, USA). A non-polar Agilent capillary column HP-5 MSI ($30 \text{ m} \times 0.25 \text{ mm i.d.}, \times 0.25 \text{ }\mu\text{m}$ film thickness) was equipped with GC. An aliquot ($1 \text{ }\mu\text{L}$) of each extract was injected in the splitless mode at an oven temperature of $35 \text{ }^{\circ}\text{C}$ for five minutes, which slowly increased to $280 \text{ }^{\circ}\text{C}$ at $10 \text{ }^{\circ}\text{C min}^{-1}$ and maintained for 10.5 minutes. The carrier gas used was helium at a flow rate of 1.0 mL min^{-1} and the mass spectra were acquired at 70 eV within a mass range of 38-550 Daltons (Da) during a scan time of $0.73 \text{ scans sec}^{-1}$. Volatiles were identified by comparing their mass spectral data with those from commercial GC/MS library databases Adams, 1995, and NIST Mass Spectrometry Data Center, 2008. The retention indices (RIs) of the compounds were determined using n-alkane standards (C8-C31) and calculated using the equation described below by van den Dool and Dec. Kratz (1963), and compared with published RIs values from the literature.

4.3 Statistical Analyses

All data analyses were performed using R (version 4.3.3) statistical software packages (R Core Team, 2024) and PAST, version 5.1. In the Y-tube olfactometer, *S. recurvalis* females' preference for odors was achieved through comparison of the recorded

frequencies of choice of either side of the olfactometer arm using Chi-square (χ^2) goodness-of-fit test. To analyze the concentration of volatile organic compounds between non-inoculated, inoculated, non-inoculated infested and inoculated infested *A. dubius* plants, the data was checked for normality using Shapiro-Wilk's test (Shapiro & Wilk, 1965). The data was not normally distributed and therefore a non-parametric analysis was conducted using Kruskal-Wallis test. Principal Components Analysis (PCA) and Non-metric Multi-Dimensional Scaling (NMDS) were employed to visualize the profiles of the identified headspace volatiles. Similarity percentage (SIMPER) analysis was performed on peak areas of volatile organic compounds to determine the relative contribution of different compounds to the dissimilarity among volatiles of the different treatments.

4.4 Results

4.4.1 Endophytic Colonization of *Amaranthus dubius* Plant by *Hypocrea lixii* F3ST1 and *Trichoderma asperellum* M2RT4

In colonization assessment, both fungal isolates *H. lixii* F3ST1 and *T. asperellum* M2RT4 were able to colonize *A. dubius* plant tissues (roots, stems and leaves). *Hypocrea lixii* F3ST1 successfully colonized 90% of the roots and stems, and 80% of the leaves, whereas *T. asperellum* M2RT4 achieved colonization rates of 85% in the roots, 100% in the stems, and 85% in the leaves (Figure 4.1). There was no fungal colonization observed in the control treatment, as well as in the incubated last rinsed water and imprinting plant materials.

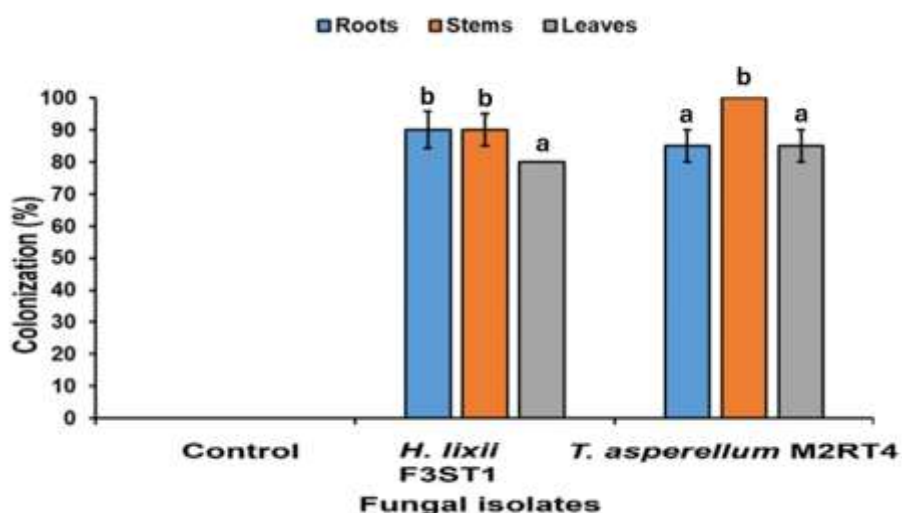


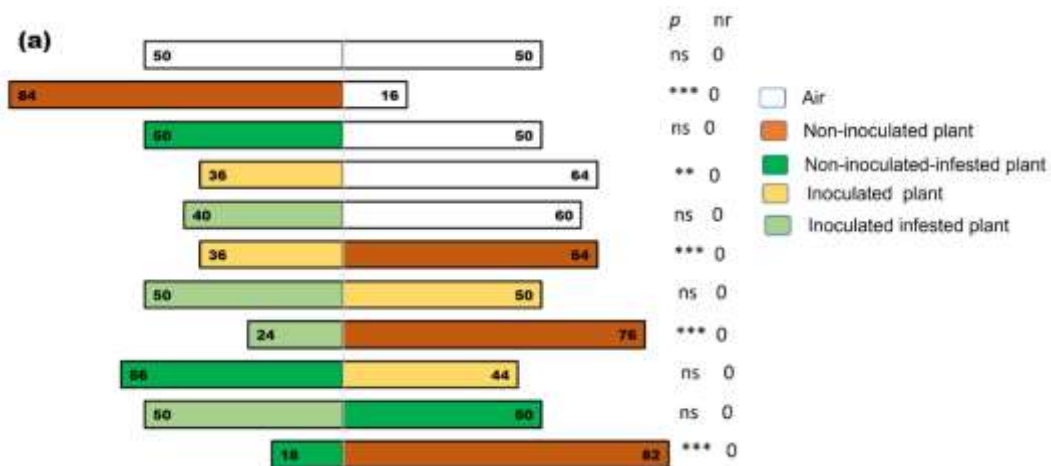
Figure 4.1: Endophytic Colonization of *Amaranthus dubius* by *Hypocrea* LIXII F3ST1 and *Trichoderma asperellum* M2RT4 4-5 Weeks Post Inoculation

Bar chart represents means $\pm$ SE (standard error) at 95% CI ($P < 0.05$; $n = 4$).

4.4.2 Behavioral Response of Female *Spoladea recurvalis* to Inoculated *Amaranthus dubius* Plants by *Trichoderma asperellum* M2RT4 and *Hypocrea lixii* F3ST1 Volatiles

Female *S. recurvalis* showed a significant preference for volatiles emitted by non-inoculated amaranth plants compared to clean air (control) ($\chi^2 = 46.24$, $df = 1$, $P < 0.001$) (Figure 4.2a & 4.2b). Conversely, volatiles from inoculated plants were significantly avoided ($\chi^2 = 23.04$, $df = 1$, $P < 0.001$; $\chi^2 = 7.84$, $df = 1$, $P < 0.01$ for *T. asperellum* M2RT4 and *H. lixii* F3ST1, respectively) by the female moths (Figure 4.2a & 4.2b). No significant attraction was observed toward volatiles from either non-inoculated infested ($\chi^2 = 0$, $df = 1$, $P = 1$) or inoculated infested plants ($\chi^2 = 5.76$, $df = 1$, $P = 0.16$; $\chi^2 = 4$, $df = 1$, $P = 0.86$ for *T. asperellum* M2RT4 and *H. lixii* F3ST1, respectively) relative to clean air (Figure 4.2a & 4.2b). In dual-choice assays, females of *S. recurvalis* were significantly more attracted to odors from non-inoculated plants than those from inoculated plants ($\chi^2 = 37.58$, $df = 1$, $P < 0.001$; $\chi^2 = 7.84$, $df = 1$, $P < 0.001$ for *T. asperellum* M2RT4 and *H. lixii* F3ST1, respectively) (Figure 4.2a & 4.2b). Likewise, females significantly preferred volatiles from non-inoculated plants over

both non-inoculated infested plants ($\chi^2 = 64$, $df = 1$, $P < 0.001$) and inoculated infested plants ($\chi^2 = 51.84$, $df = 1$, $P < 0.001$; $\chi^2 = 27.04$, $df = 1$, $P < 0.001$ for *T. asperellum* M2RT4 and *H. lixii* F3ST1, respectively) (Figure 4.2a & 4.2b). No significant differences in attraction were found between non-inoculated infested and inoculated infested plants ($\chi^2 = 0.64$, $df = 1$, $P = 0.423$; $\chi^2 = 0$, $df = 1$, $P = 1$ for *T. asperellum* M2RT4 and *H. lixii* F3ST1, respectively), and between inoculated and inoculated infested plants ($\chi^2 = 1.44$, $df = 1$, $P = 0.23$; $\chi^2 = 0$, $df = 1$, $P = 1$ for *T. asperellum* M2RT4 and *H. lixii* F3ST1, respectively), or between inoculated and non-inoculated infested plants ($\chi^2 = 4$, $df = 1$, $P = 0.455$; $\chi^2 = 1.44$, $df = 1$, $P = 0.23$ for *T. asperellum* M2RT4 *H. lixii* F3ST1, respectively) (Figure 4.2a & 4.2b).



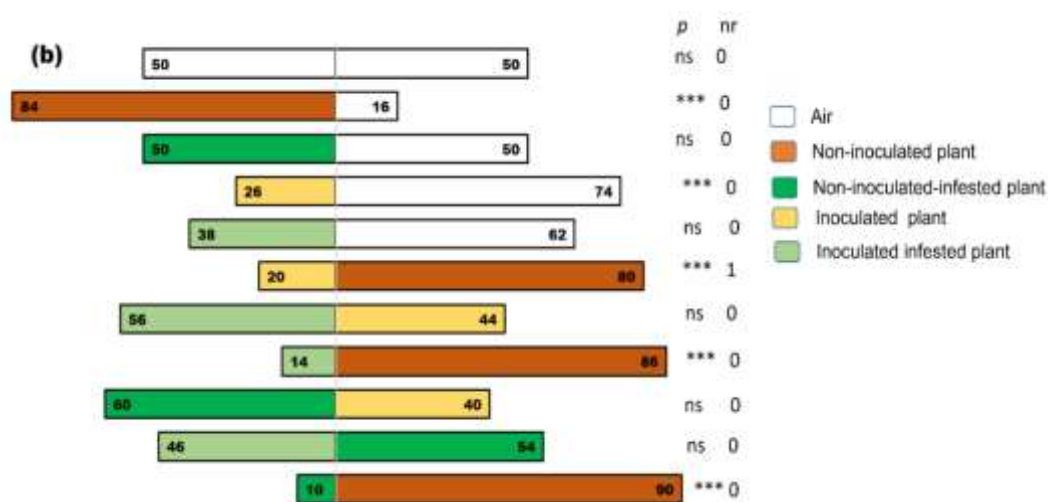


Figure 4.2 : (a) *Trichoderma asperellum* M2RT4 and (b). *Hypocrea lixii* F3ST1. Responses (%) of Females of *Spoladea recurvalis* to Volatiles from Non-Inoculated, Inoculated, Non-Inoculated Infested, and Inoculated Infested Amaranth Plants in a y-tube olfactometer choice test. Nr, number of Non-Respondent Insects (i.e., No Choice).

P stands for statistical significance levels with ns, no significant difference ($P > 0.05$); *, **, ***, significant differences at $P < 0.05$; $P < 0.01$; and $P < 0.001$, respectively, from the Chi-square test: $P = 0.05$.

4.4.3 Analysis of *Amaranthus dubius* Host Plant Volatiles

Gas Chromatography-Mass Spectrometry analysis revealed 81 VOCs emitted from amaranth plants endophytically colonized by *H. lixii* F3ST1 representing 11 chemical classes namely: alkanes (24), alcohols (13), benzenoids (11), esters (10), sesquiterpenes (6), ketones (5), aldehydes (4), monoterpenes (3), alkenes (2), amide (1) and phenol (1) (Table 4.2 and Figure 4.3a). Quantitative and qualitative differences in volatile emissions were observed among non-inoculated, inoculated, non-inoculated-infested, and inoculated-infested plants for the two treatments (Table 4.2 and Figure 4.3a). For instance, in plants colonized by *H. lixii* F3ST1, the findings indicate that specific classes of volatiles particularly alcohols, esters and benzenoids responded significantly to fungal inoculation and/or herbivory. The alcohols Methyl-3-buten-2-ol was only observed in inoculated but not in non-inoculated plants, and

infested treatments (refer to the figure). Esters, including isopentyl acetate, exhibited significant variation in response to inoculated and non-inoculated infested plants (refer to the figure). Benzenoids, e.g., 1,3-dimethylbenzene, showed a clear suppression under infestation for both inoculated and non-inoculated infested plants ($p < 0.042$) significantly (Table 4.2). the four treatments were separated into distinct clusters according to their VOC profiles (NMDS Bray Curtis Index (Figure 4.3b). The percentage contribution of predominant compounds to the dissimilarity among VOC profiles of the four treatments (non-inoculated, inoculated, non-infested, and infested plants) was determined to identify the compounds responsible for treatment differences (Figure 4.3c). Additionally, Principal Component Analysis (PCA) was employed to reduce the complexity of the volatile organic compound (VOC) identified from GC-MS analysis and to identify key compounds (Figure 4.3d).

Table 4.2: Mean Amount of Volatile Compounds (ng/ μ L) Identified in the Headspace of Non-Inoculated, Inoculated, Non-Inoculated Infested, And Inoculated Infested Amaranth Plants (n = 5)

Peak no	RT	RI _{alk} ^a	Compound ^b	Chemical class	Non-inoculated plant	Inoculated plant	Non-inoculated infested plant	Inoculated infested plant	P-value ^c
1	3.15	307	Amylene hydrate	Alcohol	nd	0.27 $\pm$ 0.12	Nd	nd	NA
2	3.28	504	2-Pentanone	Ketone	2.41 $\pm$ 1.9 a	1.75 $\pm$ 0.72 a	2.13 $\pm$ 0.98 a	3.52 $\pm$ 5.01 a	0.915
3	3.6	514	3-Buten-2-ol	Alcohol	nd	0.18 $\pm$ 0.05	Nd	nd	0.005
4	3.98	412	2,4,4-Trimethyl-1-pentene	Alkene	nd	0.44 $\pm$ 0.24	Nd	0.07	0.143
5	5.17	557	Toluene	Benzenoid	nd	0.05 $\pm$ 0.04	Nd	0.46 $\pm$ 0.77	0.6434
6	5.57	465	4-Methyl-2-pentanol	Alcohol	nd	0.33 $\pm$ 0.02	0.23	0.06	0.259
7	6.01	805	Hexanal	Aldehyde	0.35	nd	1.22 $\pm$ 0.6	0.04	0.168
8	6.21	785	Hexan-2-ol	Alcohol	nd	0.34 $\pm$ 0.16	Nd	nd	NA
9	6.49	810	Butyl acetate	Ester	0.28	nd	0.22 $\pm$ 0.16	nd	1
10	6.59	804	n-Octane	Alkane	0.46 $\pm$ 0.25	Nd	Nd	1.06	0.179
11	7.05	801	2,2,4-Trimethyl-3-pentanone	Ketone	nd	0.13 $\pm$ 0.06	Nd	nd	0.902
12	7.05	804	2,4-Dimethyl-3-pentanone	Ketone	0.3 $\pm$ 0.31	0.6	Nd	nd	0.221
13	7.12	802	2,4-Dimethyl-1-heptene	Alkene	0.17 $\pm$ 0.01	0.08 $\pm$ 0.04	Nd	nd	0.083
14	7.39		(2E)-Hexenal	Aldehyde	nd	nd	2.93 $\pm$ 0.83	0.2	0.143
15	7.50	863	(Z)-3-Hexen-1-ol	Alcohol	nd	2.9	3.58 $\pm$ 2.08	1.23	0.675
17	7.56	801	Ethylbenzene	Benzenoid	4.22 $\pm$ 4.28	0.51	2.06 $\pm$ 1.22	1.07 $\pm$ 1.33	0.368
18	7.66	802	4-Methyloctane	Alkane	0.2	nd	Nd	0.07 $\pm$ 0.04	0.157
19	7.75	802	1,3-Dimethyl benzene	Benzenoid	5.57 $\pm$ 0.73 a	0.69 $\pm$ 0.59 ab	2.01 $\pm$ 1.74 ab	0.09 $\pm$ 0.02 b	0.042
20	7.8	801	(2Z)-Hexen-1-ol	Alcohol	nd	nd	0.58 $\pm$ 0.32	0.14 $\pm$ 0.12	0.165
21	8.26	808	Isopentyl acetate	Ester	nd	0.15 $\pm$ 0.03 b	6.41 $\pm$ 5.51 a	0.42 $\pm$ 0.24 ab	0.032
22	8.29	800	o-Xylene	Benzenoid	13.78	0.55 $\pm$ 0.04	2.14 $\pm$ 1.57	0.79 $\pm$ 1.07	0.271
23	8.52	806	P-xylene	Benzenoid	1.74 $\pm$ 0.61	0.25	Nd	0.25	0.259
24	8.67	795	(3E)-Hept-2-en-1-one	Ketone	0.39 a	0.53 $\pm$ 0.31 a	Nd	0.54 $\pm$ 0.62 a	1
25	8.71	803	2-Heptanol	Alcohol	nd	nd	0.39	0.12 $\pm$ 0.1	0.221
26	8.75	801	Santolina triene	Sesquiterpene	nd	0.12 $\pm$ 0.01	0.98	nd	0.221
27	8.89	803	2-Pentenal	Aldehyde	0.27	0.81 $\pm$ 0.26	Nd	nd	0.221
28	9.11	901	Pentyl_isobutanoate	Ester	nd	0.11	0.15 $\pm$ 0.01	nd	0.221
29	9.22	905	α -Pinene	Monoterpene	0.49 $\pm$ 0.46	0.16 $\pm$ 0.08	0.22 $\pm$ 0.13	nd	0.2521
30	9.49	914	3,3-Dimethyl-2-pentanol	Alcohol	nd	0.27	0.38	1	0.368
31	9.49	900	1-Butyl methyl carbonate	Ester	nd	0.42	0.24 $\pm$ 0.11	nd	0.221
32	9.65	908	Propyl_Benzene	Benzenoid	0.61 $\pm$ 0.16	0.27	Nd	nd	0.179
33	9.84	910	Cumene	Benzenoid	nd	0.5 $\pm$ 0.14	0.29	nd	0.221
34	10.45	903	Mesitylene	Benzenoid	0.69 $\pm$ 0.48	0.39 $\pm$ 0.31	Nd	nd	0.387
35	10.45	900	1,2,4-Trimethylbenzene	Benzenoid	0.9	0.43 $\pm$ 0.16	Nd	nd	0.221
36	10.45	900	1,2,4-Trimethylbenzene	Benzenoid	1.33 $\pm$ 0.33	0.67	Nd	nd	0.179
37	10.90	910	(3E)-Hexenyl acetate	Ester	nd	0.69 $\pm$ 0.58	Nd	0.29	0.179
38	10.90	1001	δ -2-carene	Monoterpene	nd	0.9	1.15 0.9	nd	1

Peak no	RT	RI _{alk} ^a	Compound ^b	Chemical class	Non-inoculated plant	Inoculated plant	Non-inoculated infested plant	Inoculated infested plant	P-value ^c
39	10.90	910	3-Hexenyl acetate	Ester	nd	0.84 ± 0.45	4.55 ± 2.32	1.2	0.089
40	11.14	996	2-Ethylhexan-1-ol	Alcohol	nd	0.78 ± 0.19	2.36	0.05	0.202
41	11.14	996	2-Ethyl-1-hexanol	Alcohol	2.13 ± 1.58	1.17 ± 0.15	Nd	nd	1
42	11.39	1010	2-Heptanol acetate	Ester	nd	nd	1.08 ± 0.52	nd	NA
43	11.49	1016	(E)-β-Ocimene	Monoterpene	nd	0.38 ± 0.05	3.54 ± 3.37	0.77 ± 0.84	0.137
44	11.65	1025	Heptadecane (C17)	Alkane	0.76	0.12	1.2 ± 1.14	1.6 ± 1.36	0.692
45	12.37	1105	Heptadecane	Alkane	nd	0.05	0.36	1.02	0.368
46	12.38	1100	Linalool	Alcohol	nd	nd	Nd	8.1 ± 6.04	NA
47	13.18	1104	Nonanal	Aldehyde	0.79 ± 0.07	0.55 ± 0.36	2.09	nd	0.184
48	14.03	1161	Decanal	Aldehyde	nd	0.43 ± 0.14	0.24	nd	0.157
49	14.03	1161	n-Decane	Alkane	0.75 ± 0.46	nd	Nd	1.01 ± 0.79	0.723
50	14.10	1167	Dodecane	Alkane	nd	0.2	Nd	0.35 0.01	0.221
51	14.57	1223	δ-Nonalactone	Ester	nd	18.68±24.91	Nd	nd	0.056
52	14.69	1223	Caprolactam	Amide	3.02 ± 3.43	0.66	0.32	nd	0.407
53	15.85	1234	Pentadecane	Alkane	nd	nd	0.7	6.09 ± 3.74	0.179
54	16.35	1310	Hexadecane (C16)	Alkane	nd	nd	Nd	6.14 ± 1.84	NA
55	16.74	1307	Nonane	Alkane	0.55 ± 0.32 ab	0.17 ± 0.02 b	Nd	1.89 ± 1.48 a	0.011
56	16.83	1305	Tetradecane (C14)	Alkane	nd	Nd	3.29 ± 1.79	3.07 ± 0.34	1
57	17.12	1379	α-Cedrene	Sesquiterpene	5.69 ± 3.5 a	1.42 ± 0.34 ab	0.78 ± 0.14 b	nd	0.043
58	17.35	1420	2-Phenylethyl alcohol	Alcohol	1.3 ± 0.41	0.14 ± 0.04	1.13 ± 1.01	0.06	0.083
59	17.64	1442	Nonadecane (C19)	Alkane	0.46 ± 0.04	0.36	1.16 ± 0.97	1.77 ± 0.65	0.243
60	17.91	1432	β-Panasinsene	Sesquiterpene	13.4 ± 6.6	1.33 ± 0.99	Nd	nd	0.083
61	17.99	1448	Germacrene D	Sesquiterpene	nd	nd	Nd	2.93 ± 0.93	NA
62	18.31	1538	Butylated hydroxytoluene	Phenol	nd	nd	0.81	1.41 ± 0.62	0.654
63	18.47	1533	7-epi-α-Selinene	Sesquiterpene	6.01 ± 4.46	0.4 ± 0.27	Nd	1.68	0.117
64	18.51	1567	δ- Amorphone	Sesquiterpene	nd	0.36	1.77 ± 1.42	nd	0.221
65	18.54	1524	Heptacosane	Alkane	nd	nd	Nd	0.77 ± 0.21	NA
66	18.61	1523	Docosane	Alkane	nd	0.11 ± .03	2.38	nd	0.221
67	18.61	1523	Docosane	Alkane	nd	0.17	1.59 ± 0.96	2.1 ± 0.61	0.235
68	18.87	1573	59.33 Tetracosane	Alkane	0.95	0.31	3.38	1.34 ± 0.84	0.361
69	19.39	1595	2-Methylpropanoic acid, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	Ester	1.27 ± 0.92	1.21 ± 0.01	Nd	0.78	0.741
70	19.53	1528	Cedrol	Alcohol	1.21 ± 0.59	0.35	Nd	nd	0.083
71	19.85	1298	Tridecane (C31)	Alkane	0.31	nd	Nd	1.28 ± 0.78	0.157
72	20.21	1500	Untriacontane	Alkane	nd	0.18	Nd	1.36 ± 0.82	0.201
73	22.30	1500	Benzenedicarboxylic acid, butyl_2_methylpropyl ester1_2	Ester	nd	nd	1.37 ± 1.79	0.22	0.179
74	22.84	1494	Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	Ketone	0.89 ± 0.39	0.55 ± 0.21	1.35 ± 0.89	1.27 ± 0.57	0.292
75	24.84	1514	Heneicosane	Alkane	nd	0.15	0.96 ± 0.14	0.06	0.259
76	24.84	1494	Pentacosane	Alkane	nd	0.24 ± 0.08	0.46	0.96 ± 1.04	0.496

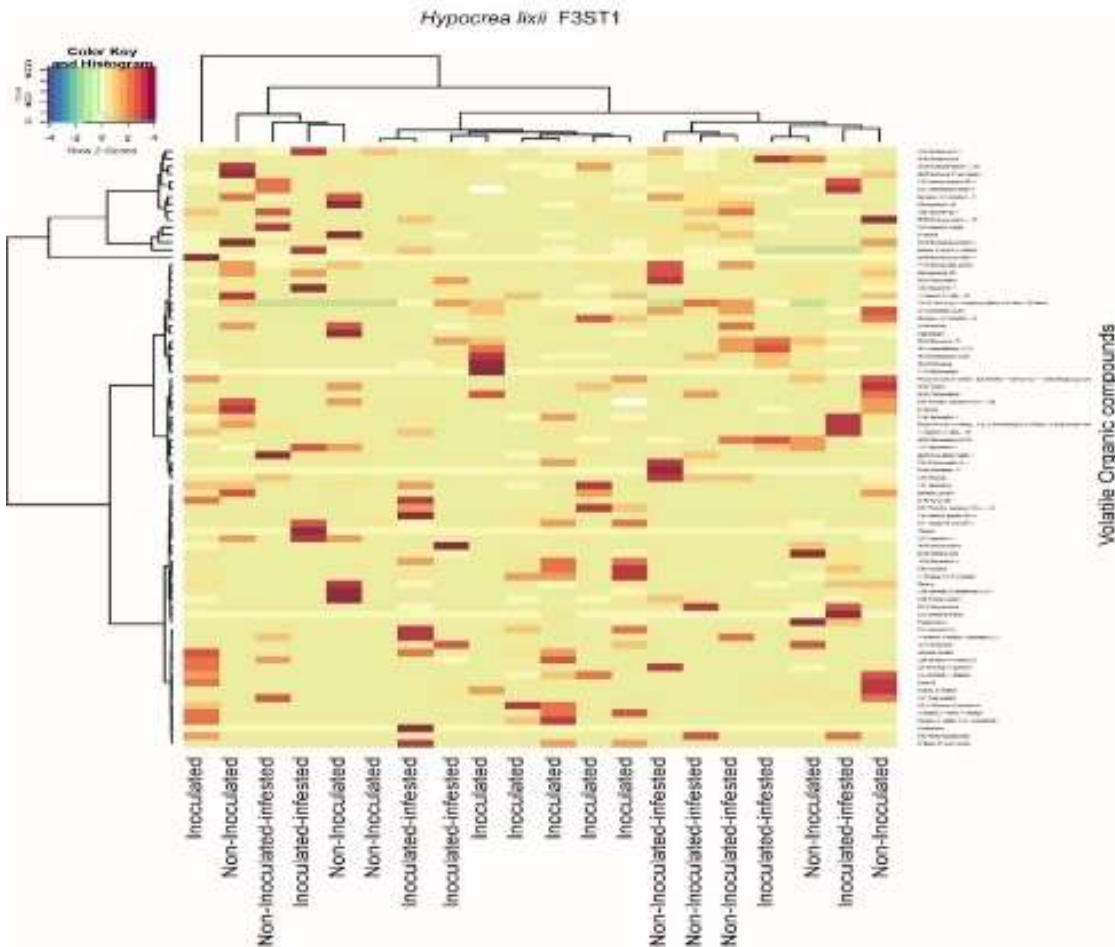
Peak no	RT	RI _{alk} ^a	Compound ^b	Chemical class	Non-inoculated plant	Inoculated plant	Non-inoculated infested plant	Inoculated infested plant	P-value ^c
77	24.91	1500	Trtriacontane	Alkane	1.27	nd	1.11	1.45	0.368
78	27.08	1500	Eicosane (C20)	Alkane	1.48 ± 1.46	0.23 ± 0.05	1.2 ± 0.78	0.52 ± 0.67	0.182
79	28.67	1500	Tricosane	Alkane	nd	0.33 ± 0.24	Nd	1.5 2.13	0.094
80	28.69	1500	Octadecane	Alkane	0.26 ± 0.19	0.23	1.56	1.82 ± 1.49	0.083
81	30.97	1500	Nonacosane	Alkane	0.28	nd	Nd	1.89 1.86	0.221

^aRetention index relative to C8–C31 n-alkanes of an Inert Cap 5MS/NP capillary column.

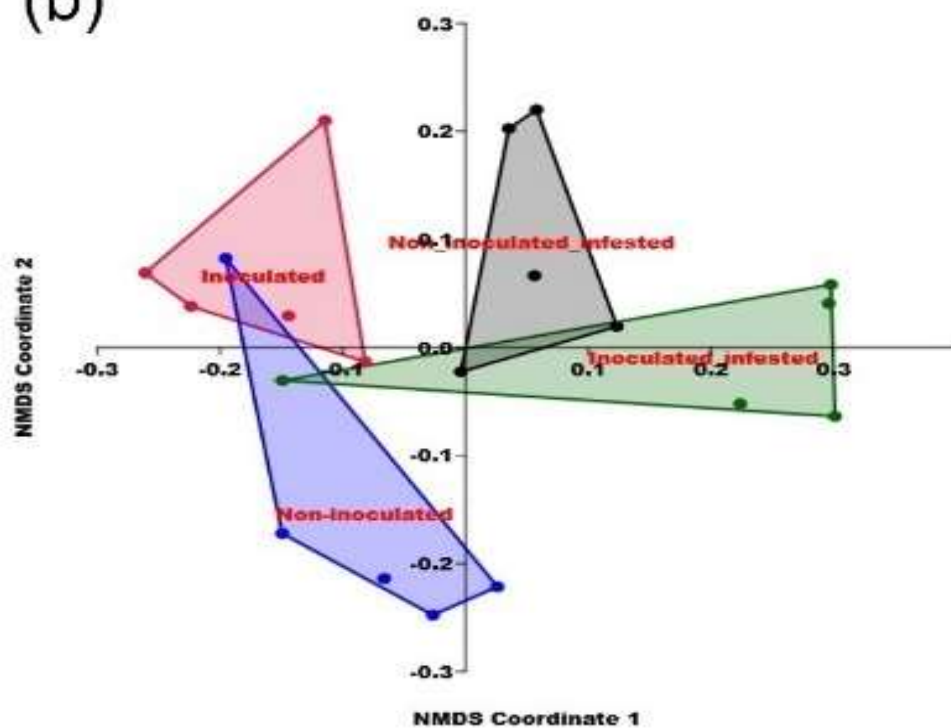
^bIdentification of compounds based on the retention time (RT) and comparison of mass spectra with published mass spectral library data from NIST11.

^cP-value of the non-parametric Kruskal Wallis test for comparison of volatile compounds between non-inoculated, inoculated, non-inoculated infested, and inoculated infested amaranth plants; means with different letters are significantly different at 5% probability level ($P \leq 0.05$), nd = not detected.

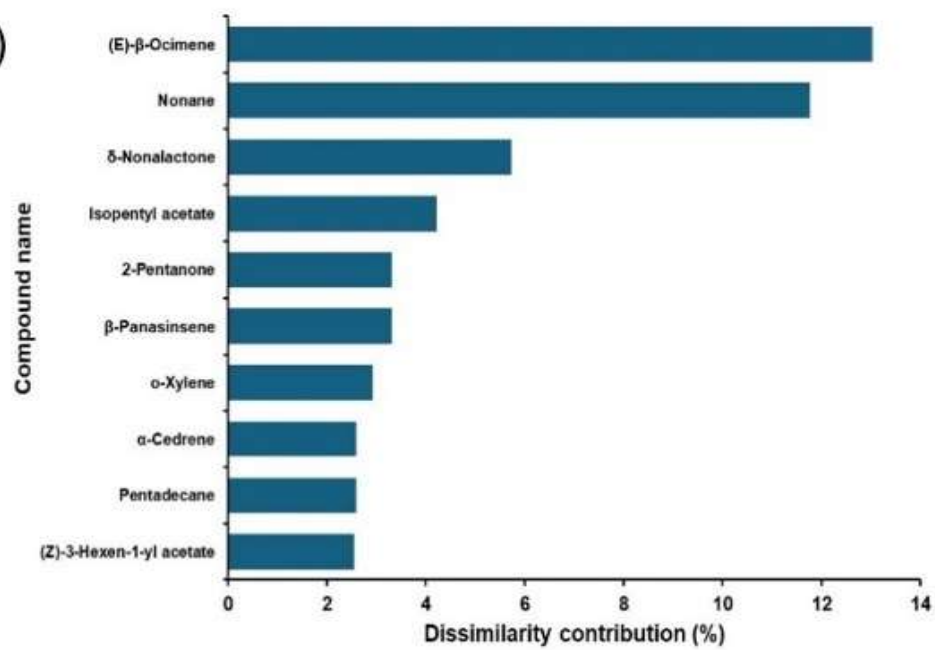
(a)



(b)



(c)



(d)

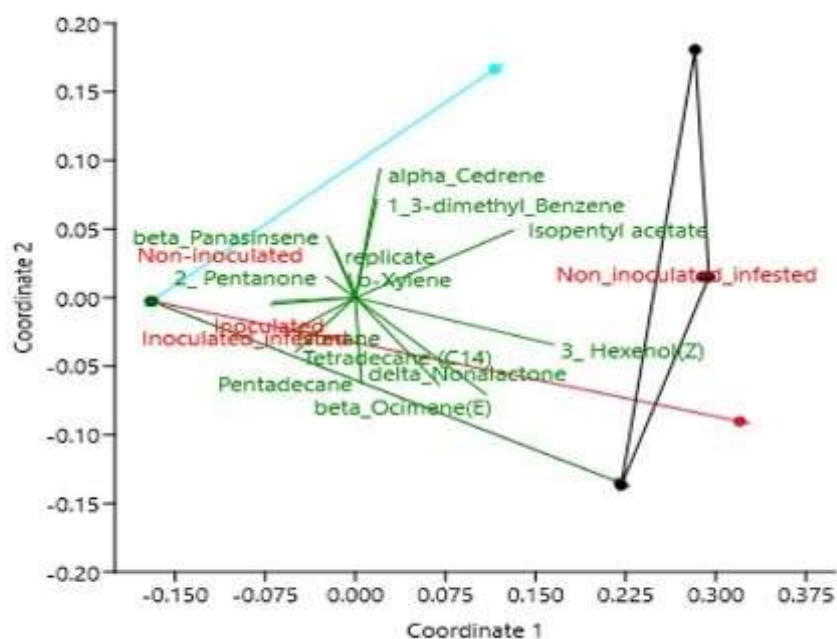


Figure 4.3: (a) Heatmap Clustering Showing the Abundance (In Decreasing Color Intensity) of the Volatile Compounds across Replicates of the Four Treatments

Variations in volatile organic compounds across the four treatments, (b) Non-metric multidimensional scaling analysis (NMDS-Bray Curtis index) of the volatile pattern of non-inoculated, inoculated, non-inoculated infested, and inoculated infested amaranth plants showing the variation in volatiles, (c) Similarity percentage analysis and the percent contribution of the predominant compounds for the dissimilarity between the four treatments, (d) Principal component analysis (PCA) of the volatile profiles produced from non-inoculated, inoculated, non-inoculated infested, and inoculated infested amaranth plants.

In addition, quantitative and qualitative differences in volatile emission were observed between non-inoculated, inoculated, non-inoculated infested, and inoculated infested plants (Figure 4.4a-d).

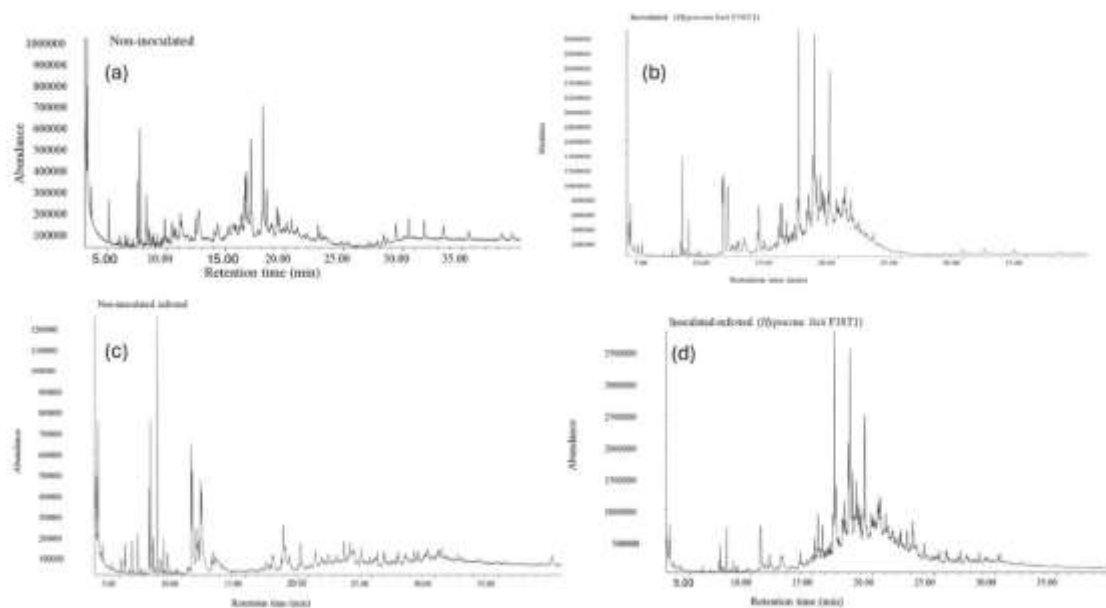


Figure 4.4: Representative Total Ion Chromatogram of (a) Non-Inoculated, (b) Inoculated, (c) Non-Inoculated Infested, and (d) Inoculated Infested Amaranths

In *T. asperellum* M2RT4 colonized amaranth plants, 74 VOCs were detected with 11 distinct chemical classes including alkanes (29), alcohols and esters (10) each, hydrocarbons (9), alkenes (6), aldehydes (3), ketones and sesquiterpenes (2) each, monoterpene, amide and benzenoid each (1) (Table 4.3, Figure 4.5a). Our results clearly showed that volatile compound profiles were influenced by both fungal inoculation and herbivore infestation, resulting in both quantitative and qualitative shifts. (Table 4.3 and Figure 4.4a). For example, in Ketones, 2-pentanone was detected in all four treatments but significantly reduced in the inoculated infested plants ($p < 0.035$), and Hepten-2-one was primarily detected in inoculated plants. Alkenes such as trimethyl-1-pentene was significantly detected in inoculated and non-inoculated infested plants ($p < 0.033$). In the case of esters, heptanol-2-acetate was detected only in non-inoculated infested plants with a statistical significance of ($p < 0.032$). Volatile alcohols such as hexanol, heptanol, and

santolina triene were mainly detected in infested plants, particularly in the non-inoculated infested treatment. However, compounds like 2-heptanol and hexenal (2E) were significantly reduced in inoculated infested plants, with p-values of $p < 0.04$ and $p < 0.009$, respectively. Aldehydes such as hexanal and nonanal showed strong responses to infestation. Notably, hexanal levels increased significantly in non-inoculated infested plants but dropped in inoculated infested plants ($p < 0.035$). A hydrocarbon trimethyl benzene (1,2,4), showed a significant reduction in the inoculated treatment compared to non-inoculated plants ($p < 0.034$). Alkanes, including compounds like nonane, docosane, eicosane, pentadecane, and triacontane, were among the most commonly detected compounds across treatments. However, most alkanes showed no significant differences in abundance, except for eicosane, which was significantly reduced in the inoculated infested plants ($p < 0.029$) (Table 4.3). Similar to *H. lixii* F3ST1 and the four treatments were separated into distinct clusters according to their VOC profiles (NMDS, Figure 4.5b). Similarly the percent contribution of the predominant compounds were dissimilar between the four treatments (non-inoculated, inoculated, non-infested and non-inoculated and inoculated infested plants) (Figure 4.5c). Furthermore, the key compounds between the VOCs of the four treatments were identified (PCA, Figure 4.5d).

Table 4.3: Mean Amount of Volatile Compounds (ng/μL) Identified in the Headspace of Non-Inoculated, Inoculated, Non-Inoculated Infested, and Inoculated Infested Amaranth Plants (n = 5)

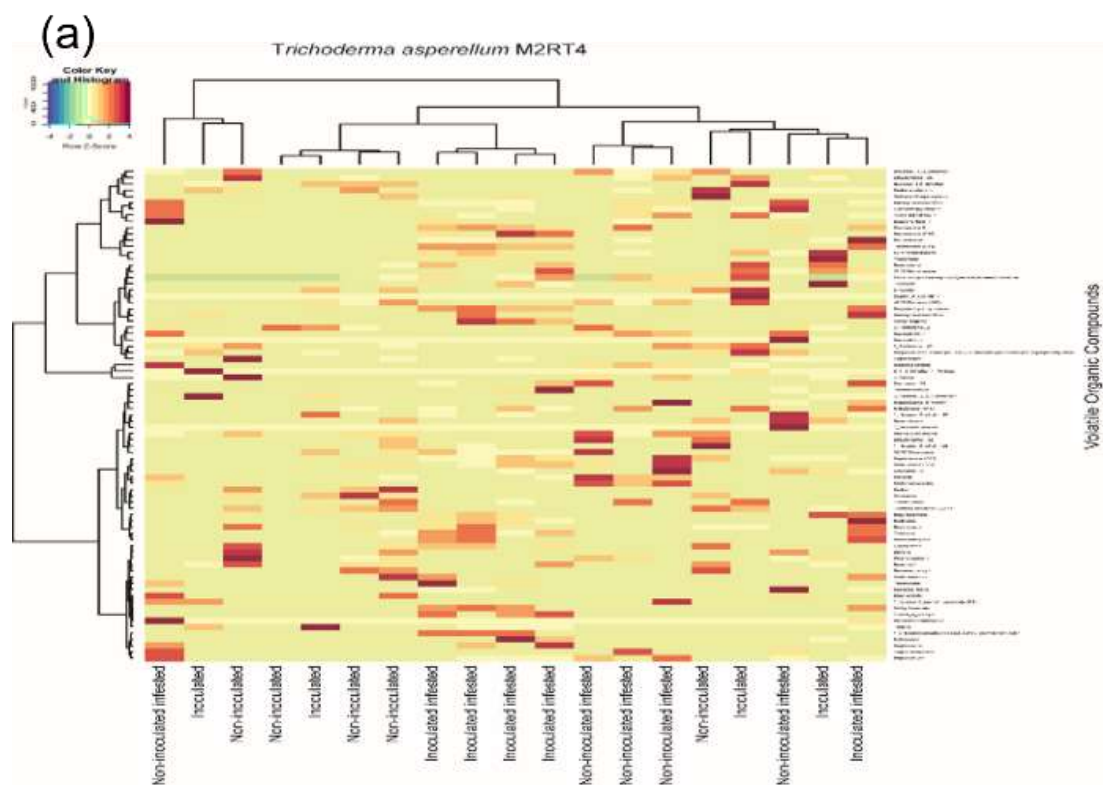
Peak no	RT	RI _{alk} ^a	Compound ^b	Chemical class	Non-inoculated plant	Inoculated plant	Non-inoculated infested plant	Inoculated infested plant	P-value ^c
1	3.28	504	2-Pentanone	Ketone	2.41 ± 1.9 a	2.06 ± 1.2 ab	2.13 ± 0.98 a	0.27 ± 0.2 b	0.035
2	3.98	412	2,3,4-Trimethyl-2-pentene	Alkene	nd	1.18 ± 1.34 a	Nd	0.08	0.179
3	4.28	608	2,4,4-Trimethyl-1-pentene	Alkene	nd	7.44 ± 9.94 b	Nd	0.06 ± 0.08 a	0.033
4	4.29	812	(3E)-2-Hepten-1-one	Ketone	0.39 a	4.29 ± 0.4 a	Nd	0.11 ± 0.9 a	0.259
5	5.15	549	2-Phenylethanol	Hydrocarbon	1.34 ± 0.41 a	nd	1.31 ± 1.01 a	nd	0.462
6	5.17	557	Toluene	Hydrocarbon	nd	0.52 ± 0.36 a	Nd	0.02 ± 0.01 a	0.064
7	5.49	580	Methyl isovalerate	Ester	nd	nd	1.08 ± 0.52 a	0.05 a	0.157
8	6.06	805	Hexanal	Aldehyde	0.35 ab	nd	1.22 ± 0.6 b	0.12 ± 0.07 a	0.035
9	6.49	809	Butyl acetate	Ester	0.28 a	nd	0.22 ± 0.16 a	nd	1
10	6.59	803	n-Octane	Alkane	0.46 ± 0.25 a	nd	nd	0.27 ± 0.05 a	0.512
11	6.62	806	4-Methyl-2-pentanol	Alcohol	nd	nd	0.23a	0.02 ± 0.01 a	0.221
12	7.39	806	(2E)-Hexenal	Aldehyde	nd	nd	2.93 ± 0.83 b	0.19 ± 0.07 a	0.009
13	7.50	863	(3Z)-Hexen-1-ol	Alcohol	nd	5.52 a	3.58 2.08 a	1 ± 0.65 a	0.213
14	7.56	801	Ethylbenzene	Hydrocarbon	4.22 ± 4.28 a	2.83 ± 2.39 a	2.06 ± 1.22 a	nd	0.867
15	7.66	802	4-Methyloctane	Alkane	nd	nd	nd	0.04 ± 0.01 a	NA
16	7.75	802	1,3-Dimethylbenzene	Hydrocarbon	5.57 ± 0.73 a	2.44 ab	2.01 ± 1.74 ab	0.09 ± 0.11 a	0.063
17	7.75	802	1,3-Dimethylbenzene	Hydrocarbon	2.75 ± 0.22 a	3.84 ± 3.1 a	nd	0.23 a	0.304
18	7.87	801	n-Hexanol	Alcohol	nd	nd	5.14 a	0.03 ± 0.03 a	0.221
19	8.02	809	Isopentyl acetate	Ester	nd	0.64 ± 0.36 a	6.41 ± 5.51 a	0.38 ± 0.18 a	0.183
20	8.29	800	o-Xylene	Alkene	13.78 a	1.5 ± 0.34 ab	2.14 ± 1.57 b	0.08 ± 0.3 a	0.045
21	8.52	873	p-Xylene	Benzenoid	1.74 ± 0.61	2.55 ± 1.44 a	nd	0.06 a	0.301
22	8.52		n-Decane	Alkane	0.75 ± 0.46 a	0.36 a	nd	0.98 ± 0.38 a	0.319
23	8.71	802	2- Heptanol	Alcohol	nd	nd	0.58 ± 0.32 b	0.11 ± 0.06 a	0.04
24	8.75	801	Santolina Triene	Alcohol	nd	nd	0.98 a	0.09 ± 0.03 a	0.179
25	9.22	936	α-Pinene	Monoterpene	0.49 ± 0.46 a	0.27 a	0.22 ± 0.13 a	nd	0.752
26	9.49	900	2:1 Butanol–methyl carbonate mixture	Alcohol	nd	0.19 a	0.24 ± 0.11 a	nd	1
27	9.65	886	n-Propylbenzene	Hydrocarbon	0.61 ± 0.16 a	nd	nd	nd	0.356
28	10.45	903	Mesitylene	Hydrocarbon	0.68 ± 0.48 a	0.65 a	nd	nd	1
29	10.45	900	1,2,4-Trimethylbenzene	Hydrocarbon	1.22 ± 0.34 a	0.65 ± 0.2 b	nd	nd	0.034
30	10.90	910	(3E)-Hexenyl acetate	Ester	nd	0.7 a	nd	3.36 ± 0.53 a	0.221
31	10.90	910	(3Z)-Hexenyl acetate	Ester	nd	nd	3.64 ± 2.86 a	1.19 ± 0.42 a	NA
32	11.14	996	2-Ethyl-1-hexanol	Alcohol	2.13 ± 1.58 a	1.06 ± 0.85 a	2.36 a	0.18 ± 0.08 a	0.117
33	11.39	1010	2-Heptanol acetate	Ester	nd	nd	1.37 ± 1.79 a	nd	NA
34	11.49	1050	(E)-β-Ocimene	Alkene	nd	0.5 ± 0.08 ab	3.54 ± 3.37 b	0.34 ± 0.16 a	0.084
35	11.65	1025	Heptadecane (C17)	Alkane	0.76 a	0.23 a	1.2 ± 1.14 a	0.54 ± 0.18 a	0.367
36	12.29	1056	Methyl benzoate	Ester	nd	nd	nd	0.1 ± 0.01 a	NA
37	13.18	1104	n-Nonanal	Aldehyde	0.79 ± 0.07 a	0.71 ± 0.00 a	2.09 a	nd	0.259
38	14.10	1167	Dodecane	Alkane	Nd	nd	nd	0.62 ± 0.6 a	NA

39	14.69	1223	Caprolactam	Amide	3.02 ± 3.43 a	0.49 a	0.32 a	0.53 a	0.284
40	15.24	1205	n-Undecane	Alkane	0.96 a	nd	nd	0.56 ± 0.09 a	0.221
41	15.85	1234	Pentadecane	Alkane	Nd	0.8 a	0.7 a	2.55 ± 4.19 a	0.765
42	15.86	1232	8-Methylheptadecane	Alkane	nd	nd	1.86 ± 2.1 a	1.36 a	1
43	15.86	1282	Octadecane (C18)	Alkane	0.26 ± 0.19 a	2. 24 a	1.56 a	0.83 ± 0.92 a	0.486
44	16.35	1310	Hexadecane (C16)	Alkane	nd	nd	nd	2.96 ± 2.46 a	NA
45	16.54	1322	Butyl butanoate	Ester	nd	1.142 a	nd	0.77 ± 0.36 a	0.157
46	16.65	1313	α -Copaene	Sesquiterpene	nd	nd	0.96 ± 0.48 a	nd	NA
47	16.74	1307	n-Nonane	Alkane	0.55 ± 0.32 a	0.18 ± 0.05 a	nd	0.66 a	0.153
48	16.83	1305	Tetradecane (C14)	Alkane	nd	nd	nd	4.13 ± 0.72 a	NA
49	17.06	1374	Methyl eugenol	Alcohol	nd	nd	nd	2.29 ± 1.08 a	NA
50	17.13	1379	α -Cedrene	Alkene	5.69 ± 3.5 a	2.59 ± 1.26 ab	0.78 ± 0.14 ab	0.67-0.15 b	0.087
51	17.64	1441	Nonadecane (C19)	Alkane	0.46 ± 0.04 a	nd	1.16 ± 0.97 a	0.51 ± 0.046 a	0.607
52	17.74	1428	epi-Nootkatol	Alcohol	nd	nd	nd	1.51 ± 0.18 a	0.695
53	17.99	1448	Germacrene D	Alkene	nd	nd	3.29 ± 1.79 a	2.54 ± 0.45 a	1
54	18.31	1538	Butylated hydroxytoluene	Hydrocarbon	nd	nd	nd	2.36 ± 0.43 a	0.315
55	18.44	1585	β -Copaene	Sesquiterpene	nd	nd	4.55 ± 5.69 a	2.23 a	1
56	18.47	1447	7-epi- α -Selinene	Alkene	6.01 ± 4.46 a	nd	nd	1.39 a	0.221
57	18.54	1524	Heptacosane	Alkane	nd	nd	0.81 a	0.98 ± 0.63 a	0.583
58	18.61	1523	Docosane	Alkane	nd	nd	2.38a	1.08 ± 0.1 a	0.179
59	18.61	1523	Docosane	Alkane	nd	0.47 a	1.59 ± 0.96 a	0.21 ± 0.07 a	0.165
60	18.87	1573	Tetracosane	Alkane	0.95 a	1.53 a	3.38 a	0.68 ± 0.62 a	0.392
61	18.87	1573	Tetracosane	Alkane	nd	nd	0.37 a	0.65 ± 0.53 a	1
62	19.39	1595	2-Methylpropanoic acid, 1,1-dimethylethyl ester	Ester	1.32 a	2.81 ± 2.17 a	1.38 ± 1.17 a	nd	0.539
63	19.53	1528	Cedrol	Alcohol	1.21 ± 0.59	nd	nd	nd	NA
64	19.85	1298	Tridecane	Alkane	0.31 a	nd	nd	1.13 ± 0.46 a	0.413
65	21.03	1499	Tetratriacontane	Alkane	nd	nd	0.24 a	1.81 ± 1.97 a	0.221
66	21.12	1652	Octacosane	Alkane	nd	0.22 a	nd	1.03 ± 0.63 a	0.221
67	22.30	1500	Benzenedicarboxylic acid, butyl 2-methylpropylester	Ester	nd	nd	nd	0.98 ± 0.02 a	NA
68	24.84	1513	Heneicosane	Alkane	nd	4.98 ± 2.85 a	0.96 ± 0.14 ab	0.98 ± 1.07 a	0.117
68	24.84	1494	Pentacosane	Alkane	nd	2.26 ± 0.14 a	0.46 a	0.93 ± 1.32 a	0.498
70	24.91	1500	Trtriacontane	Alkane	1.27 a	1.22 a	1.11 a	0.2 a	0.392
71	27.08	1499	Eicosane(C20)	Alkane	1.48 ± 1.46 a	2.1 ± 1.48 a	1.2 ± 0.78 a	0.19 ± 0.1 b	0.029
72	28.67	1499	Tricosane	Alkane	nd	4.63 a	1.11 a	1.02 ± 0.59 a	0.407
73	29.348	1500	Triacotane	Alkane	nd	6.71 a	nd	1.57 ± 0.2 a	0.221
74	30.97	1500	Nonacosane	Alkane	0.28 a	3.11 ± 0.48 a	nd	1.07 ± 0.88 a	0.717

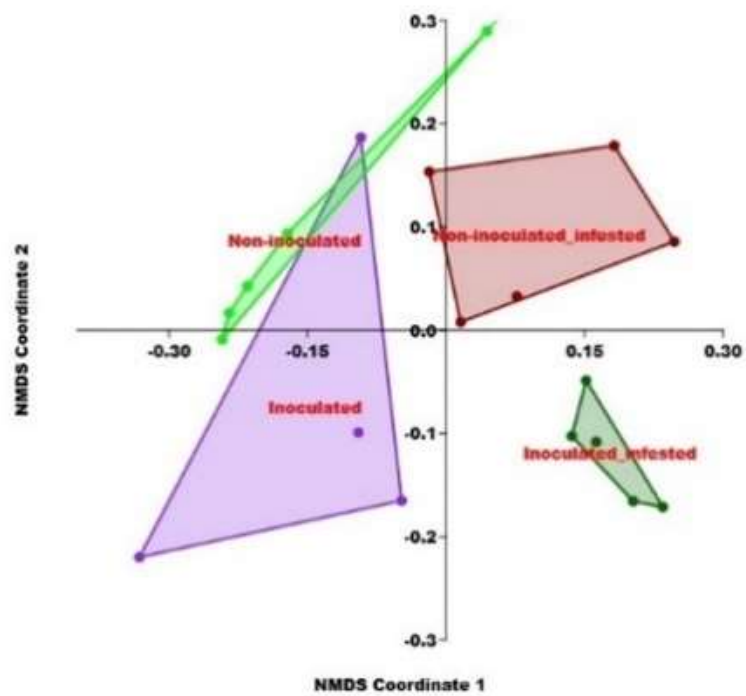
^aRetention index relative to C8–C31 n-alkanes of an Inert Cap 5MS/NP capillary column.

^bIdentification of compounds based on the retention time (RT) and comparison of mass spectra with published mass spectral library data from NIST11

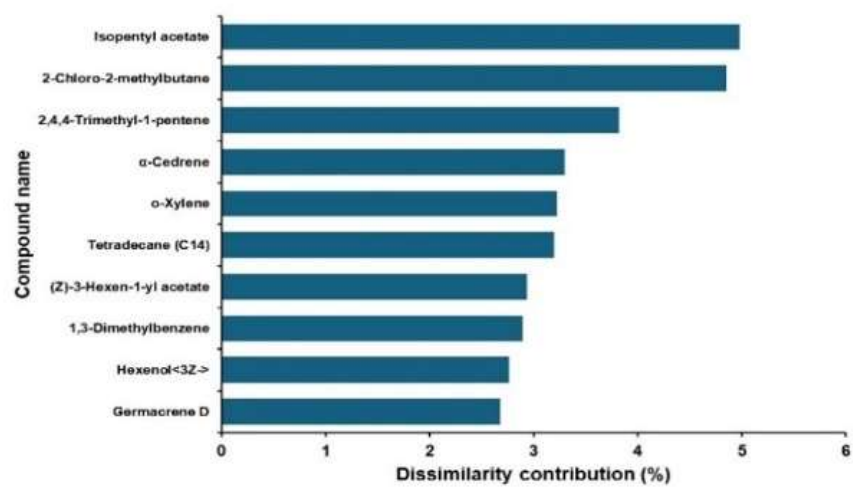
^cP-value of the non-parametric Kruskal Wallis test for comparison of volatile compounds between non-inoculated, inoculated, non-inoculated infested, and inoculated infested amaranth plants, means with different letters are significantly different at 5% probability level ($P \leq 0.05$), nd = not detected



(b)



(c)



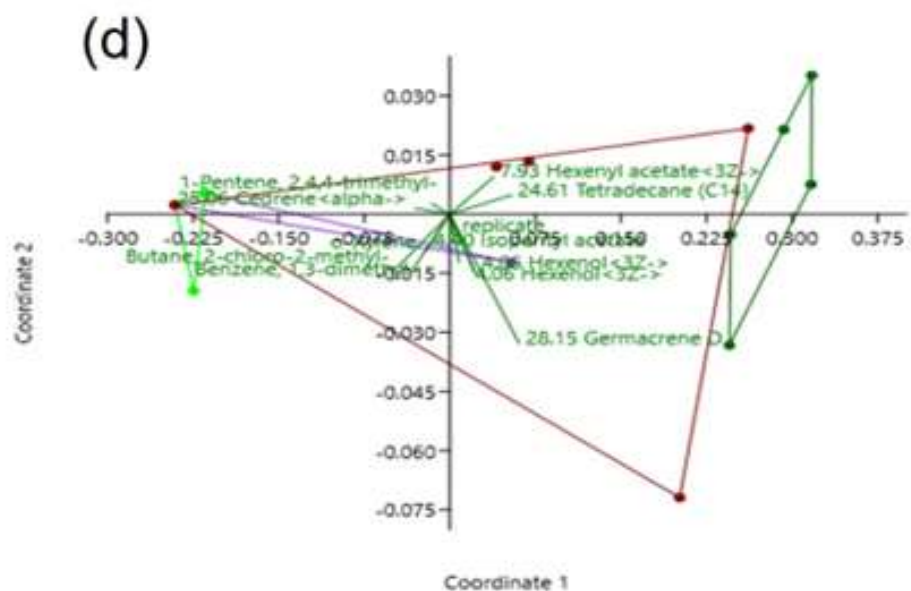


Figure 4.5: Assessment of Variations in Volatile Organic Compounds across Four Treatments

Key: (a) Heatmap Clustering Showing the Abundance (In Decreasing Color Intensity) of the Volatile Compounds across Replicates of the Four Treatments. Variations in Volatile Organic Compounds Across the Four Treatments, (b) Non-metric Multidimensional Scaling Analysis (NMDS) of the Volatile Pattern of Non-Inoculated, Inoculated, Non-Inoculated Infested, and Inoculated Infested Amaranth Plants Showing the Variation in Volatiles, (c) Similarity Percentage Analysis and the Percent Contribution of the Predominant Compounds for the Dissimilarity between the Four Treatments, (d) Principal component analysis (PCA) of the Volatile Profiles Produced from Non-Inoculated, Inoculated, Non-Inoculated Infested, and Inoculated Infested Amaranth Plants.

Furthermore, the composition and the emission levels of volatiles varied among the non-inoculated, inoculated, non-inoculated-infested, and inoculated-infested plants (Fig. 4.6a-d).

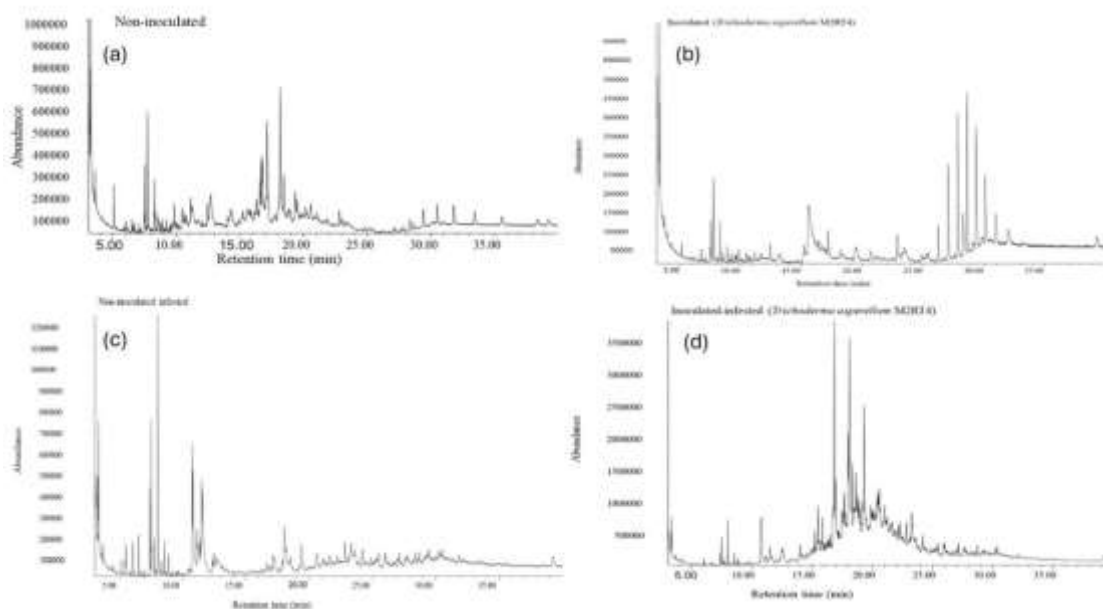


Figure 4.6: Representative Total Ion Chromatogram of (a) Non-Inoculated, (b) Inoculated, (c) Non-Inoculated Infested and, (d) Inoculated Infested Amaranths Plants

4.5 Discussion

The colonization of plants by endophytic fungi plays a significant role in shaping plant-insect interactions, particularly in mitigating herbivory by insect pests (Islam et al., 2021). In this study, the effects of two endophytic fungal isolates, *H. lixii* F3ST1 and *T. asperellum* M2RT4 on the behavior and herbivory of the beet webworm moth *S. recurvalis* were investigated. The results showed successful colonization of *A. dubius* plants by *H. lixii* F3ST1 and *T. asperellum* M2RT4 achieved through artificial seed inoculation. *Hypocrea lixii* F3ST1 exhibited successful colonization in 90% of root and stem tissues and 80% of leaf tissues, whereas *T. asperellum* M2RT4 colonized 85% of root tissues, 100% of stem tissues, and 85% of leaf tissues. This suggests a high level of host compatibility and effective colonization mechanisms possessed by both fungal endophytes. Furthermore, their widespread presence across different plant tissues highlights their potential for use as bioinoculants with the aim of promoting amaranth

growth and induce systemic resistance to herbivory attacks (Nyamamba et al., 2025). Herbivores are sensitive to HIPVs and tend to prefer plants that do not release these odors (Kessler & Baldwin, 2001).

Female insects enhance their fitness by selecting host plants for oviposition that offer optimal conditions for the survival and development of their offspring, the risk of predation and competition (Zhu et al., 2023).

In the Y-tube olfactometer (behavioral) bioassay, it was identified that *S. recurvalis* females were not attracted to *T. asperellum* M2RT4 and *H. lixii* F3ST1-inoculated plants. This could be attributed to the production of secondary metabolites by the endophytically colonized amaranth plants which consequently influence insect oviposition behavior by modifying oviposition cues signalling reduced host suitability that might have an implication for herbivore deterrence (Rasool et al., 2021; Agbessenou et al., 2022). Moreover, this study found that female *S. recurvalis* showed a significant preference for volatiles emitted by non-inoculated amaranth plants over clean air, indicating strong attraction. However, no significant attraction was observed toward infested plants, whether inoculated or non-inoculated, relative to clean air.

Our results also revealed that female *S. recurvalis* were not attracted to either non-inoculated infested or inoculated infested amaranth plants. This response may be attributed to the females sensing infestation-related chemical cues, which signal reduced host quality and risk of competition for the successful development of their offspring (Kohandani et al., 2017). Similar to the present findings, previous studies by Agbessenou et al. (2022) reported that *T. absoluta* (Meyrick) (Lepidoptera: Gelchiidae) females were not attracted to neither non-inoculated infested nor *T. asperellum* M2RT4-inoculated infested tomato plants.

Plants continuously emit volatile organic compounds (VOCs), which herbivores can exploit to locate their host plants through olfactory cues (Ling et al., 2022). Upon herbivore attack, the plant alters the composition of its emitted volatiles to signal

neighboring plants (plant-plant communication) and to attract the natural enemies of the herbivore (de Vos & Jander, 2010). Herbivore-induced plant volatiles (HIPVs) facilitate communication at multiple levels i.e. between the plant and the herbivore, among different parts of the same plant (intra-plant signaling), and between an infested plant and nearby uninfested plants (inter-plant signaling) (Arimura et al., 2009). The results from the chemical analysis from volatiles trapped from non-inoculated, inoculated, non-inoculated infested and inoculated infested endophytically colonized *H. lixii* F3ST1 and *T. asperellum* M2RT4 amaranth plants revealed emission of 81 and 74 volatile organic compounds respectively. Plants colonized by *H. lixii* M2RT4 chemical analysis identified esters, alcohols and benzenoids as the most abundant volatile compound classes that were significantly influenced by fungal inoculation and herbivore infestation, while *T. asperellum* M2RT4-colonized plants emitted esters, aldehydes, and alcohols as the most responsive groups to infestation, likely reflecting active defense signaling. Alkanes and aldehydes showed more stable patterns, in *H. lixii* F3ST1-colonized amaranth plants while in *T. asperellum* M2RT4 alkanes and hydrocarbons remained relatively stable, indicating baseline roles in both endophytic fungal isolates. Endophytically colonized plants, particularly under infestation, suppressed some VOCs emissions, suggesting a modulatory effect on plant defense pathways. These patterns provide strong evidence that these fungal endophytes influence not only plant growth (Nyamamba et al., 2025; Paradza et al., 2021) but also chemical defenses (Agbessenou et al., 2022), with important implications for pest management.

We conducted Similarity percentage analysis and the percent contribution of the predominant compounds for the dissimilarity between the four treatments for both fungal endophytes. For *H. lixii* F3ST1 we identified, (Z)-3-Hexenyl acetate, pentadecane, α -Cedrene, o-Xylene, β -Panasinsene, 2-Pentanone, Isopentyl acetate, δ -Nonalactone, Nonane and (E)- β -Ocimene as the most discriminating compounds while in *T. asperellum* M2RT4 Germacrene D, (Z)-3-hexenol, 1,3-Dimethylbenzene, (Z)-3-Hexenyl acetate, Tetradecane (C14), o-Xylene, α -Cedrene, 2,4,4-Trimethyl-1-pentene, 2-Chloro-2-methylbutane and Isopentyl acetate were identified.

Plants emit a limited number of volatile organic compounds that contribute directly to protection against herbivores. One well-studied example is the green-leaf volatile (Z)-3-hexenyl acetate (often produced together with (Z)-3-hexenol), which is released almost immediately after tissue damage. This compound interferes with host-plant location by insects such as aphids and lepidopteran pests and, importantly, attracts parasitoids and predators that attack the herbivores (Dicke & Baldwin, 2010). Another key signal is the monoterpene (E)- β -ocimene, a classic herbivore-induced plant volatile that functions in indirect defence by recruiting natural enemies and priming nearby plants to activate defence pathways (Heil & Karban, 2010). Among sesquiterpenes, germacrene D and α -cedrene are associated with feeding and oviposition deterrence and may negatively affect insect growth and survival, contributing to direct resistance (Bruce et al., 2005). Compounds such as 2-pentanone and δ -nonalactone also act as behavioral repellents that discourage insect settling on leaves (Serdo, 2024). In addition, leaf surface hydrocarbons including pentadecane and tetradecane alter the plant's chemical signature and reduce acceptance for feeding or egg laying by herbivorous insects (Eigenbrode & Espelie, 1995). Collectively, these compounds illustrate how plants rely on both direct deterrence and indirect biological control, lowering pest pressure by repelling herbivores while simultaneously attracting their natural enemies. This chemical diversity also aligns with previous studies in different crops that have reported complex VOCs profiles in endophyte-colonized plants (Agbessenou et al., 2022). For instance, Agbessenou et al. (2022) reported the emission of a complex blend of VOCs, with monoterpenes and sesquiterpenes being the predominant components in colonized tomato plants with the aim of controlling *P. absoluta*. Also, Adams et al. (2023) in their study on how non-host plant influence the trophic interaction between tomato, its pest *P. absoluta* and mirid predator *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae), revealed the emission of monoterpenes, sesquiterpenes, ketones, aldehydes, amides, alkanes, esters, and alcohols in cultivated tomato, wild tomato nightshade, marigold and blackjack. However, to further support the application of *H. lixii* F3ST1 and *T. asperellum* M2RT4 in sustainable *S. recurvalis* management, it is important to confirm the behavioral and herbivory effects of the emitted volatiles with the synthetic versions using a Y-tube olfactometer bioassay.

This would clearly establish the defense mechanisms involved in endophytically colonized amaranth plants and elaborate more on the key pathways inducing defenses against *S. recurvalis*. This integrated approach will lay the groundwork for developing eco-friendly, endophytically-based biopesticides that can be effectively incorporated into sustainable *S. recurvalis* management and amaranth cropping systems. It is important to note that insect behavioral responses are mediated by the overall blend and relative composition of VOCs rather than by individual compounds alone.

CHAPTER FIVE

GENERAL DISCUSSION CONCLUSIONS AND RECOMMENDATIONS

5.1 General Discussion

Amaranth has gained considerable research attention due to its agronomic, nutritional, and medicinal value. However, its production is constrained by both abiotic and biotic factors, particularly insect pests. Among these, *Spoladea recurvalis* is a major lepidopteran pest that causes substantial yield losses, primarily through feeding by second-instar larvae on amaranth leaves (Smith et al., 2018; Jeyasankar & Gokilamani, 2016). Although chemical pesticides remain the primary control strategy, their adverse effects on biodiversity, human health, and the environment underscore the need for sustainable and environmentally friendly alternatives.

Therefore, to address the challenges posed by pests, the use of microbial strains has emerged as a sustainable, eco-friendly, and non-toxic solution (Kumari et al., 2022; Akutse et al., 2020). Entomopathogenic fungi (EPF) are regarded as one of the most promising alternative management methods, as they are actively being developed as biological control agents against a wide range of insect pest species (Sheeba et al., 2001). The use of EPF to reduce pest population density plays a key role in sustainable pest management programs (Charnley & Collins, 2007). These EPFs can also be used as plant endophytes, where they function as bioagents, inducing systemic resistance against herbivore insect pests (Akutse et al., 2013). In contrast to chemical pesticides, EPF are target specific, safe to non-target organisms, highly virulent, persistent and safe to human and environmental health (Mantzoukas et al., 2021). Therefore, the purpose of this study was to evaluate the effectiveness of selected fungal isolates with endophytic properties with the goal of incorporating them into a sustainable management strategy for managing *S. recurvalis*.

We screened 15 endophytic fungal isolates to check for their ability to systemically colonize *A. dubius* plant through seed inoculation. Our results indicate that only *H. lixii* F3ST1 and *T. asperellum* MRT4 were able to successfully colonize and establish in *A. dubius* seedlings without causing any visible symptoms or damage, similar to the control treatments, suggesting a harmonious symbiotic association between the fungi and the host plant. The two endophytes have previously been reported to exhibit endophytic properties in tomato and nightshade (Agbessenou et al., 2020; Paradza et al., 2021), French bean (Paradza et al., 2021), cocoa (Ambele et al., 2020), maize (Kinyungu et al., 2023), and faba and rose coco beans (Akutse et al., 2013), to mention a few. For instance, our study showed that isolates of *B. bassiana* ICIPE 621, ICIPE 273, ICIPE 676, ICIPE 609, ICIPE 283, ICIPE 706, and ICIPE 279, *Fusarium. proliferatum* NF2S51, *Trichoderma. atroviride*, *Bionectria. ochroleuca* F3R21 colonized only the root tissue while isolates of *Mettarrhizium. anisopliae* ICIPE and ICIPE 41, *T. harzianum* KF2R4 failed to colonize any part of *A. dubius* plant (Nyamamba et al., 2025). It is reported that some endophytic fungi display tissue specificity, adapting to the unique conditions present in each plant organ (Guo et al., 2008). Moreover, inoculation method is also crucial to fungal colonization pattern (Tefera & Vidal, 2009).

Additionally, soil microbial communities, temperature, relative humidity, growth medium, plant age and species, inoculum density, and the fungal species involved can all influence the successful colonization of endophytic fungi in different plant tissues (Parsa et al., 2013; Bamisile et al., 2018). Work by Ambele et al. (2020) and Mutune et al. (2016) demonstrates that *M. anisopliae* isolates failed to be recovered as endophytes in cocoa and French beans, respectively. This is somewhat different from Liu et al. (2022), who reported the colonization and establishment of *M. anisopliae* in maize. Further, *H. lixii* F3ST1 and *T. asperellum* M2RT4 induced systemic resistance against *S. recurvalis* by negatively impacting its life history parameters (Nyamamba et al., 2025). A laboratory bioassay revealed that the number of egg masses obtained from the endophytically colonized amaranth plants were fewer compared to the control (endophyte-free plants). A similar observation was recorded in the number of larvae that hatched, increased larval

mortality, low pupal formation, few adult emergence, and eventually a reduction in the number of days that F1 progeny survived compared to the control treatment (Nyamamba et al., 2025). Although there was strong evidence of insect mortality on plants colonized endophytically, no fungal outgrowth was observed on the cadavers of both larvae and F1 progenies, as also reported by Akutse et al. (2013). This suggests that the exact cause of death remains unestablished in our case, although it has been reported that the endophytes stimulated the host plant to produce some secondary metabolites which affect the life history or reproduction parameters of the pests (Behie & Bidochka, 2014; Bamisile et al., 2018). It is hypothesized that fungal endophytes produce antimicrobial compounds with potent antifungal properties which can act antagonistically against plant pests and pathogens (Mai et al., 2020). Thus, the negative effects on herbivorous insects appear to involve antibiosis and feeding deterrence mechanisms, which are mediated by endophytes within the host plant and may trigger the production of secondary bioactive metabolites (Behie & Bidochka, 2014). Therefore, further research is essential to validate these promising results under field conditions and to integrate these two endophytes into management options/programs for *S. recurvalis* in amaranth cropping systems.

Fungal endophytes expand their uses not only as biocontrol agents but also as biostimulants and biofertilizers (Baron & Rigobelo, 2022; Bamisile et al., 2018). It is worth highlighting that the endophytic colonization of the amaranth plants by *H. lixii* F3ST1 and *T. asperellum* M2RT4 during the weekly measurements significantly improved plant height, the number of leaves, leaf length, leaf width and dry shoot biomass. Numerous studies have also demonstrated that endophytes can enhance growth across diverse plant species like soya bean (Russo et al., 2019), red chill (Saragih, 2019) broad bean (Jaber & Enkerli, 2016), tomato (Paradza et al., 2021) and maize (Kinyungu et al., 2023). The growth enhancement by endophytes is attributed to their ability to stimulate the production of phytohormones which have a direct effect on promoting plant growth (Baron & Rigobelo, 2022). Moreover, the growth-promoting effects of these endophytic fungal isolates may also be linked to increased nutrient uptake (Yang et al., 2021). Additionally, endophytic fungi can enhance a plant's self-defense mechanisms by

activating induced systemic resistance (ISR) pathways. These pathways may overlap with those of acquired systemic resistance (ASR), as both contribute to improved plant growth (Busby et al., 2016).

Furthermore, the role of VOCs emitted by inoculated, non-inoculated, inoculated infested, and non-inoculated amaranth plants was investigated using a Y-tube olfactometer. Our results indicated that *S. recurvalis* female moths preferred non-colonized amaranth plants over those colonized by endophytes. The findings indicated that *S. recurvalis* did not exhibit attraction to either infested non-colonized nor infested endophyte colonized amaranth plants. This behavioral response may be explained by the *S. recurvalis* females' ability to detect chemical cues associated with infestation, which signal diminished host quality and heightened competition, that could hinder the successful development of their offspring (Kohandani et al., 2017). Gas Chromatography-Mass Spectrometry analysis revealed different volatiles emitted by plants colonized by *H. lixii* F3ST1 and *T. asperellum* M2RT4 which have the potential of priming plant defense during attack by herbivores. The two endophytes *H. lixii* F3ST1 and *T. asperellum* M2RT4 demonstrate strong potential as endophytic fungal-based biopesticides for the sustainable management of *S. recurvalis* in amaranth cropping systems, while also promoting the host plant growth. This finding is consistent with previous studies reporting similar outcomes in comparable cropping systems (Agbessenou et al., 2022; Kinyungu et al., 2023). To further support their application in *S. recurvalis* management, it is paramount to confirm the behavioral effects of the emitted volatiles with the synthetic versions using a Y-tube olfactometer bioassay. This integrated approach will lay the scientific groundwork for developing eco-friendly, plant-based biopesticides that could be effectively adopted as part of sustainable *S. recurvalis* and amaranth farming systems.

5.2 Conclusion

In our study, we have demonstrated that out of the 15 fungal endophytes screened, only *H. lixii* F3ST1 and *T. asperellum* M2RT4 were successfully recovered as endophytes in amaranth plants through seed inoculation. The two endophytes also conferred systemic

resistance against *S. recurvalis*. Our findings highlight the strong potential of *H. lixii* F3ST1 and *T. asperellum* M2RT4 as effective biocontrol agents for managing *S. recurvalis* on amaranth. Their efficacy was evident through the significant reduction in the number of egg masses, egg hatching, increased larval mortality, low number of pupae formation and adult emergence and reduction in the survival of F1 progenies when compared to the control. Additionally, both endophytes remained persistent in the host plant for five weeks and contributed to enhanced plant growth. Overall, GC-MS results indicate that endophytic colonization alters the volatile emission profile of amaranth in a manner that diminishes its attractiveness to *S. recurvalis* females, highlighting a potential role of these two fungal endophytes in herbivore deterrence and could be used in integrated pest management (IPM).

5.3 Recommendations

1. Adopt *H. lixii* F3ST1 and *T. asperellum* M2RT4 as biological control agents for the sustainable management of *S. recurvalis* in amaranth production, as they effectively suppressed the pest while promoting plant growth.
2. Integrate endophyte-based seed inoculation into integrated pest management (IPM) programmes to reduce reliance on synthetic insecticides, thereby minimizing pesticide residues, environmental contamination, and adverse effects on beneficial organisms.
3. Promote the use of endophytic fungi among amaranth growers through extension and advisory services as an environmentally friendly pest management strategy that enhances crop protection and productivity while contributing to sustainable vegetable production.
4. To confirm the functional roles of these emitted volatiles identified synthetics should be used in, conducting behavioral bioassays using a Y-tube olfactometer. This will provide a scientific basis for formulating environmentally friendly, plant-based biopesticides that can be integrated into sustainable management strategies of *S. recurvalis*.

5. It is also important to establish the key defense pathways involved in the endophytically colonized amaranth plants and determine the expression of the defense genes involved in this systemic resistance mechanism.
6. Further studies are also warranted to validate these findings under field conditions for effective use of both endophytes in the management of *S. recurvalis* in different agroecological systems.

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