

**The effect of fungal endophytes on thrips and tospovirus epidemiology**

Doctoral Thesis

Approved by the Faculty of Natural Sciences of  
Gottfried Wilhelm Leibniz Universität Hannover

for acquisition of the degree of

**Doctor of Horticultural Sciences**

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**2015**

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**Date of doctoral degree conferment:** 24<sup>th</sup> June 2015

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## **DEDICATION**

**This work is dedicated to**  
**my parents (Daniel Nyumbu and Alice Matheka),**  
**wife (Faith),**  
**son (Ryan)**  
**and**  
**daughter (Reanna)**

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## ABSTRACT

Onion thrips, *Thrips tabaci* Lindeman are one of the key pests of onion, *Allium cepa* L., an economically important agricultural crop cultivated worldwide. Thrips are commonly managed through indiscriminate application of synthetic pesticides. However, most of these pesticides can lead to serious environmental hazards and are ineffective due to a number of factors including development of resistance, pest presence in cryptic habitats and overlapping generations. To remain effective, control programmes have to integrate several pest and disease management tactics including the use of beneficial micro-organisms like endophytic fungi that stimulate the plant defence responses. Endophytic fungi in recent evidence suggest that they can play symbiotic roles in nature, such as antagonists of plant pests and diseases, increased drought tolerance and plant-growth. However, information on endophyte colonization of onions and their impacts on the biology of onion thrips, influence on thrips behaviour and on induction of resistance against thrips and virus replication are lacking.

Therefore, the objectives of this study were to determine, (1) colonization of onions by endophytic fungi and their impacts on the biology of *Thrips tabaci*, (2) behavioral responses of *T. tabaci* to endophyte inoculated onion plants, and (3) if endophytic colonization of onions induces resistance against thrips and virus replication.

Colonization of onion plants by selected fungal endophyte isolates was tested using two inoculation methods whilst evaluating their effects on biology of *T. tabaci*. Seven fungal endophytes used in our study were able to colonize onion plants either by the seed or seedling inoculation methods. Seed inoculation resulted in 1.47 times higher mean percentage post inoculation recovery of all the endophytes tested as compared to seedling inoculation. Fewer thrips were observed on plants inoculated with *Clonostachys rosea* ICIPE 707, *Trichoderma asperellum* M2RT4, *Trichoderma atroviride* ICIPE 710, *Trichoderma harzianum* 709, *Hypocrea lixii* F3ST1 and *Fusarium* sp. ICIPE 712 isolates as compared to those inoculated

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with *Fusarium* sp. ICIPE 717 and the control. Onion plants colonized by *C. rosea* ICIPE 707, *T. asperellum* M2RT4, *T. atroviride* ICIPE 710 and *H. lixii* F3ST1 had significantly lower feeding punctures as compared to the other treatments while the lowest numbers of eggs were laid by *T. tabaci* on *H. lixii* F3ST1 and *C. rosea* ICIPE 707 inoculated plants.

To study behavior of thrips on endophytically colonized onion plants, choice experiments were conducted in a screen house and the laboratory. Female *T. tabaci* preferred endophyte-free (E-) over endophyte-inoculated (E+) plants. The number of feeding punctures and eggs were more on E- than on E+ plants. Oviposition was reduced six fold on E+ plants within a 72 h experimental period. In Y-tube olfactometer assay, thrips showed about 3.6 fold preference for E- plants. In individual larval choice experiments, significantly more first-instar and second preferred to feed on leaf sections of E- as compared to the E+ plants. In a settlement preference assay with groups of second-instars, larvae preferred leaf sections from E- over E+ plants with incremental time.

To study the effect of endophytic colonization of onions on induced resistance against thrips and virus replication, we conducted greenhouse trials in which a colony of viruliferous thrips were studied for feeding and transmission of *iris yellow spot virus* (IYSV) on E- and E+ onion plants. The numbers of feeding punctures were significantly lower in E+ as compared to the E- plants. Disease level sampled weekly for four weeks following thrips exposure was significantly lower in E+ as compared to E- plants. IYSV transmission was reduced 2.5-fold by the endophytic fungus on both whole plant and leaf disc assays. Our results suggest potential utility of endophytes to colonize and confer protection on onion plants against thrips damage and virus infection. Further studies should be conducted to determine whether such endophyte-mediated protection against thrips in onion extends to other agricultural crops.

**Keywords:** Endophytic fungi, Colonization, Onions, *Thrips tabaci*, *Iris yellow spot virus*, Feeding, Oviposition, Induced systemic resistance, Choice test, Multi-trophic interactions

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## ZUSAMMENFASSUNG

Der Zwiebelthrips, *Thrips tabaci* Lindeman, gehört zu den wichtigsten Schädlingen an Zwiebeln, *Allium cepa* L., einer bedeutenden Kulturpflanze mit weltweiter ökonomischer Bedeutung. Im Allgemeinen werden Thripse durch planlose Anwendung von synthetischen Pflanzenschutzmitteln kontrolliert. Allerdings können viele dieser Pflanzenschutzmittel zu ernststen Umweltrisiken führen und sind aufgrund einer Reihe von Faktoren zu denen Resistenzen, kryptisches Verhalten der Schädlinge sowie überlappende Generationen zählen nicht effektiv genug. Um die Effektivität von Kontroll-programmen zu gewährleisten ist es daher notwendig verschiedene Bekämpfungsstrategien inklusive dem Einsatz von Mikroorganismen, wie endophytischen Pilzen, zur Stimulierung der Pflanzenabwehr gegenüber Schädlingen und Krankheiten, zu integrieren.

In den letzten Jahren gibt es vermehrt Hinweise darauf, dass endophytische Pilze eine symbiotische Rolle in der Natur einnehmen, als Antagonisten von Pflanzenschädlingen und Pflanzenkrankheiten fungieren sowie die Trockentoleranz und das Pflanzenwachstum fördern. Jedoch sind Informationen zur Besiedlung von Zwiebeln mit Endophyten und ihre Auswirkungen auf die Biologie vom Zwiebelthrips, Beeinflussung des Thrips-Verhaltens und Resistenzinduktion gegenüber Thripsen sowie Replikation von Pflanzenviren in der Literatur nicht zu finden. Aus diesem Grund waren die Zielsetzungen dieser Arbeit (1) die Kolonisierung von Zwiebeln mit endophytischen Pilzen und ihre Auswirkungen auf die Biologie von *Thrips tabaci* zu bestimmen (2) die Verhaltensreaktionen von *T. tabaci* gegenüber inokulierten Zwiebelpflanzen zu untersuchen und (3) zu Klären, ob endophytische Besiedlung von Zwiebeln Resistenzen gegenüber Thripsen und Virus replikation induziert.

Die Besiedlung von Zwiebelpflanzen mit ausgewählten Isolaten endophytischer Pilze wurde mit Hilfe zweier Inokulierungs-Methoden untersucht, während gleichzeitig die Auswirkungen auf die Biologie von *T. tabaci* bestimmt wurde. Sieben der getesteten Isolate

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endophytischer Pilze waren in der Lage, Zwiebelpflanzen entweder durch Samen- oder durch Sämlings-Inokulation zu besiedeln. Sameninokulation führte bei allen Isolaten zu einer 1,47-fach höheren mittleren prozentualen Wiederfindungsrate im Vergleich zur Sämlingsinokulation. Im Vergleich zur Kontrolle und zu mit *Fusarium* sp. ICIPE 717 inokulierten Pflanzen wurden weniger Thripse auf Pflanzen beobachtet, die mit Isolaten von *Clonostachys rosea* ICIPE 707, *Trichoderma asperellum* M2RT4, *Trichoderma atroviride* ICIPE 710, *Trichoderma harzianum* 709, *Hypocrea lixii* F3ST1 oder *Fusarium* sp. ICIPE 712 inokuliert waren.

Zwiebelpflanzen, die mit *C. rosea* ICIPE 707, *T. asperellum* M2RT4, *T. atroviride* ICIPE 710 oder *H. lixii* F3ST1 besiedelt waren, wiesen signifikant weniger Saugstellen von Thripsen auf als in den anderen Behandlungen. Die geringste Anzahl Eier wurde von *T. tabaci* an *H. lixii* F3ST1 und *C. rosea* ICIPE 707 inokulierten Pflanzen abgelegt.

Um das Verhalten von Thripsen gegenüber Zwiebelpflanzen zu untersuchen die mit Endophyten besiedelt waren, wurden Wahlexperimente in einem abgeschirmten Gewächshaus und im Labor durchgeführt. Weibchen von *T. tabaci* bevorzugten dabei Endophyten freie (E-) gegenüber mit Endophyten inokulierten (E+) Pflanzen. Außerdem konnte eine höhere Anzahl an Frassstellen und Eiern an E- im Vergleich zu E+ Pflanzen festgestellt werden. Die Eiablage an E+ Pflanzen war im 72 h Versuchsintervall um das 6-fache reduziert. Im Y-Olfaktometer-Biotest zeigten Thripse eine 3,6-fach höhere Präferenz für E- Pflanzen.

In einem Wahlexperiment mit einzelnen Thripsslarven saugten signifikant mehr Erst- und Zweit-Larven an Blattstücken von E- im Vergleich zu solchen von E + Pflanzen.

In einem Test zur Besiedlungspräferenz mit Thripsslarven im zweiten Entwicklungsstadium bevorzugten die Larven mit zunehmender Zeit Blattstücke von E- gegenüber E+ Pflanzen.

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Um den Einfluss der Besiedlung von Zwiebeln mit Endophyten auf induzierte Resistenz gegenüber Thripsen und auf die Replikation von Pflanzenviren zu untersuchen, wurden Experimente in abgeschirmten Gewächshäusern durchgeführt. Dabei wurde das Frassverhalten von viruliferen Thripsen und die Übertragung von *Iris yellow spot virus* (IYSV) an E- und E+ Zwiebelpflanzen charakterisiert. Die Ergebnisse zeigen, dass die Anzahl an Frassstellen an E+ im Vergleich zu E- Pflanzen signifikant reduziert war. Das Krankheitsniveau, das wöchentlich über ein Zeitraum von 4 Wochen nach Thripsbefall erhoben wurde, war signifikant geringer an E+ im Vergleich zu E- Pflanzen. Die Übertragung von IYSV wurde durch endophytische Pilze sowohl im Biotest mit ganzen Pflanzen als auch mit Blattscheiben um den Faktor 2,5 reduziert.

Die Ergebnisse zeigen einen möglichen Nutzen von endophytischen Pilzen nach der Besiedlung von Zwiebelpflanzen und bei der Abwehr von Thripsschäden und Virus-Infektionen. Weitere Studien sind notwendig um zu untersuchen, ob dieser durch Endophyten vermittelte Schutz gegenüber Thripsen an Zwiebeln, auch auf andere landwirtschaftliche Kulturen übertragen werden kann.

**Schlüsselwörter:** endophytische Pilze, Besiedlung, Zwiebel, Zwiebel-Thrips, Iris yellow spot virus IYSV, Frassverhalten, Eiablage, induzierte systemische Resistenz, Wahlversuch, multitrophe Interaktionen



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## ABBREVIATIONS

BCAs	Biocontrol Agents
BYDV	Barley Yellow Dwarf Virus
CRBD	Randomized Complete Block Design
EPF	Entomopathogenic Fungi
HCDA	Horticultural Crops Development Authority
HSAUR	Handbook of Statistical Analyses Using R
ICIPE	International Centre of Insect Physiology and Ecology
IPM	Integrated Pest Management
IYSV	Iris Yellow Spot Virus
L.	Linnaeus
<i>Lme4</i>	Linear Mixed Effects
MASS	Modern Applied Statistics with S
NaOCL	Sodium Hypochlorite
PDA	Potato Dextrose Agar
USD	United States Dollar
ZYMV	Zucchini Yellow Mosaic Virus

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## CHAPTER 1

### GENERAL INTRODUCTION

#### 1.0 Economic importance of onion

The bulb onion, *Allium cepa* L. (Asparagales: Amaryllidaceae), is an important vegetable crop cultivated in Kenya and worldwide (Rabinowitch & Curah, 2002; HCDA, 2012). In Kenya, production of onions is estimated at 122,013 metric tonnes and valued at USD 42 million per annum (HCDA, 2012). They are important in enhanced food nutrition, employment creation, income generation in addition to providing raw material for the agro processing industries. Onion is chopped and used as an ingredient in various dishes. It contains a lachrymatory agent which contains antibiotics in addition to fungicidal, bacterial, anti cancer, anti cholesterol components and phytochemicals e.g. medicinal flavonoids (Javadzadeh et al., 2009; Narla et al., 2011).

#### 1.1 Constraints to onion production

Onion production is hindered by pests such as thrips, onion flies, army worms and cut worms and diseases such as purple blotch, powdery mildew, *iris yellow spot virus* (IYSV) among others (Schwartz, 2004; Birithia et al., 2011). Onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) is considered to be the most economically important pest of onion in Kenya and worldwide (Trdan et al., 2005; Waiganjo et al., 2008). Onion thrips causes direct damage by feeding on leaves tissues resulting in reduction of photosynthetic ability and consequently reducing onion bulb size and yield (Rueda et al., 2007; Waiganjo et al., 2008). Thrips feeding lesions also acts as a source of secondary infection by pathogenic fungi and bacteria (McKenzie et al., 1993). Onion thrips also vectors IYSV which was reported for the first time in East Africa in 2010 (Birithia et al., 2011). The virus is a major threat to both bulb and seed onion production in Eastern Africa and globally (Gent et al., 2004, 2006;

Pappu et al., 2009). Yield losses ranging from 18 to 100% have been reported on onion due to thrips and tospoviruses (Waiganjo et al., 2006; Birithia et al., 2014).

## **1.2 Endophytic fungi**

Endophytic fungi are microorganisms that live at some time of their life-cycle within tissues of healthy plants without causing apparent signs of their presence to their hosts (Petrini, 1991). Collectively, research suggests that, most, if not all, plants in natural ecosystems are symbiotic with fungal endophytes (Petrini, 1986; Arnold et al., 2003). Endophytic fungi have been reported in different climatic conditions ranging from temperate to tropical (Sánchez Márquez et al., 2011) and in different plant groups i.e. grasses (Kuldau & Bacon, 2008), agricultural crops (Akello, 2012), tropical trees (Arnold et al., 2003) and soils (Rodriguez et al., 2008). Agro-ecologically, there are differences in the distribution and abundance of fungal endophytes. In Kenya, tropical highland region harbours more endophytes diversity as compared to moist transitional and dry transitional agro-ecological zones (Akello, 2012). In agricultural systems, many abiotic and biotic factors are modified by management techniques, which strongly impact fungal communities. For instance, studies have shown that practices such as tillage, monocropping, and fertilization negatively influence the abundance and diversity of fungi (Helgason et al., 1998; Verbruggen et al., 2012).

Endophytes may colonize plants by means of horizontal transmission, when leaves accumulate numerous infections shortly after emergence by means of epiphytic germination of fungal propagules, followed by cuticular penetration or entry through stomata, or vertically when endophytes grow systematically throughout roots, stems and leaves and infect the seed progeny of an infected plant (Zabalgogeoazcoa, 2008). The relationship of endophytes with single or multiple plant hosts can be described in terms of host-specificity (endophytes restricted to a single host or group of related species), host-recurrence (the frequent

occurrence of an endophyte on a particular host), host selectivity (an endophyte forms relationship with two host species but demonstrates preference for one) (Zhou & Hyde, 2001). In fungal endophytic relationships, the plant provides the fungus with nutritional and environmental requirements (Rodriguez et al., 2009). On the other hand, endophytes elevate host protection against pathogens, herbivores, drought, stimulates seed germination and plant growth (Akello, 2012). The techniques that are routinely employed for endophyte studies generally involve three basic steps: (1) surface sterilization of plant tissue to eliminate any fungi and other microorganisms on the host surface, (2) isolation of fungal endophytes growing from samples placed onto nutrient agar, and (3) identification of the endophytes based on morphological and molecular characteristics (Muvea et al., 2014).

### **1.3 Endophyte-arthropod interactions**

Endophytic fungi play an important role in shaping of plant-herbivore interactions (Rodriguez et al., 2009). Many fungal endophytes produce secondary metabolites and some of these compounds are antifungal, antibacterial and antiviral which strongly inhibit the growth of other microorganisms (Wang et al., 2004; Vega et al., 2009; Ownley et al., 2010). Some of the mycotoxins that have been verified to be produced by endophytic microorganisms are N-formil, lolines, peramine, lolitrem B and ergovaline (Ball et al., 2011). However, different endophyte species produce different types of alkaloids and the endophytes sporulation abilities affect their impact on the levels of insect herbivory and damage caused to plants (Clement et al., 2005; Tintjer & Rudgers, 2006). Thus, an endophyte species may produce different types of alkaloid depending on the plant hosting it. For example, *Epichl e festucae* produces ergovaline and lolines in *Festuca gigantea* (L.) Vill. while, in *Festuca glauca* Vill., it produces ergovaline and peramine (Siegel & Bush, 1996). The lolines are mainly active against insects and do not affect mammals (Dahlman et al., 1991). The

pyrrolopyrazine alkaloid, peramine, is a feeding deterrent to insects (Rowan, 1993). Potential endophytes from genus *Beauveria*, *Metarhizium*, *Trichoderma* have been isolated from plants, soil and insects (Bing & Lewis, 1993; Posada & Vega, 2005, Vega et al., 2009; Akello, 2012). In insect pest management, fungal endophytes have been screened and tested on a wide variety of crops in screenhouse and/or field and found to reduce pest population, deter insect feeding as well as influence oviposition preference and performance of stem boring, sap sucking, chewing and leaf mining insects (Bing & Lewis, 1991; Cherry et al., 2004; Jallow et al., 2008; Vega et al., 2008; Akutse et al., 2013; Muvea et al., 2014). For instance, *Beauveria bassiana* isolate ARSEF 3113 significantly reduced tunneling by *Ostrinia nubilalis* Hübner and it persistent within maize plant until harvesting (Bing & Lewis, 1991). Larvae of the bluegrass webworm *Parapediasia teterrella* Zincken, were observed to prefer endophyte-free over endophyte colonized tall fescue plants in a screenhouse (Richmond et al., 2007). In another feeding assay, Faeth & Hammon (1997) observed that larvae of *Cameraria* sp. nov. Davis (Lepidoptera: Gracillariidae) develop more slowly inside leaf mines injected with *Plectophomella* sp. nov. (Coelomycetes) endophyte compared to larvae in the controls. Similarly, Akutse et al. (2013) revealed that female leaf miners had an ovipositional preference for endophyte-free plants. Apart from these, endophytic fungi are known to slow the reproductive rates as well as reduce fecundity and longevity of insects (Gurulingappa et al., 2010). Feeding of *Aphis gossypii* Glover on *Beauveria bassiana* Balsamo Vuillemin or *Lecanicillium lecanii* Zimmerman colonized cotton leaves slowed the aphid reproduction (Gurulingappa et al., 2010). In a field experiment, inoculation of maize plants with endophytic *B. bassiana* affected larval development and reduced damages caused by stem borers *O. nubilalis* and *Sesamia calamistis* Hampson (Bing & Lewis, 1991; Cherry et al., 2004). Endophyte inoculation of plants has been reported to reduce virus transmission and replication on plants. For instance, inoculation of meadow ryegrass with *Neotyphodium* sp.

endophytes has been reported to reduce the population of aphid vectors and protect the plant from *barley yellow dwarf virus* (BYDV) infections (Lehtonen et al., 2006). Whereas these findings provide insights into alternative means of combating insect pest populations and the viruses they transmit, the research done so far has focused more on clavicipitaceous endophytes in grass systems (Kuldau & Bacon, 2008; Rodriguez et al., 2009). This is possibly so because grass family has greatly contributed to development of humankind and is considered to be the most important plant family in the world (<http://www.fao.org>). They are adapted to a wide range of environmental conditions including extreme cold, warmer and drier habitats, high soil salinity, and are also tolerant to low agricultural inputs (<http://www.fao.org>). To date, there is still limited information on the role of non clavicipitaceous endophytes on insect performance. For instance, several species of *Trichoderma* and *Beauveria* isolates are currently being used as plant-growth promoters or for cross protection against plant pathogens (Harman et al., 2004; Jaber & Selim, 2014). Seed application of *B. bassiana* 11-98 resulted in endophytic colonization of tomato and cotton seedlings and protection against plant pathogenic *Rhizoctonia solani* Kühn and *Pythium myriotylum* Dreschler (Ownley et al., 2008). Yet nothing is known about endophytes biocontrol potential against onion thrips and tospoviruses they transmit. Therefore, an innovative approach for maximizing the beneficial effects of fungal endophytes needs to be developed.

#### **1.4 Problem statement and justification**

Onion thrips is regarded as the most economically important pest of onions due to their direct feeding damage and transmission of tospoviruses. Currently most farmers rely on a wide range of foliar application of insecticides to control thrips (Gachu et al., 2012). However, the technique is ineffective as is often associated with high cost of production, effects to the



environment and human health and development of pest resistance (Martin et al., 2003; Gachu et al., 2012). Moreover, large numbers of thrips are always protected between the inner leaves of onion plants and have many overlapping generations (Nault & Shelton, 2010). Hence, an integrated pest management (IPM) approach which is less reliant on insecticides needs to be developed for thrips and tospovirus management.

Entomopathogenic fungi (EPF) are considered as important biocontrol agents (BCAs). They are traditionally applied in an inundative approach (Ekesi & Maniania, 2002), but recent studies have shown that EPF play diverse roles in nature including as endophytes (Vega et al., 2009). Fungal endophytes are microorganisms that colonize internal plant tissues without causing any symptomatic effects to the host plant (Rodriguez et al., 2009). They can play symbiotic roles such as antagonists to plant diseases, beneficial rhizosphere colonizers, increased drought tolerance and plant-growth promoters (Rodriguez et al., 2009; Vega et al., 2009; Gao et al., 2010; Jaber & Selim, 2014). For instance, endophytic colonization of banana stem by *B. bassiana* has been shown to affect larval development and survivorship of banana weevil, thus reducing plant damage (Akello et al., 2008). Gurulingappa et al. (2010) also reported that feeding by *Aphis gossypii* Glover on cotton leaves colonized by either *B. bassiana* or *Lecanicillium lecanii* Zimmermann slowed aphid reproduction and consumption of wheat leaves. Lehtonen et al. (2006) reported reduction of aphids population and virus infections in endophyte-inoculated plants compared to endophyte-free plants. Jaber & Salem, (2014) evaluated mechanical transmission of *zucchini yellow mosaic virus* on cucurbits plants inoculated with *B. bassiana* isolates and reported a reduced disease incidence and severity on endophyte inoculated plants. Advantages of the application of endophytes over conventional foliar application of fungal entomopathogens are the ability to colonize plants systemically, thereby offering continuous protection and enhanced persistence (Bing & Lewis, 1991; Akello et al., 2008) and considerably low inoculum is required (Athman, 2006).

Elucidating the mechanisms of colonization of onion plants by endophytic fungi, their impacts on the biology of *T. tabaci*, influence on thrips behavior, virus transmission and virus replication are key research needs for formulation of sustainable integrated pest management (IPM) strategies. Therefore, the aim of this study is to generate information on the effect of fungal endophytes on thrips and tospovirus epidemiology. The following were the objectives and hypotheses of this study.

## **1.5 Overall objective**

This study was undertaken to determine the effect of fungal endophytes on thrips and tospovirus epidemiology

### **1.5.1 Specific objectives**

1. To determine colonization of onions by endophytic fungi and their impacts on the biology of *Thrips tabaci*.
2. To determine behavioral responses of *Thrips tabaci* Lindeman to endophyte inoculated onion plants.
3. To determine if endophytic colonization of onions induces resistance against thrips and virus replication.

## **1.6 Hypotheses**

1. Colonization of onions by endophytic fungi and their impacts on the biology of *Thrips tabaci* is the same.
2. Behavioral response of *Thrips tabaci* is not influenced by endophyte inoculation of onion plants.
3. Endophytic colonization of onions does not induce resistance against thrips and virus replication.

## CHAPTER 2

### COLONIZATION OF ONIONS BY ENDOPHYTIC FUNGI AND THEIR IMPACTS ON THE BIOLOGY OF *THRIPS TABACI*

#### ABSTRACT

Endophytic fungi, which live within host plant tissues without causing any visible symptom of infection, are important mutualists that mediate plant-herbivore interactions. *Thrips tabaci* Lindeman is one of the key pests of onion, *Allium cepa* L., an economically important agricultural crop cultivated worldwide. However, information on endophyte colonization of onions and their impacts on the biology of thrips feeding on them are lacking. We tested the colonization of onion plants by selected fungal endophyte isolates with two inoculation methods. The effects of inoculated endophytes on *T. tabaci* infesting onion were also examined. Seven fungal endophytes used in our study were able to colonize onion plants either by seed or seedling inoculation methods. Seed inoculation resulted in 1.47 times higher mean percentage post-inoculation recovery of all the endophytes tested as compared to seedling inoculation. Fewer thrips were observed on plants inoculated with *Clonostachys rosea* ICIPE 707, *Trichoderma asperellum* M2RT4, *Trichoderma atroviride* ICIPE 710, *Trichoderma harzianum* 709, *Hypocrea lixii* F3ST1 and *Fusarium sp.* ICIPE 712 isolates as compared to those inoculated with *Fusarium sp.* ICIPE 717 and the control treatments. Onion plants colonized by *C. rosea* ICIPE 707, *T. asperellum* M2RT4, *T. atroviride* ICIPE 710, and *H. lixii* F3ST1 had significantly lower feeding punctures as compared to the other treatments. Among the isolates tested, the lowest numbers of eggs were laid by *T. tabaci* on *H. lixii* F3ST1 and *C. rosea* ICIPE 707 inoculated plants. These results extend the knowledge on colonization of onions by fungal endophytes and their effects on *Thrips tabaci*.

**Keywords:** Fungal endophytes, onion thrips, colonization, feeding, oviposition

## 2.0 Introduction

In Kenya, onions *Allium cepa* L. (Asparagales: Amaryllidaceae), are grown in all regions by both large- and small-scale farmers, where they have a ready domestic and regional market (Narla et al., 2011). Onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae), is considered the most economically important pest of onion worldwide (Nawrocka, 2003; Diaz-Montano et al., 2011). In Kenya, it is present in all onion growing areas and can cause up to 59% loss in yield (Waiganjo et al., 2008). Currently, growers manage thrips by applying insecticides which are ineffective due to the cryptic feeding behavior of thrips, overlapping generations and insecticides resistance (Martin et al., 2003; Morse & Hoddle, 2006). Therefore, an integrated approach that includes the use of entomopathogens, cultural practices, host plant resistance and judicious use of insecticides is needed (Maniania et al., 2003; Shiberu et al., 2013).

Entomopathogenic fungi (EPF) are considered as important biocontrol agents (BCAs). They are traditionally applied in an inundative approach (Ekesi & Maniania, 2002), but recent studies have shown that EPF play diverse roles in nature including as endophytes (Vega et al., 2009). Indeed, the endophytic niche in a plant is a rich source of microorganisms that can directly and indirectly promote plant growth and development through plant defence against herbivorous insects (Jallow et al., 2004) and plant pathogens (Stone et al., 2000; Ownley et al., 2008) due to their ability to produce secondary metabolites with biocidal activity (Stone et al., 2004; Strobel et al., 2004). On a wide variety of crops, fungal endophytes have been reported to deter feeding, oviposition and performance of stem boring, sap sucking, chewing, and leaf mining insects (Cherry et al., 2004; Jallow et al., 2004; Qi et al., 2011; Akutse et al., 2013). For example, endophytic colonization of banana by *B. bassiana* significantly reduced larval survivorship of banana weevil, *Cosmopolites sordidus* Germar, resulting in 42–87% reduction in plant damage (Akello et al., 2008). Reduction in feeding

and reproduction by aphids, *A. gossypii* has also been reported on cotton plants which were endophytically colonized by either *B. bassiana* or *L. lecanii* (Gurulingappa et al., 2010).

Advantages of the endophytic lifestyle over conventional foliar application of fungal entomopathogens (Maniania et al., 2002, 2003) are the ability to colonize plants systemically, thereby offering continuous protection and enhanced persistence (Akello et al., 2008). Moreover, considerably low inoculum is required when applied as seed treatments (Athman, 2006). However, colonization of a host plant by an endophyte is influenced by the inoculation method, species of fungal endophytes and the host plant species itself. Based on the inoculation technique, the endophytes differ in their ability to colonize different plant parts and persist over a crop growth cycle (Bing & Lewis, 1991; Akello et al., 2007; Brownbridge et al., 2012). Akello et al. (2007) reported a higher colonization of tissue cultured bananas by *B. bassiana* through dipping roots and rhizomes in a conidial suspension as compared to injecting a conidial suspension into the plant rhizome and by growing the plants in sterile soil mixed with *B. bassiana*-colonized rice substrate. Bing & Lewis (1991) reported improved colonization of corn plants through foliar spray of conidia as compared to injection of conidia suspension. They also demonstrated the ability of *B. bassiana* to invade maize plants through the epidermis, thereafter persisting in the plant through the entire growing season which conferred crop resistance against damage by European corn borer. However, such information on endophyte colonization of onions and their impact on thrips infesting onions are not available. Hence, this study aimed to evaluate the efficacy of seed and seedling inoculation methods on colonization of onion plants by fungal endophytes and further assess their impact on infestation by onion thrips, their feeding and oviposition.

## 2.1 Materials and Methods

### 2.1.1 Fungal isolates

Five fungal isolates (*Clonostachys rosea* ICIPE 707, *Trichoderma atroviride* ICIPE 710, *Trichoderma harzianum* ICIPE 709, *Fusarium* sp. ICIPE 712, and *Fusarium* sp. ICIPE 717 with GenBank Accession Nos: KJ619987, KJ619990, KJ619989, KJ619992 and KJ619993, respectively) were used in this study. The endophytes were isolated from onion plants asymptomatic of any pathogenic infection, collected during a field survey conducted in different altitudinal gradients of Kenya namely; Nakuru (00.01 N 36.26 E, 2000 m.a.s.l.), Loitokitok (02.71 S 37.53 E, 1200 m.a.s.l.) and Kibwezi (02.25 S 38.08 E, 825 m.a.s.l.) as detailed in the GenBank Accessions mentioned above. Two fungal isolates (*Hypocrea lixii* F3ST1 and *Trichoderma asperellum* M2RT4) isolated from the above ground parts of maize and sorghum and previously reported endophytic on maize and bean seedlings (Akello, 2012) were also included. Conidia were obtained from two-week old cultures grown on potato dextrose agar (PDA) plates. The conidia were harvested by scraping the surface of sporulating cultures with a sterile scalpel. The harvested conidia were then placed in universal bottles with 10 ml sterile distilled water containing 0.05 % Triton X-100 and vortexed for 5 min to produce homogenous conidial suspensions. The conidial concentration was determined using Neubauer haemocytometer. The conidial concentration was adjusted to  $1 \times 10^8$  conidia mL<sup>-1</sup> through dilution prior to inoculation of seeds and seedlings.

To assess the viability of the conidia, 100 µL of conidial suspension was inoculated to the surface of two fresh plates of PDA for each isolate. A sterile microscope cover slip (2 × 2 cm) was placed on top of the agar in each plate before incubation. The inoculated plates were incubated for 24 h at 20°C. The percentage conidial germination was assessed by counting the number of germinated conidia out of 100 in one randomly selected field. Conidia were considered as germinated when germ tubes exceeded half of the diameter of the conidium.

The percent germination of the different isolates exceeded 90% which is recommended by (Parsa et al., 2013).

### **2.1.2 Insects**

Initial cultures of *T. tabaci* were field-collected from onion plants at the International Centre of Insect Physiology and Ecology (*icipe*) organic farm. Thrips were reared on snow peas, *Pisum sativum* L. (Fabales: Fabaceae), for over 30 generations in ventilated plastic jars at the *icipe*'s insectary at  $25 \pm 2^\circ\text{C}$ , 50–60% relative humidity (RH), 12 h L: 12 h D photoperiod.

### **2.1.3 Seed and seedling inoculation of fungal endophytes**

Onion can be established using either direct seed sowing or seedling transplanting (Infonet-biovision, 2013). Seeds of onion (var. Red Creole) were surface-sterilized in 70% ethanol and then immersed in 2% NaOCl for 2 and 3 min, respectively. The seeds were finally rinsed three times using sterile distilled water to ensure epiphytes were not carried on the seed surface. To confirm the efficiency of the surface sterilization methods, 100  $\mu\text{l}$  of the last rinse water (Schultz et al., 1998; Parsa et al., 2013) was spread onto potato dextrose agar and plates were incubated at  $20^\circ\text{C}$  for 14 days. The absence of fungal growth on the medium confirmed the reliability of the sterilization procedure. The seeds were then placed on sterile filter paper to dry for 20 min before being divided into two portions, one for the seed and the other for the seedling inoculation. For seed inoculation, 10 g of surface-sterilized seeds were subdivided into eight equal portions whereby seven portions were individually soaked in a conidial suspension of  $1 \times 10^8$  conidia  $\text{ml}^{-1}$  of each isolate for 10 hours. In the control, the eighth portion was soaked in sterile distilled water containing 0.05% Triton X 100. The inoculated seeds were air dried on a sterile paper towel for 20 min and then transferred in plastic pots (8 cm diameter  $\times$  7.5 cm height) containing sterile planting substrate. The



substrate was a mixture of red soil and livestock manure in a 5:1 ratio and was sterilized in an autoclave for 2 hr at 121°C and allowed to cool up to ambient temperature before being used. Seeds were sown 1 cm below the surface of the substrate and maintained at room temperature (~25°C and 60% RH) in the screen house. After germination, seedlings were thinned to one per pot for all the eight treatments and the four replicates. The plants were watered once per day in the evening. No additional fertilizer was added to the planting substrate.

The second portion of surface-sterilized seeds, as described earlier, was raised in a plastic bucket (30 cm diameter × 28 cm height) with sterile planting substrate and maintained in a screenhouse at room temperature (~25°C and 60% RH) for one month before transplanting. Before transplanting, seedlings (height 7 -8 cm) were watered and uprooted carefully to minimize damage to roots. After uprooting, the plants were shaken gently to dislodge excess soil on the roots, which were further washed with running tap water. Roots of four seedlings with well developed shoots were dipped in each of the seven endophyte conidial suspensions of  $1 \times 10^8$  conidia ml<sup>-1</sup> for 10 hours. Control plants were dipped in sterile distilled water containing 0.05 % Triton X-100. The inoculated seedlings were transplanted in pots containing sterile soil as described earlier. The experimental design was Completely Randomized Block Design (CRBD) with four replicates. The plants were maintained under similar conditions as those inoculated through the seeds.

#### **2.1.4 Assessment of colonization**

To determine colonization by inoculated fungal isolates, onion plants were carefully uprooted from the pots after 50 and 70 days for inoculated seeds and seedlings, respectively. Plants were then washed gently with running tap water. Leaves, stems and roots were separated from each plant. Sections of leaves were sampled from the middle and outer leaves of the plant while the whole lengths of stems and roots were used for sampling. The sampled plant parts were then surface-sterilized by dipping them in 70% ethanol and then immersing in 2% NaOCl for 2 and 3 min, respectively, and rinsed three times using sterile distilled water. The final rinse water was plated on PDA to confirm elimination of epiphytic microorganisms as described earlier. The surface-sterilized plant parts were then aseptically cut into 1 cm lengths under a laminar flow hood. Five randomly selected pieces were placed in uniform distribution on PDA plates amended with antibiotics (tetracycline and streptomycin sulfate salt at 0.05 %) (Dingle & McGee, 2003) and incubated in the dark at 25° C for 10 d, after which the presence of fungal growth was observed. Positive colonization was scored by counting the number of pieces of the different plant parts with growth of inoculated endophyte. To confirm whether the growing endophytes were the ones initially inoculated, slides prepared from the mother plates were used for comparison and morphological identification.

#### **2.1.5 Effects of endophytically-colonized onion plants on proportion of thrips observed on plants, feeding punctures and oviposition**

Seed inoculation technique was found to be effective for colonization and was therefore adopted for this study. Seeds inoculated with all fungal isolates and a control were transplanted in smaller pots (diameter 8 cm) with one plant per pot until 3- to 5-leaf stage before being used in the experiment. Plants with four fully grown leaves were exposed to one-day old presumably mated adult female thrips (10 individuals) for 72 h in Plexiglas cages

(30cm × 30cm × 25 cm) and were maintained at  $25 \pm 2^{\circ}\text{C}$ , 50 - 70% RH and 12L: 12D photoperiod. A total of four cages were used for each treatment. After 72 h, all adult thrips observed on the plants were recorded. The individual plants were cut and placed in labeled polythene paper bags for later quantification of thrips feeding and oviposition activities. Two leaves from each plant were cut each into three sections of 4 cm each; from the base, middle and tip of the leaf. The number of feeding punctures were counted under a stereo microscope and recorded. The sections were stained in boiling lactophenol-acid fuchsin solution (Nyasani et al., 2012) for 30 – 40 mins. After staining, the leaves were placed in 90 mm Petri dishes for 1 h before being destained. Destaining was done by immersing the leaves in warm water for three minutes after which the eggs were counted under a stereo microscope. Treatments were randomized in complete block design and the experiment replicated four times. Verification of colonization of onions by the endophytes was performed at the end of the experiment.

## 2.2 Data analysis

Binary data on colonization (presence or absence) were fitted in a generalized linear mixed model assuming binomial distribution error and logit using package *lme4* (Bates et al., 2013). Treatments were considered as fixed effects and the plant pieces nested within the plant as random effects. The extent of fungal colonization (%) of host plant parts was calculated as follows:

$$\textit{Colonization} = \frac{\text{Number of pieces exhibiting fungal outgrowth}}{\text{Total number of pieces plated out}} \times 100$$

The numbers of thrips observed on the onion plants were recorded for all treatments and replicates. Analysis was performed using logistic regression model which was fitted to the data on proportion of thrips recovered 72 h post exposure using package *HSAUR* (Everitt & Hothorn, 2013). The number of feeding punctures on each leaf section were determined and summed up per plant before staining the leaves for eggs count. All count data on feeding and

oviposition of *T. tabaci* were checked for normality and homogeneity of variance using Shapiro-Wilk and Levene tests, respectively, before analysis by negative binomial regression package MASS (Venables & Ripley, 2002). The negative binomial distribution was chosen, based on its biological appropriateness in handling over dispersion in count data. P-values of <0.05 were considered as significant. All the analysis were performed in R 2.15.2 statistical software (R Development Core Team, 2012).

## 2.3 Results

### 2.3.1 Seed and seedling colonization of onion plants by fungal endophytes

The viability test yielded >90% germination of conidia for all the isolates. Since the final rinse water did not show any sign of fungal growth on the media, it was concluded that the surface sterilization technique used was effective. All the tested fungal isolates were able to colonize onion plants following seed or seedling inoculation (Figs. 1, 2). However, the extent of colonization of the different plant parts depended on the inoculation method and the fungal isolate. Seed inoculation resulted in 1.47 times higher mean percentage post-inoculation recovery of all the endophytes tested as compared to seedling inoculation ( $F = 11.13$ ;  $df = 1, 3$ ;  $P = 0.002$ ). For example, mean colonization of roots by *C. rosea* ICIPE 707 isolate was  $75.00 \pm 9.7\%$  through seed inoculation and  $29.85 \pm 3.7\%$  through seedling inoculation. Seed inoculation method resulted in higher mean post-inoculation recovery of all the endophytes tested for roots, stems and leaves;  $76.06 \pm 4.1\%$ ,  $44.24 \pm 3.6\%$  and  $44.73 \pm 5.4\%$ , respectively (Fig. 1). On the other hand, seedling inoculation recorded  $55.62 \pm 4.5\%$ ,  $31.75 \pm 5.8\%$  and  $24.65 \pm 6.8\%$  for roots, stems and leaves, respectively (Fig. 2).

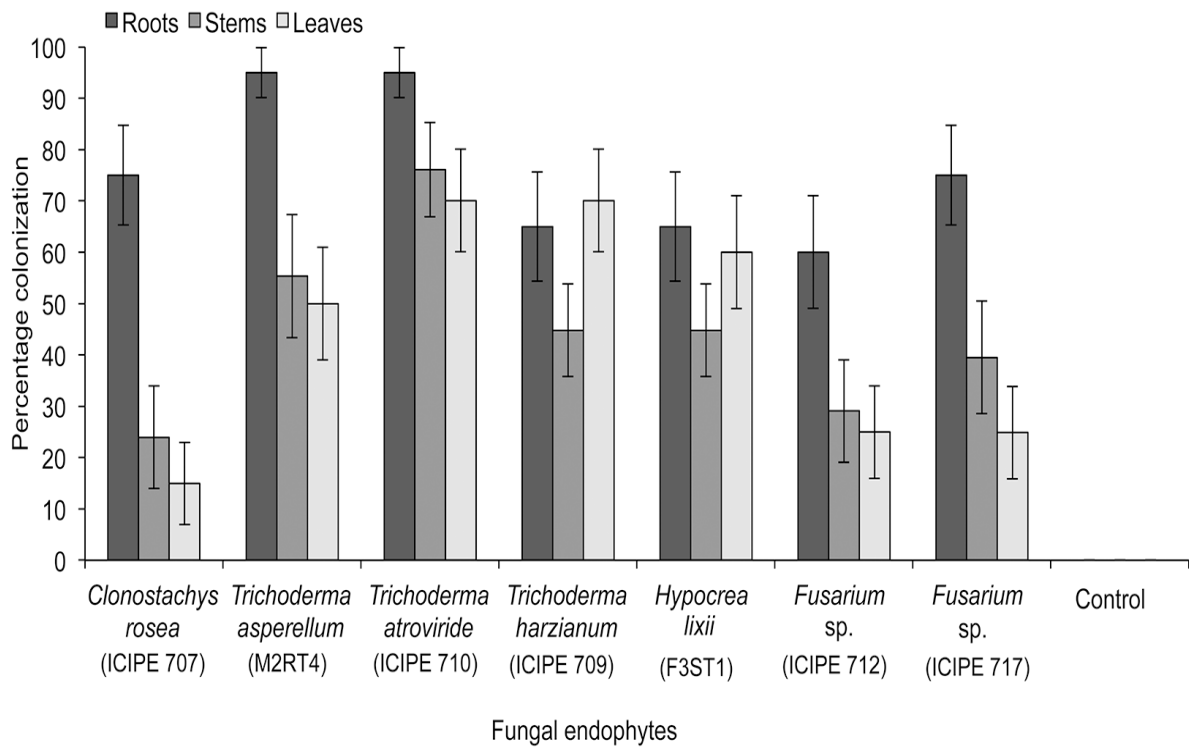


Figure 1: Endophytic colonization of onion seeds. Percentage colonization of onion plant parts (root, stem and leaves) by different fungal endophytes through seed inoculation. Data are presented as percentage mean  $\pm$  SE ( $P \leq 0.05$ ).

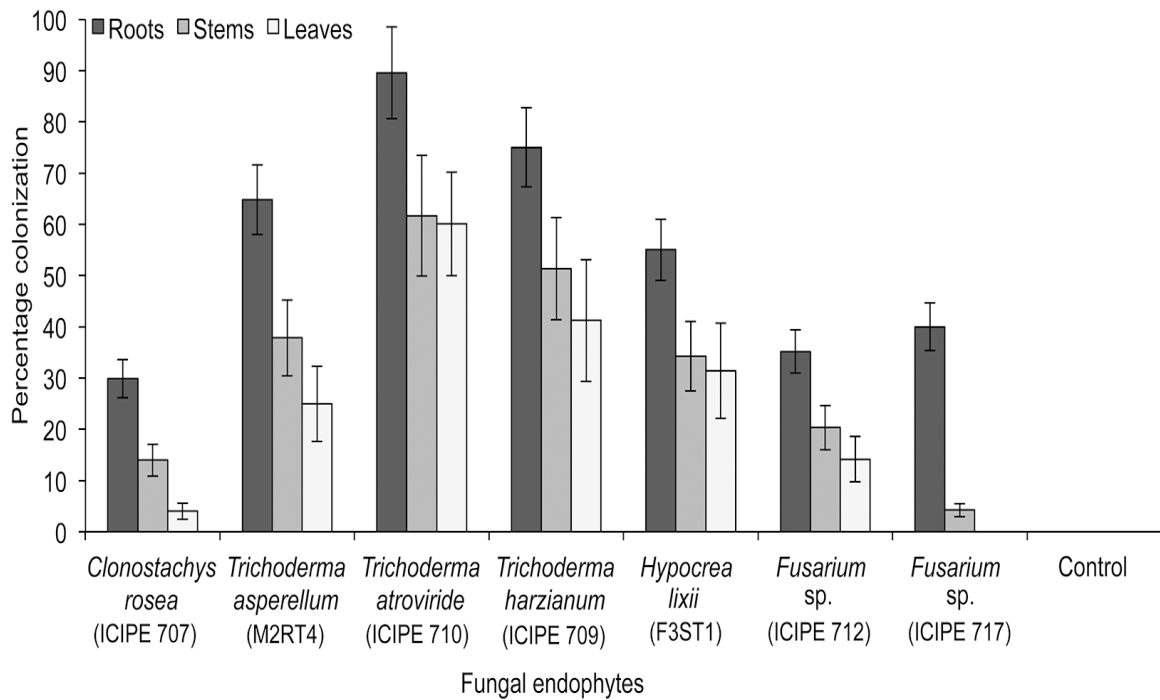


Figure 2: Endophytic colonization of onion seedlings. Percentage colonization of onion plant parts (root, stem and leaves) by different fungal endophytes through seedling inoculation. Data are mean  $\pm$  SE ( $P \leq 0.05$ ).

### 2.3.2 Effect of endophytically-colonized onion plants on thrips proportion, feeding and oviposition

The treatments had a significant effect on the proportion of thrips observed on the onion plants 72 h post-exposure ( $\chi^2 = 87.79$ ,  $df = 7$ ,  $P < 0.001$ ) (Fig. 3). Overall *Hypocrea lixii* outperformed all the other treatments in affecting the proportion of thrips on the plants. Fewer thrips were observed on plants inoculated with *C. rosea* ICIPE 707, *T. asperellum* M2RT4, *T. atroviride* ICIPE 710, *T. harzianum* ICIPE 709, *H. lixii* F3ST1 and *Fusarium sp.* ICIPE 712 isolates as compared to those inoculated with *Fusarium sp.* ICIPE 717 and the control treatments (Fig. 3). The number of feeding punctures by *T. tabaci* was significantly lower in all the endophyte inoculated plants as compared to the control treatment ( $F = 22.71$ ;  $df = 7, 21$ ;  $P < 0.001$ ;  $n = 4$ ) (Fig. 4). Plants colonized by isolates *C. rosea* ICIPE 707, *T.*

*asperellum* M2RT4, *T. atroviride* ICIPE 710, and *H. lixii* F3ST1 had significantly lower number of feeding punctures as compared to the other treatments (Fig. 4).

Highest number of eggs ( $18.6 \pm 2.2$ ) were oviposited by *T. tabaci* in the control plants than in all other endophytically-colonized plants ( $F = 16.75$ ;  $df = 7, 21$ ;  $P < 0.001$ ) (Fig. 5). Among the isolates tested, the lowest numbers of eggs were laid by *T. tabaci* on *H. lixii* F3ST1 and *C. rosea* ICIPE 707 inoculated plants. Plants inoculated with *T. asperellum* M2RT4 and *T. atroviride* ICIPE 710 isolates were equally effective in their capacity to reduce egg laying by *T. tabaci*. *Fusarium* sp. ICIPE 717 colonized plants showed about 6 times higher number of eggs as compared to *H. lixii* F3ST1 (Fig. 5).

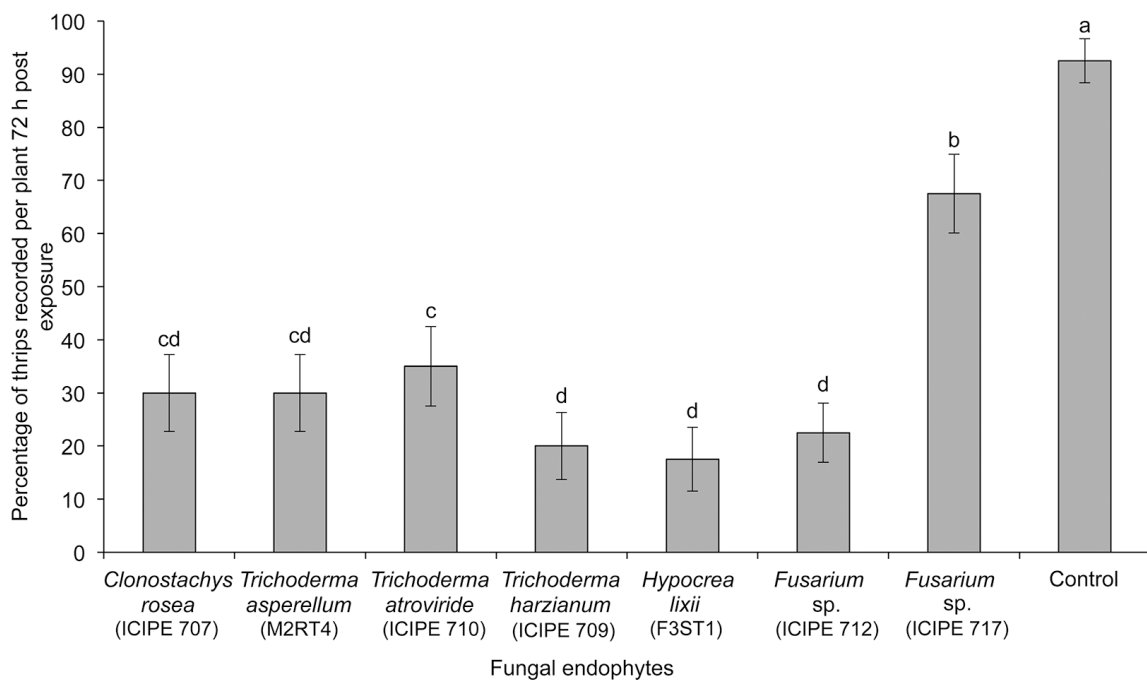


Figure 3: Effect of endophytically colonized onion plants on the proportion of adult *Thrips tabaci*. An evaluation of fungal endophytes for their effect on proportion of thrips settling on inoculated onion plants after 72 h. Bars indicate means  $\pm$  SE at 95% CI. Means followed by the same letter indicate no significant differences between treatments.

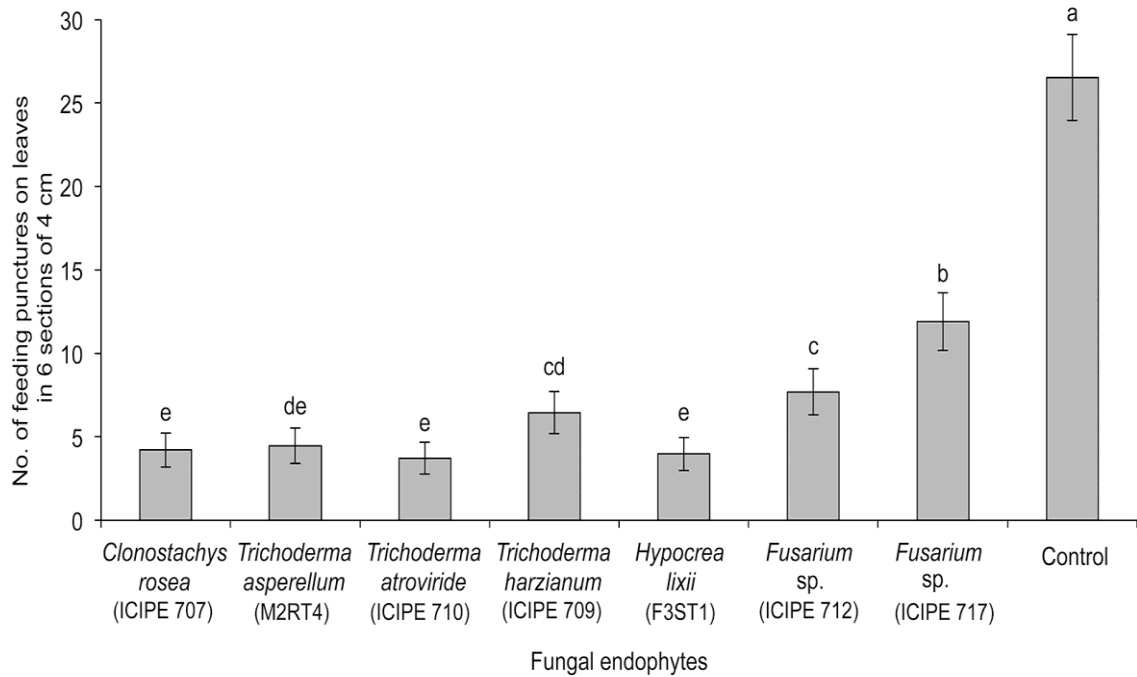


Figure 4: Effect of endophytically-colonized onion plants on feeding punctures by adult *Thrips tabaci*. The figure quantifies mean feeding activity by *Thrips tabaci* exposed for 72 h on onion plants inoculated with different fungal endophytes. Bars indicate means  $\pm$  SE at 95% CI. Means followed by the same letter indicate no significant differences between treatments.



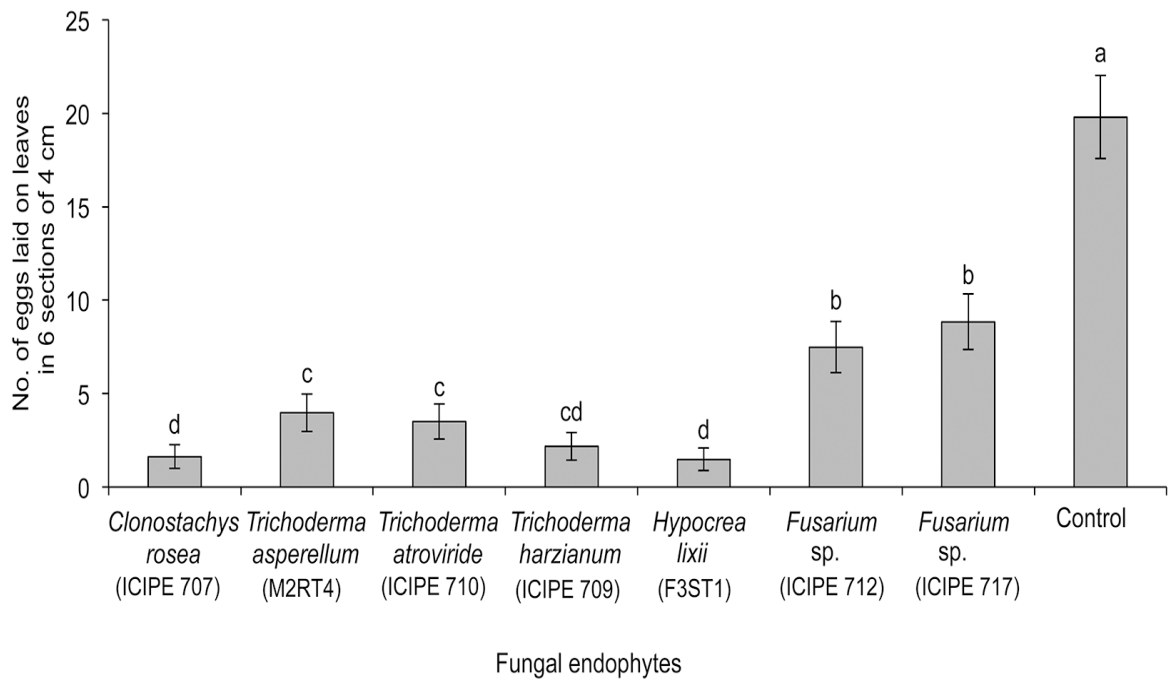


Figure 5: The figure shows the mean number of eggs laid by *Thrips tabaci* on onion plants endophytically colonized by different fungal isolates. Bars indicate means  $\pm$  SE at 95% CI. Means followed by the same letter indicate no significant differences between treatments.

## 2.4 Discussion

Plant colonization depended on inoculation method used. For instance, seed inoculation method resulted in superior colonization of onion plants as compared to the seedling inoculation. The difference in colonization between the two may be explained in part by a reduced capacity of uninoculated seedlings to enhance endophyte proliferation due to transplantation shock (Barrows & Roncadori, 2014). Moreover, endophyte inoculation at seed stage could have the advantage of colonizing both seed radical and the plumule which are close to one another in the seed. Tefera & Vidal (2009) reported that seed inoculation of sorghum plants with *B. bassiana* resulted to good endophyte colonization in vermiculate and sterile soil substrates. Seed inoculation could be advantageous in terms of low inoculum requirement as compared to augmentative sprays (Athman, 2006). Further seed treatment

could provide opportunities for endophytic fungi colonization at the young seedlings stage for early protection and enhanced seedlings health.

Backman & Sikora (2008) outlined that, integrated pest management on seeds reduces costs and environmental impact, while allowing the biological agent to build up momentum for biological control. Posada et al. (2007) found that direct injection of *B. bassiana* conidial suspensions had the highest post-inoculation recovery in coffee seedlings than foliar sprays, stem injections, or soil drenches. Our results show that there were differences in the level of colonization of different plant parts by fungal isolates. For instance, roots sections had higher colonization as compared to stems and leaves. These differences could be due to tissue specificity exhibited by endophytic fungi and their adaptation to particular physiological conditions of the plants (Guo et al., 2008). Similar results were reported on French beans and Faba beans (Akutse et al., 2013) and coffee (Posada et al., 2007).

Among the endophytes that colonized onions plants, *C. rosea* ICIPE 707, *H. lixii* F3ST1, *T. harzianum* ICIPE 709, *T. atroviride* ICIPE 710, and *T. asperellum* M2RT4 had significantly low proportion of thrips, number of feeding punctures and eggs. However, isolate *H. lixii* F3ST1 had the highest overall negative impact on *T. tabaci*. Lately, the impacts of fungal endophytes on suppression of different insects groups in different host plants are receiving increased attention (Cherry et al., 2004; Vega et al., 2008; Akutse et al., 2013). The negative effect on the proportion of thrips on the endophyte colonized plants as compared to the control could have been responsible for reduced feeding and oviposition. For instance, Akutse et al. (2013) reported that Faba beans colonized endophytically by fungal endophytes of the genus *Hypocrea* and *Beauveria* had significant negative effects on leafminer, *Liriomyza huidobrensis* (Blanchard) fitness, impacting on mortality, oviposition, emergence and longevity of the pest. Cherry et al. (2004) found a reduced number of *Sesamia calamistis* (Hampson) in *B. bassiana*-inoculated plants compared to non-inoculated plants.

Thrips are able to distinguish among plants as suitable for feeding and/or oviposition sites to ensure fitness of their progeny (Brown et al., 2002). *T. tabaci* is a key vector of *Iris yellow spot virus* (IYSV) in Kenya (Birithia et al., 2011) and the thrips densities are positively associated with IYSV incidence (Kritzman et al., 2001; Schwartz et al., 2009; Hsu et al., 2010). Hence, the reduced feeding by the thrips on endophyte colonized plants could potentially reduce the transmission of IYSV in onions. Moreover, fungal endophytes can decrease plant virus infections in plants as reported in meadow ryegrass with the *barley yellow dwarf virus* (BYDV) (Lehtonen et al., 2006). The broad array of endophyte induced defence mechanisms in plants against insect pests such as production of toxic or distasteful chemicals (Tibbets et al., 1999) and pathogenic interaction to insects (Marcelino et al., 2008) could decrease insect fitness (Akello et al., 2008) a phenomenon which needs to be further investigated.

In the present study, dead insects did not present any symptoms of mycosis. Previous studies have also revealed that dead insects recovered from endophytically-colonized plants exhibit no symptoms of fungal infection (Cherry et al., 2004; Akutse et al., 2013). The influence of endophytes colonizing onions on thrips biology in terms of observable proportion, feeding and oviposition in the present study are in accordance with the findings by (Cherry et al., 2004; Bittleston et al., 2011) on reduced feeding and by Akutse et al., (2013) on oviposition with other endophytes and pests. The reduced feeding and oviposition could have resulted due to either reduced survival of thrips or antixenotic repellence of thrips, a phenomenon that warrants further studies to unravel the underlying mechanisms such as possible release of metabolites and/or volatiles which could have effects on thrips.

## 2.5 Conclusions

In our study, isolates *C. rosea* ICIPE 707, *H. lixii* F3ST1, *T. harzianum* ICIPE 709, *T. atroviride* ICIPE 710, and *T. asperellum* M2RT4 effectively colonized the various plants parts of onion as compared to the *Fusarium* isolates. Consequently, isolate *H. lixii* F3ST1 had the most antagonistic impact on onion thrips and it could be used to develop alternative and ecologically safe management strategy for onion thrips. We conclude that, onions can be successfully inoculated especially through seeds, with different fungal endophytes. However, further studies are warranted to determine the persistence of tested endophytes in the colonized plants under natural conditions and investigate potential for vertical transmission of endophytes. Additionally, being the first report of antagonistic activity of endophytes colonizing onion against *T. tabaci*, it would be very crucial to determine the underlying mechanisms of such multi-trophic interactions.

### CHAPTER 3

#### BEHAVIORAL RESPONSES OF *THRIPS TABACI*, LINDEMAN TO ENDOPHYTE INOCULATED ONION PLANTS

##### ABSTRACT

Endophytic fungi colonize healthy plant tissues, and can in some cases induce systemic resistance to the host against biotic and abiotic stresses. In our previous study, *Hypocrea lixii* isolate F3ST1 was able to endophytically colonize onion plants and conferred to them resistance against onion thrips, *Thrips tabaci*. To further elucidate the mechanism of resistance, we examined the behavioral response of adult and larval stages of *T. tabaci* to endophyte-inoculated (E+) and endophyte-free (E-) onion plants/sections. In choice experiments, female *T. tabaci* preferred E- over E+ plants. The number of feeding punctures and eggs was more on E- than on E+ plants. Oviposition was reduced sixfold on E+ plants within a 72-h experimental period. In Y-tube olfactometer assay, thrips showed a 3.3-fold preference for E- plants. In individual larval choice experiments, significantly more first- and second-instars were found on the leaf sections of E- as compared to the E+ plants. In the settlement preference assay with groups of second-instars, more larvae preferred leaf sections from E- over E+ plants with incremental time. Our findings suggest that endophyte-colonized onion plants may trigger antixenotic repellence of *T. tabaci*, impacting on their biology. This repellence could be exploited in thrips control programmes by using endophyte-inoculated plants in the field.

**Keywords:** Endophytic fungus, *Hypocrea lixii*, thrips, choice test, induced systemic resistance

### 3.0 Introduction

The bulb onion, *Allium cepa* L. (Asparagales: Amaryllidaceae), is an important vegetable cultivated worldwide (Rabinowitch and Currah, 2002). In Kenya, production of onions is estimated at 122,013 metric tonnes and valued at USD 42 million per annum (HCDA, 2012). The major challenges to increased production of onions are unavailability of quality planting materials, diseases such as downy mildew, purple blotch and *Iris yellow spot virus*, and pests such as onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) and cut-worms (Rabinowitch and Currah, 2002; Gachu et al., 2012; BIRTHIA et al., 2014). However, *T. tabaci* is considered the most economically important pest of onion in Kenya and globally (Nawrocka, 2003; Trdan et al., 2005; Gachu et al., 2012) as it causes direct feeding and oviposition damage on plant leaves (Childers, 1997), in addition to vectoring tospoviruses (BIRTHIA et al., 2011, 2014).

Thrips are commonly managed through indiscriminate application of synthetic pesticides. However, most of these pesticides are ineffective due to a number of factors, including development of resistance, pest presence in cryptic habitats and overlapping generations (Martin et al., 2003; Cloyd, 2009; Nault and Shelton, 2010). Several sustainable methods of control have been developed in recent years to reduce thrips damage in horticultural crops. As a result, more active and selective control applications have been introduced into crop production. One sustainable method of managing this insect pest is the use of fungal endophytes (Vega et al., 2009). Fungal endophytes are microorganisms that colonize internal plant tissues without causing any symptomatic effects to the host plant (Rodriguez et al., 2009). Muvea et al. (2014) recently demonstrated that several fungal endophytes were able to endophytically colonize onion seedlings and subsequently induced resistance by reducing feeding activity (number of feeding punctures) and oviposition by thrips. However, it

remained unclear whether this resistance was due to repellent effect to the endophyte-inoculated plants or if the adults thrips died after settling on the plants (Muvea et al., 2014).

Both repellency to endophyte-inoculated plants (Sikora et al., 2008; Clement et al., 2011) and reduced survivorship of insects feeding on endophyte-inoculated plants (Gurulingappa et al., 2010; Akutse et al., 2013) in diverse insects-plants-endophyte interactions have been documented. Webber (1981) was the first to report the role of endophyte *Phomopsis oblongata* in protecting elm trees against the beetle *Physocnemum brevilineum*. The mechanisms underlying the anti-insect properties of endophytes are attributed to the production of various volatiles- and alkaloid-based defensive compounds in the plant tissue (Cardoza et al., 2002; Faeth, 2002). These defensive compounds have been shown to influence the development, survival and behavior of insects (Kaur et al., 2013). Most studies on fungal endophytes-mediated plant resistance to insects have been carried out in turf and grazing grass systems (Saikkonen et al., 1998; Clement et al., 2011) whereas relatively little is known of the nature of the interactions between crop plants and their endophytes.

Moreover, studies on the effects of secondary plant compounds on insects have generally focused on adults (Gurulingappa et al., 2010; Thakur et al., 2013; Egger and Koschier, 2014). The possible direct effects on larvae are yet to be understood explicitly. This is of considerable interest because larval stages of thrips cause more direct feeding damage than adults due to their greater abundance on a plant (Wiesenborn and Morse, 1986). Therefore, elucidating the mechanisms underlying the onion-endophyte interactions in defense against both larvae and adult thrips is critical. Hence, this study aimed at investigating the behavioral responses of *T. tabaci* to endophyte-colonized onion plants for adults and leaf sections for larvae.

### 3.1 Materials and methods

#### 3.1.1 Fungal isolate

Endophytic fungal isolate *Hypocrea lixii* F3ST1, isolated from the aboveground parts of maize obtained from a tropical highland region was selected for this study. Conidia were obtained from two-week old cultures grown on potato dextrose agar (PDA) plates. The conidia were harvested by scraping the surface of sporulating cultures with a sterile scalpel. The harvested conidia were then placed in a universal bottle with 10 ml sterile distilled water containing 0.05 % triton X-100 and vortexed for 5 min to produce homogenous conidial suspension. The conidial concentration was determined using a Neubauer haemocytometer. The conidial concentration was adjusted to  $1 \times 10^8$  conidia  $\text{mL}^{-1}$  through dilution prior to inoculation of seeds. To assess the viability of the conidia, 100  $\mu\text{L}$  of conidial suspension was inoculated to the surface of four fresh plates of PDA. Two sterile microscope cover slips ( $2 \times 2$  cm) were placed on top of the agar in each plate before incubation. The inoculated plates were incubated for 24 h at  $20^\circ\text{C}$ . The percentage conidial germination was assessed by counting the number of germinated conidia out of 100 in one randomly selected field. Conidia were considered as germinated when germ tubes exceed half of the diameter of the conidium.

#### 3.1.2 Insects

Initial cultures of *T. tabaci* were field-collected from onion plants at the International Centre of Insect Physiology and Ecology (*icipe*) organic farm at Duduville, Nairobi, Kenya. Thrips were reared on snow peas, *Pisum sativum* L. (Fabales: Fabaceae), for over 30 generations in ventilated plastic jars at the *icipe*'s insectary at  $25 \pm 2^\circ\text{C}$ , 50–60% relative humidity (RH), 12 h L: 12 h D photoperiod.



### **3.1.3 Inoculation of fungal endophyte**

Seeds of onion (var. Red Creole) were surface-sterilized in 70% ethanol and then immersed in 2% NaOCl for 2 and 3 min, respectively. The seeds were finally rinsed three times using sterile distilled water to ensure that the seed surface was sterilized free of epiphytes. To confirm the efficacy of the surface sterilization, 100  $\mu$ l of the last rinse water was spread onto four PDA plates and incubated at 20°C for 14 days. The absence of fungal growth on the medium confirmed the reliability of the sterilization procedure (Schulz & Boyle, 2005). The seeds were then placed on sterile filter paper to dry for 20 min and subdivided into two equal portions, one for inoculation and the other to serve as the control. Surface-sterilized seeds were soaked in a conidial suspension of  $1 \times 10^8$  conidia  $\text{mL}^{-1}$  for 10 hours. In the control, seeds were soaked in sterile distilled water containing 0.05% Triton X 100. The inoculated seeds were air dried on a sterile paper towel for 20 min and then transferred to plastic pots (8 cm diameter  $\times$  7.5 cm height) containing planting substrate. The planting substrate was a mixture of red soil and livestock manure in a 5:1 ratio and was sterilized in an autoclave for 2 h at 121°C and allowed to cool down to ambient temperature before being used. Four seeds per pot were sown 1 cm below the surface of the substrate and maintained at room temperature ( $\sim$ 25°C and 60% RH) in a screen house. After germination, seedlings were thinned to one per pot and watered once per day in the evening.

### **3.1.4 Choice test using endophytically-colonized and uninoculated onion plants**

The experiment was conducted using exclusion cages made of clear Perspex sheets (75  $\times$  50  $\times$  50 cm, Fig. 1). The exclusion cages were partitioned into two choice compartments measuring 37.5  $\times$  25  $\times$  50 cm with a perforated thrips release chamber (15  $\times$  15  $\times$  50 cm) in the middle (one compartment held the inoculated plant while the other one held the control treatment). Position effects were neutralised by placing the different host plants in different

directions in the different cages. Each compartment was provided with a 15-cm-diameter window secured with thrips proof nets for ventilation. The top of each choice compartment and the thrips release chamber were covered with sliding doors to enable placement of potted plants and release of insects, respectively (Fig. 1). A plant with four fully grown leaves from each treatment was placed at the centre of each compartment. Eight hours post emergence, 20 presumably mated adult female thrips were released through the thrips release chamber using 10 ml transparent plastic vials. After 72 h, all adult thrips observed on each treatment (plants) were recorded. The experiments were conducted in the insectary under room temperature conditions ( $25 \pm 2^{\circ}\text{C}$  and 50–60% RH). The individual plants were then cut and placed in labeled polythene paper bags for quantification of thrips feeding and oviposition activities in the laboratory. Two leaves were randomly selected from each plant and each leaf was cut into three sections (base, middle and tip). The sections were stained in boiling lactophenol-acid fuchsin solution for 30 – 40 mins. The stained sections were removed and placed in 90-mm Petri dishes and left to stand for 30 min before destaining. Destaining was done by immersing the leaves in warm water three times. The number of feeding punctures and eggs laid on leaves were counted and recorded under a Leica EZ4 dissecting microscope. Endophytic colonization was confirmed at the end of the experiment by sterilizing and plating the remaining plant parts to observe growth of the target endophyte.

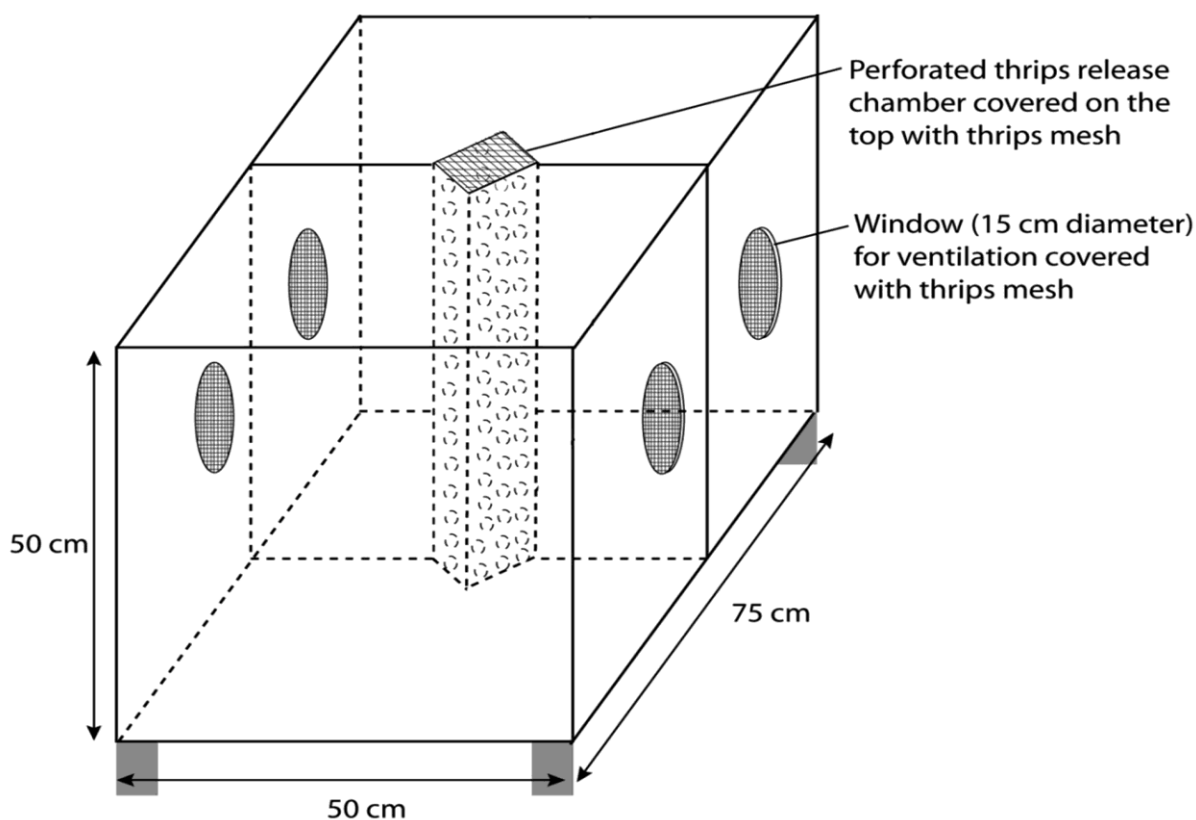


Figure 1: Cage unit for conducting choice experiments

### 3.1.5 Y-tube olfactometer bioassay to test for thrips response to E+ and E- plants

Thrips response to E+ or E- plants was assessed in a Y-tube olfactometer (Fig. 2). Soil surfaces of pots holding the plants were covered using three layers of aluminum foil to prevent the soil odor from being blown upwind. The lower arm of the Y-tube was a 15 cm length of 28 mm corning glass tubing that branched at the tip to form two upper arms of 5 cm length before becoming parallel for the final 10 cm of each arm. The interior angle of the upper arms of the Y-tube was 60°. The olfactometer design incorporated two airtight Perspex boxes (30 × 10 × 10 cm) upwind of the Y-tube for the stimulus and control. The stimulus consisted of E+ potted plant while the control consisted of E- potted plant. The airflow was set at 0.8 L/min using a flowmeter (Air Cadet<sup>®</sup> Vacuum/Pressure Pump Station, Chicago, IL).

An individual adult test insect was introduced into the Y-tube approximately 2 cm from the outlet. Trials constituting 7.5% of the total released insects were terminated after 5 min due to lack of response. A successful trial was one in which the upwind-responding insect moved entirely past the midpoint of the bifurcation in the Y-tube and into one of the arms of the 'Y'. A trial was designated unsuccessful when the insect did not make either choice. Ten tests were performed daily after which the tubes were cleaned and dried in the oven. The experiment was repeated six times with ten insects over time. The treatment and the control side of the tube were alternated to eliminate any positional effect that could impact insect response. The experiments were conducted in the laboratory under room temperature conditions ( $25 \pm 2^\circ\text{C}$  and 50–60% RH).

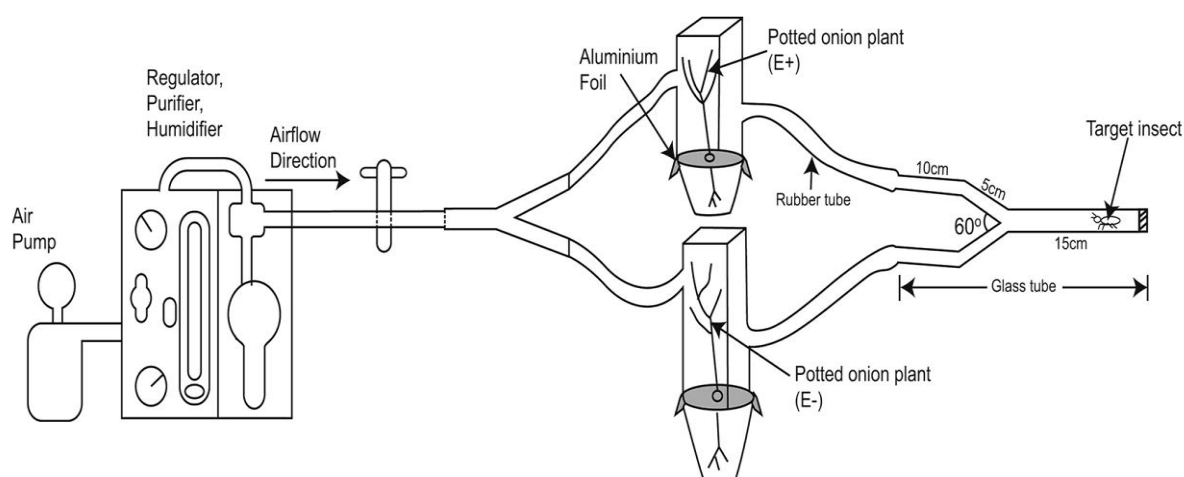


Figure 2: Schematic drawing of the Y-tube olfactometer used to measure insect behavior in the presence of endophyte-inoculated (E+) and endophyte-free (E-) plants

### 3.1.6 Choice test using endophytically-colonized leaf sections

Leaf sections (approx. 1.0 cm in length) were taken from screenhouse grown onion plants (var. Red creole) presumably colonized by *H. lixii* (E+) or uninoculated onion plants (E-). Four leaf sections, two each from E+ and E- plants, were placed in a 90-mm plastic Petri dish

lined with moist filter paper in a circular, equidistant and alternating pattern. Position effects were neutralised by placing E+ or E- leaf sections in different directions in the different Petri dishes. The identity of each section (E+ or E-) was marked on the bottom of the Petri dish directly below each section using a permanent marker to aid in observations. An individual first or second-instar larva of *T. tabaci* was then placed in the middle of the Petri dish and allowed 12 h to select a host. Larvae were chosen over adults for this experiment due to the small experimental arena. Further, larvae could not be used on whole plant choice test as described earlier due to their slow mobility. The location of each larva was then recorded as E+, E- or uncommitted and the experiment was repeated using 33 larvae of each instar. Larvae were deemed uncommitted if they did not make either choice of E+ or E- leaf sections. A host selection criterion was fulfilled if, after 12 h, a larva had settled itself on a leaf section.

### **3.1.7 Choice experiment to test for settlement preferences of second instar *T. tabaci***

Another choice experiment was conducted to test for settlement preferences of second-instar of *T. tabaci* over time. The experimental procedure was the same as the one described above, with the exception that a group of 10 *T. tabaci* second-instar was used. The dish was sealed with a perforated plastic sealing film and after 0, 5, 10, 20, 40 and 60 mins, the positions of the larvae on either leaf sections or elsewhere in the Petri dish were recorded. The experiment was replicated with 15 groups of larvae.

### **3.2 Data analysis**

All data were checked for normality of distribution and equality of variance where appropriate. The numbers of thrips observed on the onion plants was recorded for the treatments and replicates. Analysis was performed using logistic regression model which was fitted to the data on proportion of thrips recovered 72 h post exposure using package *HSAUR*

(Everitt & Hothorn, 2013). The number of feeding punctures on each leaf section was determined and summed up per plant before staining the leaves for eggs count. All count data on feeding and oviposition of *T. tabaci* were checked for normality and homogeneity of variance using Shapiro-Wilk and Levene tests, respectively, before analysis by negative binomial regression using package *MASS* (Venables & Ripley, 2002). The negative binomial distribution was chosen, based on its biological appropriateness in handling over dispersion in count data. Comparisons for each category (E+, E-, uncommitted) for first and second instar larvae in the Petri dish assay and the Y-tube olfactometers experiment were performed using a chi-squared test except that in the Y-tube insects that did not make a decision were not included in the analysis. Data on settlement preference of second instar was analysed using a repeated measures ANOVA and a Bonferroni post hoc test using package *multcomp* (Hothorn et al., 2008). All data were analysed using R 2.15.3 (R Development Core Team, 2013). P-values <0.05 were considered as significant.

### **3.3 Results**

#### **3.3.1 Choice assays with female thrips on whole plants**

In the choice experiment, *T. tabaci* females displayed a stronger preference to settle on E- as compared to the E+ plants ( $\chi^2 = 70.48$ ,  $df = 1$ ,  $P < 0.001$ ; Fig. 3). Moreover, *T. tabaci* showed a clear preference for E- in its feeding activity and oviposition ( $\chi^2 = 58.57$ ,  $df = 1$ ,  $P = < 0.001$  and  $\chi^2 = 72.28$ ,  $df = 1$ ,  $P < 0.001$ , respectively; Fig. 4). Oviposition was reduced sixfold on E+ plants within a 72 h experimental period.

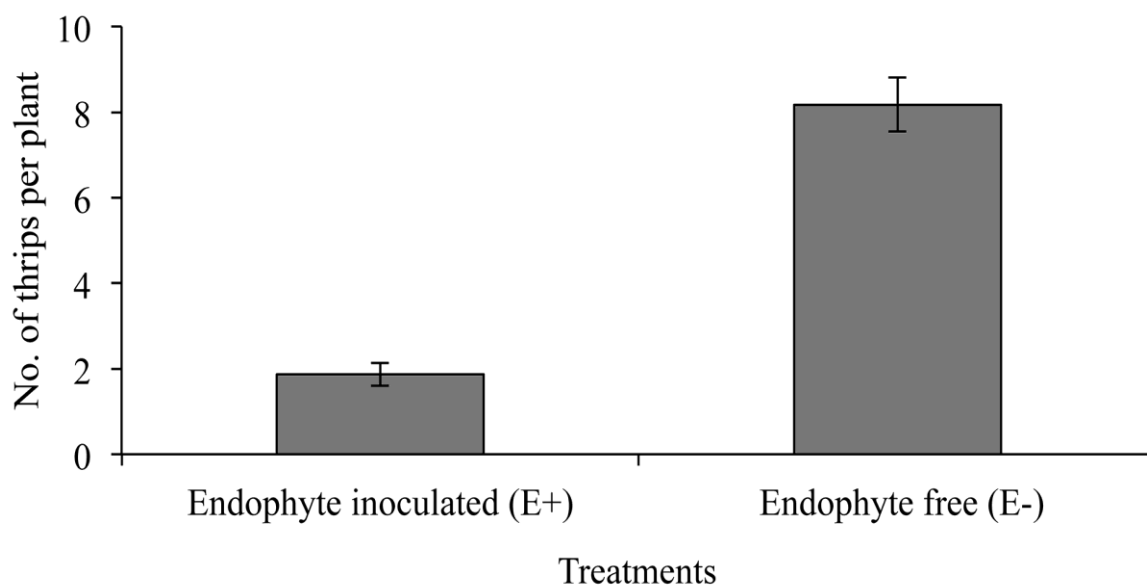


Figure 3: Number of female adult *Thrips tabaci* recorded on endophyte-inoculated (E+) and endophyte-free (E-) onion plants after 72 hours in a choice test. Bars indicate means  $\pm$  standard error (SE) ( $n = 25$ ).

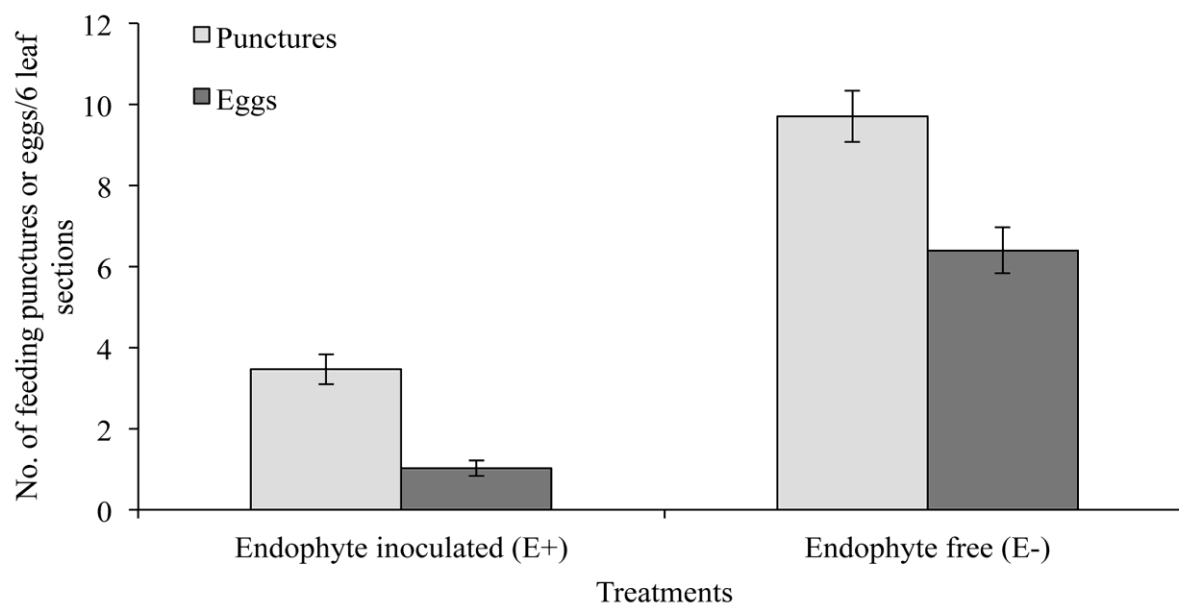


Figure 4: Number of feeding punctures and eggs laid on endophyte inoculated (E+) and endophyte-free (E-) onion plants after 72 h in a choice test. Bars indicate means  $\pm$  standard error (SE). Means followed by the same upper or lower case letters indicate no significant differences between host plants for feeding damage and oviposition, respectively, by *post hoc* comparisons using chi-square test ( $n = 25$ ).

### 3.3.2 Repellency of adult *Thrips tabaci* by endophyte-inoculated plants in a Y-olfactometer

*Hypocrea lixii* (F3ST1)-inoculated plants in the bioassay tests in the Y-tube olfactometer showed repellency to adult thrips. When E+ and E- plants were used as stimuli sources in Y-tube olfactometer, thrips showed about 3.3 fold preference for E- plants ( $\chi^2 = 20.46$ ,  $df = 1$ ,  $P < 0.001$ ; Fig. 5).

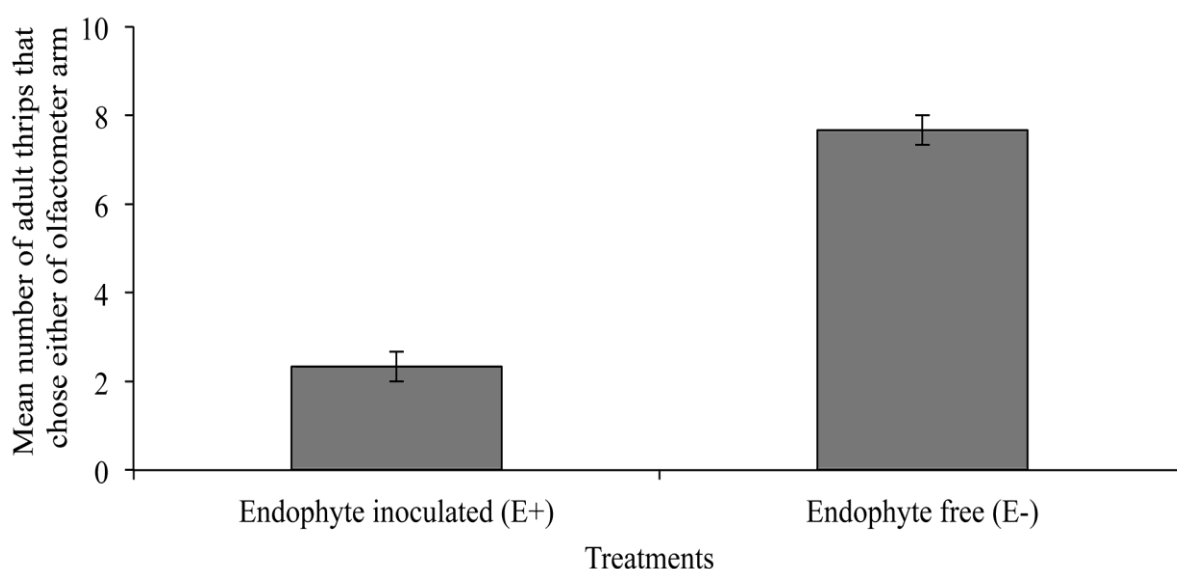


Figure 5: Mean number of adult *Thrips tabaci* response to endophyte-inoculated (E+) and endophyte-free (E-) onion plants in a Y-tube olfactometer ( $n = 6$ )

### 3.3.3 Choice assay using first and second instars

Using individual preference test, both first and second-instar *T. tabaci* preferred E- over E+ plants. In the choice assays, after 12 h, significantly more first-instars were found on or underneath leaf sections of E- as compared to the E+ leaf sections ( $\chi^2 = 32.18$ ,  $df = 1$ ,  $P < 0.001$ ; Fig. 6). There was no difference between the uncommitted and *T. tabaci* larvae found on E+ treatments. For second-instar, insects significantly preferred E- as compared to the E+



leaf sections ( $\chi^2 = 38.02$ ,  $df = 1$ ,  $P < 0.001$ ; Fig. 6). Furthermore, the number of uncommitted larvae was marginally lower for second-instar as compared to first instar.

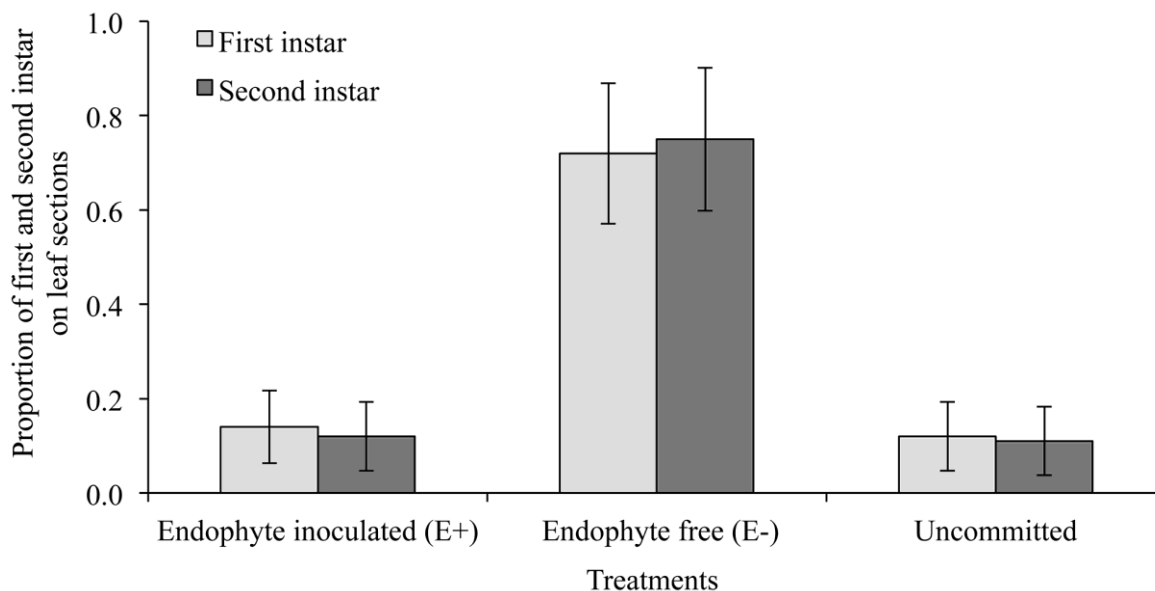


Figure 6: Proportion of first and second instar *T. tabaci* residing on leaf sections taken from endophyte-inoculated (E+) and endophyte-free (E-) onion plants, or not residing on any leaf section (uncommitted) after 12 h in Petri dish choice assays. Bars indicate means  $\pm$  standard error (SE) ( $n = 33$ ).

### 3.3.4 Settlement preference

There was a gradual response in the number of *T. tabaci* larvae settling on any of the two treatments leaf sections in the bioassay unit. Using groups of second-instars, more than 30 and 96% of the larvae had chosen one of the leaf sections after 5 and 40 mins of release, respectively. After 20 mins most thrips made a decision and there was no significant difference between the preference for E+ and uncommitted insects (Fig. 7). With incremental time, more larvae preferred E- over E+ plants (Fig. 7). There were significant differences in the preference for leaf sections at all time intervals ( $F_{2, 28} = 32.06$ ,  $P < 0.001$ ;  $F_{2, 28} = 12.5$ ,  $P < 0.001$ ;  $F_{2, 28} = 52.02$ ,  $P < 0.001$ ;  $F_{2, 28} = 55.99$ ,  $P < 0.001$ ;  $F_{2, 28} = 55.93$ ,  $P < 0.001$ ) for 5, 10, 20, 40 and 60 mins, respectively.

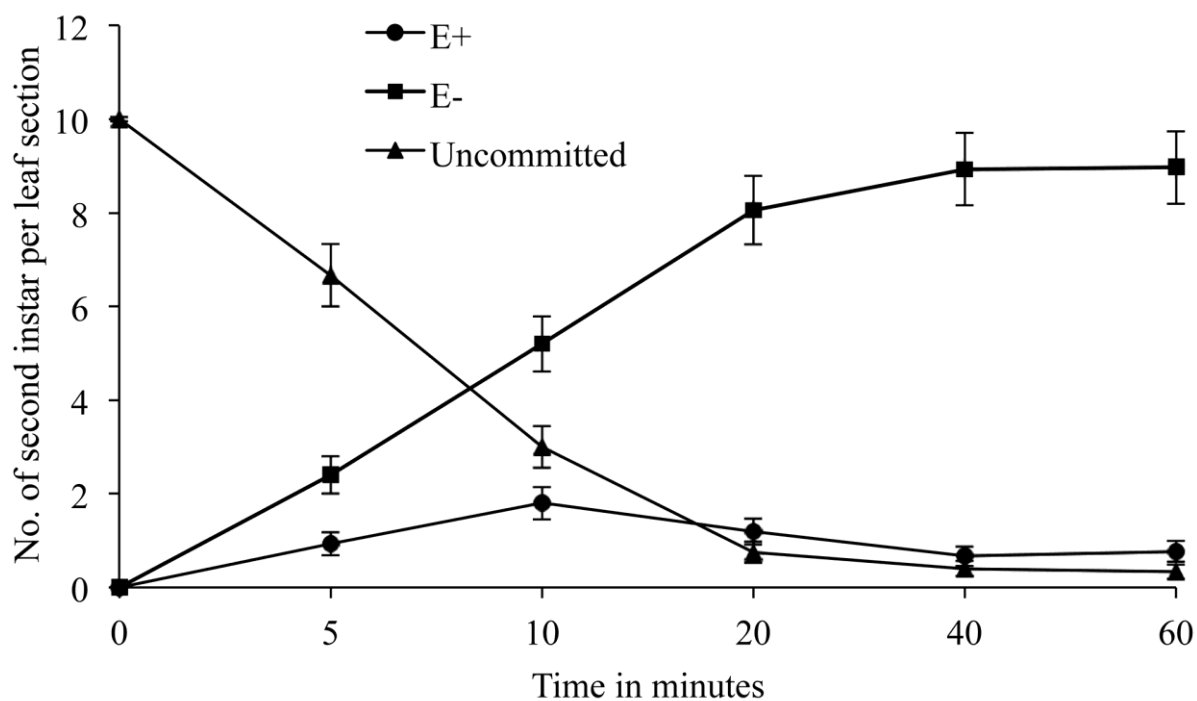


Figure 7: Mean number ( $\pm$ SE) of settled second instar *Thrips tabaci* on either endophyte-inoculated (E+) or endophyte-free (E-) leaf sections, or uncommitted in the bioassay unit. Settlement preference recorded at 0, 5, 10, 20, 40 and 60 mins after the release of groups of 10 second instar per unit for 15 times.

### 3.4 Discussion

The results of the present study demonstrate that an endophytic fungus can play a mediating role in plant-insect interactions. Host selection by phytophagous insects involves a linked sequence of behaviors and discrimination events (Miller & Strickler, 1984). Results from our choice experiments with whole plants showed that the number of adult thrips was lower in endophyte-colonized (E+) as compared to endophyte-free (E-) onion plants indicating antixenosis of E+ plants. Similarly, Cherry et al. (2004) reported a reduced number of stem borer insects on E+ as compared to E- plants. Such repellence of E+ plants could be due to emissions of volatiles influencing insect preferences. Results from the Y-tube olfactometer assays using whole plants and adult thrips confirmed the involvement of volatiles in influencing increased preference for E- plants over E+ plants. In an experiment using E- and E+ peppermint plants, Mucciarelli et al. (2007) observed an increase of terpenoids compounds in leaves in the presence of the PGP-HSF endophyte. Analysis of volatile secondary metabolites in *Festuca arundinacea* (Poaceae) infected with the fungal endophyte *Neotyphodium coenophialum* revealed emission of volatiles from plants with potential of influencing insect preferences (Qawasmeh et al., 2011). Compounds like  $\beta$ -myrcene produced from plants inoculated with arbuscular mycorrhizal fungi and *Beauveria bassiana* are believed to be involved in the plants induced resistance against herbivory (Shrivastava, 2011) as they are utilized by insects for different purposes such as repellents to some insects, as well as, attractants to aphidophagous hoverflies in the orchids (Stökl et al., 2011).

Significant reduced feeding and oviposition activities of adult thrips on E+ plants were also observed in choice experiments. Similar results were obtained by Akello et al. (2008) where they reported that endophytic *B. bassiana* negatively affected banana weevil feeding and development, resulting in reduced plant damage. The reduced feeding and oviposition could have been as a result of either reduced survival of thrips or antixenotic repellence of thrips as

demonstrated in our earlier results. The influence of endophytes on feeding and oviposition in the present study is in accordance with the findings of Muvea et al. (2014) with *T. tabaci* on onions, Cherry et al. (2004) with *Sesamia calamistis* Hampson on maize, Bittleston et al. (2011) with *Atta colombica* Guérin-Méneville on *Cordia alliodora* Ruíz and Pavón seedlings and Akutse et al. (2013) with *Liriomyza huidobrensis* Blanchard on beans.

Results from the Petri dish choice assays with first and second-instar *T. tabaci* also indicated a strong preference of larval thrips for the endophyte-free (E-) leaf sections as compared to endophyte-inoculated (E+) onion leaf sections. These findings were further confirmed by the settling preference tests where more second-instar avoided settling on the E+ leaf sections. Similar observations were made by Richmond, (2007) who reported that second-instars *Parapediasia teterrella* Zincken displayed a stronger preference for E- than E+ plant materials. As with the adults, plant volatiles may be a signal used by larvae to discriminate between inoculated and endophyte-free plants or plant tissues. However, the possibility of changes in contact chemoreception and mechanoreception cues due to the endophyte infection leading to observed effects on larvae selection behavior, and the adult feeding and oviposition behavior cannot be discounted. Duffey & Felton (1991) and Jallow et al. (2004, 2008) reported that fungal endophytes can exert indirect effects by altering plant morphological traits, thus reducing acceptability of plant tissues by insects. Another mechanism underlying anti-herbivore properties of endophytic fungi is attributed mainly to the production of various alkaloid-based defensive compounds in the plant tissue (Faeth, 2002). The types and concentrations of alkaloids present in E+ plants are likely to be the most important determinant of insect response, and there are several factors which may influence variation in the expression of these alkaloids. While the particular types of alkaloids produced in E+ plants are mainly functions of the fungal strain or genotype (Popay & Bonos, 2005), individual alkaloids may vary with respect to their activity against insect herbivores. For

instance, loline alkaloids may be toxic and/or deterrent to many insects (Popay & Bonos, 2005), but apparently have little or no direct impact on others (Richmond et al., 2004) which warrants further studies. Akutse et al. (2013) reported that Faba beans colonized endophytically by fungal endophytes of the genus *Hypocrea* and *Beauveria* had significant negative effects on leafminer, *L. huidobrensis* fitness, impacting on mortality, oviposition, emergence and longevity of the pest. Fungal endophytes have therefore, a potential of inducing direct and indirect defenses against herbivores (Leckie, 2002; Ownley et al., 2010).

### **3.5 Conclusion**

Our study is the first to demonstrate the role of antixenotic factors, especially volatiles, repelling *T. tabaci* adults and larvae away from endophyte inoculated plants/leaf sections. However, further studies to elucidate potential semiochemicals that could be involved in inducing repellency against onion thrips by *H. lixii* needs to be undertaken. Studies on the changes in contact chemoreception and mechanoreception cues induced by endophytes and their influence on host seeking behavior of adult and larval *T. tabaci* could aid in further unraveling this multi-trophic interaction. Outcomes of such studies could open up opportunities for further refining of integrated pest management strategies for *T. tabaci* and the tospoviruses that they transmit on plants.

## CHAPTER 4

### ENDOPHYTIC COLONIZATION OF ONIONS INDUCES RESISTANCE AGAINST THRIPS AND VIRUS REPLICATION

#### ABSTRACT

*Iris yellow spot virus* (IYSV) vectored by *Thrips tabaci* Lindeman is a major hindrance to onion production in eastern Africa. Control measures often rely on insecticides which have deleterious effects. Endophytes are one key alternative as they can play important role in mediating induced systemic resistance. Hence, we examined the potential effect of endophytic fungus *Hypocrea lixii* (F3ST1) on feeding and propagation of IYSV on endophyte-inoculated (E+) and endophyte-free (E-) onion plants. Transmission was also tested in leaf disc bioassays. The numbers of feeding punctures were significantly lower in E+ as compared to the E- plants. Disease level sampled weekly for four weeks following thrips exposure was significantly lower in E+ as compared to E- plants. IYSV transmission was reduced 2.5-fold by the endophytic fungus (*Hypocrea lixii* F3ST1) on both whole plant and leaf disc assays. Our results suggest potential utility of endophytic *Hypocrea lixii* F3ST1 to reduce virus infection on onion plants. Further studies should be conducted to determine whether such endophyte-thrips-virus mediated interaction extends to other onion varieties and viruses.

**Keywords:** *Hypocrea lixii*, onions; thrips; *iris yellow spot virus*; multi-trophic interactions

#### 4.0 Introduction

Onion, *Allium cepa* L. (Asparagales: Amaryllidaceae), is an important vegetable crop grown for subsistence or commercial farming. In Kenya, onions are grown in all counties by both large- and small-scale farmers (Narla et al., 2011). The major factors limiting onion production are pests and diseases (Gachu et al., 2012). Onion thrips (*Thrips tabaci* Lindeman) is considered to be the most economically important pest of onion in Kenya and worldwide (Trdan et al., 2005; Waiganjo et al., 2008). They cause direct damage by feeding on leaves tissues resulting in a reduction of photosynthetic ability and consequently reducing onion bulb size and yield (Rueda et al., 2007; Biriithia et al., 2014). Bulb onion yield losses of up to 60% have been reported in Kenya due to thrips damage alone (Waiganjo et al., 2008). Thrips feeding lesions also acts as a source of secondary infection by pathogenic fungi and bacteria (McKenzie et al., 1993). Onion thrips also vectors a tospovirus, i.e. *iris yellow spot virus* (IYSV), which is a major threat to both bulb and seed onion production in eastern Africa and globally (Gent et al., 2004, 2006; Pappu et al., 2009).

Insecticides application is the commonly used strategy to manage onion thrips vectoring IYSV (Gachu et al., 2012). However, they can lead to serious environmental hazards in addition to causing pesticide resistance in onion thrips populations (Martin et al., 2003). To remain effective, control programmes have to integrate several disease management tactics including the use of beneficial micro-organisms that stimulate the plant defence responses (Vega et al., 2009). Endophytes are one of such organisms that inhabit and live inside plant tissues without inducing apparent symptoms in their hosts (Rodriguez et al., 2009). In primed plants with endophytes, defense responses are accelerated upon pathogen or insect attack, resulting in enhanced resistance to the attacker (Brotman et al., 2010).

Published evidence suggests that endophytic fungi can play symbiotic roles in nature, such as antagonists of plant disease, beneficial rhizosphere colonizers, increased drought tolerance

and plant-growth promoters (Rodriguez et al., 2009; Vega et al., 2009; Jaber & Selim, 2014). When endophytes colonize plants, they produce enzymes which have the function to suppress plant pathogen activities directly and have the capability of degrading the cell walls of such pathogens (Gao et al., 2010). Emission of secondary metabolites is considered to play an important role during plant defense activities against insects and pathogen attack. Moreover, plant colonization by endophytes influences the population dynamics of herbivory vectors. For instance, endophytic isolates of the genus *Neotyphodium* protected meadow ryegrass (*Lolium pratense* = *Festuca pratensis*) from herbivory by bird cherry oat aphid (Lehtonen et al., 2006). A reduction of tunneling in maize by *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) (Bing & Lewis, 1991) and *Sesamia calamistis* Hampson (Lepidoptera: Pyralidae) (Cherry et al., 2004) were attributed to endophytic *Beauveria bassiana* Balsamo (Hypocreales: Clavicipitaceae). Similarly, endophytic strain of *B. bassiana* reduced the population of *Iraella luteipes* Thompson (Hymenoptera: Cynipidae) feeding on *Papaver somniferum* L. (Quesada-Moraga et al., 2009). Several isolates of endophytic fungi have been reported to colonize onion plants and confer protection against thrips (Muvea et al., 2014). These authors demonstrated that endophytic fungus, *Hypocrea lixii* F3ST1 had the ability to reduce thrips population, feeding activities and oviposition on onion plants.

So far, very few studies have examined the potential effect of endophytes as a plant disease antagonist (Lehtonen et al., 2006; Ownley et al., 2010). Despite a scarcity of such studies, substantial evidence indicates that endophytic fungi can provide plants with protection against plant pathogens. For instance, *Chaetomium* and *Phoma* endophytes inoculated in wheat plants reduced severity of foliar disease caused by *Puccinia* and *Pyrenophora* spp. by releasing antifungal compounds (Dingle & McGee, 2003). The mechanism of increased disease tolerance in endophyte-inoculated plants is largely speculative, but it is hypothesized that secondary compounds produced by endophytes may play a partial role in this



phenomenon (Yue et al., 2000). One important case of plant pathogens in onions is *iris yellow spot virus* (Birirthia et al., 2014). The host plant properties can affect viral diseases both directly through host metabolites and indirectly via effects of plant quality on insect vectors transmitting the viruses. Other mechanisms implicated in plant-endophyte-virus interactions on induced resistance to viral infection may include inhibition of viral multiplication or accumulation (Loebenstein, 1972). A case study evaluating the transmission and multiplication of *zucchini yellow mosaic virus* (ZYMV) found lower virus titer levels on endophyte-inoculated plants with *Beauveria bassiana* isolates as compared to the endophyte-free plants (Jaber & Salem, 2014). Endophytic systemic colonization through intercellular spaces and vascular xylem elements can inhibit or interfere with the systemic movement of plant viruses from cell to cell, which eventually results in delayed multiplication in inoculated plants (Loebenstein, 1972; Martelli, 1980). Lehtonen et al. (2006) reported a lower percentage of *barley yellow dwarf virus* (BYDV) infections in endophyte-inoculated meadow ryegrass (*Lolium protense*) compared to endophyte-free plants, indicating that endophyte inoculation can protect plants from virus infections and eventual multiplication. However, there is no report yet available on the potential of endophytes to protect onion plants against IYSV. Therefore, the aim of this study was to examine how endophytically colonized onion plants induces resistance against thrips and the virus they transmit.

## 4.1 Material and methods

### 4.1.1 Insects rearing

Initial cultures of *T. tabaci* were field-collected from onion plants at the International Centre of Insect Physiology and Ecology (*icipe*) organic farm, Duduville, Nairobi, Kenya. Thrips were reared on snow peas, *Pisum sativum* L. (Fabales: Fabaceae), for over 35 generations in ventilated plastic jars at the *icipe*'s insectary at  $25 \pm 1^\circ\text{C}$ , 50–60% relative humidity (RH), 12 h L: 12 h D photoperiod. Adults for the experiment were allowed to lay eggs on snow pea pods for 3 days and were then removed. Neonate first instars (8 h-old) were used in the subsequent experiments.

### 4.1.2 Fungal isolate

Endophytic fungal isolate *Hypocrea lixii* F3ST1, isolated from the aboveground parts of maize obtained from tropical highland region in Kenya was selected for this study based on its antagonistic effects against thrips (Muvea et al., 2014). Conidia were obtained from two-week old cultures grown on PDA plates. The conidia were harvested by scraping the surface of sporulating cultures with a sterile scalpel. The harvested conidia were then placed in a universal bottle with 10 ml sterile distilled water containing 0.05 % triton X-100 and vortexed for 5 min to produce homogenous conidial suspension. The conidial concentration was determined using a Neubauer hemocytometer. The conidial concentration was adjusted to  $1 \times 10^8$  conidia  $\text{mL}^{-1}$  through dilution prior to inoculation of seeds. To assess the viability of the conidia, 100  $\mu\text{L}$  of conidial suspension was inoculated to the surface of four fresh plates of PDA. Two sterile microscope cover slips were placed on top of the agar in each plate before incubation. The inoculated plates were incubated for 24 h at  $20^\circ\text{C}$ . The percentage conidial germination was assessed by counting the number of germinated conidia out of 100 in one randomly selected field.

### 4.1.3 Seed inoculation and endophyte colonization

Seeds of onions (var. Red Creole, East Africa Seed Co. Ltd, Tanzania) were surface-sterilized in 70% ethanol and then immersed in 2% NaOCl for 2 and 3 min, respectively. The seeds were finally rinsed three times using sterile distilled water to ensure that the seed surface was sterilized free of epiphytes. To confirm the efficacy of the surface sterilization, 100 µl of the last rinse water was spread onto four PDA plates and incubated at 20°C for 14 days. The absence of fungal growth on the medium confirmed the reliability of the sterilization procedure (Schulz & Boyle, 2005). The seeds were then placed on sterile filter paper to dry for 20 mins and subdivided into two equal portions one for inoculation and the other to serve as the control. Surface-sterilized seeds were soaked in a conidial suspension of  $1 \times 10^8$  conidia mL<sup>-1</sup> for 10 h. In the control, seeds were soaked in sterile distilled water containing 0.05% Triton X 100. The inoculated seeds were air dried on a sterile paper towel for 20 mins and then transferred to plastic pots (8 cm diameter × 7.5 cm height) containing planting substrate. The substrate was a mixture of red soil and livestock manure in a 5:1 ratio and was sterilized in an autoclave for 2 h at 121°C and allowed to cool down to ambient temperature before being used. Four seeds per pot were sown 1 cm below the surface of the substrate and maintained at room temperature (~25°C and 60% RH) in a screen house.

Endophytic colonization of the inoculated plants was confirmed using the technique described by Muvea et al. (2014). Plants were randomly selected and carefully removed from the pots 50 days after inoculation and the roots washed with running tap water. Seedling leaves, stems and roots were cut into different sections. Five randomly selected leaf, stem and root sections from each plant were surface-sterilized as described above. The different plant parts were then aseptically cut into ~1cm pieces before placing the pieces 4 cm apart from each other, on PDA plates amended with a 0.05% solution of antibiotic (streptomycin sulfate salt). Plates were incubated at  $25 \pm 2^\circ\text{C}$  for 10 days, after which the presence of endophyte

was determined. Prior to incubation of the different plant parts, the last rinse water was also plated out to assess the effectiveness of the surface sterilization procedure. The colonization of the different plant parts was recorded by counting the number of pieces that showed the inoculated fungal growth. Only the presence of the endophyte used for inoculation was scored. After testing for colonization, the remaining seedlings were thinned to one per pot and watered once per day.

#### **4.1.4 Acquisition and transmission of IYSV**

Virus transmission using thrips was selected for this study because mechanical inoculation has been found unsuccessful on onions (Bulajic et al., 2008; Srinivasan et al., 2010; Naveed and Pappu, 2012). A cohort of 500 first instar (8 h-old) of *T. tabaci* obtained from *icipe*'s insectary were allowed an acquisition access period (AAP) of 16 h to acquire virus on IYSV-infected *Allium cepa* var. Red creole (plants maintained at *icipe* as virus inoculum source). The thrips acquire the virus by feeding on infected plants. The virus infection in the plants used for virus acquisition was confirmed using IYSV-specific ELISA Flashkit (Agdia Biofords, Netherlands) (Birithia et al., 2013). Thrips were then transferred and reared on snow pea pods until adults emerged. Four plants, two each from E+ and E- plants and which were 10 weeks old were placed in an equidistant and alternating pattern in 44 thrips-proof cages (40 × 30 cm). Virus transmission was performed by releasing 20 viruliferous adults thrips per cage and allowed to feed for 48 h after which all the thrips were removed from the plants using a Carmel brush. The cages were then randomly divided equally into four groups (Group 1, 2, 3 and 4) whereby for instance; group one represented samples for week one in that order. This was considered important to enable assessment of virus propagation over time. To exclude bias in our results on virus transmission on E+ and E- treatments, samples for the test were cut from sections of the plant with visible feeding punctures. This was

necessary because random selection would imply that E+ will have less titer as feeding punctures are positively correlated with virus transmission (Jiang et al., 2000). Transmission by whole plant i.e. leaves was evaluated after 2 weeks post thrips exposure using IYSV-specific DAS-ELISA (Agdia Biofords). Control healthy plants (without endophyte and virus) were tested simultaneously for baseline titers. To obtain a more precise assessment, transmission of IYSV was additionally analyzed using leaf disc assays and individual thrips. The assay was done by allowing viruliferous individual adult thrips which were reared and infected as described earlier to feed on 2 cm<sup>2</sup> onion leaf discs placed in Petri dishes (9 cm diameter). The leaf discs were obtained from E+ and E- plants. The top of the petri dishes was sealed with Parafilm to prevent escape of thrips. A single adult thrip was allowed to feed on each leaf disc and the treatments (E+ and E- leaf discs) were replicated 22 times. After 48 h, leaf discs were tested for the presence of the virus as described earlier. To determine IYSV transmission on plants, double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was carried out following the manufacturer's instructions. ELISA readings were considered positive when the absorption (OD = 405 nm) of the sample wells was at least two times greater than the mean absorption of negative control samples.

#### **4.2 Statistical analysis**

All data were checked for normality and homogeneity of variance before analysis. The number of feeding punctures were determined and summed up per treatment for all the 44 cages before analysis by negative binomial regression using package *MASS* (Venables & Ripley, 2002). Virus titer levels on whole plants over time was analysed using a repeated measures analysis of variance (ANOVA) and a Bonferroni post hoc test using package *multcomp* [17]. Tukey HSD multiple comparisons of means was used to separate the means at the significance level of 0.05. Treatments were considered as fixed effects and the cages as

replicates. Petri dish experiment analysis was performed using a chi-squared test.  $P$ -values were based on type III chi-square values in all the analyses. All statistical analyses were performed in R 2.15.3 (R Development Core Team, 2013).

### 4.3 Results

The effect of endophyte colonization in plants on the feeding preference of viruliferous *T. tabaci* was evaluated. The number of feeding punctures were significantly lower in endophyte-inoculated plants as compared to the control treatment ( $\chi^2 = 19.67$ ,  $df = 1$ ,  $P < 0.001$ ,  $n = 44$ ; Fig. 1). There was about a 2-fold decrease in feeding activities on E+ plants. Since feeding was assessed on random section of the plant, the data was collected and recorded before picking samples for IYSV test.

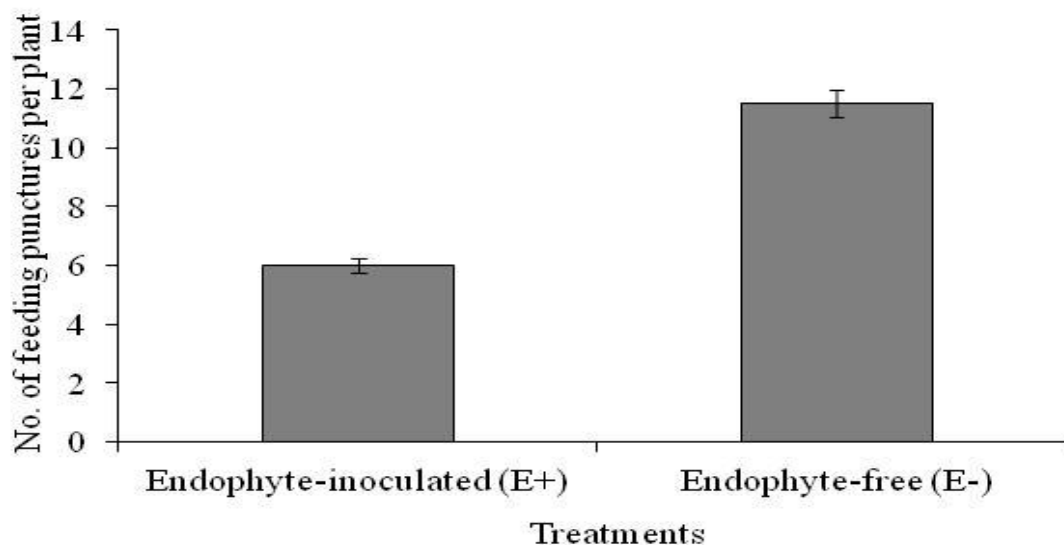


Figure 1: Effect of endophytically colonized onion plants on feeding punctures by viruliferous adult *Thrips tabaci*. The figure quantifies mean feeding activity by *T. tabaci* exposed for 48 h on endophyte inoculated (E+) and endophyte-free (E-) onion plants. Bars indicate means  $\pm$  SE at 95% CI ( $n = 44$ ).

*Iris yellow spot virus* transmission and replication was reduced 2.5-fold on endophytically colonized onion plants. Endophyte-inoculated plants recorded lower IYSV titer levels of  $0.23 \pm 0.07$  as compared to  $0.58 \pm 0.11$  from the endophyte-free plants ( $F = 5.98$ ;  $df = 1, 10$ ;  $P < 0.001$ ; Fig. 2). The effect of time in regard to virus propagation was significant for E- plants ( $F = 10.98$ ;  $df = 3, 10$ ;  $P < 0.001$ ). However, there was no significant difference in the level of IYSV multiplication on E+ plants over time ( $F = 1.02$ ;  $df = 3, 10$ ;  $P = 0.39$ ) (Fig. 2). The average ELISA values for healthy controls for four weeks (without endophyte and virus) was  $0.11 \pm 0.003$  which was 2 and 5-fold lower than the readings for E+ and E- plants, respectively.

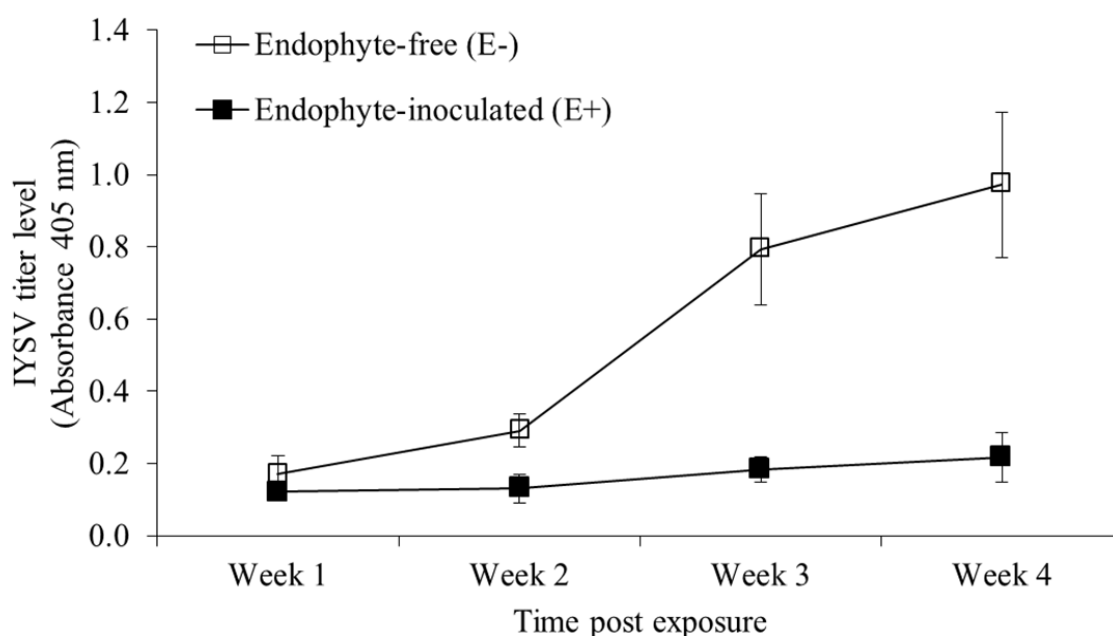


Figure 2: Effect of endophytically colonized onion plants by *Hypocrea lixii* F3ST1 on IYSV propagation overtime. An evaluation of endophytic fungus for its effect on IYSV transmission by viruliferous thrips after 48 h as measured on a whole plant. Means  $\pm$  (standard error) SE at 95% confidence interval ( $n = 11$ ).

To obtain a more precise assessment of virus transmission and propagation, IYSV was additionally analyzed using leaf disc assays and individual thrips. In this experiment, leaf discs were obtained from E+ and E- plants and they were exposed to viruliferous thrips. Results from the leaf disc assay, showed a 2.5 fold reduction of IYSV transmission on endophyte inoculated onion plants ( $\chi^2 = 4.65$ ,  $df = 1$ ,  $P = 0.03$ ) (Fig. 3).

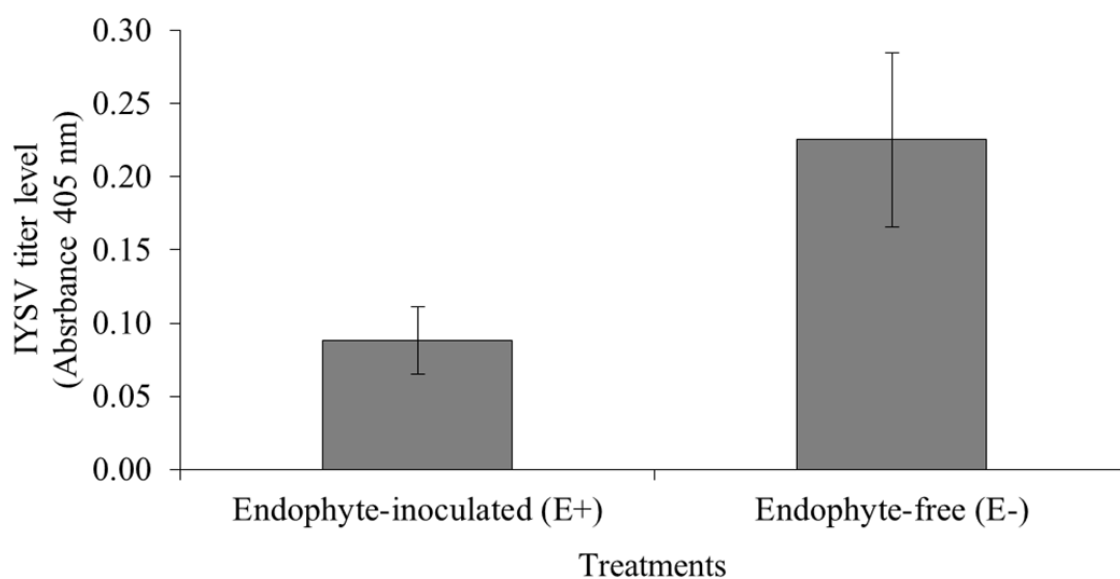


Figure 3: Effects of endophytic fungi on IYSV transmission by *Thrips tabaci* on leaf discs obtained from endophyte inoculated (E+) and endophyte-free (E-) onions plants ( $n = 22$ ).



#### 4.4 Discussion

This is the first study of viruliferous *T. tabaci* on feeding, *Iris yellow spot virus* transmission and propagation on endophytically-colonized onion plants. The specificity of *T. tabaci* to feed and transmit IYSV in onions is well documented (Pappu et al., 2009; Birithia et al., 2014). Our findings show that there was a reduced feeding activity of viruliferous *T. tabaci* on endophyte-inoculated (E+) as compared to endophyte-free (E-) onion plants. This implies that the endophyte colonization of the onion plants could have caused deterrence effects to the thrips. Similar results were obtained by Guy & Davis, (2002) where they reported protection of New Zealand tall fescue colonized by *Neotyphodium* endophytes through deterrence of aphid feeding. In a previous study using non viruliferous *T. tabaci*, feeding activities were reduced on E+ onion plants and it was speculated that antibiosis and/or antixenosis repellency of thrips could have played a key role (Muvea et al., 2014).

Our results provide evidence that the endophytic colonization of onion plants by *Hypocrea lixii* (F3ST1) reduces transmission and multiplication of IYSV. Endophyte-inoculated onion plants harbored less viral infections both in whole plants over time and leaf discs than endophyte-free plants. This implies that *H. lixii* F3ST1 colonization of onions is able to successfully control part of the propagation of IYSV, as it caused a reduction of the virus titers produced by the pathogen in the plants compared to the endophyte-free ones. Multiple mechanisms of endophytes in plant defence vary with plant pathogen in disease suppression (Vega et al., 2009; Ownley et al., 2010). It has been reported that biological control agents may produce secondary metabolites that directly attacks the pathogens or that induce the systemic resistance which, in turn, reduce the pathogen incidence in the plant host (Gao et al., 2010; Ownley et al., 2010). For instance, endophytic inoculation of *Pinus halepensis* Mill seedlings with fungal endophytes (*Trichoderma* spp., *Aureobasidium pullulans*, *Aureobasidium* spp., endophyte 20.1 and *Leotiomycece* spp.) significantly reduced leaf

necrosis length caused by a plant pathogen, *Gremmeniella abietina* (Lagerberg) Morelet (Romeralo et al., 2015).

Our results on the first week sampling don't present much difference in titer level. However, the differences become apparent from there onwards. The important part to note is that, in E+ plants, the virus propagation was remarkably kept low whereas in the control it was on contrary. The possible explanation to occurrence of this phenomenon could be that the viruliferous thrips were triggered to feed and transmit the virus indiscriminately while the endophyte reduced its propagation. Similar observation were made by Jaber & Selim (2014) where they reported *zucchini yellow mosaic virus* incidence and severity, sampled weekly over a period of four weeks was significantly lower in *B. bassiana*-inoculated plants as compared to the non-inoculated control plants. These authors speculated a possible induced systemic resistance that may inhibit virus multiplication and accumulation in a host plant. Moreno-Delafuente et al. (2013) while using viruliferous whiteflies speculated that, virus infection to a vector may provoke whiteflies to focus their attention on feeding activities as soon as they land and settle on the plant. The results of this study are concordant with those reported by Lehtonen et al. (2006) on meadow ryegrass using *Neotyphodium uncinatum* Gams, petrini, Schmidt endophytes. The authors reported reduced transmission of *barley yellow dwarf virus* (BYDV) on E+ as compared to E- plants. They speculated that the effects on feeding activities of aphids on E+ plants was the main reason for the lower virus infection frequency in endophyte-inoculated meadow ryegrass.

The failure of IYSV to propagate on E+ could also be attributed to induced plant tolerance and/or resistance. The fact that leaf samples from both E+ and E- were picked from section with signs of feeding, the possible role of a biochemical factor in endophyte-IYSV interactions could also play part in reducing virus multiplication. For instance, some alkaloids released by endophytic fungi have been reported to possess antiviral activities (Selim et al.,

2012). Moreover, endophytes and the chemicals they produce may be toxic or distasteful to insects (Vega et al., 2009), pathogenic to insects and/or could decrease insect fitness (Akutse et al., 2013). Baseline IYSV titer level for healthy controls (plants without virus and endophytes) was more than 2-fold lower than for E+ and E- plants. Although virus titers may largely vary within families, the titer levels obtained in our study were similar to those obtained by other workers for IYSV (Naveed and Pappu, 2012). However, the virus infection was positive for both E+ and E- treatments. Nevertheless, the utility of endophytic inoculation for reducing the virus propagation as compared to plants without fungal inoculation cannot be underrated.

#### **4.5 Conclusions**

Our study suggests that viruliferous thrips are efficient transmitters of IYSV and that endophyte-inoculation reduces virus transmission and multiplication. This knowledge has clear implications for understanding the epidemiology of insect-transmitted plant diseases and improving their management options under integrated agricultural systems. Further screening of other endophyte isolates for resistance and/or tolerance to thrips and viruses are warranted.

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## CHAPTER 5

### GENERAL DISCUSSION

Onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae), are considered the most economically important pest of onion in Kenya and globally. The pest causes direct damage on plant leaves during feeding and oviposition. Indirectly, *T. tabaci* vectors *iris yellow spot virus* (IYSV) (Bunyaviridae: Tospovirus) (Birithia et al., 2011) which is known to cause up to 100% leaf drying in onions under unmanaged conditions. In Kenya, control of onion thrips is based on the use of insecticides with insufficient evaluation of their effects. This technique has been rendered ineffective because thrips are always protected between the inner leaves of an onion plant, the pupal stage is spent in the soil, they have many overlapping generations, polyphagy and pesticides resistance (Brewster, 1994; Nault & Shelton, 2010).

Several sustainable methods of control e.g. use of entomopathogens and intercropping have been developed in recent years to reduce thrips damage in onion crop in Kenya (Ekesi & Maniania, 2003, Gachu et al., 2012). One sustainable method of managing this insect pest is the use of endophytic fungi which have been utilized for different insects in many parts of the world (Gurulingappa et al., 2010; Ownley et al., 2010; Akutse et al., 2013). For instance, consumption of wheat leaves colonized by either *B. bassiana* or *Aspergillus parasiticus* Speare slowed the growth of *Chortoicetes terminifera* Walker nymphs while feeding by *A. gossypii* on cotton leaves colonized by either *B. bassiana* or *Lecanicillium lecanii* slowed aphid reproduction (Gurulingappa et al., 2010). An endophytic strain of *B. bassiana* reduced the population of *Iraella luteipes* (Thompson) (Hymenoptera: Cynipidae) feeding on *Papaver somniferum* L. (Quesada-Moraga et al., 2009). Similarly, an increased adult mortality and reduced larval damage by *Cosmopolites sordidus* (Germar) were attributed to the endophytic colonization of *B. bassiana* in banana (Akello et al., 2008). *Thrips tabaci* is the main vector

of IYSV on onions (Birithia et al., 2010). Transmission of this and some other plant viruses is mediated by the piercing-sucking mouthparts of insects, named stylets, when they penetrate through the intercellular spaces and establish feeding sites in phloem sieve elements (Stafford et al., 2012). In a study using *T. tabaci*, it was demonstrated that feeding on IYSV infected plants did not influence its mortality. Studies have shown that viruliferous insects behave differently when compared to those without the virus (Stafford et al., 2011). These authors suggested that the direct effects of virus infection might increase feeding, development time and survival of the vector consequently leading to increased transmission efficiency and spread of virus (Moreno-Delafuente et al., 2013). Unlike other viruses e.g. *Zucchini yellow mosaic virus* (ZYMV) which can be transmitted both mechanically and through aphid vectors (Gal-On, 2007), IYSV can only be transmitted through insect vectors (Naveed & Pappu, 2012). Studies have revealed that fungal endophytes can be utilized for management of viruses infection on economical crops. For instance, Squash plants (*Cucurbita pepo* L.) inoculated with *B. bassiana* endophytes were protected from damage (symptoms expression) by ZYMV through a reduction of virus multiplication (Jaber & Selim, 2014). Other evidence for induced systemic resistance of fungal endophytes includes a reduction in disease symptoms. For example, *B. bassiana* 11-98 induced systemic resistance in cotton against *Xanthomonas axonopodis* pv. *Malvacearum* (Bacterial blight). However, information on endophytic colonization of onions and its antagonistic effects on thrips vectors infesting onions is not available.

Therefore, to study colonization of onions by endophytic fungi and further assess their impacts on biology of *T. tabaci*, seed and seedling inoculation methods were conducted in a screen house while feeding and oviposition activities were determined using cages in an insectary. To study behavioral responses of *T. tabaci* to endophyte-colonized onion plants, choice experiments were conducted using whole plants for adults and leaf discs for larvae. To

study how endophytic colonization of onions induces resistance against thrips and virus replication, choice experiments of endophyte-inoculated and endophyte-free onion plants were conducted using viruliferous adult *T. tabaci* in the insectary.

In the colonization experiment, all fungal isolates were able to colonize onion plants following seed or seedling inoculation. However, the extent of colonization of the different plant parts depended on the inoculation method and the fungal isolate used. Seed inoculation resulted in 1.47 times higher mean percentage post-inoculation recovery of all the endophytes tested as compared to seedling inoculation. Endophytic fungi treatments had negative effect on the proportion of thrips observed on the onion plants 72 h post-exposure. Fewer thrips were observed on endophyte-inoculated (E+) as compared endophyte-free (E-) control plants. Moreover, endophyte inoculation reduced feeding and oviposition with *Hypocrea lixii* F3ST1 outperforming all the other isolates. In studies looking at behavioral responses of *T. tabaci* to endophyte-colonized onion plants in a choice experiment, *T. tabaci* females displayed a stronger preference for E- as compared to the E+ plants in settling, feeding and oviposition. *Hypocrea lixii* (F3ST1)-inoculated plants in a Y-tube olfactometer showed repellency to adult thrips. More larvae were found on or underneath leaf sections of E- as compared to the E+ leaf sections. The potential role of endophytic fungus *Hypocrea lixii* (F3ST1) in reducing feeding and transmission of IYSV on onion plants was evaluated in a screenhouse using viruliferous thrips. Feeding and virus transmission were significantly reduced on endophyte-inoculated plants and leaf sections.

The outcome from our study show that inoculation through seeds results to superior colonization as compared to seedling. This may be explained in part by a reduced capacity of seedlings to enhance endophyte proliferation due to transplantation shock (Barrows & Roncadori, 2014). Higher seed colonization could also have occurred because the endophytes have an advantage of colonizing both seed radical and the plumule, which are close to one

another in the seed. This outcome could provide opportunities for endophytic colonization at the young seedling stage for early protection and enhanced seedling health (Backman & Sikora, 2008). The ability to establish fungal endophytes in plants following artificial inoculation has been demonstrated for Faba and French beans (Akutse et al., 2013), banana (Akello et al., 2008; 2012), and cocoa (Posada & Vega, 2005). Studies evaluating endophytic colonization of *Pinus radiata* D. Don by *B. bassiana* reported superior colonization of the plants through seed inoculation as compared to seedling root dip (Brownbridge et al., 2012). Fungal isolates in our study colonized plant parts differently which could be attributed to tissue specificity and/or chemistry and adaptation to particular physiological condition of a plant (Arnold et al., 2003; Guo et al., 2008; Wearn et al., 2012). For instance, Wearn et al., (2012) reported fungal species diversity in roots was on average twice as high as that in the leaves. The number of thrips, feeding punctures and eggs were lower on E+ as compared to endophyte-free plants. The reduced feeding and oviposition could have been a result of either reduced survival of thrips or antixenotic repellence of thrips. The results of this study are concordant with those of a screenhouse study carried out by Akutse et al. (2013) who reported that Faba beans colonized endophytically by fungal endophytes of the genera *Hypocrea* and *Beauveria* had significant negative effects on leafminer, *Liriomyza huidobrensis* (Blanchard) fitness, impacting on oviposition, emergence, longevity and causing 100% mortality within 15 days. These authors speculated a possible role of metabolites in deterrence and reduction of the insect fitness. *Helicoverpa armigera* Hübner larvae reared on tomato plants raised in a greenhouse colonized by *Acremonium strictum* Gams suffered significant reduction in growth rate, prolonged development times, suppressed moulting, and produced smaller pupae, and emerged adults were less fecund compared to larvae reared on control plants (Jallow et al., 2004). Larvae of bluegrass

webworm *Parapediasia teterrella* Zincken fed more on diets with endophyte-free plants of *L. perenne* L. and *Festuca arundinacea* Schreb whereas if only plants infected with *Acremonium* spp. were available the insects would starve to death. In the field, endophyte-free plants were severely attacked by the insects, whereas those infected with *Acremonium* spp. stayed almost free of insect larvae (Kanda et al., 1994). These negative effects imply that, endophytic colonization could be utilized for a long term effects on thrips biology. This phenomenon warrants further studies to unravel the underlying mechanisms such as possible release of metabolites and/or volatiles which could have effects on thrips.

Behavioral study of *T. tabaci* as influenced by endophytes reveals antixenosis of E+ plants. Y-tube olfactometer assays using whole plants and adult thrips confirmed the possible involvement of volatiles in influencing increased preference for E- plants over E+ plants. This implies that, the endophyte colonization could be responsible for triggering the plant defence system through release of volatiles. Analysis of volatile secondary metabolites in *Festuca arundinacea* (Poaceae) infected with the fungal endophyte *Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon and Hanlin revealed emissions of volatiles from plants with potential of influencing insect preferences (Qawasmeh et al., 2011). In an experiment using E- and E+ peppermint plants, Mucciarelli et al. (2007) observed an increase of terpenoids volatile compounds in leaves in the presence of the PGP-HSF endophyte. The authors speculated that the general improvement of plant growth and the glandular tissue differentiation were the most convincing explanations for the observed promotion of terpenoid biosynthesis in E+ peppermints. These outcomes further strengthens the functional role of endophytic colonization i.e. improving plant growth to facilitate emission of volatiles something that warrants further studies. *T. tabaci* larvae also showed preference for E- leaf sections as compared to E+ leaf sections. These findings were further



confirmed by the settling preference tests where more second-instar avoided settling on the E+ leaf sections. Similar observations were made by Richmond, (2007) who reported that, *Parapediasia teterrella* Zincken second instar displayed a stronger preference for E- over E+ plant materials. The authors implied a possible role of secondary metabolites in the non preference.

This study also shows that endophytic colonization reduces feeding and virus propagation by viruliferous thrips in onion plants. The observed reduced feeding activities were probably caused by deterrent effects due the endophyte colonization of onion plants. These results were related to previous outcome reported by Muvea et al. (2014). However, mean feeding by viruliferous thrips was higher than in non infected thrips. Similar results were reported by Stafford et al. (2011) that the feeding behavior of virus-infected *Frankliniella occidentalis* Pergande is modified to enhance persistent feeding. Guy & Davis, (2002) reported protection of New Zealand tall fescue colonized by *Neotyphodium* sp. through deterrence of aphid feeding. Endophyte-inoculated onion plants harbored less viral infections both in whole plants and leaf discs than endophyte-free plants. IYSV transmission increased gradually over time and it was lower in E+ plants as compared to the E- control plants. Compared to the baseline titer level, this implies that endophyte treated plants could offer benefits in regard to managing onion thrips and IYSV. Similar observation were made by Jaber & Selim (2014) where they reported *zucchini yellow mosaic virus* incidence and severity, sampled weekly over a period of four weeks, were significantly lower in *B. bassiana*-inoculated plants as compared to the non-inoculated control plants. These authors speculated a possible induced systemic resistance that may inhibit virus multiplication and accumulation in a host plant. Moreover, the possible role of a biochemical factor in endophyte-IYSV interactions could also play part in deterring virus propagation. For instance, some alkaloids released by endophytic fungi have been reported to posses antiviral activities (Selim et al. 2012). This

endophyte-virus interaction mechanism can be used to play a vital role in management of plant virus transmission on important agricultural crops. Based on research findings from this study, onions can be successfully inoculated through seeds with different fungal endophytes. *H. lixii* F3ST1 may be utilized to develop alternative and ecologically safe management strategy for onion thrips due to its antagonistic impact on onion thrips. However, further studies are warranted to determine the persistence of tested endophytes in the colonized plants under natural conditions and investigate potential for vertical transmission of endophytes and their multi-trophic interactions. Endophytic fungi could be utilized due to its role in thrips repellency and subsequent reduction of virus transmission. Further studies to elucidate potential semiochemicals that could be involved in inducing repellency against onion thrips and reduced virus transmission by *H. lixii* F3ST1 needs to be undertaken together with changes in contact chemoreception and mechanoreception cues induced by endophytes. Outcomes of such studies could open up opportunities for further refining of management strategies for thrips and the tospoviruses that they transmit.

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**ACKNOWLEDGEMENTS**

This study was funded by the BMZ (The German Federal Ministry for Economic Cooperation and Development) through GIZ (Deutsche Gesellschaft für Internationale Zusammenarbeit) through a project grant entitled “Implementation of integrated thrips and tospovirus management strategies in small-holder vegetable cropping systems of Eastern Africa “ (Project number: 11.7860.7-001.00 Contract number: 81141840) to which we are grateful. My sincere gratitude goes to my supervisors Prof. Dr. Hans-M. Poehling, Dr. Rainer Meyhöfer and Dr. Sevgan Subramanian for awarding me the Dissertation Research Internship Program (DRIP) fellowship and providing me with all the necessary support that I required during my study. Their great input through critical comments and valuable suggestion helped shape this research work. I would like to thank Drs. Maniania and Ekesi for their invaluable input in the manuscripts published from this thesis. It is my pleasure to thank Capacity building for all their support during my stay at *icipe*. I am indebted to Dr. Johnson Nyasani whose constant ideas, suggestion and corrections have had a great impact on the success of this work and Dr. Daisy Salifu for her guidance on data analysis. My sincere gratitude to colleagues at the thrips IPM and Arthropod Pathology Unit (APU) (Dr. Saliou, Mr. Muia, Mr. Mulwa, Mr levi, Ms Peris, Ms Catherine and Ms Matilda) for their endless support. Finally, I would like to thank my beloved wife (Faith Mukila), Son (Ryan Muvea), Daughter (Reanna Kanini), Brothers, Sisters, Parents (Daniel and Alice) and my Aunt (Vicky) for their spiritual support, love, patience, care, understanding and encouragement.

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Nielsen, M.-C., Worner, S., Chapman, B., de Kogel, W.-J., Perry, N., Sansom, C., Murai, T., Muvea, A.M., Subramanian, S., Davidson, M. & Teulon, D. (2010) Optimising the use of allelochemicals for thrips pest management. Proceedings of the International Society of Chemical Ecology Conference, 26<sup>th</sup> Annual Meeting, July 31<sup>st</sup> - August 4<sup>th</sup> 2010, Tours, France, pp 324.

## **AWARDS**

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**DECLARATION**

I, Alexander Mutua Muvea declare that this thesis, entitled ‘The effect of fungal endophytes on thrips and tospovirus epidemiology’ is an original piece of my work conducted by myself and has not been submitted for a degree in any other University.

Alexander Mutua Muvea

Hannover, 2015

Gottfried Leibniz Universität Hannover