Potential of integrating entomopathogenic and endophytic fungal based biopesticides for sustainable management of the South American tomato pinworm, *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

by

Ayaovi Agbessenou

Submitted in partial fulfilment of the requirements for the degree
PhD in Entomology

In the
Department of Zoology and Entomology
Faculty of Natural and Agricultural Sciences

UNIVERSITY OF PRETORIA

January 2023

Declaration

I, **Ayaovi Agbessenou** declare that the dissertation/thesis, which I hereby submit for the degree Doctor of Philosophy at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature 

Ayaovi Agbessenou

16th January 2023

Dedication

This dissertation is dedicated to my mother, my father (late), my beloved wife (Kékéli) and our children (Elinam and Dzidula) for their constant love, prayers, and support which drove me positively and enabled the successful completion of my PhD degree. May the Almighty God continue to protect you.

Ethics statement

The author, whose name appears on the title page of this dissertation/thesis declares that he has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research.

Signature 

Student name: Ayaovi Agbessenou

Month Year: January 2023

Disclaimer

This thesis consists of chapters that have been prepared as stand-alone papers already published (Chapters 2, 3 and 4) or as manuscripts. Consequently, there is some overlap of information and references in the thesis.

Acknowledgements

I would like to start by quoting the founder of the International Centre of Insect Physiology and Ecology (*icipe*), Professor Thomas Risley Odhiambo who said: "Get the very best people and then if you have more resources, put buildings and equipment around them". Throughout my PhD journey, I was really surrounded by high profile scientists who provided me with the mentorship that I needed to achieve this significant milestone. I am therefore indebted to my supervisors: Dr Komivi Akutse, Dr Fathiya Khamis and Prof. Abdullahi Yusuf for their guidance, motivation, mentorship and insightful comments and suggestions which greatly improved my research work and outputs.

I would like to sincerely thank *icipe* for awarding me the scholarship to pursue my PhD studies through the Dissertation and Research Internship Program (DRIP) under the African Union (AU) Tuta-IPM project, (Contract number: AURG II-2-123-2018). I also appreciate the financial support from UK's Foreign, Commonwealth & Development Office (FCDO) (DFID Biopesticide Project, B2291A-DFID-BIOPESTICIDE) through *icipe*. I wish to express my profound gratitude to the University of Pretoria (UP) for giving me the opportunity to pursue my doctoral studies and for providing me with the academic, administrative, and financial supports during my PhD journey.

My gratitude goes to Capacity Building and Institutional Development (CBID) staff especially, Dr Sunday Ekesi, Dr Rob Skilton, Vivian Atieno, Esther Ndung'u and Margaret Ochanda for their administrative support.

I would also like to thank the Arthropod Pathology Unity (APU) team especially Sospeter Wafula, Jane Kimemia, Levi Ombura, Carolyne Akal, Cynthia Opany, Maureen Adhiambo, Gasper Kirui and Kelvin Kariuki for their invaluable suggestions, administrative and technical support.

My thanks also go to the *icipe* Scholars Association (IScA) for giving me the opportunity to lead the association which has greatly improved my leadership skills. Thanks to IScA executive committee members for the period of 2020-2021 especially Stella Gachoki, Fairo Dzekashu, Sahadatou Mama Sambo, Evelyn Night Okello, Birhanu Sisay and Ferdinand Ong'wen.

I am also thankful to my big brothers Drs Bruce Anani, Komi Fiaboe, Saliou Niassy, Guillaume Ezui, Mawufe Agbodzavu, and Bonoukpoe Sokame for their constant support.

I am also thankful to my friends Drs Pascal Ayelo, Pascal Aigbedion-Atalor, Steve Baleba, Souleymane Diallo, Abdelmutalab Azrag, Akua Antwi-Agyakwa, Iman Hassaballa,

Olabimpe Olaide, Ritter Guimapi, and Inusa Ajene for the various interactions we had which improved my work. I am also thankful to Rodrigue Fiaboe, Komi Agboka, and Emmanuel Peter for their continuous support.

My heartfelt gratitude also goes to my tutor (Innocent Amedekagna), my uncle (Marc Attiley) and my siblings (Joseph, Mawussi, Esther, Philippe, and Jojo) for always supporting me.

Research outputs from this thesis

Journal articles

Agbessenou, A., Akutse, K. S., Yusuf, A. A., Ekesi, S., Subramanian, S. & Khamis, F. M. (2020). Endophytic fungi protect tomato and nightshade plants against *Tuta absoluta* (Lepidoptera : Gelechiidae) through a hidden friendship and cryptic battle. *Scientific Reports*, 10, 22195. https://doi.org/10.1038/s41598-020-78898-8

Agbessenou, A., Akutse, K. S., Yusuf, A. A., Wekesa, W. S. & Khamis, F. M. (2021). Temperature-dependent modelling and spatial prediction reveal suitable geographical areas for deployment of two *Metarhizium anisopliae* isolates for *Tuta absoluta* management. *Scientific Reports*, 11, 23346. https://doi.org/10.1038/s41598-021-02718-w

Agbessenou, A., Akutse, K. S., Yusuf, A. A. & Khamis, F. M. (2022). The Endophyte *Trichoderma asperellum* M2RT4 induces the systemic release of methyl salicylate and (*Z*)-jasmone in tomato plant affecting host location and herbivory of *Tuta absoluta*. *Frontiers in Plant Science*, 13, 860309. https://doi.org/10.3389/fpls.2022.860309

Conference presentations

Oral presentations

Agbessenou, A., Akutse, K. S., Yusuf, A. & Khamis, F. M. (2021). *Trichoderma asperellum* M2RT4 induces systemic release of methyl salicylate and (*Z)*-jasmone in tomato plant affecting host location and herbivory of the tomato leafminer, *Tuta absoluta*. Annual General Meeting, Department of Zoology and Entomology, University of Pretoria, 02nd – 03rd December 2021.

Agbessenou, A., Akutse, K. S., Yusuf, A. Wekesa, W. S. & Khamis, F. M. (2021). Making the right decision: Temperature-dependent modelling approach and spatial prediction reveal suitable areas for deployment of two *Metarhizium anisopliae* isolates for sustainable management of *Tuta absoluta*. Proceeding of the International Congress on Invertebrate Pathology and Microbial Control, the 53rd Annual Meeting of the Society for Invertebrate Pathology (SIP 2021) Virtual Conference on Invertebrate Pathology and Microbial Control, 28th June – 02nd July 2021. Fungi division, pp. 115.

Poster presentations

Agbessenou, A., Akutse, K. S., Yusuf, A. Wekesa, W. S. & Khamis, F. M. (2021). Temperature-dependent modelling approach and spatial prediction reveal suitable areas for deployment of two *Metarhizium anisopliae* isolates for sustainable management of *Tuta absoluta*. *icipe* Governing Council Annual meeting, Naivasha, Kenya, 18th November 2021.

Agbessenou, A., Akutse, K. S., Yusuf, A. A., Ekesi, S., Subramanian, S., & Khamis, F. M. (2020). The hidden friendship: Endophytic fungi protect tomato and nightshade plants against the tomato leafminer, *Tuta absoluta* through a cryptic battle. *icipe* Governing Council Annual meeting, Nairobi, Kenya, 20th November 2020.

Awards

Award of Excellence by *icipe* Governing Council 18th November 2021. **Best published science paper by an *icipe* Scholar, Winner**. Title: Endophytic fungi protect tomato and nightshade plants against *Tuta absoluta* (Lepidoptera: Gelechiidae) through a hidden friendship and cryptic battle. *Scientific Reports*, 10, 22195. https://doi.org/10.1038/s41598-020-78898-8.

Award of Excellence by *icipe* Governing Council 18th November 2021. **Best science poster by an *icipe* Scholar, First Runner Up.** Title: Temperature-dependent modelling approach and spatial prediction reveal suitable areas for deployment of two *Metarhizium anisopliae* isolates for sustainable management of *Tuta absoluta*.

Potential of integrating entomopathogenic and endophytic fungal based biopesticides for sustainable management of the South American tomato pinworm, *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

by

Ayaovi Agbessenou

Supervisor: **Prof. Abdullahi A. Yusuf**

Co-supervisors: **Dr. Komivi S. Akutse**
and
Dr. Fathiya M. Khamis
Plant Health Theme
International Centre of Insect Physiology and Ecology (*icipe*)
P.O. Box 30772-00100, Nairobi, Kenya

Department: **Zoology and Entomology**

University: **University of Pretoria**

Degree: **Doctor of Philosophy in Entomology**

Thesis summary

The South American tomato pinworm, *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the most devastating insect pests of tomato and other solanaceous crops (nightshade and potato) in Africa, yet control options are limited with the management of the pest heavily relying on indiscriminate use of synthetic insecticides leading to adverse effects on the environment and health of humans. Entomopathogenic fungi (EPF) offer an effective and viable alternative to synthetic insecticides, as they cause significant epizootics in the target host populations through an inundative approach and can also be used as plant endophytes whose presence within host plants are beneficial to the host. Even though promising beneficial effects due to endophytes are reported, the mechanism of plant-endophyte-*P. absoluta* communication is still poorly understood. Besides, the efficacy of three *Metarhizium anisopliae* (Metchnikoff) Sorokin fungal strains (ICIPE 18, ICIPE 20 and ICIPE 665) has been previously reported as the most potent isolates with high potential as biocontrol agents of adult *P. absoluta*. However, the interactions between their performance and abiotic factors that could affect their efficacy in the field need to be determined for an effective selection of the most virulent fungal isolate(s) best suited for mass-production prior to formulations and field deployment. This study assessed the efficacy of 18 candidate entomopathogenic- and endophytic-fungal isolates with the aim of integrating them into a sustainable management programme to control the pest. Chapter one provides an overview of current knowledge on the ecology and management practices of *P. absoluta*, and it identifies potential research gaps. Chapter two assessed the thermotolerance and virulence of the three candidate *M. anisopliae* isolates against adult *P. absoluta* under different temperature regimes. The findings revealed that over 90% of conidia germinated at 20, 25 and 30 °C while no germination occurred at 15 °C. In addition, growth of the three isolates occurred at all temperatures but was slower at 15, 33 and 35 °C compared to 20, 25 and 30 °C. Optimum temperatures for mycelial growth and spore production were 30 and 25 °C, respectively. Furthermore, ICIPE 18 produced 34.87 and 58.96% higher amount of spores than ICIPE 20 and ICIPE 665, respectively. The highest mortality of *P. absoluta* moths occurred at 30 °C for all the three isolates and was 91, 90 and 78% for ICIPE 18, ICIPE 20 and ICIPE 665, respectively. While the LT_{50} values were 30.69 and 45.63% for ICIPE 18 and ICIPE 20, respectively, significantly lower at 25 °C and 34.87% and 32.72% for ICIPE 18 and ICIPE 20, respectively, lower at 30 °C than those of ICIPE 665. Subsequently, Logan-4 and Logan-1 models gave the best fit to the mortality data to model the virulence of ICIPE

18 and ICIPE 20, against adult *P. absoluta* using the Entomopathogenic Fungi Application (EPFA) software. Spatial prediction revealed suitable locations for ICIPE 18 and ICIPE 20 deployment against *P. absoluta* in Kenya, Tanzania, and Uganda. Chapter three investigated the endophytic properties of 15 fungal isolates on tomato and nightshade and evaluated their insecticidal activity against adult and immature stages of the pest. The results showed that twelve isolates were endophytic to both host plants with varying colonisation rates. *Hypocrea lixii* F3ST1 and *T. asperellum* M2RT4 colonised more than 85% of all the plant tissues of both host plants while *B. bassiana* ICIPE 706 colonised 60, 40 and 15% of roots, stems and leaves of tomato, respectively; and 70, 35 and 15% of roots, stems and leaves of nightshade, respectively. *Trichoderma atroviride* F2S21 successfully-colonised 100, 100 and 75% of roots, stems and leaves of tomato plant respectively, and 100, 95 and 55% of roots, stems and leaves in nightshade, respectively. Tomato and nightshade host plants endophytically-colonised by *Trichoderma asperellum* M2RT4, *Beauveria bassiana* ICIPE 706 and *Hypocrea lixii* F3ST1 outperformed all the other isolates, significantly reducing the number of eggs laid, mines developed, pupae formed, and adults emerged. *Trichoderma asperellum* M2RT4 endophytically-colonised tomato plants recorded the lowest number of eggs (30.0 ± 4.51 eggs), followed by *Beauveria bassiana* ICIPE 706 (31.25 ± 5.88 eggs), *Hypocrea lixii* F3ST1 with (63.25 ± 2.66 eggs) compared to (111.0 ± 13.32 eggs) in the control. Upon egg hatching, *T. asperellum* M2RT4-endophytically-colonised tomato plants recorded the lowest number of mines (24.0 ± 5.4 mines) compared to (107.33 ± 13.32 mines) in the control. The lowest number of eggs was laid on *T. asperellum* M2RT4 endophytically-colonised nightshade plants (33.25 ± 3.97 eggs) compared to (109.33 ± 23.31 eggs) in the control. The lowest number of mines (24.5 ± 5.55 mines) was recorded on *T. asperellum* M2RT4 endophytically-colonised nightshade plants compared to the control (107.33 ± 23.31 mines). In endophytically-colonised tomato plants, fewer *P. absoluta* pupae (20.75 ± 4.05 pupae) were produced in *B. bassiana* ICIPE 706 followed by *T. asperellum* M2RT4 (21.25 ± 5.22 pupae) which were significantly different from the control (103.67± 12.55 pupae). *Phthorimaea absoluta* adult emergence varied significantly among the fungal isolates, where the highest number of moths (148.0 ± 24.57) emerged from *Fusarium proliferatum* F2S51 endophytically-colonised tomato plants, followed by the control (101.67 ± 11.46) while the lowest number (17.0 ± 6.34 moths) was recorded on *T. asperellum* M2RT4 endophytically-colonised tomato plants. The highest number of pupae was obtained in the control (102.33 ± 22.93 pupae) and the lowest (19 ± 4.12 pupae) was recorded in *T. asperellum* M2RT4

endophytically-colonised nightshade plants. Further, the number of adults that emerged from the control (99.33 ± 22.98 moths) was significantly higher than the lowest number (15.5 ± 3.2 moths) that was obtained in *T. asperellum* M2RT4 endophytically-colonised nightshade plants. Furthermore, the survival of exposed adults and F1 progeny was significantly reduced by *Trichoderma* sp. F2L41 and *B. bassiana* isolates ICIPE 35(4) and ICIPE 35(15) compared to other endophytic isolates. Chapters four and five unravelled the underlying chemical and molecular mechanisms by which the presence of the most potent endophyte *Trichoderma asperellum* M2RT4 within tomato host plant affects *P. absoluta* host selection and herbivory, respectively. Chemical analysis revealed the emission of methyl salicylate in endophytically-colonised tomato plant while for non-colonised infested plants monoterpenes were significantly higher. *Phthorimaea absoluta* females were attracted to monoterpernes including α-pinene, 2-carene, and β-phellandrene but repelled by methyl salicylate. Additionally, it was found that upon herbivory, *T. asperellum* M2RT4 modulates tomato plant chemistry through the production of (*Z*)-jasmone thus activating both salicylic and jasmonic acid defense pathways. Transcriptome analysis showed that *in planta* colonisation of tomato plant by *T. asperellum* M2RT4 activates tomato host plant defense pathways through the expression of *N*-methyltransferase. The findings not only reveal suitable locations for deployment of EPF-based biopesticides against *P. absoluta* in East Africa, but also contribute to a better understanding of host plant-*P. absoluta* interactions mediated by endophytic fungi, which might potentially be used in the development of effective biopesticides and their integration into a sustainable *P. absoluta* IPM management programme.

Table of Contents

List of tables

Chapter two

Chapter three

Chapter four

Chapter five

List of figures

Chapter one

Chapter two

Chapter three

Chapter four

Chapter five

List of abbreviations

ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
DCM	Dichloromethane
DEG	Differentially expressed gene
EPF	Entomopathogenic fungi
EPFA	Entomopathogenic fungi application
GLM	Generalised linear model
HAMP	Herbivore-associated molecular patterns
icipe	International Centre of Insect Physiology and Ecology
IPM	Integrated pest management
ISR	Induced systemic resistance
JA	Jasmonic acid
MAMP	Microbe-associated molecular patterns
NIST	National Institute of Standards and Technology
NMDS	Non-metric multi-dimensional scaling
PAST	Paleontological Statistics
PCA	Principal component analysis
RT	Retention time
SA	Salicylic acid
SAR	Systemic acquired resistance
SIMPER	Similarity percentage
VOCs	Volatile organic compounds
VPD	Vapour pressure deficit

CHAPTER ONE

General introduction

1.1.Background

Africa's population continues to grow steadily and it is projected to increase from the current 1.04 billion to 1.30 billion by 2030, subsequently reaching 2.12 billion by 2050 (United Nations, 2019). This rapid population growth generates an increased demand for food while agricultural productivity is currently below its potential in most countries on the continent (Afari-Sefa et al., 2012). Africa is also a continent where food and nutrition insecurity are constant challenges as more than 2 million people may not get a single meal per day (Olson et al., 2021). In an effort to boost food and nutrition security, the vegetable sector has been identified as having the potential to address these challenges on the continent (Weinberger and Lumpkin, 2007). Vegetables represent an important source of micronutrients, fibre, minerals and vitamins that contribute to achieve a balanced and healthy diet (Afari-Sefa et al., 2012). Tomato, (*Solanum lycopersicum* L.) (Solanales: Solanaceae) is one of the most valued vegetable crops in Africa with an annual production estimated at 22 million tons in 2020 (FAOSTAT, 2022) providing a wide range of nutritional and health benefits (Ochilo et al., 2019). However, its production is hindered by many biotic and abiotic factors (Tumwine et al., 2002). Amongst these constraints are the lack of access to improved agricultural technologies (agro-chemical inputs, improved seed varieties, irrigation, etc.) coupled with climate change and threats of emerging/invasive/transboundary pests and diseases that limit smallholder farmers from generating desired outputs. Invasive species are organisms that are introduced outside their natural range either via human-mediated (e.g. contaminants, corridors, stowaways) or natural dispersal (IUCN, 2000). Furthermore, climate change is another major factor contributing to the successful spread of invasive insect species into areas that were previously unfavourable, hence presenting significant threats to global agriculture (Paini et al., 2016), especially in SSA where food systems are already fragile and yield and crop quality are low (Savary et al., 2019). Many invasive insect species, across a diverse range of taxa, are now present in the eastern African region and with detrimental impacts on crop production (Pratt et al., 2017). Among these is, the South American tomato pinworm, *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae) a native pest to South America. It is currently one of the most devastating insect pests of tomato and other solanaceous crops of economic importance such as black nightshade, *Solanum nigrum* L.; eggplant, *Solanum melongena* L. and potato, *Solanum tuberosum* L.

(Idriss et al., 2018). In East Africa alone, annual losses due to *P. absoluta* in mixed small farming systems are estimated to be 69.6-79.4 million USD (Pratt et al. 2017). To overcome these losses, current *P. absoluta* management strategies rely heavily on indiscriminate use of synthetic insecticides causing adverse environmental and human health impacts and for which the insect has developed resistance (Desneux et al., 2021). The use of some insecticide groups is ineffective against larvae due to their leafmining behaviour (Desneux et al., 2021). Hence, the need to search for sustainable pest management methods to manage this pest with great interest placed on biological control approaches using natural enemies (predators, parasitoids or pathogens).

1.2.The South American tomato pinworm, *Phthorimaea absoluta*

1.2.1. Biology, host range, and damage

Although *P. absoluta* prefers tomato as its main host (Mansour et al., 2018), it also attacks other solanaceous crops including nightshade (*Solanum nigrum* L.), eggplant (*Solanum melongena* L.), potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.), and African eggplant (*Solanum aethiopicum* L.) (Brévault et al., 2014; Mohamed et al., 2015). The main biological characteristics leading to a successful invasion by *P. absoluta* are its high reproductive capacity as well as the short generation time with occurrence of several generations per year (Mansour et al., 2019). *Phthorimaea absoluta* has holometabolous life cycle (Figure 1.1). Female moths usually lay their yellow eggs on the upper surface of the leaves or stems (Biondi et al., 2018; Brévault et al., 2014; Desneux et al., 2010). Upon hatching, young larvae penetrate leaves, stems, and aerial fruits on which they feed and develop, causing significant damage to the crops. Young larvae (1^{st}-2^{nd} instar) bore into the plant and, once mature (3^{rd}-4^{th} instar), they leave their mines and move to new locations for feeding. Pupation occurs mainly on the leaves and in the soil (20 cm) depending on environmental and growing conditions (Uchoa-Fernandes et al., 1995). When *P. absoluta* does not pupate in the soil, a cocoon is usually built. Pupae (length: 5-6 millimetres) are cylindrical in shape and greenish when just formed becoming darker in colour as they near adult emergence (Figure 1.1). Upon emergence, females mate only once a day and are able to mate up to six times during their lifespan, with a single mating lasting about 4-5 h. Adult lifespan ranges between 10 and 15 days for females and 6-7 days for males under laboratory conditions (Uchoa-Fernandes et al., 1995). Females live longer than males, and they also reach sexually maturity before males (Tropea Garzia et al., 2012). The majority of

oviposition occurs 7 days after the first mating under laboratory conditions, and during this period a single female lays more than 70% of its eggs during the afternoon and twilight hours. During its lifetime, a single female may produce up to 260 eggs (Uchoa-Fernandes et al., 1995). Larvae do not enter diapause if food is available, and there may be 10-12 generations per year with the life cycle completed in 29-38 days depending on environmental conditions (Desneux et al., 2010). The pest is nocturnal (active at night), and adults usually remain hidden during the day, showing morning-crepuscular activity with adults spreading across fields by flying (Desneux et al., 2010). Recently, Silva et al. (2021) reported that *P. absoluta* preferred to lay eggs on domesticated tomato plants over non-cultivated solanaceous plant species.

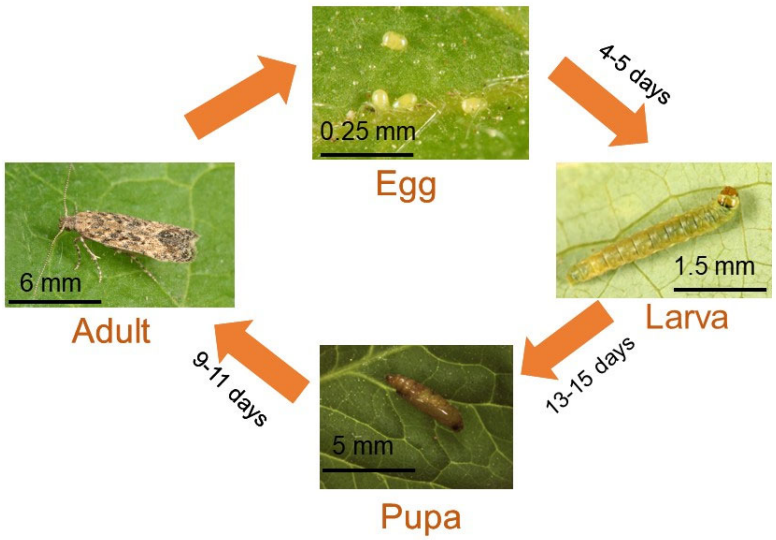

Figure 1.1. Life cycle of *Phthorimaea absoluta* showing the developmental stages through egg, larva, pupa and adult. ©*icipe*

1.2.2. Geographical distribution of the South American tomato pinworm, *Phthorimaea absoluta* in Africa

Phthorimaea absoluta originates from South America and the pest invaded Africa in 2008 through Spain, causing serious damage on Solanaceae crops (Figure 1.2) (Mansour et al., 2018). The pest has spread throughout Africa, with several reports confirming its presence in the continent (Mansour et al., 2018). Since the first detection in North Africa – Tunisia,

Algeria, and Morocco – in 2008, *P. absoluta* has spread both east and southwards to other sub-Saharan African countries, where it has established and become a major pest of tomato (Mansour et al., 2018). Currently, *P. absoluta* is reported in 41 of the 54 countries on the continent (Mansour et al., 2018). It is speculated that the spread of *P. absoluta* from Northern to Eastern Africa seems to have transited through Sudan (Mansour et al., 2018). Subsequently, the pest has migrated to Kenya via Ethiopia where it was first reported in 2014 (Mansour et al., 2018). From Kenya, the pest invaded Tanzania (2014) and Uganda (2015) (Tumuhaise et al., 2016). The rapid spread of *P. absoluta* over long distances supports the human-aided hypothesis of the dispersal of insect pests. Additionally, the absence of adequate and effective surveillance mechanisms, lack of or poor specific phytosanitary expertise to intercept infested leaves or fruits destined to and from international markets has contributed to the spread of the pest (Desneux et al., 2021).

Figure 1.2. Occurrence and years of first report of *Phthorimaea absoluta* invasion in Africa, as of August 2018, based on the published literature. Years of first report are indicated as follows: 08 (2008), 09 (2009), 10 (2010), 12 (2012), 13 (2013), 14 (2014), 15 (2015), 16 (2017), 17 (2017) (Mansour et al., 2018).

1.2.3. Economic importance of *Phthorimaea absoluta*

Phthorimaea absoluta is responsible for high yield losses and is listed as a quarantine pest in the European Union (EU) (Desneux et al., 2021). The pest causes yield losses of up to 100% on tomato crops (Biondi et al., 2018; Desneux et al., 2010). Highest losses are mostly experienced during early invasion owing to inadequate mitigation measures related to lack

of preparedness (Tarusikirwa et al., 2020). For example, in Nigeria, the impacts of *P. absoluta* has resulted in countrywide destruction of tomato farms and the shutdown of the Dangote tomato processing factory (Borisade et al., 2017) in what locals refer to as the 'Tomato tsunami'. As such, tomato yield losses due to *P. absoluta* invasion in Nigeria has been estimated at 720,000 metric tons (Sanda et al., 2018). It was reported that economic losses due to this pest reaches as high as US$ 59.3 million worldwide annually (Aigbedion-Atalor et al., 2019). Since its detection outside its native ranges in 2006 and subsequent spread and invasion, *P. absoluta* has resulted in significant reduction of the marketability of tomatoes (Tarusikirwa et al., 2020).

1.2.4. Management strategies

Since its first detection on the African continent in 2008, vegetable growers have been using synthetic insecticides to manage *P. absoluta*, but this practice has attracted growing public concerns associated with environmental and human health impacts (Niassy et al., 2020). Other management strategies including biological control (parasitism, predation and microbial agents), host plant resistance, trapping, and use of pheromones have been reported to suppress pest population with varying degrees of success (Aigbedion-Atalor et al., 2020; Akutse et al., 2020a; Ayelo et al., 2021).

1.2.4.1.Monitoring, mass trapping, and mating disruption

Monitoring of *P. absoluta* populations in tomato crops is usually achieved by trapping males or by sampling eggs and larvae infesting the host plants (Biondi et al., 2018). The density of males in a population tends to be negatively correlated with tomato production (Megido et al., 2013). During the last decade, a variety of traps have been designed and tested in the field-for their attractiveness to *P. absoluta*. One of such is the red Delta trap which is dubbed 'the most cost-effective'and recommended design (Desneux et al., 2021; Uchoa-Fernandes et al., 1995). These traps are typically made from paper or plastic shaped into a triangular prism, left open at both ends, with placement of a sticky panel insert at the bottom part of the trap and a pheromone lure placed on the sticky panel (Figure 1.3). Components of *P. absoluta* female pheromones are (3E, 8Z, 11Z)–3, 8, 11–tetradecatrien–1–yl acetate or TDTA (1) and (3E, 8Z)–3, 8–tetradecadien–1–yl acetate or TDDA (Megido et al., 2013). Also, water-filled bowls in combination with the pheromone lure can be used for controlling *P. absoluta* populations (Desneux et al., 2021).

In general, mass trapping methods for reducing the population densities of *P. absoluta* involve the use of lures (to attract males) (Witzgall et al., 2010). However, mass trapping alone is rarely sufficient to control males and should be used in combination with other control measures to reach an acceptable level of crop protection (Mansour and Biondi, 2021). The mating disruption strategy aims to interfere with the mate-searching efficacy of males by saturating the environment with a synthetic female pheromone (Desneux et al., 2021). Cocco et al. (2013) reported that the release of large amounts of sex pheromone is necessary to achieve significant results, with 500 to 1,000 pheromone dispensers per hectare being deployed for reducing pest infestations. Even if the efficiency of this method has been improved, its viability could be limited by the costs necessary for the production of pheromones,their field distribution andapplication (Desneux et al., 2021).

Figure 1.3. A Delta trap showing adults *P. absoluta* that have been caught in a tomato greenhouse. *©icipe*

1.2.4.2.Biological control of *Phthorimaea absoluta*

a. Predators and parasitoids

Since the invasion of *P. absoluta* in Africa, studies aimed at finding and assessing indigenous natural enemies that could potentially be used as biological control candidates have been ongoing (Aigbedion-Atalor et al., 2020). In Kenya, a number of hymenopteran parasitoids belonging to different families Chalcididae, Bethylidae, Braconidae, Eulophidae have been found attacking various developmental stages of *P. absoluta* (Mansour et al., 2018). Similarly, Idriss et al. (2018) reported in Sudan the performance of two native braconid parasitoids, *Bracon nigricans* Szépligeti and *Dolichogenidea appellatory* (Telenga)

(Hymenoptera: Braconidae), against different immature stages of *P. absoluta* under laboratory conditions. Recently, Aigbedion-Atalor et al. (2020) reported that the solitary koinobiont endoparasitoid *Dolichogenidea gelechiidivoris* Marsh (Hymenoptera: Braconidae), imported from Peru into Kenya, has shown potential for classical biological control of *P. absoluta* by parasitising early (first and second) instar larvae of the pest. Predatory insects commonly used against *P. absoluta* in Sudan are those from the family Miridae *Nesidiocoris tenuis* (Reuter) and *Macrolophus pygmaeus* (Rambur) (Mansour et al., 2018).

b. Entomopathogens

Entompathogenic microorganisms and their products have proven to be highly effective, species-specific and eco-friendly leading to their adoption in biological control strategies around the world (Akutse et al., 2020b). Among the pathogens (bacteria, viruses, protozoa and fungi), entomopathogenic fungi (EPFs) offer the best prospect against *P. absoluta* microbial control agents (Akutse et al., 2020a). Entomopathogenic fungi especially *Metarhizium anisopliae* Sorokin (1883) and *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) have been used with variable success against insect pests occupying different habitats (Inglis et al., 2001). These fungi kill insects by attaching to their cuticular surface, germinating and forming specialised infection structures (appressoria), penetrating through the cuticle and colonising internal host tissues resulting in host death and subsequent conidiation on the surface of the cadaver (Kreutz et al., 2007). Strains of *M. anisopliae* (ICIPE 18, ICIPE 20 and 665) were identified as the most effective candidate biopesticides against *P. absoluta* (Akutse et al., 2020a). An increasing number of recent studies demonstrate that EPFs including endophytes play additional roles in nature, viz. plant growth promotion, and rhizosphere colonisation (Barelli et al., 2016; Jaber and Ownley, 2018; Vega, 2008). Over several decades, the International Centre of Insect Physiology and Ecology (*icipe*), in partnership with the private sector have developed and commercialised entomopathogenic and endophytic fungal-based biopesticides for the management of major insect pests of economic importance in Africa (Akutse et al., 2020b). These ecological roles provide opportunities for the multiple use of EPFs in integrated pest management strategies (Lacey et al., 2015a).

c. Endophytic fungi

The term "endophytes" was first coined in 1809 by the German scientist Heinrich Friedrich (Hardoim et al., 2015). At that time, they were termed "*Entophytae*" and were described as a distinct group of partly parasitic fungi living in plants. Galipe (1887) reported the occurrence of bacteria and fungi within the tissues of vegetable plants and postulated that these microorganisms are derived from the soil and migrate into the plant, where they might play a beneficial role for the host plant. The most recent definition was by Petrini (1991) who termed endophytes as "all organisms inhabiting plant organs that at some time in their life cycle can colonise internal plant tissues without causing any apparent harm to their host". Plant-endophyte interactions may vary from mutualistic symbiosis (beneficial endophytes) to commensalism (non-beneficial/virulent endophytes) and rarely parasitism (virulent endophytes) (Mattoo and Nonzom, 2021). Various genera of entomopathogenic fungi such as *Beauveria, Metarhizium, Trichoderma, Hypocrea* have so far been reported to act as plant endophytes in a variety of host plants (Akello et al., 2009; Akutse et al., 2013; Bing and Lewis, 1991; Muvea et al., 2014; Ownley et al., 2008). Therefore, emerging multiple roles played by fungal entomopathogens provide promising potential for their indirect, multi-faceted and cost-effective use in sustainable agriculture. For instance, they can be used as biofertilisers (Jaber and Enkerli, 2016) and dual microbial agents of plant diseases and insect pests (Bamisile et al., 2018; Ownley et al., 2010; Vega, 2008).

d. Plants resistance against *Phthorimaea absoluta*

Breeding host plants resistant to *P. absoluta* is one of the most promising strategies for the sustainable management of the pest (de Oliveira et al., 2020). Although genetic sources of resistance to *P. absoluta* were detected among germplasm bank accessions of *S. lycopersicum* (Sohrabi et al., 2017), the most promising ones are from wild tomato (*Lycopersicon esculentum var. cerasiforme*) (Ecole et al., 2001; Leite et al., 2001). Constitutive tomato resistance to *P. absoluta* has been the focus of attention in breeding programs relying on resistance related to leaf allelochemicals or trichome density (De Azevedo et al., 2003). Glandular trichomes (type I, IV, VII) exhibit high deterrent activity against *P. absoluta* (Bleeker et al., 2012; De Azevedo et al., 2003). These compounds impair egg laying and larva feeding, leading to antixenosis, and larval toxicity, or antibiosis (Bleeker et al., 2012; De Azevedo et al., 2003).

e. Endophyte-mediated plant resistance against herbivores

Since the discovery that entomopathogenic fungi can live within plants as endophytes, researchers have been trying to understand how this affects mainly plants and herbivorous insect pests (Jensen et al., 2019). The mechanisms behind negative effects on herbivores by endophytic fungi have been suggested to be caused by an upregulation of the plants' defense and/or fungal metabolites that could be transported through the plants' vascular tissue and, thereby affecting herbivores directly (Jaber and Ownley, 2018). Induced systemic resistance (ISR) and systemic acquired resistance (SAR) are the two resistance patterns which have attracted the most attention of researchers (Fadiji and Babalola, 2020). Induced systemic resistance is modulated by ethylene or jasmonic acid (JA) which cannot be linked with the building up of pathogenesis-related (PR) proteins. For example, Hao et al. (2017) reported that root colonisation by the endophytic fungus *M. anisopliae* in peanuts (*Arachis hypogaea*) triggered differential expression of genes involved in ethylene responsive transcription factors, dehydration-responsive element-binding proteins, nitrate transporters, and transcription factors. On the other hand, SAR which is caused by infections from pathogens is mediated by salicylic acid. In addition, endophytic fungi are known to produce secondary metabolites that are involved in mechanisms of signalling, defense, and genetic regulation of the establishment of symbiosis (Hardoim et al., 2015).

f. Integration of entomopathogenic- and endophytic fungi as successful components of IPM

Integrated pest management (IPM) is built on the concept that pest populations can be maintained cost-effectively below economic threshold levels by combining a wide range of management techniques that have little negative impact on the environment and ecosystem in general (Skinner et al., 2014). According to the United States Environmental Protection Agency (EPA), biopesticides are pesticides derived from natural materials such as animals, plants, bacteria and certain minerals, and can be classified in three classes: (1) microbial pesticides, which consist of a microorganism as the active ingredient; (2) biochemical pesticides, which are naturally occurring substances that control pests by non-toxic mechanisms such as insect sex pheromones and plant extracts; and (3) plant-incorporated protectants, which are pesticidal substances that plants produce from genetic material that has been added to the plant (Akutse et al., 2020b). Due to their eco-friendly and cont-effective nature, biopesticides needs to be fully integrated into an IPM approach for a successful management of *P. absoluta*. Already, biopesticides are being developed to target

adults as well as induce systemic resistance through endophytic activity against larvae (Akutse et al., 2020a; Akutse et al., 2020b). Thus in order to ensuring a more sustainable and efective pest management, irrespective of the invasion area, these biopesticides need to be integrated into a package that includes monitoring and mass trapping, resistant cultivars, protected cultivation, field sanitation and release of parasitoids/predators (Desneux et al., 2021). Additionally, some critical factors must be assessed when considering an entomopathogenic fungus for IPM, including: virulence; mass production potential; compatibility with chemical insecticides, fungicides and natural enemies; persistence; shelf life; and ease of application (Skinner et al., 2014).

1.3.Problem statement and rationale

The South American tomato pinworm, *P. absoluta* is ranked as the most economically important pest of tomato and solanaceous crops (nightshade, potato and eggplant) worldwide (Idriss et al., 2018). The pest can cause up to 100 percent yield loss on tomatoes, reducing fruit quality. The economic losses have been estimated at up to $79.4 million per year in Africa (Aigbedion-Atalor et al., 2019). The most common control method used against the pest are synthetic chemical insecticides which have negative impacts on human and environment health and often leads to the development of insecticide resistance (Niassy et al., 2020). Therefore, there is an urgent need to promote environmentally friendly control measures to sustainably manage *P. absoluta* (Niassy et al., 2020). Microbial-based biopesticides, especially entomopathogenic fungi have long been considered in IPM strategies. They are known to cause significant epizootics in the target host population with minimum impact on beneficial and other non-target organisms (Akutse et al., 2020a; Mweke et al., 2020), justifying their choice as promising candidates in IPM programs. Entomopathogenic fungi can target both larval and adult stages of a pest as either direct sprays or through “lure-and-infect” application. They can also be used as plant endophytes whose presence in the host is beneficial (Akutse et al., 2013; Akutse et al., 2020a; Mkiga et al., 2020; Opisa et al., 2019). Among the fungal entomopathogens, *Beauveria bassiana* (Hypocreales: Clavicipitaceae) and *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) have been used to control several insect pests of economic importance. Strains belonging to these genera are relatively easy to mass-produce, formulate and apply, and have been found to be among the most virulent against insect pests in Africa (Akutse et al., 2020b). Recently, Akutse et al. (2020a) reported the potential of some fungal isolates for the control of *P.*

absoluta moths and identified three *M. anisopliae* strains (ICIPE 18, ICIPE 20 and ICIPE 665) as the most potent candidate biopesticides for use against the pest. Although these *M. anisopliae* strains were pathogenic against *P. absoluta* in laboratory experiments, the selection of suitable strains coupled with the study of their spatial prediction are a prerequisite to optimise their efficacy and formulations before field deployment. Additionally, given the concealed feeding behaviour of immature stages of *P. absoluta*, endophytes are more likely to be effective against the larval stage of the pest. Endophytes possess remarkable insecticidal properties and confer protection to host plants through the production of secondary metabolites and the activation of antagonism mechanisms such as antibiosis and induced systemic resistance (Pieterse et al., 2014; Poveda, 2021b). However, the mechanism behind plant-endophyte-pest interactions is still poorly understood. Therefore, understanding the chemical and molecular mechanisms that shape these interactions between host plants, endophytes, and *P. absoluta* is crucial for developing novel approaches aimed at promoting their use in sustainable management of the South American tomato pinworm.

1.4.General aim and objectives of the thesis

1.4.1. General aim

This thesis was aimed at assessing the efficacy of candidate entomopathogenic- and endophytic-fungal isolates, with the goal of integrating them into a sustainable management program to control the South Amercian tomato pinworm, *P. absoluta*.

1.4.2. Specific objectives

i) To assess the thermotolerance of three candidate isolates of entomopathogenic fungus *M. anisopliae* (ICIPE 18, ICIPE 20 and ICIPE 665) and their virulence against *P. absoluta* adults under different temperature regimes;

ii) To evaluate the endophytic properties of 15 fungal isolates in both tomato and nightshade host plants and assess their insecticidal activity against both adult and immature stages of the pest;

iii) To unravel the underlying chemical mechanism by which the presence of the endophyte *Trichoderma asperellum* M2RT4 within tomato host plant affects *P. absoluta* moths' oviposition selection and herbivory;

iv) To investigate the transcriptional changes elicited in tomato plant upon *T. asperellum* M2RT4 colonisation, with or without *P. absoluta* infestation.

1.5.Research questions

The main research questions of this thesis were as follows:

i) Do environmental factors modulate the performance/efficacy of entomopathogenic fungi in *P. absoluta* management?

ii) Could endophytic fungi successfully establish themselves within tomato and nightshade host plants and induce systemic resistance in the host plants against *P. absoluta*?

iii) Does the endophyte *T. asperellum* M2RT4 alter quantitatively and qualitatively tomato host plant volatile composition?

iv) Does the endophyte *T. asperellum* M2RT4 induce transcriptional reprogramming in tomato host plant leading to the activation of defense pathways?

1.6. Hypotheses

To achieve the objectives in this thesis, the following hypotheses were formulated:

i) Environmental factors do not modulate the performance/efficacy of entomopathogenic fungi

ii) Endophytic fungi cannot successfully establish themselves within tomato and nightshade host plants and cannot induce systemic resistance in the host plants against *P. absoluta*

iii) The endophyte *T. asperellum* M2RT4 does not alter quantitatively and qualitatively tomato host plant volatile composition

iv) The endophyte *T. asperellum* M2RT4 does not induce transcriptional reprogramming in tomato host plant.

References

Afari-Sefa, V., Tenkouano, A., Ojiewo, C. O., Keatinge, J. D. H., and Hughes, J. d. A. (2012). Vegetable breeding in Africa: Constraints, complexity and contributions toward achieving food and nutritional security. *Food Secur.* 4, 115–127. doi:10.1007/s12571-011-0158-8.

Aigbedion-Atalor, P. O., Hill, M. P., Zalucki, M. P., Obala, F., Idriss, G. E., Midingoyi, S.-K., Ekesi, S. and Mohamed, S. (2019). The South America tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae), spreads its wings in Eastern Africa: distribution and socioeconomic impacts. *J. Econ. Entomol.* 112, 2797–2807. doi:10.1093/jee/toz220.

Aigbedion-Atalor, P. O., Mohamed, S. A., Hill, M. P., Zalucki, M. P., Azrag, A. G. A.,

Srinivasan, R., and Sunday, E. (2020). Host stage preference and performance of *Dolichogenidea gelechiidivoris* (Hymenoptera: Braconidae), a candidate for classical biological control of *Tuta absoluta* in Africa. *Biol. Control* 144, 1–8. doi:10.1016/j.biocontrol.2020.104215.

Akello, J., Dubois, T., Coyne, D., and Hillnhutter, C. (2009). *Beauveria bassiana* as an endophyte in tissue-cultured banana plants: A novel way to combat the banana weevil *Cosmopolites sordidus*. *Acta Hortic.* 828, 129–138. doi:10.17660/ActaHortic.2009.828.12.

Akutse, K. S., Maniania, N. K., Fiaboe, K. K. M., Van den Berg, J., and Ekesi, S. (2013). Endophytic colonization of *Vicia faba* and *Phaseolus vulgaris* (Fabaceae) by fungal pathogens and their effects on the life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Fungal Ecol.* 6, 293–301. doi:10.1016/j.funeco.2013.01.003.

Akutse, K. S., Subramanian, S., Khamis, F. M., Ekesi, S., and Mohamed, S. A. (2020a). Entomopathogenic fungus isolates for adult *Tuta absoluta* (Lepidoptera: Gelechiidae) management and their compatibility with *Tuta* pheromone. *J. Appl. Entomol.*, 1–11. doi:10.1111/jen.12812.

Akutse, K. S., Subramanian, S., Maniania, N. K., Dubois, T., and Ekesi, S. (2020b). Biopesticide research and product development in Africa for sustainable agriculture and food security – Experiences from the International Centre of Insect Physiology and Ecology (icipe). *Front. Sustain. Food Syst.* 4, 563016. doi:10.3389/fsufs.2020.563016.

Ayelo, P. M., Yusuf, A. A., Pirk, C. W. W., Chailleux, A., Mohamed, S. A., and Deletre, E. (2021). Terpenes from herbivore-induced tomato plant volatiles attract *Nesidiocoris tenuis* (Hemiptera: Miridae), a predator of major tomato pests. *Pest Manag. Sci.* 77, 5255–5267. doi:10.1002/ps.6568.

Bamisile, B. S., Dash, C. K., Akutse, K. S., Keppanan, R., and Wang, L. (2018). Fungal endophytes: Beyond herbivore management. *Front. Microbiol.* 9. doi:10.3389/fmicb.2018.00544.

Barelli, L., Moonjely, S., Behie, S. W., and Bidochka, M. J. (2016). Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi. *Plant Mol. Biol.* 90, 657–664. doi:10.1007/s11103-015-0413-z.

Bing, L. A., and Lewis, L. C. (1991). Suppression of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. *Environ. Entomol.* 20, 1207–1211. doi:10.1093/ee/20.4.1207.

Biondi, A., Guedes, R. N. C., Wan, F.-H., and Desneux, N. (2018). Ecology, worldwide spread, and management of the invasive South American tomato pinworm, *Tuta absoluta*: past, present, and future. *Annu. Rev. Entomol.* 63, 239–258. doi:10.1146/annurev-ento-031616-034933.

Bleeker, P. M., Mirabella, R., Diergaarde, P. J., VanDoorn, A., Tissier, A., Kant, M. R., et al. (2012). Improved herbivore resistance in cultivated tomato with the sesquiterpene biosynthetic pathway from a wild relative. *Proc. Natl. Acad. Sci. U. S. A.* 109, 20124–20129. doi:10.1073/pnas.1208756109.

Borisade, O. A., Kolawole, A. O., Adebo, G. M., and Uwaidem, Y. I. (2017). The tomato leafminer (*Tuta absoluta*) (Lepidoptera : Gelechiidae) attack in Nigeria: effect of climate change on over-sighted pest or agro-bioterrorism? *J. Agric. Ext. Rural Dev.* 9, 163–171. doi:10.5897/JAERD2017.0856.

Brévault, T., Sylla, S., Diatte, M., Bernadas, G., and Diarra, K. (2014). *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae): A new threat to tomato production in Sub-Saharan Africa. *African Entomol.* 22, 441–444. doi:10.4001/003.022.0202.

Cherif, A., Mansour, R., and Grissa-lebdi, K. (2013). Biological aspects of tomato leafminer *Tuta absoluta* (Lepidoptera : Gelechiidae) in conditions of Northeastern Tunisia : possible implications for pest management. 179–184.

Cocco, A., Deliperi, S., and Delrio, G. (2013). Control of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in greenhouse tomato crops using the mating disruption technique. *J. Appl. Entomol.* 137, 16–28. doi:10.1111/j.1439-0418.2012.01735.x.

De Azevedo, S. M., Ventura Faria, M., Maluf, W. R., Barneche De Oliveira, A. C., and De Freitas, J. A. (2003). Zingiberene-mediated resistance to the South American tomato pinworm derived from *Lycopersicon hirsutum var. hirsutum*. *Euphytica* 134, 347–351. doi:10.1023/B:EUPH.0000005007.14924.d2.

de Oliveira, J. R. F., de Resende, J. T. V., Filho, R. B. de L., Roberto, S. R., da Silva, P. R., Rech, C., and Nardi, C. (2020). Tomato breeding for sustainable crop systems: High levels of zingiberene providing resistance to multiple arthropods. *Horticulturae* 6, 1–14. doi:10.3390/horticulturae6020034.

Desneux, N., Han, P., Mansour, R., Arnó, J., Brévault, T., Campos, M. R., Chailleux, A., Guedes, R. N., Karimi, J., Perez-Hedo, M., Urbaneja, A., Verheggen, F. J., Zappalà, L., Abbes, K., Bayram, Y., Cantor, F., Cuthbertson, A. G., Firake, D. M., Ismoilov, K., Messelink, G., Nofemela, R., Oke, A., Ramos, C., Ricupero, M., Roditakis, E., Zhang,

Y-B., and Biondi, A. (2021). Integrated pest management of *Tuta absoluta*: practical implementations across different world regions. *J. Pest Sci.* 1–23. doi:10.1007/s10340-021-01442-8.

Desneux, N., Wajnberg, E., Wyckhuys, K. A. G., Burgio, G., Arpaia, S., Narváez-Vasquez, C. A., Cabello, T., and Urbaneja, A. (2010). Biological invasion of European tomato crops by *Tuta absoluta*: ecology, geographic expansion and prospects for biological control. *J. Pest Sci.* 83, 197–215. doi:10.1007/s10340-010-0321-6.

Ecole, C. C., Picanço, M. C., Guedes, R. N. C., and Brommonschenkel, S. H. (2001). Effect of cropping season and possible compounds involved in the resistance of *Lycopersicon hirsutum f. typicum* to *Tuta absoluta* (Meyrick) (Lep., Gelechiidae). *J. Appl. Entomol.* 125, 193–200. doi:10.1046/j.1439-0418.2001.00525.x.

EPPO (2022). EPPO Global Database. *Eur. Plant Prot. Organ.* Available at: https://gd.eppo.int/taxon/GNORAB/distribution [Accessed July 11, 2022].

Fadiji, A. E., and Babalola, O. O. (2020). Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects. *Front. Bioeng. Biotechnol.* 8, 00467. doi:10.3389/fbioe.2020.00467.

FAOSTAT (2022). Statistical database of the Food and Agriculture Organization of the United Nations. *Online*. Available at: www.fao.org.

Galipe, V. (1887). Note sur la présence de micro-organismes dans les tissus végétaux. *Soc Biol Fil* 39, 410–416.

Hao, K., Wang, F., Nong, X., McNeill, M. R., Liu, S., Wang, G., and Zhang, Z. (2017). Response of peanut *Arachis hypogaea* roots to the presence of beneficial and pathogenic fungi by transcriptome analysis. *Sci. Rep.* 7, 964. doi:10.1038/s41598-017-01029-3.

Hardoim, P. R., Overbeek, L. S. Van, Berg, G., Pirttilä, M., Compant, S., Campisano, A., and Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.* 79, 293–320. doi:10.1128/mmbr.00050-14.

Idriss, G. E. A., Mohamed, S. A., Khamis, F., Du Plessis, H., and Ekesi, S. (2018). Biology and performance of two indigenous larval parasitoids on *Tuta absoluta* (Lepidoptera: Gelechiidae) in Sudan. *Biocontrol Sci. Technol.* 28, 614–628. doi:10.1080/09583157.2018.1477117.

Inglis, G. D., Goettel, M. S., Butt, T. M., and Strasser, H. (2001). Use of hyphomycetous

fungi for managing insect pests. In: Butt, T.M., Jackson, C., Magan, N. (Eds.), Fungi as Biocontrol Agents: Progress, Problems and Potential. CABI, pp. 23–69.

IUCN (2000). Guidelines for the prevention of biodiversity loss caused by alien invasive species.

Jaber, L. R., and Enkerli, J. (2016). Effect of seed treatment duration on growth and colonization of *Vicia faba* by endophytic *Beauveria bassiana* and *Metarhizium brunneum*. *Biol. Control* 103, 187–195. doi:10.1016/j.biocontrol.2016.09.008.

Jaber, L. R., and Ownley, B. H. (2018). Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? *Biol. Control* 116, 36–45. doi:10.1016/j.biocontrol.2017.01.018.

Jensen, R. E., Enkegaard, A., and Steenberg, T. (2019). Increased fecundity of *Aphis fabae* on *Vicia faba* plants following seed or leaf inoculation with the entomopathogenic fungus *Beauveria bassiana*. *PLoS One* 14, 1–12. doi:10.1371/journal.pone.0223616.

Kreutz, J., Zimmermann, G., and Vaupel, O. (2007). Horizontal transmission of the entomopathogenic fungus *Beauveria bassiana* among the spruce bark beetle, *Ips typographus* (Col., Scolytidae) in the laboratory and under field conditions . *Biocontrol Sci. Technol.* 14, 837–848. doi:10.1080/788222844.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., and Goettel, M. S. (2015). Insect pathogens as biological control agents: Back to the future. *J. Invertebr. Pathol.* 132, 1–41. doi:10.1016/j.jip.2015.07.009.

Leite, G. L. D., Picanço, M., Guedes, R. N. C., and Zanuncio, J. C. (2001). Role of plant age in the resistance of *Lycopersicon hirsutum f. glabratum* to the tomato leafminer *Tuta absoluta* (Lepidoptera: Gelechiidae). *Sci. Hortic. (Amsterdam).* 89, 103–113. doi:10.1016/S0304-4238(00)00224-7.

Mansour, R., and Biondi, A. (2021). Releasing natural enemies and applying microbial and botanical pesticides for managing *Tuta absoluta* in the MENA region. *Phytoparasitica* 49, 179–194. doi:10.1007/s12600-020-00849-w.

Mansour, R., Brévault, T., Chailleux, A., Cherif, A., Grissa-Lebdi, K., Haddi, K., Mohamed, S., Tonnang, H. E., Desneux, N., and Biondi, A. (2018). Occurrence, biology, natural enemies and management of *Tuta absoluta* in Africa. *Entomol. Gen.* 38, 83–112. doi:10.1127/entomologia/2018/0749.

Mansour, R., Cherif, A., and Attia-barhoumi, S. (2019). *Tuta absoluta* in Tunisia: ten years of invasion and pest management. *Phytoparasitica* 47, 461–474.

Mattoo, A. J., and Nonzom, S. (2021). Endophytic fungi: understanding complex cross-talks. *Symbiosis* 83, 237–264. doi:10.1007/s13199-020-00744-2.

Megido, R. C., Haubruge, E., and Verheggen, F. J. (2013). Pheromone-based management strategies to control the tomato leafminer, *Tuta absoluta* (Lepidoptera : Gelechiidae). A review. *Biotechnol. Agron. Soc. Environ.* 17, 475–482.

Mkiga, A. M., Mohamed, S. A., du Plessis, H., Khamis, F. M., Akutse, K. S., Nderitu, P. W., Niassy, S., and Sunday, E. (2020). Compatibility and efficacy of *Metarhizium anisopliae* and sex pheromone for controlling *Thaumatotibia leucotreta*. *J. Pest Sci.* 94, 393–407. doi:10.1007/s10340-020-01281-z.

Mohamed, E. S. I., Mahmoud, M. E. E., Elhaj, M. A. M., Mohamed, S. A., and Ekesi, S. (2015). Host plants record for tomato leaf miner *Tuta absoluta* (Meyrick) in Sudan. *EPPO Bulletin 45*, 108-111.

Muvea, A. M., Meyhöfer, R., Subramanian, S., Poehling, H. M., Ekesi, S., and Maniania, N. K. (2014). Colonization of onions by endophytic fungi and their impacts on the biology of *Thrips tabaci*. *PLoS One* 9, 1–7. doi:10.1371/journal.pone.0108242.

Mweke, A., Akutse, K. S., Ulrichs, C., Fiaboe, K. K. M., Maniania, N. K., and Ekesi, S. (2020). Integrated management of *Aphis craccivora* in cowpea using intercropping and entomopathogenic fungi under field conditions. *J. Fungi* 6, 1–16. doi:10.3390/jof6020060.

Niassy, S., Ekesi, S., Migiro, L., and Otieno, W. (2020). *Sustainable management of invasive pests in Africa*. Springer International Publishing doi:10.1007/978-3-030-41083-4.

Ochilo, W. N., Nyamasyo, G. N., Kilalo, D., Otieno, W., Otipa, M., Chege, F., Karanja, T., and Lingeera, E. K. (2019). Characteristics and production constraints of smallholder tomato production in Kenya. *Sci. African* 2, e00014. doi:10.1016/j.sciaf.2018.e00014.

Olson, R., Gavin-smith, B., Ferraboschi, C., and Kraemer, K. (2021). Food fortification: The advantages, disadvantages and lessons from sight and life programs. *Nutrients* 13, 1118.

Opisa, S., du Plessis, H., Akutse, K. S., Fiaboe, K. K. M., and Ekesi, S. (2019). Horizontal transmission of *Metarhizium anisopliae* between *Spoladea recurvalis* (Lepidoptera: Crambidae) adults and compatibility of the fungus with the attractant phenylacetaldehyde. *Microb. Pathog.* 131, 197–204. doi:10.1016/j.micpath.2019.04.010.

Ownley, B. H., Griffin, M. R., Klingeman, W. E., Gwinn, K. D., Moulton, J. K., and Pereira, R. M. (2008). *Beauveria bassiana*: Endophytic colonization and plant disease control.

J. Invertebr. Pathol. 98, 267–270. doi:10.1016/j.jip.2008.01.010.

Ownley, B. H., Gwinn, K. D., and Vega, F. E. (2010). Endophytic fungal entomopathogens with activity against plant pathogens: Ecology and evolution. *Ecol. Fungal Entomopathog.* 55, 113–128. doi:10.1007/s10526-009-9241-x.

Paini, D. R., Sheppard, A. W., Cook, D. C., De Barro, P. J., Worner, S. P., and Thomas, M. B. (2016). Global threat to agriculture from invasive species. *Proc. Natl. Acad. Sci. U. S. A.* 113, 7575–7579. doi:10.1073/pnas.1602205113.

Petrinin, O. (1991). "Fungal endophytes of tree leaves," in *Microbial ecology of leaves*, eds. J. Andrews and S. Hirano (New York, NY: Springer-Verlag), 179–197. doi:10.1007/978-1-4612-3168-4_9.

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M., and Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* 52, 347–375. doi:10.1146/annurev-phyto-082712-102340.

Poveda, J. (2021). *Trichoderma* as biocontrol agent against pests: New uses for a mycoparasite. *Biol. Control* 159, 104634. doi:10.1016/j.biocontrol.2021.104634.

Pratt, C. F., Constantine, K. L., and Murphy, S. T. (2017). Economic impacts of invasive alien species on African smallholder livelihoods. *Glob. Food Sec.* 14, 31–37. doi:10.1016/j.gfs.2017.01.011.

Sanda, N. B., Sunusi, M., Hamisu, H. S., Wudil, S. B., Sule, H., and Abdullahi, A. M. (2018). Biological invasion of tomato leaf miner, *Tuta absoluta* (Meyrick) in Nigeria : Problems and management strategies optimization: A review. *Asian J. Agric. Hortic. Res.* 1, 1–14. doi:10.9734/AJAHR/2018/41959.

Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., and Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nat. Ecol. Evol.* 3, 430–439. doi:10.1038/s41559-018-0793-y.

Silva, G. A., Queiroz, E. A., Arcanjo, L. P., Lopes, M. C., Araújo, T. A., Galdino, T. S. V., Rodrigues-Silva, N. and Picanco, M. C. (2021). Biological performance and oviposition preference of tomato pinworm *Tuta absoluta* when offered a range of Solanaceous host plants. *Sci. Rep.* 11, 1153. doi:10.1038/s41598-020-80434-7.

Skinner, M., Parker, B. L., and Kim, J. S. (2014). Role of entomopathogenic fungi in integrated pest management. *Integrated pest management*, 169-191.

Smith, J. D., Dubois, T., Mallogo, R., Njau, E.-F. F., Tua, S., and Srinivasan, R. (2019). Host range of the invasive tomato pest *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) on

solanaceous crops and weeds in Tanzania. *Florida Entomol.* 101, 573. doi:10.1653/024.101.0417.

Sohrabi, F., Nooryazdan, H. R., Gharati, B., and Saeidi, Z. (2017). Plant resistance to the moth *Tuta absoluta* (Meyrick) (Lepidoptera:Gelechiidae) in tomato cultivars. *Neotrop. Entomol.* 46, 203–209. doi:10.1007/s13744-016-0441-7.

Tarusikirwa, V. L., Machekano, H., Mutamiswa, R., Chidawanyika, F., and Nyamukondiwa, C. (2020). *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) on the "Offensive" in Africa: Prospects for integrated management initiatives. *Insects* 11, 764.

Tropea Garzia, G., Siscaro, G., Biondi, A., and Zappalà, L. (2012). *Tuta absoluta*, a South American pest of tomato now in the EPPO region: Biology, distribution and damage. *EPPO Bull.* 42, 205–210. doi:10.1111/epp.2556.

Tumuhaise, V., Khamis, F. M., Agona, A., Sseruwu, G., and Mohamed, S. A. (2016). First record of *Tuta absoluta* (Lepidoptera: Gelechiidae) in Uganda. *Int. J. Trop. Insect Sci.* 36, 135–139. doi:10.1017/S1742758416000035.

Tumwine, J., Frinking, H. D., and Jeger, M. J. (2002). Integrating cultural control methods for tomato late blight (*Phytophthora infestans*) in Uganda. *Ann. Appl. Biol.* 141, 225–236. doi:10.1111/j.1744-7348.2002.tb00214.x.

Uchoa-Fernandes, M. A., Della Lucia, T. M. C., and Vilela, E. F. (1995). Mating, oviposition and pupation of *Scrobipalpuloides absoluta* (Meyr.) (Lepidoptera: Gelechiidae). *An. da Soc. Entomológica do Bras.* 24, 159–164. doi:10.37486/0301-8059.v24i1.1007.

United Nations (2019). *[World population prospects 2019]*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12283219.

Vega, F. E. (2008). Insect pathology and fungal endophytes. *J. Invertebr. Pathol.* 98, 277–279. doi:10.1016/j.jip.2008.01.008.

Weinberger, K., and Lumpkin, T. A. (2007). Diversification into horticulture and poverty reduction: A Research Agenda. *World Dev.* 35, 1464–1480. doi:10.1016/j.worlddev.2007.05.002.

Witzgall, P., Kirsch, P., and Cork, A. (2010). Sex pheromones and their impact on pest management. *J. Chem. Ecol.* 36, 80–100. doi:10.1007/s10886-009-9737-y.

CHAPTER TWO

Temperature-dependent modelling and spatial prediction reveal suitable geographical areas for deployment of two *Metarhizium anisopliae* isolates for *Phthorimaea absoluta* management

This chapter has been published as:

Agbessenou, A., Akutse, K. S., Yusuf, A. Wekesa, W. S. & Khamis, F. M. (2021). Temperature-dependent modelling and spatial prediction reveal suitable geographical areas for deployment of two *Metarhizium anisopliae* isolates for *Tuta absoluta* management. *Scientific Reports*, 11:23346. https://doi.org/10.1038/s41598-021-02718-w

Abstract

Phthorimaea absoluta is one of the most devastating pests of tomato and nightshade in Africa. We previously demonstrated the efficacy of *Metarhizium anisopliae* isolates ICIPE 18, ICIPE 20 and ICIPE 665 against *P. absoluta* moths. However, adequate strain selection and accurate spatial prediction are fundamental to optimize their efficacy and formulations before field deployment. This study therefore assessed the thermotolerance, conidial yield and virulence (between 15 and 35 °C) of these potent isolates. Conidia of the three *M. anisopliae* isolates germinated at all tested temperatures (ranging from 5 to 100%), except at 15°C where no germination was observed 18 h post-incubation. Growth of the three isolates occurred at all temperatures, but was slower at 15, 33 and 35 °C as compared to 20, 25 and 30 °C. Optimum temperatures for mycelial growth and spore production were 30 and 25 °C, respectively. Furthermore, ICIPE 18 produced higher number of spores than ICIPE 20 and ICIPE 665. The highest mortality occurred at 30 °C for all the three isolates, while the LT50 values of ICIPE 18 and ICIPE 20 were significantly lower at 25 and 30 °C compared to those of ICIPE 665. Subsequently, several nonlinear equations were fitted to the mortality data to model the virulence of ICIPE 18 and ICIPE 20 against adult *P. absoluta* using the Entomopathogenic Fungi Application (EPFA) software. Spatial prediction revealed suitable locations for ICIPE 18 and ICIPE 20 deployment against *P. absoluta* in Kenya, Tanzania, and Uganda. Our findings suggest that ICIPE 18 and ICIPE 20 could be considered as effective candidate biopesticides for an improved *P. absoluta* management based on temperature and location-specific approaches.

2.1.Introduction

Tomato, *Solanum lycopersicum* L., is one of the most valuable cultivated vegetable crops in sub-Saharan Africa providing a source of direct and indirect employment for many people (Sibomana et al., 2016). In Kenya, tomato is a food and nutrition security vegetable crop mostly cultivated by smallholder farmers for both domestic and export markets (Ochilo et al., 2019). The rapid growth of the tomato industry has been coupled with the emergence of devastating indigenous and invasive insect pests and diseases (Pratt et al., 2017). As a result of these pests infestations, significant annual losses of up to 70% of tomato production have been estimated in Africa (Pratt et al., 2017), providing a clear constraint on current and future yields. Among these biotic constraints, the South American tomato pinworm, *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is ranked as the most devastating insect pest of tomatoes (Aigbedion-Atalor et al., 2019; Brévault et al., 2014). *Phthorimaea absoluta* is an invasive species native to South America (Guedes and Picanço, 2012), which was first detected in 2008 on the African continent following an introduction in the Mediterranean Basin (Guillemaud et al., 2015) with subsequent invasion of tomato fields in 2006 in Spain (Abbes et al., 2012; Desneux et al., 2010). More than a decade after its first detection in Africa, the pest has since spread to nearly every country on the continent destroying thousands of hectares of tomato fields and other solanaceous crops (e.g. potato, *Solanum tuberosum* L. and black nightshade, *Solanum nigrum* L.), frequently causing total crop losses (Mansour et al., 2018). The management of invasive pests on the African continent, especially for the South American tomato pinworm has taken a reactive approach leading to sporadic and uncoordinated actions to control the pest which has over the years gained a foothold on the continent (Niassy et al., 2020). The extent of damage and the associated alarming level of economic losses (estimated at US$ 1.1 billion) being reported annually due to *P. absoluta* are enormous and are likely to increase significantly if left uncontrolled, leading to additional production costs to manage the pest (Pratt et al., 2017).

In response to this challenging situation, smallholder vegetable farmers have been desperately applying cocktails of synthetic insecticides (Mansour et al., 2018), largely driven by government-subsidised agrochemical input schemes, aggressive marketing by insecticide companies and their representatives, and out of desperation to reduce the pest infestation levels. Yet, the widespread use of synthetic insecticides has rarely delivered a satisfactory level of control due to the cryptic feeding behaviour of the pest's immature stages and the

rapid development of resistance to the classes of insecticides used against *P. absoluta* population in many parts of the world (Guedes et al., 2019; Guedes and Picanço, 2012; Lietti et al., 2005), and consequently jeopardising the pest control efforts. Additionally, insecticides resistance resulting from indiscriminate applications has caused unprecedented disruption of the resilience of natural ecosystems and has attracted growing public concerns over effects on non-target organisms, environmental and human health (Lichtenberg and Zimmerman, 1999). This has resulted in the search for alternatives to insecticides to manage *P. absoluta* infestations with a great interest in developing biological control approaches using natural enemies (predators, parasitoids or microbials) (Agbessenou et al., 2020; Aigbedion-Atalor et al., 2020; Akutse et al., 2020; Soares et al., 2019). Among the microbials being explored, entomopathogenic fungi (EPF) offer effective and viable alternative to control insect pests of economic importance, as they cause epizootics in the target host population while minimising impacts on beneficial and other non-target organisms as well as increasing the quality of agricultural products (Chandler et al., 2011; Lacey et al., 2015b). These make them also potential option as biopesticides for controlling the South American tomato pinworm, *P. absoluta* (Akutse et al., 2020).

The mode of action of EPF against insects starts with spore adhesion to the host, followed by formation of appressoria that penetrate the cuticle, which later reach and invade the hemocoel and finally interferes with the host immune system (Shang et al., 2015). However, the level of infection depends on the physiological properties (virulence, sporulation and persistence) of fungal strains which are regarded as a major obstacle to the success of their development as biocontrol agents (Bayissa et al., 2017; Jackson et al., 2010). Additionally, under natural conditions, fungal infection is increasingly associated with stressful abiotic factors such as UV, humidity and temperature which modulate the virulence of the pathogen (Fang et al., 2012). Indeed, there is increasing evidence that temperature is the dominant abiotic factor that has a significant influence on the infectivity profiles of fungal strains (Dimbi et al., 2004; Ekesi et al., 2003; Onsongo et al., 2019; Tumuhaise et al., 2018) whose application in the field as biopesticides result sometimes in inconsistent performance and efficacy, which limits their use (Jaronski, 2010). It is therefore important to explore the effect of this key abiotic stress, on the efficacy of the identified potent EPF isolates (Akutse et al., 2020) to sustainably manage *P. absoluta* in different agroecological systems, especially under the continuous climate change scenario. Further, different nonlinear and linear models are used to estimate fungal growth over a wide range of temperature regimes and to predict

the effect of EPF virulence in epizootic development among target insect pest populations (Klass et al., 2007a, 2007b). Consequently, accurate prediction of the potential ecological fitness of these virulent fungal isolates is fundamental to optimize their efficacy in field application against the South American tomato pinworm.

Akutse et al. (2020) recently reported the efficacy of three *Metarhizium anisopliae* (Metchnikoff) Sorokin fungal strains (ICIPE 18, ICIPE 20 and ICIPE 665) as the most potent isolates which hold promise as biocontrol agents for managing *P. absoluta* moths. However, the interactions between their performance and abiotic factors that could affect their field efficacy are paramount to be established for an effective selection of the most virulent fungal isolate(s) best suited for mass-production prior to its/their formulations and field deployment. To achieve this, it is important to simulate the effects of different temperature regimes on *M. anisopliae* ICIPE 18, 20 and 665 mass-production, their efficacy and virulence against *P. absoluta*, and subsequently predict potential suitable areas of application of these biopesticides or strengthen their efficacy through appropriate new formulations. Therefore, this chapter aimed to (i) assess the germination, growth and conidial production of the three candidate isolates, (ii) evaluate their virulence against adult *P. absoluta* under different temperature regimes, (iii) determine mass-production indices for the three fungal isolates and (iv) develop spatial predictions on potential areas where the candidate fungal isolates could cause significant epizootics in *P. absoluta* populations.

2.2.Materials and methods

Insects

Source colony of *P. absoluta* was initially established from wild adults and larvae collected from infested tomato leaves and fruits in Mwea (0°36′31.3″S 037°22′29.7″E), Kirinyaga county, Kenya in June 2019. The moths were kept in ventilated, sleeved Perspex cages (40 × 40 × 45 cm) and fed *ad libitum* with 10% honey solution placed on the top side of each cage (Agbessenou et al., 2020). Four potted tomato plants (*Solanum lycopersicum* L. cv. "Money maker" grown from seeds obtained from Simlaw Seeds Company Ltd., Nairobi, Kenya) were placed in the cages for oviposition. The plants were removed 24 h post-exposure to female moths and transferred to separate wooden cages (50 × 50 × 60 cm) with ventilated openings on its both sides and top covered with netting material until the eggs hatched. After three days, leaves with larvae were removed from these plants and transferred into clean sterile plastic containers (21 cm long × 15 cm wide × 8 cm high) lined with paper

towel to absorb excess moisture and fine netting infused lid for ventilation. The larvae were supplied daily with fresh tomato leaves as food until pupation. The pupae were collected from the plastic containers using a fine camel hair-brush and placed inside a clean plastic container (21 cm long × 15 cm wide × 8 cm high) for adult emergence. The colony was rejuvenated every three months through infusion, with infested tomato leaves collected from the field to reduce inbreeding (Agbessenou et al., 2020; Akutse et al., 2020). Insects were maintained under rearing conditions of 28 ± 2°C, 48% relative humidity (RH) and 12:12 L:D photoperiod at the Animal Rearing and Quarantine Unit (ARQU) of *icipe* for five generations prior to bioassays (Agbessenou et al., 2020).

Fungal isolates and viability assessment

The three *M. anisopliae* fungal isolates (ICIPE 18, ICIPE 20 and ICIPE 665) used in this study were obtained from the International Centre of Insect Physiology and Ecology (*icipe*)'s Arthropod Pathology Unit Germplasm (Table 2.1). The isolates were cultured on Sabouraud dextrose agar (SDA) (OXOID CM0041, Oxoid Ltd., Basingstoke, UK), and maintained at 25 ± 2°C in complete darkness. Conidia were harvested by scraping the surface of two- to three-week-old sporulated cultures using a sterile spatula. The harvested conidia were then suspended in 10 ml sterile distilled water containing 0.05% (w/v) Triton X-100 (MERCK KGaA, Darmstadt, Germany) and vortexed for five minutes at about 700 rpm to break conidial clumps and ensure a homogenous suspension. Conidial concentrations were quantified using an improved Neubauer hemocytometer under a light microscope (Goettel and Inglis, 1997). The conidial suspension was adjusted to a concentration of 3×10^6 conidia mL^{-1} through serial dilution.

Prior to commencement of the bioassays, spore viability was determined by plating evenly 0.1 mL of 3×10^6 conidia mL^{-1} onto 9-cm Petri dishes containing SDA. Three sterile microscope cover slips (2 × 2 cm) were placed randomly on the surface of each inoculated plate. Plates were sealed with Parafilm membrane and incubated in complete darkness at 25 ± 2°C and were examined after 16-20 h (Goettel and Inglis, 1997). The percentage germination of conidia was determined from 100 randomly selected conidia on the surface area covered by each cover slip under a light microscope (400×) using the method described by Goettel and Inglis (1997). Conidia were considered to have germinated when the length of the germ tube was at least twice the diameter of the conidium (Goettel and Inglis, 1997). Four replicates were made for each isolate making a total of 12 plates for all isolates.

Table 2.1. Source and identity of the three *Metarhizium anisopliae* isolates used in the study and their germination rates.

Fungal species	Isolates	Source	Locality/Country	Year of isolation	Germination rate (%)
Metarhizium anisopliae	ICIPE 18	Soil	Mbita (Kenya)	1989	97.3 ± 2.5
	ICIPE 20	Soil	Migori (Kenya)	1989	96.8 ± 1.4
	ICIPE 665	Soil	Kenya	2008	96.2 ± 0.8

Effect of temperature on spore germination of *Metarhizium anisopliae* fungal isolates

Aliquots (0.1 mL) of a 3×10^6 conidia/mL conidial suspension were spread with a sterile glass spreader over the surface of 9-cm Petri dishes containing SDA. Three sterile microscope cover slips were placed on each plate, and the plates were securely sealed with Parafilm membrane as described above. The plates were then incubated in complete darkness at constant temperatures of 15, 20, 25, 30, 33 and 35°C. At 18 h post-incubation, plates were flooded with lactophenol aniline cotton blue to halt germination and to stain the spores for easy visibility. Percentage germination of 400 conidia, as four randomly selected counts of 100 conidia, on each plate was assessed under the 400× objective of a Leica DM500 light microscope (Leica Microsystems, Wetzlar, Germany) using the method described by Goettel and Inglis (1997). A conidium was considered to have germinated when the germ tube length was equal to or greater than the length of the conidia. Each plate served as a replicate with four replicates per fungal isolate making a total of 72 plates.

Effect of temperature on radial growth and sporulation

Conidial suspensions of the three isolates (ICIPE 18, ICIPE 20 and ICIPE 665) were prepared from two-week-old sporulated cultures and adjusted at a concentration of 1×10^7 conidia mL^{-1} prior to subculture. Aliquots (0.1 mL) were spread-plated on 9-cm Petri dishes containing SDA. Inoculated plates were then incubated in complete darkness at 25°C for three days to obtain mycelial mats. Mycelial mats were cut from culture plates into round agar plugs using an eight-mm diameter cork-borer. Each agar plug (ca. five mm thick) was then transferred onto the center of a fresh SDA medium plate from which a similar size plug of media had been previously removed using the same cork-borer. The plates with implanted mycelial plugs were sealed with Parafilm membrane and incubated in complete darkness at

15, 20, 25, 30, 33 and 35°C. Radial growth was recorded daily for 12 days using two cardinal diameters, through two orthogonal axes previously drawn on the bottom of each Petri dish to serve as a reference (Fargues et al., 1992). The experiment was replicated four times with each replicate originating from a different culture plate. Twelve days post-incubation, conidia were then harvested by scraping the surface of the sporulated cultures from each plate using a sterile spatula. The harvested conidia were then suspended in 10 mL sterile distilled water containing 0.05% Triton X-100 and vortexed for five min at about 700 rpm to break conidial clumps and ensure a homogenous suspension. Conidial concentrations were quantified using an improved Neubauer hemocytometer under a light microscope as described above (Goettel and Inglis, 1997).

Effect of temperature on the virulence of *Metarhizium anisopliae* fungal isolates against *Phthorimaea absoluta* moths

Temperatures above 30°C (33 and 35°C) were fatal to the insect given that all the adults in the control completely died during the first two days following their introduction into the incubator. Therefore, the bioassay was conducted at 10, 15, 20, 25 and 30°C which are the representative temperature range at which the pest occurs in the field (Santana et al., 2018). Twenty one-day-old virgin (unmated newly emerged moths) *P. absoluta*, male and female (at the ratio of 1:1) were inoculated with dry conidia of the *M. anisopliae* isolates using velvet-coated plastic jars (60 × 40 cm) following the method described by Migiro et al. (2010) and Akutse et al. (2020). For each isolate, the device was contaminated with 0.3 g dry conidia (equal to 0.15×10^9 conidia/g), after which moths were introduced into the device for three minutes to pick up fungal spores. Evidence of infection was confirmed through visual observation of fungal spores strongly attached to the body of the insects. Control insects were exposed to fungus-free velvet-coated plastic jars. Three (3) minutes post-exposure, contaminated insects were transferred into clean ventilated Perspex cages (15 × 15 × 15 cm) and provided with 10% honey solution placed on the top side of each cage as food source daily up to the end of the experiment. Each treatment consisted of 20 treated insects per replicate and incubated at 10, 15, 20, 25 and 30°C with four replicates per isolate making a total of 1,200 insects. Mortality was recorded daily for 12 days. Dead insects were surface-sterilised using 1% sodium hypochlorite solution, then put in 70% alcohol for five seconds and followed by three rinses in sterile distilled water. Insects were placed in Petri dishes lined with moistened filter paper to promote fungal growth on the cadaver surface.

Petri dishes were securely sealed with Parafilm membrane and incubated at 25°C. Mortality as a result of fungal infection was confirmed by the presence of hyphae and conidia on the surface of the cadaver. Each treatment consisted of 10 treated insects per replicate and incubated at 25°C with four replicates per isolate making a total of 120 insects.

Conidia production, harvest and assessment of mass production indices

Dry aerial conidia of *M. ansiopliae* ICIPE 18, ICIPE 20 and ICIPE 665 were produced on long-grain rice substrate (Tumuhaise et al., 2018). One kilogram of precooked parboiled rice substrate was transferred into breathable bio control PP bags (24 cm long × 14 cm wide) with double B filter (Unicorn Imp. & Mfg. Corp, Plano, Texas, US) and autoclaved at 121°C for 60 min. After which the substrate was allowed to cool to 28°C and inoculated with 50 ml of a three-day-old culture of blastospores/mycelia under a laminar flow cabinet. The bag was sealed under aseptic condition and incubated for 21 days at ambient conditions (26 ± 1°C and 60-70 % RH), after which the contents were transferred into sterile plastic buckets (33 × 25 × 13 cm) to allow the culture to dry for seven days at 26 ± 1°C. Conidia were harvested by sifting through a mesh sieve (295 µm mesh size). Six replicated production sets were ran for each isolate. At harvest, conidial powder from each bag and isolate was taken to estimate the following mass-production parameters: (i) weight of conidia powder per kg of rice, (ii) number of conidia per gram of powder, (iii) number of conidia per kg of powder, (iv) percentage water content (based on weight loss of 1 g powder dried at 120°C for 2 h), (v) percentage viability based on counts of germinated conidia, and (vi) percentage consumed substrate in each bag (based on the weight of the dry rice substrate before inoculation and that of the dry substrate residues immediately after harvesting the conidia) (Tumuhaise et al., 2018).

Modelling the effect of temperature on the radial growth rate

For temperature-dependent models, both linear and nonlinear were fitted to the calculated radial growth data. A linear function was fitted to the data to determine the relationship between growth rate and temperature (Smits et al., 2003). The linear model expressed as y (t) = a + bt was used to estimate the relationship between relevant temperatures and growth rate of fungal isolates, where y is the rate of growth, t is ambient temperature, and intercept (a) and slope (b) as the model parameters. The minimum temperature threshold (T_{min}) and standard error (SE_{Tmin}) were calculated using Equations 1 and 2:

$$T_{min} = \frac{-a}{b} \quad (1)$$

$$SE_{T_{min}} = \frac{y_m}{b}\sqrt{\frac{S^2}{N \times y_m^2} + \left[\frac{SE_b}{b}\right]^2} \quad (2)$$

Where y_m is the average value of the growth rate, b is estimated slope of fitted line, S^2 is the residual mean square of the linear model, and N is the sample size (Campbell et al., 1974). However, linear function cannot accurately capture the growth rate at extreme temperatures (Smits et al., 2003). Many empirical nonlinear models such as Logan and Brière models are fitted to fungal growth rate (Bayissa et al., 2017; Smits et al., 2003). This allowed determining the minimum temperature (T_{min}), optimum temperature (T_{opt}) and upper temperature (T_{max}) thresholds. Optimum temperature threshold (T_{opt}) is defined as the temperature when the fungal growth rate is observed to be maximal, while T_{max} is referred to threshold temperatures above which growth does not occur (Smits et al., 2003). Among the nonlinear models evaluated, the nonlinear regression model of Brière-1 (Equation 3) was fitted to the data so as to describe the fungal radial growth rate at the various temperature thresholds, $r(T)$ (Brière et al., 1999).

$$r(T) = n \times T \times (T - T_{min}) \times \sqrt{T_{max} - T} \quad (3)$$

Where, r is considered as the radial growth rate, derived as a function of temperature T, n being an empirical constant, T_{min} being the lower developmental temperature threshold and T_{max} the upper temperature threshold. The optimum temperature (T_{opt}) of the fungal growth was estimated using Equation 4 (Brière et al., 1999):

$$T_{opt} = \frac{2mT_{max} + (m+1)T_{min} + \sqrt{(4m^2T_{max}^2 + (m+1)^2T_{min}^2 - 4m^2T_{min}T_{max}}}{4m+2} \quad (4)$$

Where m is an empirical constant (Brière et al., 1999).

Goodness of fit and selection criteria of the model

The best-fitted model was selected based on the residuals and comparing Akaike's Information Criterion (AIC) and the Model Selection Criterion (MSC). The accuracy of different linear models in fitting the data was determined by comparing the coefficients of determination (R^2). Goodness-of-fit of the model was assessed using the coefficient of determination (for linear model; R^2) or the coefficient for nonlinear regression (for nonlinear models; R^2) and the residual sum of squares (RSS). Higher values of R^2 and lower values for RSS suggest a better fit (Archontoulis and Miguez, 2015). For the linear regression, the data points at 33°C and 35°C which deviated from the straight line through the other points were omitted for correct calculation of regression (Bayissa et al., 2017; Smits et al., 2003).

Modelling the effect of temperature on the virulence of fungal strains against *Phthorimaea absoluta* moths

An open-source computer-aided tool built on R-codes and Java interface, the entomopathogenic fungi application (EPFA) software version 1.0 (Guimapi et al., 2020) was used for modelling the virulence of the fungal strains (ICIPE 18 and ICIPE 20) against *P. absoluta* moths. The recorded mortality was plotted against the corresponding temperature values from which nonlinear models were fitted function to the observed data (Guimapi et al., 2020). The model parameters were estimated by fitting equations to the recorded mortality and the corresponding temperature values. Eighty two (82) models were fitted to the data and the best-fitted models were selected based on their coefficient of determination R^2, adjusted R^2, Akaike's information criteria (AIC), the root-mean-squared error (RMSE) and residual sum of squares (RSS) (Guimapi et al., 2020). In addition, nonlinear models allowed the assessment of the minimum temperature threshold (T_{min}) and the maximum temperature threshold (T_{max}). The Logan-4 model (Logan et al., 1976) predicted well the effect of temperature on virulence of ICIPE 18 against *P. absoluta* moths while the Logan-1 model gave the best fit to the virulence of ICIPE 20 and ICIPE 665. The mathematical expressions of the models are presented in Table 2.2.

Table 2.2. Mathematical equations describing the relationship between temperature and virulence of *Metarhizium anisopliae* fungal isolates.

Model	Equation	References
Logan-1	$m(T) = Y * \left(e^{p*T} - e^{\left(p*T_{max}\frac{(T_{max}-T)}{v}\right)}\right)$	(Logan et al., 1976)
Logan-4	$m(T) = \alpha\left(\frac{1}{1+k*\ e^{-b(T-T_{min})}} - e^{\frac{(T_{max}-(T-T_{min}))}{Dt}}\right)$	

For Logan models α, Y, k, b, Dt, and v are the model parameters, T_{min} the minimum temperature threshold and T_{max} the upper temperature threshold (°C).

Spatial prediction of the virulence of the most potent fungal isolates

Metarhizium anisopliae isolates (ICIPE 18 and ICIPE 20) were selected for the spatial prediction study based on germination, viability and growth patterns, the speed of kill (LT_{50} value) and the mortality rates they caused to *P. absoluta* moths across all tested temperature ranges. To predict the spatial virulence of each fungal isolate, the temperature-dependent mathematical expression obtained during the modelling step was run at each grid of the raster files of Kenya, Tanzania and Uganda using the monthly minimum and maximum temperature datasets obtained from WorldClim (http://www. worldclim.org/). The gridded temperature datasets were loaded into EPFA software, simultaneously extracted from the database and then organised in matrix format using longitude as column and latitude as a row (Guimapi et al., 2020). A point object picks the temperature-dependent mathematical expression of the virulence for the isolates and this is consecutively applied in each geographical coordinate of the grid. The results were converted into ASCII file format (.asc) and transferred into an open source software Q-GIS (Steiniger and Hunter, 2012) for visualisation (Guimapi et al., 2020). The virulence map was produced for Kenya, Tanzania and Uganda after completing the fitting process.

Data analyses

Conidial germination data were analysed with generalised linear model (GLM) assuming a binomial distribution with the log link function. Percent mortality was corrected for control mortality using Abbott's formula (Abbott, 1925). Mortality data were analysed using logistic regression in a GLM for a binomial distribution using the logit link function. Time-mortality data were analysed with GLM using the function "dose.p" from the MASS library, to generate LT_{50} estimates, along with slopes of the regression curves. GLM analysis was ran for each replication, and the resultant LT_{50} values and their respective slopes were subjected

to ANOVA to generate means. Additionally, data on sporulation (conidia production) and number of conidia per gram of powder were analysed using GLM with negative binomial error distribution taking into account overdispersion. Data on weight of conidia powder per kg of rice were analysed using GLM with gamma distribution. Percentage water content and percentage consumed substrate data were analysed with beta regression. Whenever a significant difference was found, multiple means comparison was made using Tukey's HSD *post-hoc* test to assess pairwise comparison, adjustment for LS means with $\alpha = 0.05$.

All statistical analyses were performed using R (version 3.6.3) statistical software packages (R Core Team, 2019) and all statistical results were considered significant at the confidence interval of 95% ($P < 0.05$).

2.3.Results

Effect of temperature on conidial germination

Conidia of the three *M. anisopliae* isolates germinated at all tested temperatures (ranging from 5 to 100%), except at 15°C where no germination was observed 18 h post-incubation. Temperature significantly affected conidial germination ($\chi^2 = 724.62$; df = 5; $P < 0.001$) while fungal isolates did not have any effect on the germination ($\chi^2 = 1.34$; df = 2; $P = 0.51$) (Figure 2.1). Significant differences in germination were observed at 20°C ($\chi^2 = 13.54$; df = 2; $P < 0.01$) and 25°C ($\chi^2 = 14.51$; df = 2; $P < 0.001$), with *M. anisopliae* ICIPE 20 achieving the highest germination rate. However, there were no significant differences among all the fungal isolates at 30°C ($\chi^2 = 3.30$; df = 2; $P = 0.19$), 33°C ($\chi^2 = 2.89$; df = 2; $P = 0.24$) and 35°C ($\chi^2 = 1.49$; df = 2; $P = 0.47$). The optimum temperatures for conidial germination were observed at 20°C, 25°C and 30°C for all the three isolates (Figure 2.1). The interaction between fungal isolates and temperature significantly affected conidial germination ($\chi^2 = 18.6$; df = 10; $P = 0.04$).

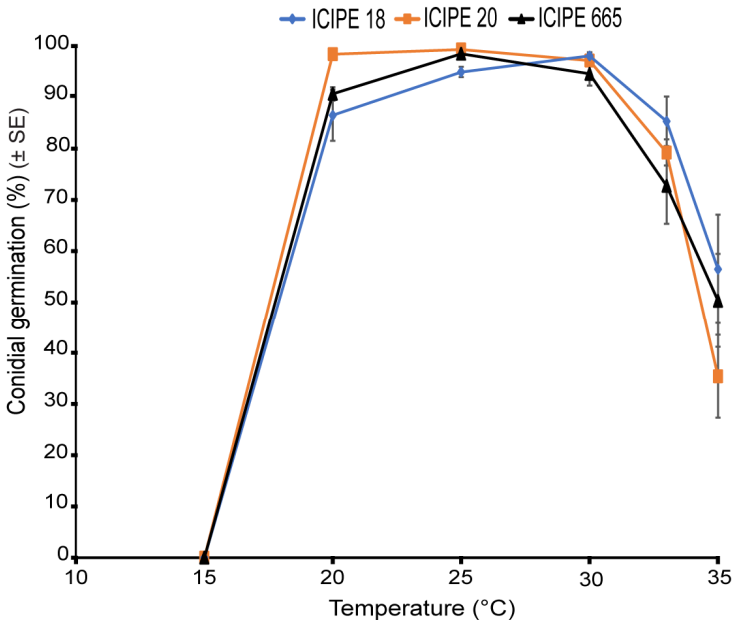

Figure 2.1. Effect of temperature on conidial germination of the three *Metarhizium anisopliae* fungal isolates ICIPE 18, 20 and 665. Error bars indicate the standard error of the mean at 95% CI (n = 72; N = 4; Tukey's HSD test).

Effect of temperature on fungal growth

The growth of the three isolates occurred at all temperatures, but was slower at 15, 33 and 35°C as compared to 20, 25 and 30°C (Figure 2.2A). Temperature (F = 339.01; df = 5, 54; $P < 0.001$) and fungal isolates (F = 234.33; df = 2, 54; $P < 0.001$) significantly affected the mean radial growth. Mean radial growth differs significantly among the three fungal isolates at 15°C (F = 14.57; df = 2, 9; $P < 0.01$), 20°C (F = 42.33; df = 2, 9; $P < 0.001$), 25°C (F = 15.28; df = 2, 9; $P < 0.01$), 30°C (F = 369; df = 2, 9; $P < 0.001$), 33°C (F = 224.8; df = 2, 9; $P < 0.001$) and 35°C (F = 53.17; df = 2, 9; $P < 0.001$) (Figure 2.2A). The highest growth rate occurred at 30°C for both ICIPE 18 and ICIPE 20 and at 25°C for ICIPE 665 (Figure 2.2A). At all temperature regimes, isolates ICIPE 18 and ICIPE 20 grew faster than ICIPE 665 (Figure 2.2A). Like the conidial germination, the interaction between temperature and fungal isolates significantly affected the radial growth (F = 13.43; df = 10, 54; $P < 0.001$). Parameter estimates obtained from the nonlinear Brière-1 model and linear models fitted to the radial growth rate are presented on Table 2.3. The fitted models for radial growth rate versus temperature for all the three fungal isolates are presented in Figures 2.2 B, C, D. The minimum temperature threshold (T_{min}) estimated using the Brière-1 model was lower as compared to estimates of the linear regression model for the three fungal isolates (Table 2.3).

The Brière-1 nonlinear model had a good fit to the data and predicted a lower temperature threshold of 4.45, 8.04 and -8.27°C, for ICIPE 18, ICIPE 20 and ICIPE 665, respectively. The upper temperature threshold (T_{max}) was 35.11, 35.16 and 35.01°C, for ICIPE 18, ICIPE 20 and ICIPE 665, respectively with an optimum temperature of 29.86, 29.31 and 29.55°C, for ICIPE 18, ICIPE 20 and ICIPE 665, respectively (Table 2.3). Using the linear model, T_{min} of the fungal isolates was estimated at 9.1°C, 8.45° and 1.25°C for ICIPE 18, ICIPE 20 and ICIPE 665, respectively. The optimum temperature threshold (T_{opt}) was estimated at 29.86, 29.31 and 29.55°C for ICIPE 18, ICIPE 20 and ICIPE 665, respectively (Table 2.3). The linear regression model showed a strong positive relationship between temperature and radial growth rate (R^2 = 0.82 and R^2 = 0.87 for ICIPE 18 and ICIPE 20, respectively) (Table 2.3).

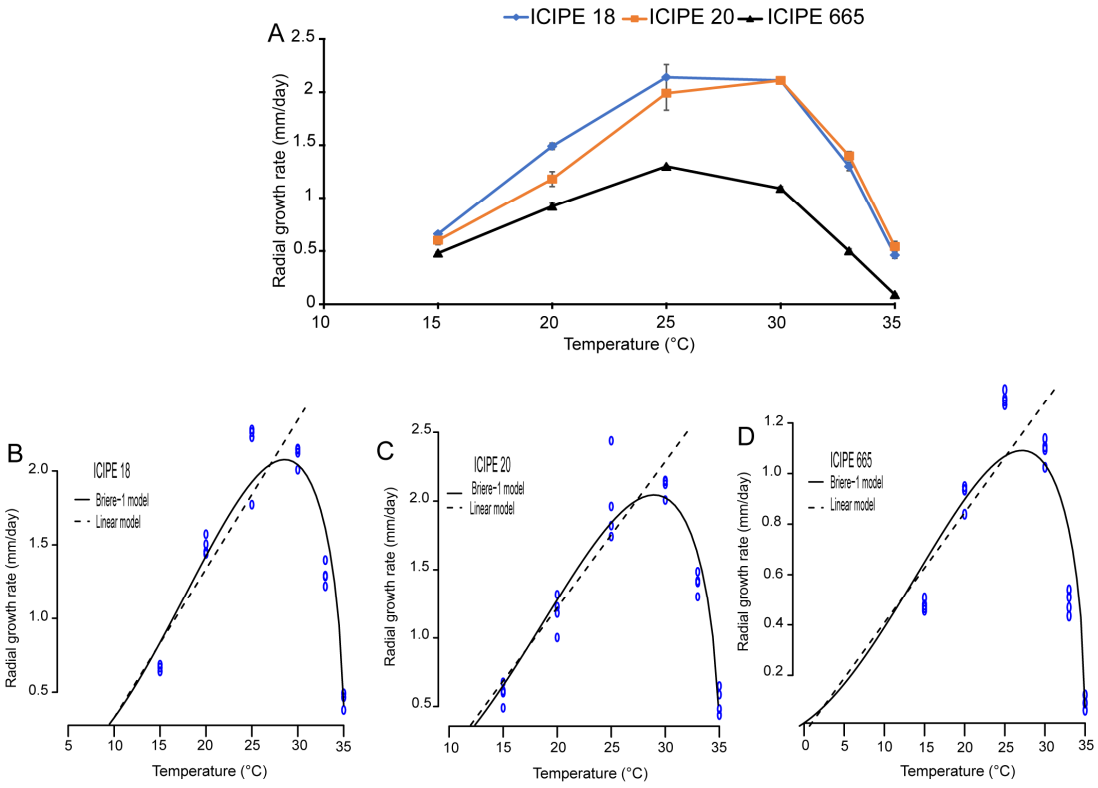

Figure 2.2. Relationship between temperature and radial growth rate of the three *Metarhizium* anisopliae fungal isolates. (A) Relative growth rates of *Metarhizium anisopliae* isolates ICIPE 18, ICIPE 20 and ICIPE 665 on SDA medium between 15 and 35 °C. Linear (dashed lines) and Brière-1 (continuous lines) nonlinear models fitted to observed values of the radial growth rate of *Metarhizium anisopliae* isolates (B) ICIPE 18, (C) ICIPE 20 and (D) ICIPE 665 at constant temperatures (n = 72; N = 4; Tukey's HSD test).

Table 2.3. Estimated parameters and their approximative standard errors for linear and Brière-1 nonlinear model describing the relationship between temperature and growth of *Metarhizium ansiopliae* isolates.

Model	Parameters	Fungal isolates		
		ICIPE 18	**ICIPE 20**	**ICIPE 665**
Linear	a	-0.64 ± 0.28	-0.93 ± 0.24	-0.05 ± 0.19
	b	0.1 ± 0.01	0.11 ± 0.01	0.04 ± 0.01
	T_{min}	9.1	8.45	1.25
	R^2	0.82 ± 0.27	0.87 ± 0.23	0.64 ± 0.19
Brière-1	T_{min}	4.45 ± 2.03	8.04 ± 1.35	-8.27 ± 8.15
	T_{max}	35.11 ± 0.06	35.16 ± 0.06	35.01 ± 0.04
	T_{opt}	29.86	29.31	29.55

Effect of temperature on conidial production or sporulation

Conidial production varied from a low rate of 2.4×10^4 conidia/ml (ICIPE 18, 35°C) to a maximum of 1.06×10^8 conidia/ml (ICIPE 20, 25°C) (Figure 2.3). Temperature ($\chi^2 = 3195.5$; df = 5; $P < 0.001$) and fungal isolates ($\chi^2 = 69.4$; df = 2; $P < 0.0001$) significantly affected conidial production. Also, the interaction between temperature and fungal isolates affected conidial yield ($\chi^2 = 119.9$; df = 10; $P < 0.001$). Isolates ICIPE 18 and ICIPE 20 yielded the highest conidial density at 15, 20, 25 and 30°C. The best sporulation rate for all the three isolates was obtained at 25°C (Figure 2.3).

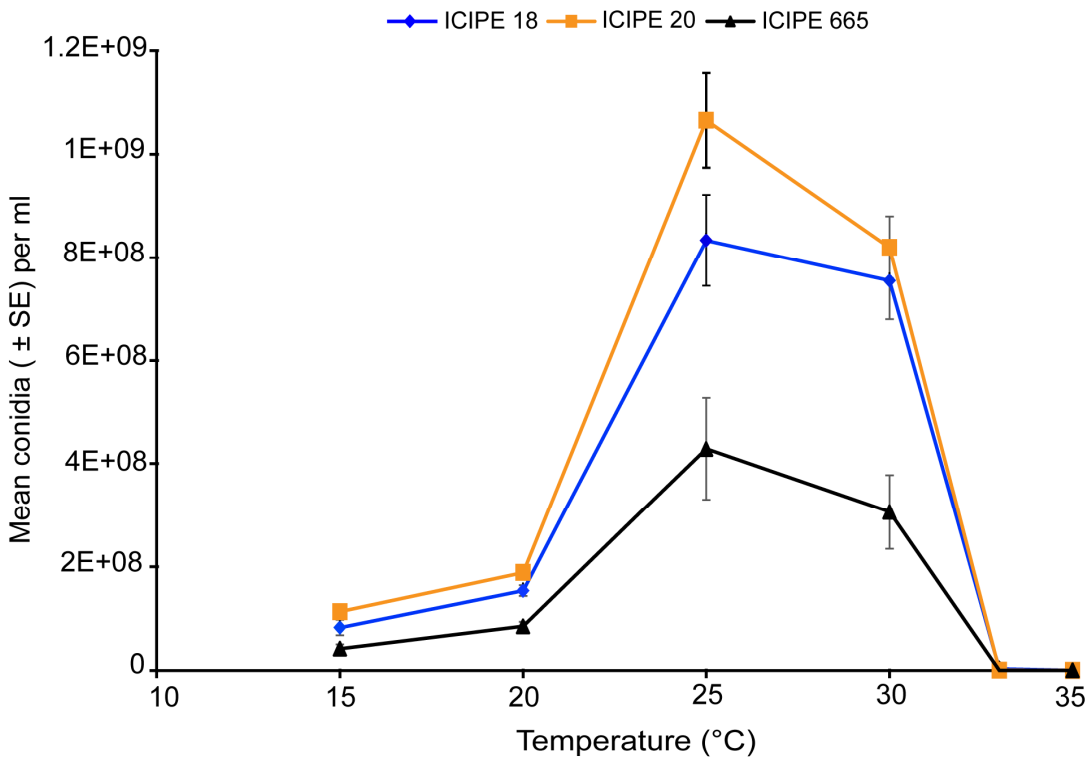

Figure 2.3. Effect of temperature on conidial production of the three *Metarhizium anisopliae* isolates ICIPE 18, 20 and 665. Error bars indicate the standard error of the mean at 95% CI (n = 72; N = 4; Tukey's HSD test).

Effect of temperature on the virulence of *Metarhizium anisopliae* fungal isolates against *Phthorimaea absoluta* moths

All the three isolates were virulent against *P. absoluta* moths variably, with percentage mortality ranging from 18–91%, 20–90% and 25–78% for ICIPE 18, ICIPE 20 and ICIPE 665, respectively across temperature regimes of 10–30 °C (Figure 2.4). Mortality increased significantly (χ^2 = 422.54, df = 4, P < 0.001) with increase in temperature. The highest mortality occurred at 30°C for all the three fungal isolates. Isolates had significant effect (χ^2 = 15.23, df = 2, P < 0.001) on *P. absoluta* moth mortality. At 10°C ICIPE 665 caused the highest mortality rate while at 15 and 20°C, ICIPE 18 caused the highest mortality followed by isolates ICIPE 20 and ICIPE 665 (Figure 2.4). However, no significant difference in mortality was observed among the fungal isolates at 25 (χ^2 = 0.18, df = 2, P = 0.91) and 30°C (χ^2 = 5.77, df = 2, P = 0.06). The interaction between temperature and fungal isolates significantly affected percentage mortality (χ^2 = 45.96, df = 8, P < 0.001). The lethal time required for 50% of insects to die (LT_{50}) was calculated for all the three isolates that caused

>50% mortality 12 days post-treatment (Table 2.4). The speed of infection is faster at 25 and 30°C than at low temperatures (10 and 15°C) for all the three isolates. The LT_{50} values of ICIPE 18 and ICIPE 20 were significantly lower at 25 and 30°C compared to ICIPE 665 (at 25°C F = 4.92; df = 2, 9, P = 0.036; and at 30°C F = 5.13, df = 2, 9, P = 0.033) (Table 2.4). The Logan models had a good fit to the mortality data (Figure 2.5). The Logan-4 nonlinear model gave the best fit to the data and predicted a minimum and maximum temperature threshold of 9.10 and 33.11°C, respectively for ICIPE 18. The Logan-1 predicted a maximum temperature threshold (T_{max}) of 33.27 and 33.36°C for ICIPE 20 and ICIPE 665, respectively (Table 2.5).

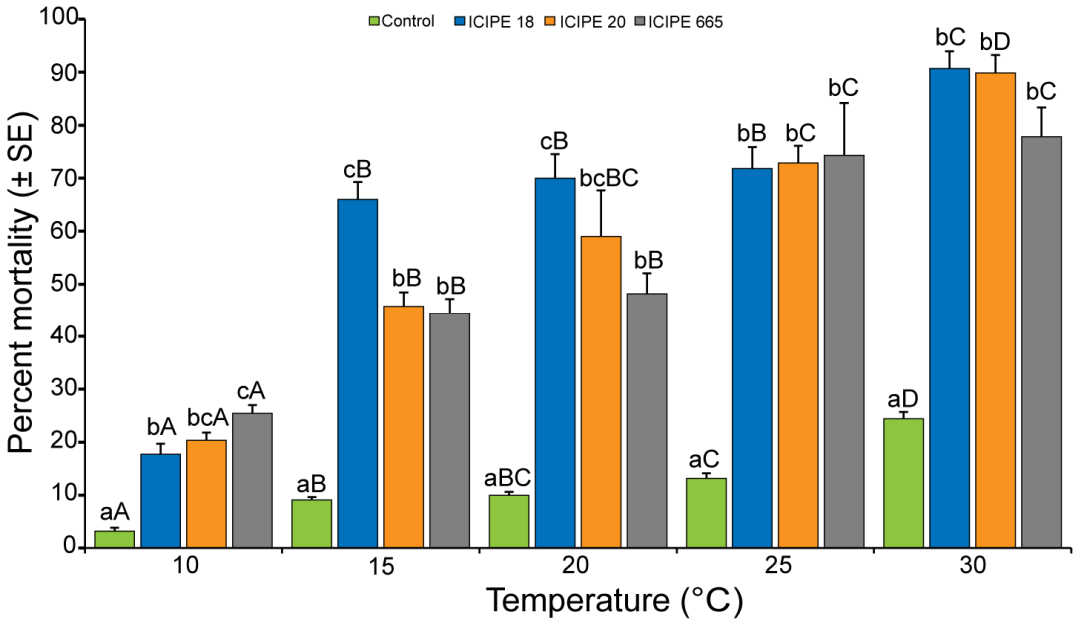

Figure 2.4. Effect of temperatures on virulence of the three isolates of *Metarhizium anisopliae* against *Phthorimaea absoluta* moths, 12 days post-inoculation. Different lowercase letters show significant difference (GLM, P ≤ 0.05) in mortality among fungal isolates across the different temperature regimes. Different uppercase letters denote significant difference (GLM, $P \leq 0.05$) in mortality across the different temperature regimes for each isolate (n = 60; N = 4; Tukey's HSD test).

Table 2.4. LT_{50} values 12 days post-exposure of *Phthorimaea absoluta* moths to *Metarhizium anisopliae* fungal isolates dry conidia under different temperatures.

Temperature (°C)	LT_{50} ± SE (Days)		
	ICIPE 18	ICIPE 20	ICIPE 665
10	-	-	-
15	2.88 ± 1.19	-	-
20	3.59 ± 1.04 a	4.93 ± 0.93 a	3.21 ± 0.26 a
25	3.41 ± 0.97 ab	2.40 ± 0.47 b	6.43 ± 1.23 a
30	1.41 ± 0.13 b	1.48 ± 0.30 ab	2.92 ± 0.56 a

Means within a row followed by the same lower letter case are not significantly different (Tukey's HSD test, P = 0.05). Dash (-) means the LT_{50} value was not estimated for cumulative mortality less than 50% at 12 days post-treatment.

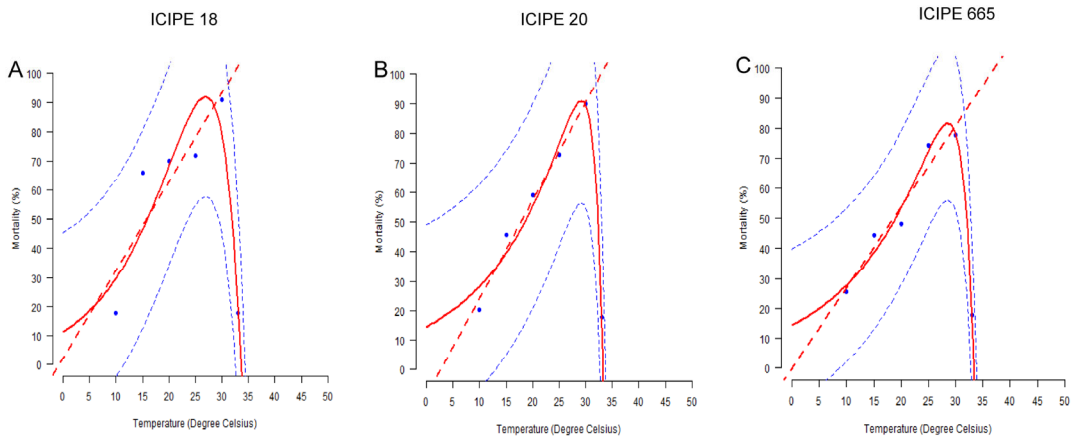

Figure 2.5. Observed and predicted mortality of *Phthorimaea absoluta* moths by *Metarhizium anisopliae* isolates ICIPE 18, ICIPE 20 and ICIPE 665 in relation to temperature using the linear and nonlinear models. The blue dots on the graph represent the cumulative values of the proportion of *Phthorimaea absoluta* moths that were killed by the three isolates during the experiments at the respective temperatures. The curve represents the Logan-4 and Logan-1 nonlinear models that best fits the experimental data points and were used to predict the level of efficacy of isolates (A) ICIPE 18, (B) ICIPE 20 and (C) ICIPE 665 against *Phthorimaea absoluta* moths. Fitted models are the dashed straight lines for linear regression and solid lines for the Logan models. Dashed lines above and below represent the upper and lower 95% confidence interval (n = 60; N = 4; Tukey's HSD test).

Table 2.5. Model parameters of Logan models describing the effect of temperature on virulence of fungal isolates against *Phthorimaea absoluta* moths.

Fungal isolates	Model		Parameters	*F* value	*df* 1, 2	*P* value	Adj R^2	AIC
ICIPE 18	Logan-4	α	4119.195	9.11	5, 17	< 0.001	0.70	12.90
		k	1561.87					
		b	0.12					
		T_{min}	9.10					
		T_{max}	33.11					
		Dt	8.17					
ICIPE 20	Logan-1	Y	0.14	20.60	3, 5	0.04	0.92	-9.81
		T_{max}	33.27					
		p	0.07					
		v	1.66					
ICIPE 665	Logan-1	Y	0.10	27.75	3, 5	0.03	0.94	-13.42
		T_{max}	33.36					
		p	0.48					
		v	15.17					

F, *F*-test statistic; *df*, degree of freedom; *P*, probability value; R^2, coefficient of determination; AIC, Akaike's information criterion.

Mass production indices

Metarhizium anisolpiae isolate ICIPE 18 had the most yield with a conidia powder weight of 86.30 ± 16.56 g/kg of rice compared to ICIPE 20 at 41.67 ± 5.95 g/kg and ICIPE 665 with 22.28 ± 5.54 g/kg ($\chi^2 = 22.65$, df = 2, $P < 0.001$) (Figure 2.6A). In addition, both the number of conidia per gram of powder and the number of conidia per kg of powder were significantly higher for ICIPE 18 compared to ICIPE 20 and ICIPE 665 (conidia per gram: $\chi^2 = 20.43$, df = 2, $P < 0.001$; conidia per kg: $\chi^2 = 20.48$, df = 2, $P < 0.001$) (Figure 2.6B, C). The percentage of water content was significantly higher for ICIPE 18 compared to ICIPE 20 and ICIPE 665 (F = 29.16, df = 2, $P < 0.0001$) (Figure 2.6D). Regardless of the fungal isolates, the percentage of conidial viability was more than 90% and not significantly different among the fungal isolates ($\chi^2 = 2.70$, df = 2, $P = 0.26$) (Figure 2.6E). The weight of the rice residues

after conidia harvest was significantly lower (F = 7.13, df = 2, $P < 0.01$) in ICIPE 18 than in ICIPE 20 and ICIPE 665 (Figure 2.6F).

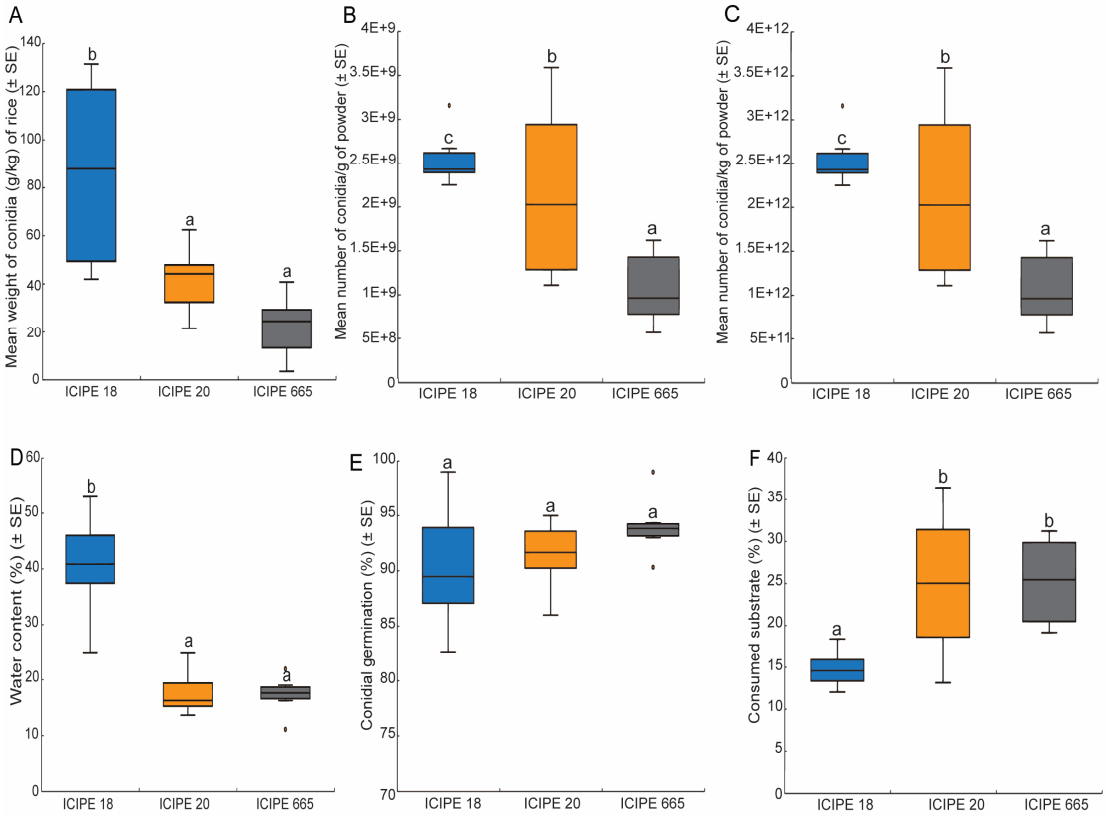

Figure 2.6. Mass-production indices of *Metarhizium anisopliae* isolates ICIPE 18, ICIPE 20 and ICIPE 665. **A**. Mean weight of conidia powder/kg of rice. **B.** Mean number of conidia/g of powder. **C.** Mean number of conidia/kg of powder. **D.** Water content (%) of conidia. **E.** Percentage conidial germination and **F.** Percentage consumed substrate. Different lowercase letters above error bars indicate a significant difference across the treatments (n = 18; N = 6; Tukey's HSD test). Middle quartile (line that divides the box into two parts) shows midpoint of the data. Middle box represents 50% of the scores for each treatment and the middle 50% values fall within the inter-quartile range.

Spatial prediction of the virulence of *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 20 against *Phthorimaea absoluta* moths in East Africa

Spatial predictions using EPFA for the performance or virulence of *M. anisopliae* isolates ICIPE 18 and ICIPE 20 against *P. absoluta* mothsare shown in Figures 2.7, 2.8 and 2.9. The pathogen performance model predicted that a deployment of *M. anisopliae* ICIPE 18 and ICIPE 20 in Kenya would result in high mortality (more than 70%) of adult *P. absoluta* in locations in the coastal part such as Taita Taveta, Kwale, Kilifi; and moderate mortality (45-

63%) for locations in central Kenya such as Laikipia (Figures 2.7A,B); unlike in some parts of Nakuru and Nyandarua where the model predicted very low performance of the fungal pathogen ranging from 16 to 34% mortality (Figures 2.7A,B). Moreover, the model predicted a very high probability of mortality of adult *P. absoluta* in several regions in Tanzania (Lindi, Morogoro, Tabora, Tanga) for ICIPE 18 and a moderate mortality in Iringa and Njombe (Figure 2.8A). Similarly, for ICIPE 20, the model predicted a high mortality pattern in regions of Singida, Dodoma, Mbeya, Manyara; but predicted moderate virulence pattern of ICIPE 20 in Iringa and Njombe (Figure 2.8B). In Uganda, the virulence pattern was almost similar for the two isolates with high probability of mortality predicted in Lango, West Nile, Teso and Acholi; and very low to moderate mortality predicted in Elgon (Figures 2.9A, B). In general, environmental conditions appeared to be conducive to the pathogens' virulence across the three countries (Kenya, Tanzania, and Uganda).

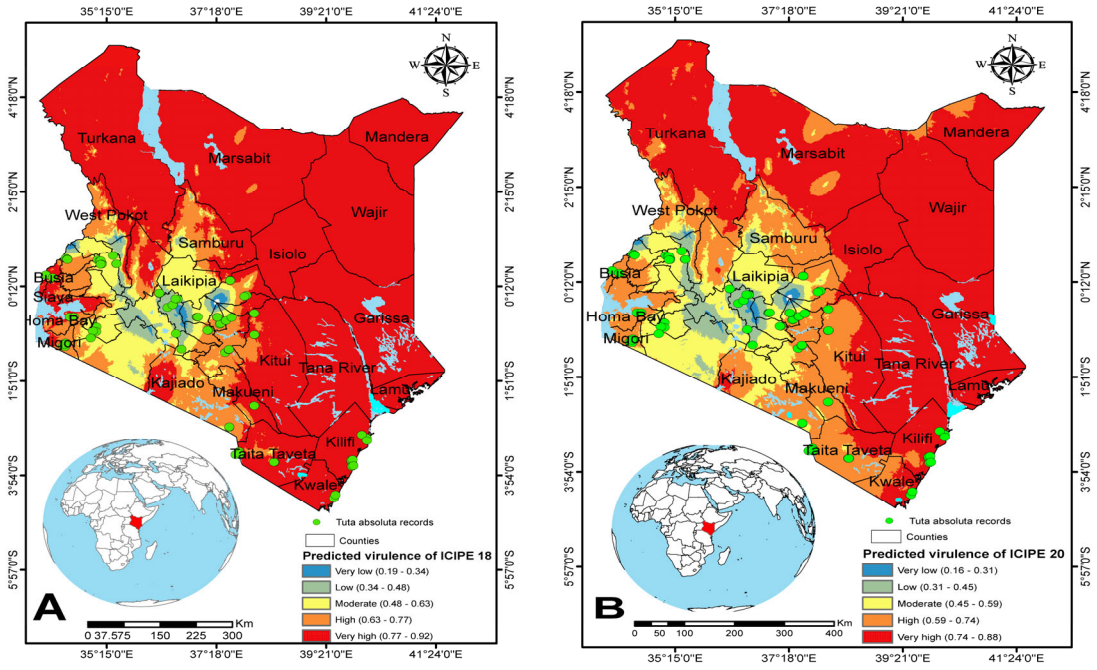

Figure 2.7. Spatial patterns of predicted virulence of *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 20 against *Phthorimaea absoluta* moths in Kenya: (A) ICIPE 18 and (B) ICIPE 20. The dots in green indicate *Phthorimaea absoluta* records in the three countries. The figures were generated using the QGIS 3.10.2 software (https://qgis.org/downloads/) (Allen and Mehler, 2019).

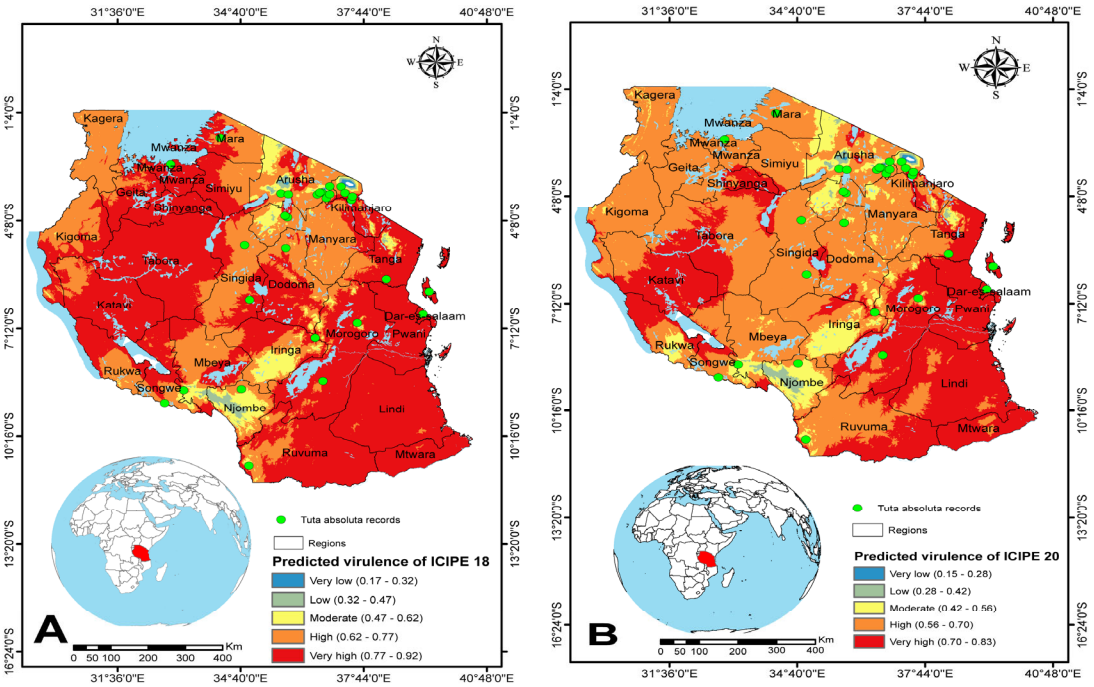

Figure 2.8. Spatial patterns of predicted virulence of *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 20 against *Phthorimaea absoluta* moths in Tanzania: (A) ICIPE 18 and (B) ICIPE 20. The dots in green indicate *Phthorimaea absoluta* records in the three countries. The figures were generated using the QGIS 3.10.2 software (https://qgis.org/downloads/) (Allen and Mehler, 2019).

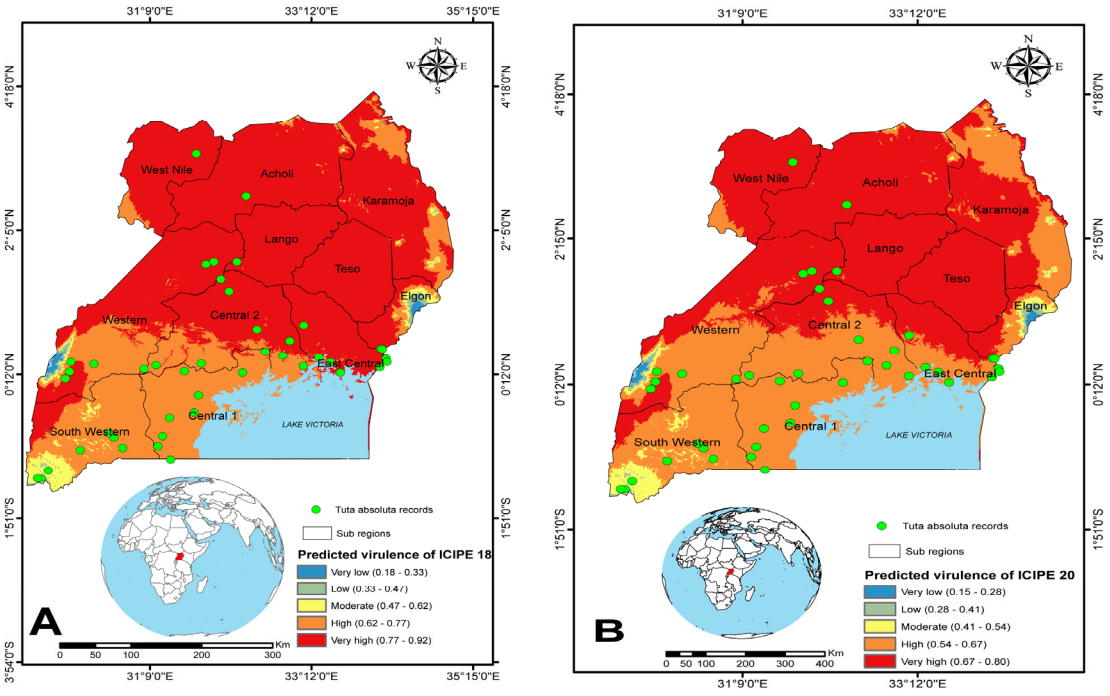

Figure 2.9. Spatial patterns of predicted virulence of *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 20 against *Phthorimaea absoluta* moths in Uganda: (A) ICIPE 18 and (B) ICIPE 20. The dots in green indicate *Phthorimaea absoluta* records in the three countries. The figures were generated using the QGIS 3.10.2 software (https://qgis.org/downloads/) (Allen and Mehler, 2019).

2.4.Discussion

All the three *M. anisopliae* isolates (ICIPE 18, ICIPE 20 and ICIPE 665) tested in this study showed significant variation in germination, radial growth, conidial production and virulence against adult *P. absoluta* across the various temperature regimes with ICIPE 18 and ICIPE 20 showing their superiority as the best candidate biopesticides for sustainable management of the pest. Our results also showed that Kenya, Tanzania and Uganda where these potent isolates are expected to be registered, commercialised and upscaled for *P. absoluta* control exhibit different patterns in spatial virulence of the two candidate isolates (ICIPE 18 and ICIPE 20); which clearly demonstrates the importance of using spatial modelling as a decision-support tool for the optimization of biopesticides deployment in different agroecological zones. Furthermore, our study indicated that *M. anisolpiae* ICIPE 18 yielded the highest weight of conidia powder followed by ICIPE 20 and ICIPE 665 when using rice as growth substrate for mass-production of the candidate isolates.

The ability of an EPF isolate to germinate under given environmental temperature regimes is a critical determinant of its efficacy (McCammon and Rath, 1994). Here, our findings

revealed that over 90% of conidia germinated at 20, 25 and 30°C, while only between 35 and 50% germinated at 35°C. At 15°C, no spore germination was recorded after 18 h incubation, but low germination was observed after longer time (delayed germination) which consequently translates into low hyphal growth and low sporulation. This is in agreement with previous studies that revealed that no germination occurred at low temperatures (< 15°C) for *M. anisopliae* fungal isolates (Bayissa et al., 2017; Tumuhaise et al., 2018). Ekesi et al. (1999) also observed an absence or delayed conidial germination at low temperatures indicating that conditions were not favourable for spores to initiate germination within 18 h post-incubation. In contrast, Dimbi et al. (2004) recorded spores germination at 15°C after 24 h inoculation for several *M. anisopliae* isolates including ICIPE 18 and ICIPE 20. Similarly, De Croos and Bidochka (1999) found that some *M. anisopliae* isolates were cold-active due to their ability to germinate and grow at temperatures as low as 8°C. This suggests that spore germination may be related to the geographical origin of the strains or influenced by the conditions under which the spores are formed, highlighting the significant intra-specific variation in the germination among *M. anisolpliae* strains (Acheampong et al., 2020; Dahlberg and Etten, 1982; De Croos and Bidochka, 1999; Hywel-Jones and Gillespie, 1990). Importantly, germination occurred at 20°C for all the three isolates; which marks the transition from a resting state to active development mostly driven by metabolic changes (Allen, 1965). Our findings also showed that *M. anisopliae* isolates ICIPE 18 and ICIPE 20 had the highest germination rate at all temperature regimes compared to ICIPE 665 with an optimum at 25-30°C. Hywel-Jones and Gillespie (1990) also reported an intra-specific variation in the germination among *M. anisopliae* strains with the highest germination rate achieved at 25 and 30°C.

Growth of the three fungal isolates was adversely affected at 15, 33 and 35°C. This finding concurs with previous studies that reported growth inhibition of fungal pathogens exposed to extreme temperatures (Dimbi et al., 2004; Onsongo et al., 2019). The extremely low growth rate of the isolates observed at 15, 33 and 35°C indicates that these temperatures were unsuitable for spores' development and were also close to the insect's lower and upper thermal limits. Indeed, *P. absoluta* has a lower thermal threshold ranging from 5.37 to 7.38°C while its upper thermal threshold varies between 33.82 to 35.69°C (de Campos et al., 2020). However, fungal growth for the three isolates became evident at 20°C reaching an optimum at 30°C predicted by the linear and Brière-1 models, even though ICIPE 665 grew more slowly than the other two isolates. This is in an agreement with Bayissa et al. (2017)

and Ekesi et al. (2003) who reported that *M. ansiopliae* species are mesophilic fungi that grow well between 15 and 30°C with an optimal temperature range of 20-30°C. Vidal et al. (1997) reported similar growth pattern for *Paecilomyces fumosoroseus* fungal species exhibiting high growth rate between 8 and 30°C with thermal optima ranging from 20 to 30°C. It is important to note that for fungal isolates of tropical origin, growth does not occur below a lower thermal threshold (10°C) and gradually the growth increases with increase in temperature to a maximum at optimal temperature (25-28°C), and finally decreases rapidly to zero at an upper threshold (32-35°C) which is considered as the lethal temperature (Smits et al., 2003; Vidal et al., 1997). Although the germination and fungal growth rate reached their optimum at 30°C for isolates ICIPE 18 and ICIPE 20, we observed a significant decrease in spore production at the same temperature. Nevertheless, isolates ICIPE 18 and ICIPE 20 yielded the highest conidia production at all the temperature regimes compared to ICIPE 665 with an optimum at 25°C. The poor yield of conidia observed at 33 and 35°C illustrates the requirement/importance of optimum temperature to sustain conidia production and consequently boost fungal mass-production. This finding is consistent with the observation that optimum temperature for spore production is at 25°C (Cabanillas and Jones, 2009).

Phthorimaea absoluta moths exposed to dry conidia showed a sharp increase in mortality at temperatures between 10 and 30°C. The highest level of infection was observed at 25 and 30°C for all the three fungal isolates, presumably representing the optimum temperatures at which fungal infection is most affecting the insect. This virulence pattern observed is therefore important in the selection of key application zones of these fungal candidates since their virulence is showed to be temperature dependent. The effect of temperature on virulence of *M. anisopliae* dry conidia against adult stages of insect pests has previously been reported by Onsongo et al. (2019) in adult Tephritid fruit flies *Zeugodacus cucurbitae* (Coquillet) (Diptera: Tephritidae) which succumbed to fungal infection over a wide temperature range (15-30°C), with an optimum of 25°C. A similar mortality pattern was observed with *M. anisopliae* isolate ICIPE 69 which was found effective against adult legume pod borer *Maruca vitrata* Fabricius (Lepidoptera: Crambidae) at temperature ranging from 15 to 33°C, with an optimal temperature infection ranging between 25-30°C (Tumuhaise et al., 2018). The lowest LT_{50} values were recorded at 25 and 30°C for the two most virulent isolates, ICIPE 18 and ICIPE 20. The rapid germination and growth of these two fungal isolates at almost all the temperature ranges could probably explain the fastest

death they induced to the insects observed in this study. This confirms the observation that fungal isolates tend to kill most rapidly at the optimum temperature of their vegetative growth (Tumuhaise et al., 2018). These two candidate isolates could therefore be deployed in the field under different temperature ranges (20-30°C) using an autodissemination device against adult *P. absoluta* through an "attract-and-infect" strategy, as they were found to be compatible with the commercial *Phthorimaea* pheromone lure (TUA-Optima®) (Akutse et al., 2020).

Mathematical models have become an important tool for understanding and predicting the virulence and suitable application areas of entomopathogenic fungi against insect pests of economic importance often occurring in unpredictable environmental conditions (Guimapi et al., 2020; Klass et al., 2007a). Here, we used *P. absoluta* occurrence in East Africa (Aigbedion-Atalor et al., 2019) to model the virulence of the candidate isolates (ICIPE 18 and ICIPE 20) against the pest. The decision-support tool revealed that in some areas across Kenya, Tanzania and Uganda (where the potent fungal-based biopesticides are planned to be registered, commercialised and upscaled), the candidate fungal isolates might be effective as an excellent control and management tool for *P. absoluta* while in other areas the predicted level of virulence tend to be very low. For example, in the southern and northern part of Kenya, the models predicted higher virulence of both isolates while very low to moderate virulence has been predicted in the Central part of the country. Interestingly, tomato production is highly concentrated in Kirinyaga, Kajiado, Bungoma, Kwale and Taita Taveta counties (Aigbedion-Atalor et al., 2019) where ICIPE 18 and ICIPE 20 are expected to perform well by inducing high infection to the pest. In Tanzania, tomato is mainly cultivated in Iringa, Morogoro and Tanga regions (Smith et al., 2019) while in Uganda, the main tomato production areas are Central, Eastern and Western regions (Tumuhaise et al., 2016) where the model predicted high mortality pattern of the pest for the two candidate isolates. As such, deploying fungal-based biopesticides in these locations will have a high probability of managing *P. absoluta*. However, the microclimate conditions in some counties of Kenya (e.g. Naivasha in Nakuru county) where tomato is produced in greenhouses could be suitable for the infection of the pest by the fungal isolates and this requires further investigation. Klass et al. (2007b) predicted the effects of temperature on performance of a fungus-based biopesticide for controlling locusts and grasshoppers. The authors also predicted considerable spatial variation in *M. anisopliae* var. *acridum* and its virulence across different regions where the two voracious pests (locusts and grasshoppers) occur.

These three countries (Kenya, Tanzania and Uganda) are among the countries where most biopesticides developed by *icipe* are also registered and commercialized. With a pest like *P. absoluta*, which is present permanently on tomato and other solanaceous crops, fungal isolates which can infect the pest under different agro-ecologies and remaining viable for an extended period would be more economical for smallholder farmers. Although these candidate biopesticides hold considerable promise, unpredictable weather conditions in the field could seriously undermine their performance leading to poor delivery. Therefore, the main challenge of predicting the spatial virulence of fungal pathogens lies in the high variability of the environmental factors. Climatic factors like relative humidity (more specifically "vapour pressure deficit", VPD) which was not explored in this study have been shown to have great impacts on fungal growth and virulence. It is therefore important to assess the combined impact of temperature and relative humidity (VPD) in future studies.

One of the primary prerequisites for a pathogen to be developed as a biopesticide is its ease for mass-production (Kassa et al., 2008; Tumuhaise et al., 2018). We found that *M. anisopliae* ICIPE 18 outperformed the other two isolates ICIPE 20 and ICIPE 665, as it produced the highest conidial yield, consumed less substrate and displayed high moisture content indicating that this isolate is capable of colonising the rice substrate and yields high conidia. This high sporulation capacity is an interesting feature, which would definitely contribute to fast-track the registration of ICIPE 18 when field validation trials are conclusive. Interestingly, we recorded conidia yield higher than 2×10^9 conidia/g of powder for both ICIPE 18 and ICIPE 20, which indicates that ICIPE 20 can equally grow and sporulate very well on rice substrate producing large number of infective spores therefore making it highly desirable for commercialisation. Rice is considered as the most suitable substrate for fungal spores mass-production as it is locally available and provides a large surface area for sporulation (Jenkins et al., 1998). Using rice as growth substrate, Barra et al. (2018) also reported a high production of conidia per gram (2.1×10^9) for *Purpureocillium lilacinum* isolate JQ926212. Besides, we recorded a high level of moisture content (40%) for isolate ICIPE 18. Moisture plays a crucial role in conidial production of fungal isolates in solid-state fermentation as fungal spores require free moisture during the germination and host penetration process (Jackson, 1997).

In conclusion, *M. anisopliae* isolates ICIPE 18 and ICIPE 20 were found to be effective against *P. absoluta* moths and could be developed as biopesticides based on their efficacy across a broad range of temperature regimes (germination, growth and sporulation), speed

of kill (LT_{50}) and virulence against the pest. In addition, both isolates can successfully be mass-produced on rice using a simple, fast and cost-effective mass-production technique (especially for private sector for business incubation) suitable for deployment in the field. However, the successful deployment of these two biopesticides requires field validation trials under different agroecological zones for which the decision-support tool has provided us with tangible information related to the suitable locations where the two candidate fungal isolates are expected to cause significant epizootics in *P. absoluta* populations. Adequate fungal strains selection and their accurate spatial prediction are therefore fundamental approach to optimize their efficacy prior to field deployment and could consequently guide decision-making for private sector, farmer-based organisations and policy in promoting effective use of candidate fungal pathogens against *P. absoluta* moths in East Africa and beyond.

References

Abbes, K., Harbi, A., and Chermiti, B. (2012). The tomato leafminer *Tuta absoluta* (Meyrick) in Tunisia: Current status and management strategies. *EPPO Bull.* 42, 226–233. doi:10.1111/epp.2559.

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18, 265–267.

Acheampong, M. A., Coombes, C. A., Moore, S. D., and Hill, M. P. (2020). Temperature tolerance and humidity requirements of select entomopathogenic fungal isolates for future use in citrus IPM programmes. *J. Invertebr. Pathol.* 174, 107436. doi:10.1016/j.jip.2020.107436.

Agbessenou, A., Akutse, K. S., Yusuf, A. A., Ekesi, S., Subramanian, S., and Khamis, F. M. (2020). Endophytic fungi protect tomato and nightshade plants against *Tuta absoluta* (Lepidoptera: Gelechiidae) through a hidden friendship and cryptic battle. *Sci. Rep.* 10, 22195. doi:10.1038/s41598-020-78898-8.

Aigbedion-Atalor, P. O., Hill, M. P., Zalucki, M. P., Obala, F., Idriss, G. E., Midingoyi, S.-K., Sunday, E., and Mohamed, S. (2019). The South America tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae), spreads its wings in Eastern Africa: distribution and socioeconomic impacts. *J. Econ. Entomol.* 112, 2797–2807. doi:10.1093/jee/toz220.

Aigbedion-Atalor, P. O., Mohamed, S. A., Hill, M. P., Zalucki, M. P., Azrag, A. G. A.,

Srinivasan, R., and Sunday, E. (2020). Host stage preference and performance of *Dolichogenidea gelechiidivoris* (Hymenoptera: Braconidae), a candidate for classical biological control of *Tuta absoluta* in Africa. *Biol. Control* 144, 1–8. doi:10.1016/j.biocontrol.2020.104215.

Akutse, K. S., Subramanian, S., Khamis, F. M., Ekesi, S., and Mohamed, S. A. (2020). Entomopathogenic fungus isolates for adult *Tuta absoluta* (Lepidoptera: Gelechiidae) management and their compatibility with *Tuta* pheromone. *J. Appl. Entomol.*, 1–11. doi:10.1111/jen.12812.

Allen, C., and Mehler, D. M. A. (2019). Open science challenges, benefits and tips in early career and beyond. *Plos Biol.* 17, 1–14.

Allen, P. J. (1965). Metabolic aspects of spores germination in fungi. *Annu. Rev. Phytopathol.* 3, 313–342.

APRD Arthropod Pesticide Resistance Database, Michigan State University. https://www.pesticideresistance.org/display.php?pa.

Archontoulis, S. V., and Miguez, F. E. (2015). Nonlinear regression models and applications in agricultural research. *Agron. J.* 107, 786–798. doi:10.2134/agronj2012.0506.

Barra, P., Barros, G., Etcheverry, M., and Nesci, A. (2018). Mass production studies in solid substrates with the entomopathogenic fungus, *Purpureocillium lilacinum*. *Int. J. Adv. Agric. Res.* 6, 78–84.

Bayissa, W., Ekesi, S., Mohamed, S. A., Kaaya, G. P., Wagacha, J. M., Hanna, R., and Maniania, N. K. (2017). Selection of fungal isolates for virulence against three aphid pest species of crucifers and okra. *J. Pest Sci.* 90, 355–368. doi:10.1007/s10340-016-0781-4.

Brévault, T., Sylla, S., Diatte, M., Bernadas, G., and Diarra, K. (2014). *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae): A new threat to tomato production in Sub-Saharan Africa. *African Entomol.* 22, 441–444. doi:10.4001/003.022.0202.

Brière, J.-F., Pracros, P., Le Roux, A.-Y., and Pierre, J.-S. (1999). A novel rate model of temperature-dependent development for arthropods. *Environ. Entomol.* 28, 22–29. doi:10.1093/ee/28.1.22.

Cabanillas, H. E., and Jones, W. A. (2009). Effects of temperature and culture media on vegetative growth of an entomopathogenic fungus *Isaria* sp. (Hypocreales: Clavicipitaceae) naturally affecting the whitefly, *Bemisia tabaci* in Texas. *Mycopathologia* 167, 263–271. doi:10.1007/s11046-008-9176-2.

Campbell, A., Frazer, B. D., Gilbert, N., Gutierrez, A. P., and Mackauer, M. (1974). Temperature requirements of some aphids and their parasites. *J. Appl. Ecol.* 11, 431–438.

Chandler, D., Bailey, A. S., Tatchell, G. M., Davidson, G., Greaves, J., and Grant, W. P. (2011). The development, regulation and use of biopesticides for integrated pest management. *Philos. Trans. R. Soc. B Biol. Sci.* 366, 1987–1998. doi:10.1098/rstb.2010.0390.

Dahlberg, K. R., and Etten, J. L. V (1982). Physiology and biochemistry of fungal sporulation. *Annu. Rev. Phytopathol.* 20, 281–301. doi:10.1146/annurev.py.20.090182.001433.

de Campos, M. R., Béarez, P., Amiens-Desneux, E., Ponti, L., Gutierrez, A. P., Biondi, A., and Desneux, N. (2020). Thermal biology of *Tuta absoluta*: demographic parameters and facultative diapause. *J. Pest Sci.* 94, 829-842 doi:10.1007/s10340-020-01286-8.

De Croos, J. N. A., and Bidochka, M. J. (1999). Effects of low temperature on growth parameters in the entomopathogenic fungus *Metarhizium anisopliae*. *Can. J. Microbiol.* 45, 1055–1061. doi:10.1139/cjm-45-12-1055.

Desneux, N., Wajnberg, E., Wyckhuys, K. A. G., Burgio, G., Arpaia, S., Narváez-Vasquez, C. A., Cabello, T., and Urbaneja, A. (2010). Biological invasion of European tomato crops by *Tuta absoluta*: ecology, geographic expansion and prospects for biological control. *J. Pest Sci.* 83, 197–215. doi:10.1007/s10340-010-0321-6.

Dimbi, S., Maniania, N. K., Lux, S. A., and Mueke, J. M. (2004). Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. *BioControl* 49, 83–94. doi:10.1023/B:BICO.0000009397.84153.79.

Ekesi, S., Maniania, N. K., and Ampong-Nyarko, K. (1999). Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Sci. Technol.* 9, 177–185. doi:10.1080/09583159929767.

Ekesi, S., Maniania, N. K., and Lux, S. A. (2003). Effect of soil temperature and moisture on survival and infectivity of *Metarhizium anisopliae* to four tephritid fruit fly puparia. *J. Invertebr. Pathol.* 83, 157–167. doi:10.1016/S0022-2011(03)00069-7.

Fang, W., Azimzadeh, P., and St. Leger, R. J. (2012). Strain improvement of fungal insecticides for controlling insect pests and vector-borne diseases. *Curr. Opin.*

Microbiol. 15, 232–238. doi:10.1016/j.mib.2011.12.012.

Fargues, J., Maniania, N., Delmas, J., and Smits, N. (1992). Influence de la température sur la croissance in vitro d'hyphomycètes entomopathogènes. *Agronomie* 12, 557–564. doi:10.1051/agro:19920708.

Goettel, M. S., and Inglis, D. G. (1997). *Fungi: Hyphomycetes*. doi:10.1016/B978-012432555-5/50013-0.

Guedes, R. N. C. C., Roditakis, E., Campos, M. R., Haddi, K., Bielza, P., Siqueira, H. A. A. A., and Nauen, R. (2019). Insecticide resistance in the tomato pinworm *Tuta absoluta*: patterns, spread, mechanisms, management and outlook. *J. Pest Sci.* 92, 1329–1342. doi:10.1007/s10340-019-01086-9.

Guedes, R. N. C., and Picanço, M. C. (2012). The tomato borer *Tuta absoluta* in South America: Pest status, management and insecticide resistance. *EPPO Bull.* 42, 211–216. doi:10.1111/epp.2557.

Guillemaud, T., Blin, A., Le Goff, I., Desneux, N., Reyes, M., Tabone, E., Tsagkarakou, A., Nino, L. and Lombaert, E. (2015). The tomato borer, *Tuta absoluta*, invading the Mediterranean Basin, originates from a single introduction from Central Chile. *Sci. Rep.* 5, 8371. doi:10.1038/srep08371

Guimapi, R. A., Mohamed, S. A., Biber-Freudenberger, L., Mwangi, W., Ekesi, S., Borgemeister, C., et al. (2020). Decision support system for fitting and mapping nonlinear functions with application to insect pest management in the biological control context. *Algorithms* 13, 1–21. doi:10.3390/A13040104.

Hywel-Jones, N. L., and Gillespie, A. T. (1990). Effect of temperature on spore germination in *Metarhizium anisopliae* and *Beauveria bassiana*. *Mycol. Res.* 94, 389–392. doi:10.1016/S0953-7562(09)80363-8.

Jackson, M. A. (1997). Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. *J. Ind. Microbiol. Biotechnol.* 19, 180–187. doi:10.1038/sj.jim.2900426.

Jackson, M. A., Dunlap, C. A., and Jaronski, S. T. (2010). Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. *BioControl* 55, 129–145. doi:10.1007/s10526-009-9240-y.

Jaronski, S. T. (2010). Ecological factors in the inundative use of fungal entomopathogens. *BioControl* 55, 159–185. doi:10.1007/s10526-009-9248-3.

Jenkins, N. E., Heviefo, G., Langewald, J., Cherry, A. J., and Lomer, C. J. (1998).

Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News Inf.* 19, 21–32.

Kassa, A., Brownbridge, M., Parker, B. L., and Skinner, M. (2008). Whey for mass production of *Beauveria bassiana* and *Metarhizium anisopliae*. *Mycol. Res.* 112, 583–591. doi:10.1016/j.mycres.2007.12.004.

Klass, J. I., Blanford, S., and Thomas, M. B. (2007a). Development of a model for evaluating the effects of environmental temperature and thermal behaviour on biological control of locusts and grasshoppers using pathogens. *Agric. For. Entomol.* 9, 189–199. doi:10.1111/j.1461-9563.2007.00335.x.

Klass, J. I., Blanford, S., and Thomas, M. B. (2007b). Use of a geographic information system to explore spatial variation in pathogen virulence and the implications for biological control of locusts and grasshoppers. *Agric. For. Entomol.* 9, 201–208. doi:10.1111/j.1461-9563.2007.00336.x.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., and Goettel, M. S. (2015). Insect pathogens as biological control agents: Back to the future. *J. Invertebr. Pathol.* 132, 1–41. doi:10.1016/j.jip.2015.07.009.

Lichtenberg, E., and Zimmerman, R. (1999). Adverse health experiences, environmental attitudes, and pesticide usage behavior of farm operators. *Risk Anal.* 19, 283–294. doi:10.1023/A:1006929812285.

Lietti, M. M. M., Botto, E., and Alzogaray, R. A. (2005). Insecticide resistance in Argentine populations of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Neotrop. Entomol.* 34, 113–119. doi:10.1590/s1519-566x2005000100016.

Logan, J. A., Wollkind, D. J., Hoyt, S. C., and Tanigoshi, L. K. (1976). An analytic model for description of temperature dependent rate phenomena in arthropods. *Environ. Entomol.* 5, 1133–1140.

Mansour, R., Brévault, T., Chailleux, A., Cherif, A., Grissa-Lebdi, K., Haddi, K., Mohamed, S., Oke, A., Tonnang, H. E., Desneux, N., and Biondi, A. (2018). Occurrence, biology, natural enemies and management of *Tuta absoluta* in Africa. *Entomol. Gen.* 38, 83–112. doi:10.1127/entomologia/2018/0749.

McCammon, S. A., and Rath, A. C. (1994). Separation of *Metarhizium anisopliae* strains by temperature dependent germination rates. *Mycol. Res.* 98, 1253–1257. doi:10.1016/S0953-7562(09)80295-5.

Migiro, L. N., Maniania, N. K., Chabi-Olaye, A., and Vandenberg, J. (2010). Pathogenicity

of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales: Clavicipitaceae) isolates to the adult pea leafminer (Diptera: Agromyzidae) and prospects of an autoinoculation device for infection in the field. *Environ. Entomol.* 39, 468–475. doi:10.1603/en09359.

Niassy, S., Ekesi, S., Migiro, L., and Otieno, W. (2020). *Sustainable management of invasive pests in Africa*. Springer International Publishing doi:10.1007/978-3-030-41083-4.

Ochilo, W. N., Nyamasyo, G. N., Kilalo, D., Otieno, W., Otipa, M., Chege, F., Karanja, T., and Lingeera, E. K. (2019). Characteristics and production constraints of smallholder tomato production in Kenya. *Sci. African* 2, e00014. doi:10.1016/j.sciaf.2018.e00014.

Onsongo, S. K., Gichimu, B. M., Akutse, K. S., Dubois, T., and Mohamed, S. A. (2019). Performance of three isolates of *Metarhizium anisopliae* and their virulence against *Zeugodacus cucurbitae* under different temperature regimes, with global extrapolation of their efficiency. *Insects* 10, 1–13. doi:10.3390/insects10090270.

Pratt, C. F., Constantine, K. L., and Murphy, S. T. (2017). Economic impacts of invasive alien species on African smallholder livelihoods. *Glob. Food Sec.* 14, 31–37. doi:10.1016/j.gfs.2017.01.011.

R Core Team (2019). R: A language and environment for statistical computing R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org/.

Santana, P. A., Kumar, L., Da Silva, R. S., and Picanço, M. C. (2018). Global geographic distribution of *Tuta absoluta* as affected by climate change. *J. Pest Sci.* 92, 1373–1385. doi:10.1007/s10340-018-1057-y.

Shang, Y., Feng, P., and Wang, C. (2015). Fungi that infect insects: Altering host behavior and beyond. *PLoS Pathog.* 11, 1–6. doi:10.1371/journal.ppat.1005037.

Sibomana, M. S., Workneh, T. S., and Audain, K. (2016). A review of postharvest handling and losses in the fresh tomato supply chain: a focus on Sub-Saharan Africa. *Food Secur.* 8, 389–404. doi:10.1007/s12571-016-0562-1.

Smith, J. D., Dubois, T., Mallogo, R., Njau, E.-F. F., Tua, S., and Srinivasan, R. (2019). Host range of the invasive tomato pest *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) on solanaceous crops and weeds in Tanzania. *Florida Entomol.* 101, 573. doi:10.1653/024.101.0417.

Smits, N., Brière, J.-F., and Fargues, J. (2003). Comparison of non-linear temperature-dependent development rate models applied to in vitro growth of entomopathogenic fungi. *Mycol. Res.* 107, 1476–1484. doi:10.1017/S095375620300844X.

Soares, M. A., Campos, M. R., Passos, L. C., Carvalho, G. A., Haro, M. M., Lavoir, A.-V. V., Biondi, A., Zappala, L., and Desneux, N. (2019). Botanical insecticide and natural enemies: a potential combination for pest management against *Tuta absoluta*. *J. Pest Sci.* 92, 1433–1443. doi:10.1007/s10340-018-01074-5.

Steiniger, S., and Hunter, A. J. S. (2012). "Free and open source GIS software for building a spatial data infrastructure," in *Geospatial Free and Open Source Software in the 21st Century*, 247–261.

Tumuhaise, V., Ekesi, S., Maniania, N. K. K., Tonnang, H. E. Z. E. Z., Tanga, C. M. M., Ndegwa, P. N. N., Srinivasan, R., and Mohamed, S. (2018). Temperature-dependent growth and virulence, and mass production potential of two candidate isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin for managing *Maruca vitrata* Fabricius (Lepidoptera: Crambidae) on cowpea. *African Entomol.* 26, 73–83. doi:10.4001/003.026.0073.

Tumuhaise, V., Khamis, F. M., Agona, A., Sseruwu, G., and Mohamed, S. A. (2016). First record of *Tuta absoluta* (Lepidoptera: Gelechiidae) in Uganda. *Int. J. Trop. Insect Sci.* 36, 135–139. doi:10.1017/S1742758416000035.

Vidal, C., Fargues, J., and Lacey, L. A. (1997). Intraspecific variability of *Paecilomyces fumosoroseus*: Effect of temperature on vegetative growth. *J. Invertebr. Pathol.* 70, 18–26. doi:10.1006/jipa.1997.4658.

CHAPTER THREE

Endophytic fungi protect tomato and nightshade plants against *Phthorimaea absoluta* (Lepidoptera : Gelechiidae) through a hidden friendship and cryptic battle

This chapter has been published as:

Agbessenou, A., Akutse, K. S., Yusuf, A. A., Ekesi, S., Subramanian, S., & Khamis, F. M. (2020). Endophytic fungi protect tomato and nightshade plants against *Tuta absoluta* (Lepidoptera : Gelechiidae) through a hidden friendship and cryptic battle. *Scientific Reports*, 10:22195. https://doi.org/10.1038/s41598-020-78898-8

Abstract

Endophytic fungi live within plant tissues without causing any harm to the host, promote its growth, and induce systemic resistance against pests and diseases. To mitigate the challenging concealed feeding behaviour of immature stages of *Phthorimaea absoluta* in both tomato (*Solanum lycopersicum*) and nightshade (*Solanum scabrum*) host plants, 15 fungal isolates were assessed for their endophytic and insecticidal properties. Twelve isolates were endophytic to both host plants with varied colonisation rates. Host plants endophytically-colonised by *Trichoderma asperellum* M2RT4, *Beauveria bassiana* ICIPE 706 and *Hypocrea lixii* F3ST1 outperformed all the other isolates in reducing significantly the number of eggs laid, mines developed, pupae formed and number of emerged adults. Furthermore, the survival of exposed adults and F1 progeny was significantly reduced by *Trichoderma* sp. F2L41 and *B. bassiana* isolates ICIPE 35(4) and ICIPE 35(15) compared to other isolates. The results indicate that *T. asperellum* M2RT4, *B. bassiana* ICIPE 706 and *H. lixii* F3ST1 have high potential to be developed as endophytic-fungal-based biopesticide for the management of *P. absoluta*.

3.1.Introduction

Vegetable production is one of the most viable horticultural sub-sector in Africa and is considered an important route out of poverty for smallholder farmers (Ekesi et al., 2011). Tomato (*Solanum lycopersicum* L.; Solanaceae) is one of the most promising vegetable crops for horticultural expansion in Africa, but the crop is experiencing significant losses due to abiotic and biotic stressors threatening the livelihoods of millions of smallholder farmers (Pratt et al., 2017). Among the biotic factors, the invasive South American tomato pinworm *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae), which originated from South America and spread as far as Europe (Desneux et al., 2011) has emerged as one of the most important devastating pest of tomato during the last decade, contributing to increasing risk of malnutrition and food insecurity in Africa. In addition to tomato crop, the pest also attacks various cultivated and wild plants within the Solanaceae family such as pepper, *Capsicum annuum* L.; tobacco, *Nicotiana tabacum* L.; potato, *Solanum tuberosum* L. and black nightshade, *Solanum nigrum* L. (Idriss et al., 2020). In Kenya, both tomato and nightshade crops are the most preferred hosts for the South American tomato pinworm with high infestation levels causing up to 100% yield losses on tomato (Idriss et al., 2020). Estimates of the economic losses due to this pest reaches as high as US$ 59.3 million annually (Aigbedion-Atalor et al., 2019). Ovipositing female lays eggs on the upper surface of tomato leaves which hatch after four to five days. Neonate larvae penetrate the leaf and feed on the mesophyll resulting in the production of mines on the leaf surface compromising the photosynthetic activity of the plant that negatively affect crop productivity or yield (Biondi et al., 2018). Mature larvae bore into the tomato stems, fruits and flowers, spending most of their lifespan inside the crop than outside (Desneux et al., 2010). This concealed feeding behaviour allows the pest to escape from most of the non-systemic synthetic insecticides currently being applied hindering management of the pest. The resultant high use of synthetic pesticides causes significant short-and long-term adverse environmental and human health effects and increased resistance development in *P. absoluta* (Guedes et al., 2019). Therefore, this emphasises the need to promote environmentally-friendly control methods to curtail these problems. As a viable alternative to the use of synthetic insecticides, the development of biological control approaches using entomopathogenic fungi has shown promising results as they cause high mortality to insect pests of economic importance (Akutse et al., 2019a; Dimbi et al., 2013; Maniania et al., 2016; Mweke et al., 2018). Akutse et al. (2020) reported the potential of fungal pathogens to control *P. absoluta* and

subsequently identified three *Metarhizium anisopliae* (Metschnikoff) Sorokin strains (ICIPE 18, ICIPE 20 and ICIPE 655) as candidate biopesticides causing mortality of 95.0, 87.5 and 86.25%, respectively against the adult stage of the pest. Entomopathogenic fungi have been traditionally used to control insect pests mostly through inundative application (Inglis et al., 2001). Recent studies have begun to examine their activity as plant endophytes to systemically protect plants against herbivorous insect pests (Behie and Bidochka, 2014) and are therefore best suited to target the cryptic stages of *P. absoluta* such as larvae (Akutse et al., 2019b).

Endophytic fungi are symptomless microbial organisms that live within host plant tissues either naturally or through artificial inoculation without causing any harm to the host (Wilson, 1995). Some of the advantages of using endophytic fungi reside in the fact that, they are less exposed to the effect of environmental stresses and require little inoculum for its systemic delivery within the host plant tissues (Quesada-Moraga et al., 2009). In some cases, these ubiquitous fungi play an important role as plant growth promoters participating therefore in the acquisition of nutrients by the plants (Barelli et al., 2016). Although several inoculation methods have been reported to be effective in delivering the inoculum at the target site, insecticidal seed treatment has been termed as the most convenient, safe and cost-effective inoculation method for successful endophytic colonisation of many crop plants (Latz et al., 2018). Consequently, using this delivery technique, host-adapted endophytes have been successfully established in tomatoes (Ownley et al., 2008), Faba bean (Akello and Sikora, 2012; Akutse et al., 2013), maize (Russo et al., 2019) and cotton (Ownley et al., 2008). Upon plant colonisation, endophytic fungi help their host plants to perform better under stressful environmental conditions (drought) and withstand biotic stressors (pathogens and herbivores) through the induction of local or systemic resistance, antibiosis, phytohormones production and the stimulation of plant secondary metabolites (Lahrmann et al., 2013; Fadiji and Babalola, 2020).

In an attempt to improve the management of the pea leafminer *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae), Akutse et al. (2013) and Gathage et al. (2016) reported that through seed inoculation, endophytic fungi could successfully colonise Faba beans plant tissues and cause significant suppression of the pest. A similar study by Muvea et al. (2014) reported on the establishment of endophytic fungi within onion plant and the ability of these microorganisms in reducing the population of onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) on inoculated plants. Similarly, tomato seeds pre-treated with the

endophytic fungi *Beauveria bassiana* (Balsamo-Criv.) Vuillemin reduced larval performance of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Powell et al., 2009). Recently, Klieber and Reineke, (2016) revealed that endophytic fungi inoculated in tomato plants mediated systemic resistance against *P. absoluta* and played a significant role in reducing feeding activity of the immature stage of the pest. This pest control strategy has added a new dimension to the use of fungal entomopathogens against cryptic insect pests whose life cycle limits the effectiveness of chemical insecticides and other control methods (Quesada-Moraga et al., 2009; Resquín-romero et al., 2016). Therefore, to tackle the concealed feeding behaviour of the larval stage of *P. absoluta*, the objective of this chapter was to assess the endophytic properties of 15 fungal isolates on both tomato and nightshade plants, and evaluate their insecticidal activity or pathogenicity and their ability to induce systemic resistance against the pest with the aim to use the potent fungal endophytic-based biopesticide as a component of *Phthorimaea absoluta* IPM system/strategy.

3.2.Materials and methods

Fungal cultures

Fifteen fungal isolates belonging to five different genera (*Beauveria* (7), *Fusarium* (1), *Hypocrea* (1), *Metarhizium* (3) and *Trichoderma* (3)), obtained from *icipe*'s Arthropod Pathology Unit Germplasm, were used in this study (Table 3.1). These isolates were cultured on potato dextrose agar (PDA) (OXOID CM0139, Oxoid Ltd., Basingstoke, UK), except for *Metarhizium* which were cultured on Sabouraud dextrose agar (SDA) (OXOID CM0041, Oxoid Ltd., Basingstoke, UK), and maintained at 25 ± 2 °C in complete darkness. Conidia were harvested by scraping the surface of two to three-week-old sporulated cultures using a sterile spatula. The harvested conidia were then suspended in 10 mL sterile distilled water containing 0.05% Triton X-100 (MERCK KGaA, Darmstadt, Germany) and vortexed for five minutes at about 700 rpm to break conidial clumps and ensure a homogenous suspension (Akutse et al., 2013; Muvea et al., 2014). Conidial concentrations were quantified using an improved Neubauer hemocytometer under a light microscope (Goettel and Inglis, 1997). The conidial suspension was adjusted to a concentration of 1×10^8 conidia mL^{-1} through serial dilution prior to inoculation of tomato and nightshade seeds.

Prior to commencement of the bioassays, spore viability was determined by plating evenly 0.1 mL of 3×10^6 conidia mL^{-1} onto 9-cm Petri dishes containing SDA (OXOID CM0041, Oxoid Ltd., Basingstoke, UK) or PDA (OXOID CM0139, Oxoid Ltd., Basingstoke, UK).

Three sterile microscope cover slips (2 × 2 cm) were placed randomly on the surface of each inoculated plate. Plates were sealed with Parafilm and incubated in complete darkness at 25 ± 2 °C and were examined after 16-20 h. The percentage germination of conidia was determined from 100 randomly selected conidia on the surface area covered by each cover slip under a light microscope (400×) using the method described by Goettel and Inglis (1997). Conidia were considered to have germinated when the length of the germ tube was at least twice the diameter of the conidium (Goettel and Inglis, 1997). Four replicates were used for each isolate.

Table 3.1. List of fungal isolates used in this study.

Fungal species	Isolate	Source	Origin	Year of isolation
Beauveria bassiana	ICIPE 35(4), ICIPE 35(6), ICIPE 35(12), ICIPE 35(15)	Coffee berry	Kenya	2009
B. bassiana	ICIPE 273	Soil	Mbita (Kenya)	2006
B. bassiana	ICIPE 706	Monocotyledons	Kenya	2012
Metarhizium anisopliae	ICIPE 7	*Amblyomma variegatum*	Rusinga (Kenya)	1996
M. anisopliae	ICIPE 30	*Busseola fusca* (Lepidoptera)	Kenduba (Kenya)	1989
M. anisopliae	ICIPE 69	Soil	Matete (DRC)	1990
Trichoderma sp.	F2L41	Onion	Loitoktok (Kenya)	2012
Trichoderma atroviride	F2S21	Onion	Loitoktok (Kenya)	2012
Trichoderma asperellum	M2RT4	Maize and Sorghum	Nakuru (Kenya)	2009
Fusarium proliferatum	F2S51	Onion	Embu (Kenya)	2012
Hypocrea lixii	F3ST1	Maize and Sorghum	Nakuru, Embu and Kakamega (Kenya)	2009

Seed inoculation and colonisation assessment of endophyte isolates

Tomato (*Solanum lycopersicum* L. cv. "Moneymaker") and nightshade (*Solanum scabrum* Mill cv. "Giant nightshade") seeds (Simlaw Seeds Company Ltd., Nairobi, Kenya) were surface-sterilised successively in 70% ethanol for two minutes followed by 1.5% sodium hypochlorite for three (3) min and finally rinsed three times in sterile distilled water. The surface sterilised seeds were placed on sterile filter paper on a clean working surface in a cabinet until the residual water evapourated. Effectiveness of the surface sterilisation

technique was confirmed by plating out 0.1 mL of the last rinse water onto potato dextrose agar and also imprinting of surface sterilised seeds onto PDA (tissue imprint) supplemented with 100 mg/L Streptomycin and plates were incubated at 25 °C for 14 days (Schulz et al., 1998). Seeds were then soaked overnight for 12 h in conidial suspensions titrated at 1×10^8 conidia mL^{-1}. For the controls, sterilised seeds were soaked overnight for 12 h in sterile distilled titrated (0.05% Triton X-100) water (Akutse et al., 2013; Muvea et al., 2014). Seeds were then transferred into plastic pots (8 cm diameter × 7.5 cm high) containing the planting substrate with a volume of 2 L (mixture of manure and soil 1:5). The substrate was sterilised in an autoclave for 2 h at 121 °C and allowed to cool for 72 h prior to planting. Five seeds were sowed per pot and maintained at room temperature (25 ± 2 °C, 60% RH and 12:12 L:D photoperiod). Pots were transferred immediately after germination to the screen house (2.8 m length × 1.8 m width × 2.2 m height) at 25 ± 2 °C, 55% RH and 12:12 L:D photoperiod for four to five weeks. After germination, seedlings were thinned to two per pot and watered twice (~150 cm^3) per day (morning and evening). No additional fertiliser was added to the planting substrate. Plants of four to five-week-old were used for the various experiments.

To determine the colonisation of inoculated fungal isolates in tomato and nightshade, plants were carefully uprooted from the pots four to five weeks after inoculation and washed under running tap water to remove any soil attached to the plants. Seedlings (ca. 30 cm in height) were divided into three different sections (ca. 5 cm long): leaves, stems and root sections using a sterile scalpel (Akutse et al., 2013). Five randomly selected leaf, stem and root sections from each plant were surface-sterilised as described above. The different plant parts were then aseptically cut under a laminar flow hood into 1 × 1 cm pieces before placing the pieces, four cm apart on PDA plates supplemented with a 0.05% solution of antibiotic (streptomycin sulphate salt) (Akutse et al., 2013; Muvea et al., 2014). Plates were incubated at 25 ± 1 °C for 10 days, after which the presence of endophytes was determined. The last rinse water was also plated to assess the effectiveness of the surface sterilisation procedure as described earlier. Plate imprinting was also conducted to assess effective surface sterilisation of plant materials (Douglas Inglis et al., 2012). The colonisation of the different plant parts was recorded by counting the number of pieces of the different plant parts that showed the presence of inoculated fungal growth/mycelia according to Koch's postulates (Petrini and Fisher, 1986). Only the presence of endophytes that were inoculated was scored. Fungal isolates were identified morphologically using slides which were prepared from the mother plates. Treatments were arranged in a randomised complete block design (RCBD)

with four replicates per experiment (Akutse et al., 2013). The success rate of fungal endophyte colonisation (%) of host plant parts was calculated as follows:

$$\text{Colonisation (\%)} = \frac{\text{Number of pieces exhibiting fungal outgrowth}}{\text{Total number of pieces plated out}} \times 100$$

Insects

A colony of *P. absoluta* was established from wild moths and larvae collected from infested tomato leaves and fruits in Mwea (0°36′ 31.3″ S 037°22′ 29.7″E), Kenya in June 2019. The moths were kept in ventilated, sleeved Perspex cages (40 × 40 × 45 cm) and were fed ad *libitum* with 10% honey solution placed to the top side of each cage as food source (Aigbedion-Atalor et al., 2020). Four potted tomato plants were placed in the cages for oviposition. The plants were removed 24 h post-exposure to female moths and transferred to separate wooden cages (50 × 50 × 60 cm) ventilated with netting material at the sides and on the top until the eggs hatched. Leaves with larvae were removed from these plants, three days after the larvae hatched and placed into clean sterile plastic containers (21 cm long × 15 cm wide × 8 cm high) lined with paper towel to absorb excess moisture and fine netting infused lid for ventilation. The larvae were supplied daily with fresh tomato leaves as food until they pupated. The pupae were collected from the plastic containers using a fine camel hair-brush and placed inside clean plastic containers for adult emergence. The colony was rejuvenated every three months through infusion, with infested tomato leaves collected from the field to reduce inbreeding (Akutse et al., 2020; Aigbedion-Atalor et al., 2020). Insects were maintained under a rearing condition of 28 ± 2 °C, 48% RH and 12:12 L:D photoperiod at the Animal Rearing and Quarantine Unit (ARQU) of *icipe* for five generations prior to bioassays (Akutse et al., 2020).

Pathogenicity of endophytically-colonised tomato and nightshade plants on life history parameters of *Phthorimaea absoluta*

Based on their ability to colonise plant tissues of both host plants, nine isolates (*B. bassiana* ICIPE 273, ICIPE 35(4), ICIPE 35(15), ICIPE 706, *F. proliferatum* F2S51, *T. harzianum* F2L41, *T. atroviride* F2S21, *H. lixii* F3ST1 and *T. asperellum* M2RT4) were tested for their impact against oviposition potential, egg, larval and pupal survival, adult emergence and longevity of *P. absoluta*. Two-day-old mated adults (10 individuals at sex ratio of 1:2 male: female) were exposed for 48 h to four-week-old endophytically-colonised host plant

seedlings in Plexiglas cages (50 cm × 50 cm × 45 cm). Each cage contained four potted plants that represented a treatment, and was maintained at 25 ± 2 °C, 40% RH and 12:12 L: D photoperiod. All the treatments were arranged in a randomised complete block design and the experiment replicated four times. After 48 h post-exposure, insects were removed from the cages and introduced into clean cages (20 cm × 20 cm × 20 cm) and their survival was recorded by counting the number of live adults daily inside the cages until all moths died (Akutse et al., 2013). For each treatment, 10 female *P. absoluta* moths were monitored and the experiment was replicated four times.

Eggs that were laid on endophytically-colonised and control plants were maintained on the plants until they hatched. After hatching, larvae were allowed to feed upon their natal plants until they reached the 2nd and 3rd instars (approximately 8-10 d post-exposure). In the control, plants were not inoculated with fungal pathogens. For each treatment, the number of eggs laid on each plant was recorded as well as the number of mines and this was replicated four times. Using a fine paint brush, larvae were transferred into cages containing four potted plants that were in the same developmental stage as the one on which the caterpillars had hatched and had been feeding previously. Dead moths were placed on Petri dishes lined with damp sterilised filter paper to allow fungal growth on the surface of the cadaver (mycosis test). Caterpillars were allowed to feed freely on the potted plants in a cage until they pupated. For each treatment, pupation was recorded daily, and pupae were collected from leaves 10-11 d post-exposure, counted and then incubated at 25 ± 2 °C. Adult emergence was determined for each treatment, and non-viable pupae were also counted. Following adult emergence from the endophytically-colonised and control plants, 20 adult moths were selected per treatment and the survival of F1 progenies was recorded daily until all moths died and this was replicated four times (Oliveira et al., 2009). The moths were maintained in a cage as described in section “insects” above. A 10% honey solution was provided as food and cages maintained at 25 ± 2 °C, 48% RH and 12:12 L:D photoperiod. To confirm that the mortality of the moths was as a result of direct fungal infection, dead insects were placed on a moistened filter paper in Petri dishes and were observed for post-mortem fungal sporulation (mycosis test). Mycosis was assessed by surface sterilising the dead moths with 1% sodium hypochlorite followed by three rinses with sterile distilled water, after which the sterilised cadavers were placed on sterile wet filter paper in sterile Petri dishes that were then sealed with Parafilm and kept at room temperature. Each treatment consisted of 10 insects and replicated four times.

Statistical analyses

Colonisation rate and count data (number of eggs, mines, pupae and adults) were tested for normality using Shapiro-Wilk test (Shapiro and Wilk, 1965) and homogeneity of variance using Levene test. The data were not normally distributed and variances were not homogeneous, therefore colonisation rate and adult emergence data were analysed with generalised linear model (GLM) using binomial distribution and logit link function. Count data were analysed with generalised linear model (GLM) with negative binomial error distribution taking into account overdispersion. Whenever there was a difference, the means were separated using Tukey's honest significant difference (HSD) test using "agricolae" package in R (De Mendiburu, 2020). The survival curves were generated using Kaplan-Meier estimator method, and log-rank test was used to compare the effect of the various fungal isolates on *P. absoluta* exposed adults and F1 progenies survival using the "Survival" package (Therneau, 2020). Cox's proportional hazard was ued to test for differences in survival rate among the treatments (Crawley, 2007).

All analyses were performed using the R (version 3.6.2) statistical software packages (R Core Team, 2019) and all statistical results were considered significant at the confidence interval of 95% ($P < 0.05$).

3.3.Results

Endophytic colonisation of tomato and nightshade by fungal isolates

The results of viability tests showed that conidia germination of the different fungal isolates used in this study exceeded 90% after 18 h of incubation. Endophytic colonisation rate was determined by the recovery of the inoculated fungal strains from the roots, stems and leaves, respectively. The 15 fungal isolates differed markedly in their ability to colonise both tomato and nightshade plants. Irrespective of the host plants, *M. anisopliae* isolates ICIPE 30, ICIPE 69 and ICIPE 7 failed to colonise the various plant parts while the remaining 12 isolates were successfully recovered from tomato and nightshade host plant parts (Figures 3.1A and 3.1B). However, colonisation of the different plant tissues (roots, stems and leaves) varied depending on fungal isolates and host plants. For example, isolates of *F. proliferatum* F2S51, *Trichoderma* sp. F2L41, *T. atroviride* F2S21, *H. lixii* F3ST1, *B. bassiana* ICIPE 35(4), ICIPE 273, ICIPE 706 and *T. asperellum* M2RT4 colonised roots, stems and leaves of both host plants. *Beauveria bassiana* ICIPE 35(15) colonised the roots, stems, and leaves of

tomato plants while it colonised only the roots and stems of nightshade (Figures 3.1A and 3.1B). It is worth noting that; *B. bassiana* ICIPE 35(12), ICIPE 35(6) and ICIPE 279 colonised only roots and stems of both host plants. In addition, *H. lixii* F3ST1 and *T. asperellum* M2RT4 colonised more than 85% of all the plant tissues of both host plants while *B. bassiana* ICIPE 706 colonised 60, 40 and 15% of roots, stems and leaves of tomato, respectively (Figure 3.1A); and 70, 35 and 15% of roots, stems and leaves of nightshade, respectively (Figure 3.1B). *Trichoderma atroviride* F2S21 successfully colonised 100, 100 and 75% of roots, stems and leaves of tomato plant respectively, and 100, 95 and 55% of roots, stems and leaves in nightshade, respectively (Figures 3.1A and 3.1B). Significant differences in colonisation by isolates were observed on roots (χ^2 = 112.31, df = 11, P < 0.0001), stems (χ^2 = 204.36, df = 11, P < 0.0001) and leaves (χ^2 = 279.74, df = 11, P < 0.0001) of tomato (Figure 3.1A). Similarly, significant differences were observed in colonisation levels of plant parts of nightshade: roots (χ^2 = 114.17, df = 11, P < 0.0001), stems (χ^2 = 131.89, df = 11, P < 0.0001) and leaves (χ^2 = 297.73, df = 11, P < 0.0001) (Figure 3.1B).

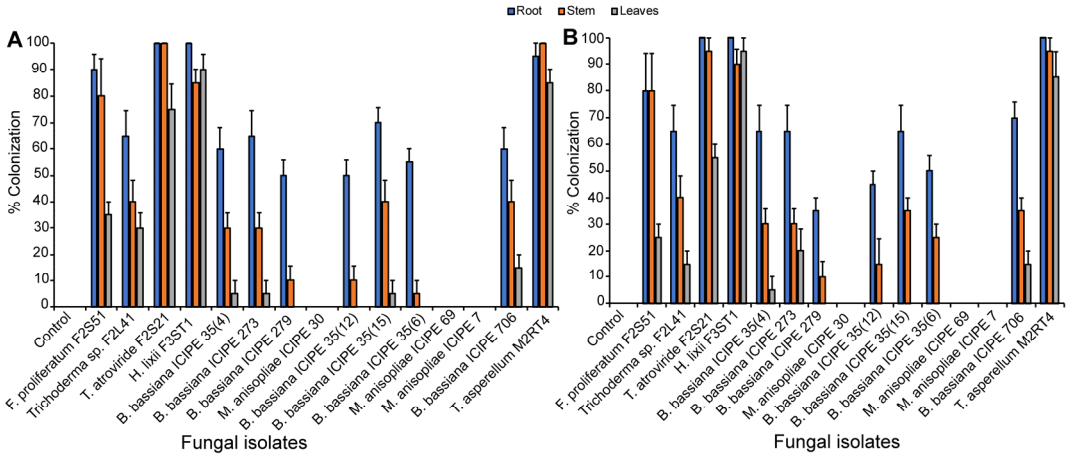

Figure 3.1. Endophytic colonisation of tomato *Solanum lycopersicum* (A) and nightshade *Solanum scabrum* (B) host plant parts by 15 fungal isolates at 4-5 weeks post-inoculation. Bar chart represents means ± SE (standard error) at 95% CI (P < 0.05; n = 4).

Effect of endophytically-colonised tomato and nightshade host plants on survival of adult *Phthorimaea absoluta*

The survival of *P. absoluta* moths exposed to endophytically-colonised tomato plants varied significantly among the treatments (Proximate log rank test, χ^2 = 168.5, df = 9, P < 0.0001). For example, at day five post-exposure, mean adult survival was 28.21% with *B. bassiana*

ICIPE 273 and 32.69% with *F. proliferatum* F2S51 compared to 52.28% in the control (Figure 3.2A). At ten days post-exposure, there was no significant difference in survival rates both amongst the treatments and against the control as mean adult survival ranged between 9.2% to 26.40% including the control, except for *B. bassiana* ICIPE 706 (30.80%). At day 15 post-exposure, the survival was less than 10% including the control. At day 20 post-exposure, there was no significant difference in survival rate among all the treatments including the control (Figure 3.2A). Similarly, there was a significant difference in the survival of *P. absoluta* moths exposed to endophytically-colonised nightshade plants (Proximate log rank test, $\chi^2 = 82.79$, df = 9, $P < 0.0001$) compared to the control. At five days post-exposure, there was no significant difference in survival rates both amongst the treatments and against the control as mean adult survival was between 39 and 54% including the control (Figure 3.2B). At ten days post-exposure, mean adult survival ranged between 10% to 26.6% including the control. At day 15 post-exposure, no survival was observed in *T. asperellum* M2RT4 while mean adult survival was below 10% in all the treatments including the control (Figure 3.2B).

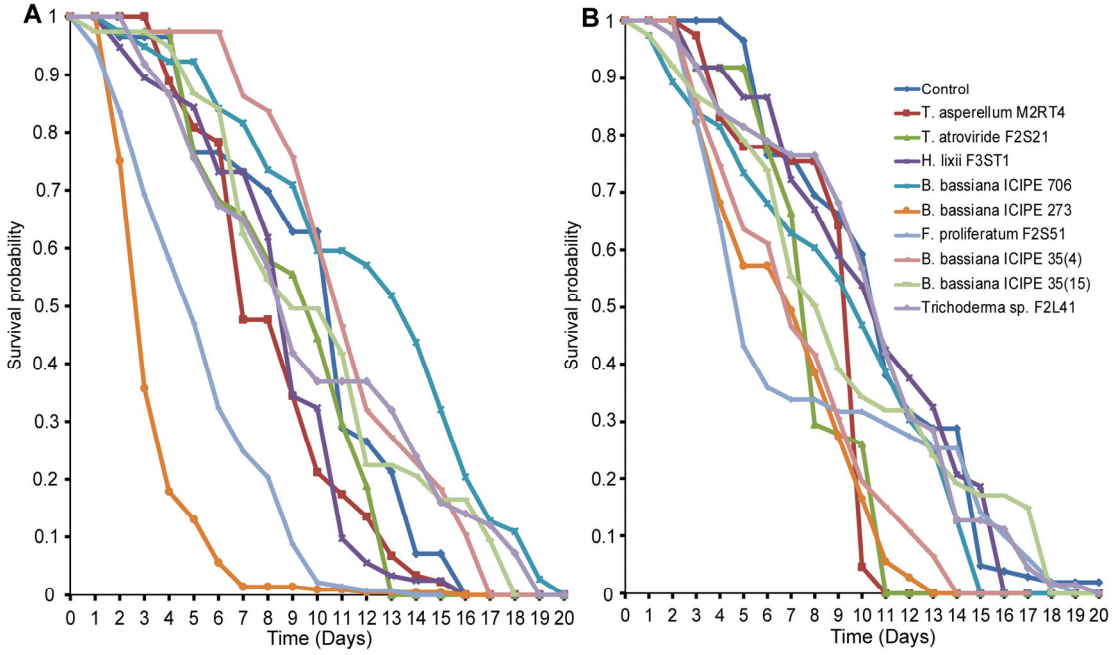

Figure 3.2. Effect of endophytically-colonised host plants by fungal isolates on survival of *Phthorimaea absoluta* moths: (A) Kaplan-Meier survival curves of *Phthorimaea absoluta* moths exposed to endophytically-colonised tomato plants, (B) Kaplan-Meier survival curves of *Phthorimaea absoluta* moths exposed to endophytically-colonised nightshade plants ($P < 0.05$, n = 4).

Effect of endophytically-colonised tomato and nightshade host plants on oviposition and leafmining of *Phthorimaea absoluta*

The number of eggs laid on endophytically-colonised tomato plants varied significantly among the treatments (χ^2 = 208.92, df = 9, P < 0.0001) (Figure 3.3A). For instance, *T. asperellum* M2RT4 endophytically-colonised tomato plants recorded the lowest number of eggs (30.0 ± 4.51 eggs), followed by *B. bassiana* ICIPE 706 (31.25 ± 5.88 eggs), *H. lixii* F3ST1 with (63.25 ± 2.66 eggs) and *T. atroviride* F2S21 with (63.5 ± 7.63 eggs), compared to (111.0 ± 13.32 eggs) in the control (Figure 3.3A). However, the highest number of eggs was recorded on *B. bassiana* ICIPE 273 (228.75 ± 24.36 eggs), followed by *F. proliferatum* F2S51 (177.0 ± 15.96 eggs), *Trichoderma* sp. F2L41 with (142.0 ± 27.67 eggs) and the control (111.0 ± 13.32 eggs) (Figure 3.3A). Upon hatching, *T. asperellum* M2RT4-endophytically-colonised tomato plants recorded the lowest number of mines (24.0 ± 5.4 mines) while *B. bassiana* ICIPE 273 recorded the highest number of mines (219.0 ± 20.92 mines) followed by *F. proliferatum* F2S51 (173.5 ± 15 mines), *Trichoderma* sp. F2L41 with (137.0 ± 24.47 mines), compared to (107.33 ± 13.32 mines) in the control (χ^2 = 216.4, df = 9, P < 0.0001) (Figure 3.3B).

Similarly, endophytically-colonised nightshade plants had a significant effect on the oviposition of *P. absoluta* (χ^2 = 91.73, df = 9, P < 0.0001) (Figure 3.3C). Among the fungal isolates, the lowest number of eggs was laid on *T. asperellum* M2RT4 endophytically-colonised nightshade plants (33.25 ± 3.97 eggs) compared to (109.33 ± 23.31 eggs) in the control (Figure 3.3C). However, the highest number of eggs (162.25 ± 20.01 eggs) was recorded on *B. bassiana* ICIPE 273, followed by *Trichoderma* sp. F2L41 with (111.75 ± 21.85 eggs) and *B. bassiana* ICIPE 706 (104.25 ± 11.38 eggs), compared to (109.33 ± 23.31) eggs in the control (Figure 3.3C). Subsequently, following egg hatchability, the lowest number of mines (24.5 ± 5.55 mines) was recorded on *T. asperellum* M2RT4 endophytically-colonised nightshade plants while the highest was recorded on *B. bassiana* ICIPE 273 (155.0 ± 19.94 mines) followed by *Trichoderma* sp. F2L41 (108.5 ± 22.02 mines) and the control (107.33 ± 23.31 mines) (χ^2 = 110.95, df = 9, P < 0.0001) (Figure 3.3D).

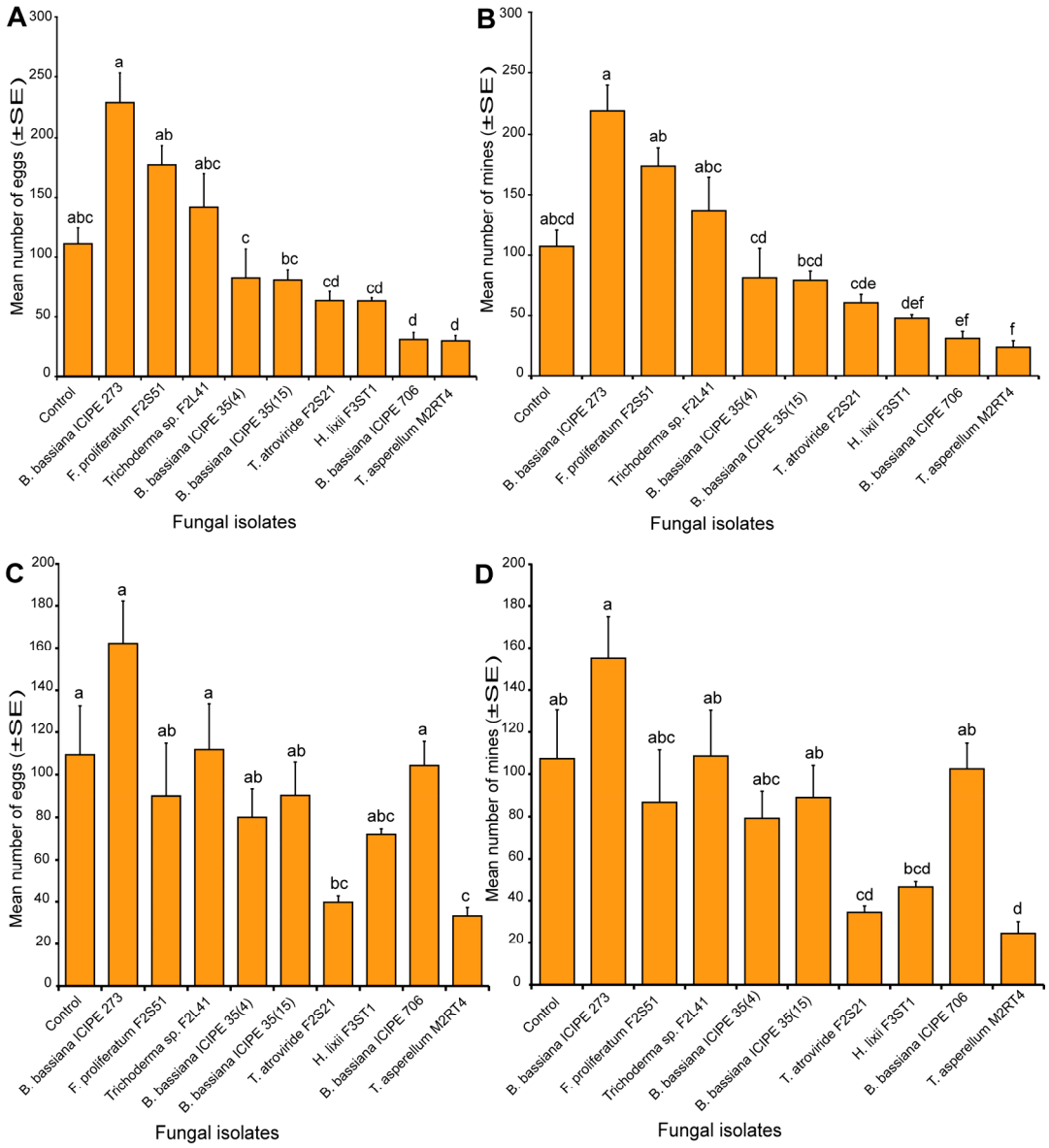

Figure 3.3. Effect of endophytically-colonised host plants by fungal isolates on oviposition and leafmining of *Phthorimaea absoluta* at 48 h post-exposure. (A) Bar chart showing mean number (± SE) of *Phthorimaea absoluta* eggs laid on endophytically-colonised tomato plants. (B) B Bar chart showing mean number (± SE) of mines produced by *Phthorimaea absoluta* on endophytically-colonised tomato plants. (C) Bar chart showing mean number (± SE) of *Phthorimaea absoluta* eggs laid on endophytically-colonised nightshade plants. (D) Bar chart showing mean number (± SE) of mines produced by *Phthorimaea absoluta* on endophytically-colonised nightshade plants. Means followed by different lowercase letters are significantly different ($P < 0.05$; n = 4; Tukey's HSD test).

Effect of endophytically-colonised tomato and nightshade host plants on *Phthorimaea absoluta* pupation and adult emergence

The pupation of *P. absoluta* larvae that survived was significantly affected (χ^2 = 131.45, df = 9, P < 0.0001) by the endophytically-colonised tomato plants (Figure 3.4A). In endophytically-colonised tomato plants, fewer *P. absoluta* pupae (20.75 ± 4.05 pupae) were produced in *B. bassiana* ICIPE 706 followed by *T. asperellum* M2RT4 (21.25 ± 5.22 pupae) which were significantly different from *F. proliferatum* F2S51 (151.25 ± 23.92 pupae) and the control (103.67 ± 12.55 pupae) (Figure 3.4A). Furthermore, *P. absoluta* moth emergence varied significantly among the fungal isolates (χ^2 = 58.34, df = 9, P < 0.01), where the highest number of moths (148.0 ± 24.57) emerged from *F. proliferatum* F2S51 endophytically-colonised tomato plants, followed by the control (101.67 ± 11.46) while the lowest number (17.0 ± 6.34 moths) was recorded on *T. asperellum* M2RT4 endophytically-colonised tomato plants (Figure 3.4B).

Pupal formation was significantly different among the treatments (χ^2 = 90.95, df = 9, P < 0.0001) where the highest number was obtained in the control (102.33 ± 22.93 pupae) and the lowest (19 ± 4.12 pupae) was recorded in *T. asperellum* M2RT4 endophytically-colonised nightshade plants (Figure 3.4C). Furthermore, the number of adults that emerged from the control (99.33 ± 22.98 moths) was significantly higher than the lowest number (15.5 ± 3.2 moths) that was obtained in *T. asperellum* M2RT4 endophytically-colonised nightshade plants (χ^2 = 44.99, df = 9, P < 0.0001) (Figure 3.4D).

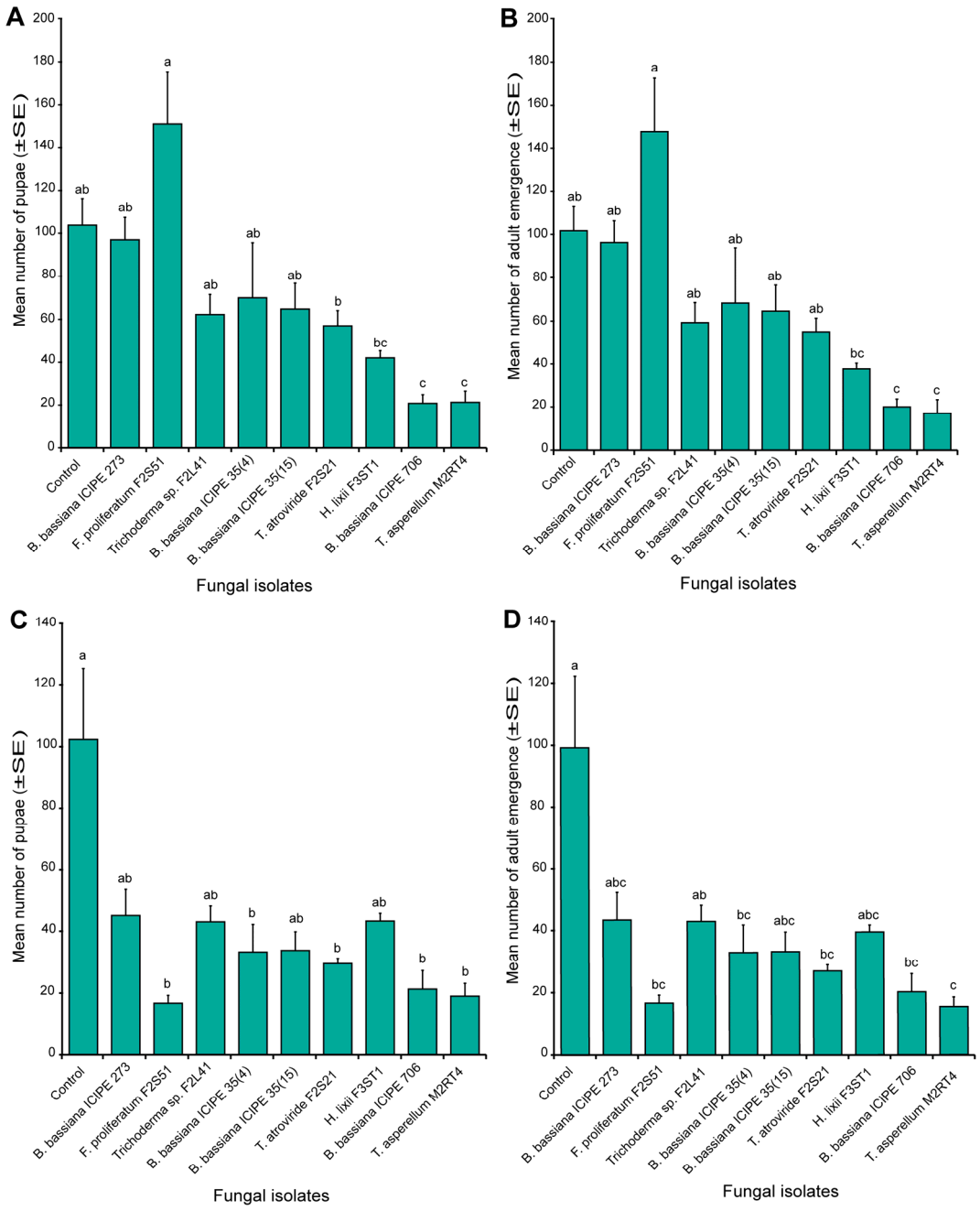

Figure 3.4. Effect of endophytically-colonised host plants by fungal isolates on *Phthorimaea absoluta* pupation and adult emergence. (A) Bar chart showing mean number (± SE) of *Phthorimaea absoluta* pupae produced on endophytically-colonised tomato plants. (B) Bar chart showing mean number of *Phthorimaea absoluta* moths emerging from endophytically-colonised tomato plants. (C) Bar chart showing mean number (± SE) of *Phthorimaea absoluta* pupae produced on endophytically-colonised nightshade plants. (D) Bar chart showing mean number of *Phthorimaea absoluta* moths emerging from endophytically-colonised nightshade plants. Means followed by different lowercase letters are significantly different ($P < 0.05$; n = 4; Tukey's HSD test).

Effect of endophytically-colonised tomato and nightshade host plants on *Phthorimaea absoluta* F1 progenies survival

The median survival time of F1 progenies from the endophytically-colonised tomato plants varied significantly among the treatments (Proximate log rank test, $\chi^2 = 180.7$, df = 9, $P < 0.0001$) (Figure 3.5A). At day five post emergence, mean survival was between 15.6 and 24% in *Trichoderma* sp. F2L41, *B. bassiana* ICIPE 35(4), *B. bassiana* ICIPE 35(15) and *F. proliferatum* F2S51 compared to 58.16% in the control (Fig. 5A). At day 10 post emergence, there was no survival in *Trichoderma* sp. F2L41, *B. bassiana* ICIPE 35(4), *B. bassiana* ICIPE 35(15) while it was between 7 and 28% in other treatments including the control (Figure 3.5A). Similarly, the survival of F1 progeny from endophytically-colonised nightshade plants revealed significant difference between treatments (Proximate log rank test, $\chi^2 = 128.9$, df = 9, $P < 0.0001$) (Figure 3.5B). At day 5 post emergence, mean survival was between 17 and 29% in *Trichoderma* sp. F2L41, *B. bassiana* ICIPE 35(4), *B. bassiana* ICIPE 35(15) and *F. proliferatum* F2S51 compared to 51.23% in the control (Figure 3.5B). At day 10 post emergence, there was no survival in *Trichoderma* sp. F2L41, *B. bassiana* ICIPE 35(4), *B. bassiana* ICIPE 35(15) and *F. proliferatum* F2S51 while it ranged between 11 and 19% in other treatments including the control (Figure 3.5B).

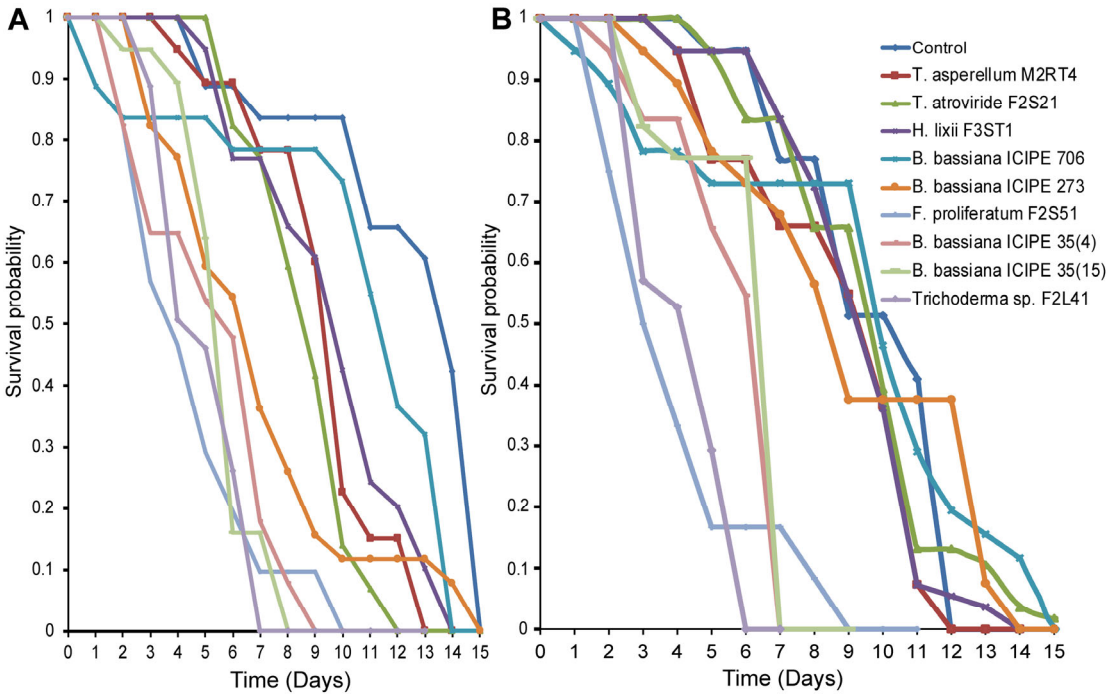

Figure 3.5. Effect of endophytically-colonised host plants by fungal isolates on *Phthorimaea absoluta* F1 progenies survival. (A) Kaplan-Meier survival curves of *Phthorimaea absoluta* F1 progenies survival emerging from endophytically-colonised tomato plants. (B) Kaplan-

Meier survival curves of *Phthorimaea absoluta* F1 progenies survival emerging from endophytically-colonised nightshade plants ($P < 0.05$, n = 4).

3.4.Discussion

This study demonstrated successful endophytic colonisation and establishment of some fungal isolates in tomato and nightshade host plants by negatively affecting *P. absoluta* through significant reduction of the pest oviposition capacity, leafmining, pupal formation, adult emergence, and survival. *Trichoderma asperellum* M2RT4, *B. bassiana* ICIPE 706 and *H. lixii* F3ST1-endophytically-colonised host plants outperformed all the other endophytes in affecting all the life-history parameters of the pest and could therefore contribute to its suppression in tomato and other solanaceous crops.

Among the 15 fungal isolates tested, 12 were endophytic to both host plants with varying colonisation rates while *M. anisopliae* isolates failed to colonise both host plants. Irrespective of the host plant, fungal isolates belonging to the genera *Fusarium* (*F. proliferatum* F2S51), *Trichoderma* (*T. asperellum* M2RT4, *T. atroviride* F2S21 and *Trichoderma* sp. F2L41) and *Hypocrea* (*H. lixii* F3ST1) have demonstrated high colonisation rates of all tomato and nightshade plant tissues. These fungal isolates except for *Trichoderma* sp. F2L41 had a similar *in planta* colonisation pattern in onion through seed inoculation as previously reported by Muvea et al. (2014). Mutune et al. (2016) also reported the potential of *T. asperellum* M2RT4, *T. atroviride* F2S21 and *H. lixii* F3ST1 to endophytically-colonise different parts of the common bean plant *Phaseolus vulgaris* L. (Fabaceae). This implies that the recovery of endophytic fungi from plant tissues (leaves, stems and roots) after seed inoculation is an indication of their ascending movement within the plant (Posada et al., 2007). Previously, such systemic spread of endophytes within the plant has been reported to occur in several crops such as maize (Bing and Lewis, 1991), *Vicia faba* and *P. vulgaris* (Akutse et al., 2013; Behie et al., 2015), tomatoes (Powell et al., 2009), bananas (Akello et al., 2007) and coffee (Posada and Vega, 2005). Some endophytic fungi have also been reported to display a differential ability to colonise and multiply in the root cortex of different plant species while others establish in the whole plant tissues (Demers et al., 2015). Recently, a survey conducted on the prevalence and distribution of fungal root endophytes occurring in tomato crop in Kenya by Bogner et al. (2016) found that the most prevalent endophytic fungi associated with tomato roots were members of *Fusarium* and *Trichoderma* genera. This confirms observations by Hardoim et al. (2015) who reported that

members of these two genera have the potential to colonise a wide range of hosts, suggesting their great metabolic and physiological adaptability.

In contrast to the successfully high colonisation rates of plant tissues by *Fusarium*, *Trichoderma* and *Hypocrea*, the level of colonisation of *B. bassiana* isolates varied according to the various plant tissues with low colonisation rate found in the leaves. A probable explanation of the low recovery of *B. bassiana* from the aerial tissues could be due to the speed of colonisation (inoculum migration) or the presence of physical barriers in the leaf which prevent the fungus from penetrating the epidermis which may contain some substances inimical to the growth of the fungus (Martin, 1964; Posada et al., 2007). However, there are several research evidence that reported the ability of *B. bassiana* to colonise a wide range of plants belonging to the monocot and dicot groups including banana (Akello et al., 2007), *V. faba* (Jensen et al., 2019), opium (Landa et al., 2013), maize (Bing and Lewis, 1992), cassava (Greenfield et al., 2016), tomato (Powell et al., 2009) and coffee (Posada et al., 2007). Nonetheless, this underscores the lack of host specificity expressed by this fungal species in both host seedlings (Card et al., 2016). In this study, the highest recovery of *B. bassiana* was from the roots of the plants which indicates that through seed inoculation this strain has gained access to the cells of the plant. This confirms the observation that many endophytic fungi originate from the rhizosphere microbiota, an environment which attracts microorganisms better due to the presence of root exudates and rhizodeposits (Philippot et al., 2013). On the other hand, Behie and Bidochka (2014) reported that endophytic fungi may display preferential tissue colonisation within their host plants owing to many factors, including plant tissue type, plant genotype, microbial taxon and strain type (Hardoim et al., 2015). Even though *M. anisopliae* isolates ICIPE 7, ICIPE 30 and ICIPE 69 were reported to be pathogenic to several arthropod pests of economic importance (Tumuhaise et al., 2015), their failure in colonising both host plants indicates their inability to establish themselves in living plant tissues of tomato and nightshade. Similar results have been reported on other host plants such as French bean and Faba bean (Akutse et al., 2013; Mutune et al., 2016). Additionally, perhaps not all insect-pathogenic fungi have the ability to establish themselves as endophytes in living plant tissues (Branine et al., 2019). However, numerous studies have documented the ability of *Metarhizium* spp. to colonise plant roots providing multiple benefits to their host plants (Barelli et al., 2018; Greenfield et al., 2016; Wyrebek et al., 2011).

In general, our results reveal that exposure of both endophytically-colonised host plants to ovipositing *P. absoluta* female moths has resulted in a significant reduction in the number of eggs laid on the inoculated plant compared to the control. Among the most potent endophytic fungal isolates, we found that *T. asperellum* M2RT4, *B. bassiana* ICIPE 706 and *H. lixii* F3ST1 significantly reduced oviposition of the pest. For instance, Muvea et al. (2015) demonstrated a sixfold reduction in oviposition of onion thrips on plants endophytically-colonised by *H. lixii* F3ST1 compared to endophyte-free plants. Also, Akutse et al. (2013) reported that Faba bean endophytically-colonised by *H. lixii* had significant effect on the egg-laying capacity of the pea leafminer, *L. huidobrensis*. It is worth noting that the female's choice to reduce egg production could be due to the absence of favorable conditions that would compromise the survival of the progeny (Slansky Jr., 1982). Furthermore, *T. asperellum* M2RT4 negatively affected leafmining activity as well as pupation and adult emergence. When the hatching larvae feed on inoculated tissue, it generally results in a decreased fitness of the herbivore (Carroll, 1988). This corroborates with Akutse et al. (2013) who reported that endophytic fungi provide systemic protection against the pea leafminer, *L. huidobrensis* and have deterrent effects on life-history parameters of the pest. In addition, several studies have also reported insecticidal activities of endophytic fungi against insects feeding on endophytically-colonised plants through antibiosis or feeding deterrence, suggesting that immature larvae were probably affected through the secretion of toxic compounds *in planta* (Allegrucci et al., 2017; Barta, 2018; Greenfield et al., 2016; Klieber and Reineke, 2016; Russo et al., 2018). The inhibition of the larval performance due to the presence of *Trichoderma* spp. within the host plants has previously been reported (Contreras-cornejo et al., 2017). The systemic activity of this fungal isolate as one of the most potent endophytic fungal strain controlling *P. absoluta* was not surprising, since similar effects have been reported in previous studies by Akello and Sikora (2012) and Muvea et al. (2014) on aphids and thrips population, respectively. The latter indicated that onion thrips feeding on onion plants inoculated by *Trichoderma* spp. performed worse and few immature stages reached the adult stage compared to the control. This suggests that *T. asperellum* M2RT4 possesses specific properties that trigger plant resistance which results in significant reduction of insect herbivory (Contreras-Cornejo et al., 2016). Similarly, Coppola *et al.* (Coppola et al., 2017) reported an enhancement of the indirect defense barriers against the aphid *Macrosiphum euphorbiae* (Hemiptera: Aphididae) feeding on tomato plants colonised by *T. harzianum* T22.

On the other hand, we found that *B. bassiana* fungal isolates (ICIPE 706 and ICIPE 273) reduced leafmining activity as well as pupation although showing low level *in planta* colonisation pattern. However, *B. bassiana* isolate ICIPE 706 had the highest negative impact on the pest oviposition, pupation and adult emergence in both host plants, while it reduced significantly the mines formation only in tomato. Since *P. absoluta* larvae continue to feed on inoculated plants after egg hatching due to their cryptic nature, the amount and quality of host diet could significantly affect the feeding behaviour of the leafmining larvae. It is therefore possible that this low colonisation level was sufficient for the plants to initiate a defense reaction (Meera et al., 1995). Klieber and Reineke (2016) reported that *P. absoluta* larvae experienced detrimental effects when feeding on tomato leaves infected with *B. bassiana*. Lewis et al. (1996) also demonstrated that when *B. bassiana* remains in the maize plant as endophyte, it provides a season-long management of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) through the reduction of the larval activity of the pest. Qayyum et al. (2015) reported that endophytic colonisation of *B. bassiana* has potential as an effective strategy to control *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in tomatoes.

Not all the fungal isolates tested in this study were able to deter the oviposition behaviour against *P. absoluta*. Of the tested isolates, three (*B. bassiana* ICIPE 273, *F. proliferatum* F2S51 and *Trichoderma* sp. F2L41) recorded high number of eggs compared to other treatments and the control (endophyte-free tomato and nightshade plants). Jensen et al. (2019) also found an increased fecundity of the second generation of *Aphis fabae* on *V. faba* plants following seed and leaf inoculation with *B. bassiana*. The authors further speculated that *B. bassiana* is responsible for the improvement of the quality of the host plant which have led the insects to increase the number of eggs laid on the inoculated plants. Similarly, Jallow et al. (2008) examined in tomato the systemic effects of the endophytic fungus *Acremonium strictum* on the oviposition behaviour of the polyphagous moth *Helicoverpa armigera* (Hübner). The authors reported that strains of *H. armigera* moths oviposited more eggs on leaves of *A. strictum*-inoculated plants as compared to endophyte-free plants. Later, Jaber and Vidal (2010) suggested that the increased oviposition preference of *H. armigera* moths to inoculated plants might be an evolutionary adaptation to the host plant. Although we have not investigated the mechanism by which these three isolates (*B. bassiana* ICIPE 273, *F. proliferatum* F2S51 and *Trichoderma* sp. F2L41) increased the attractiveness to the two host plants for egg-laying in *P. absoluta*, our results suggest that secondary metabolites

or microbial volatile organic compounds produced by these endophytes or the interaction of the plants with the fungi may play a role in influencing the host selection of *P. absoluta* for oviposition (Davis et al., 2013). The difference in the number of eggs laid on the several inoculated plants is suggestive of chemical and/or molecular mechanism(s) mediating interaction between the endophytes, insect and its host plants, calling for further studies.

The results reported here showed that females exposed to both tomato and nightshade plants lived less than 20 days. This finding is in agreement with Silva et al. (2015) who reported that females *P. absoluta* had a lifespan less than 20 days. However, our result is in contrast with those of Pereyra and Sánchez (2006) who reported that the survival of *P. absoluta* individuals could be extended until day 45 and remained high most of the lifetime but start decreasing to 50% at day 25. These variations might be due to the experimental conditions or the food source provided to the emerged adults during the survival bioassays. Further, we found a rapid decline in the survival rates of *P. absoluta* F1 progenies that emerged from larvae that fed on *Trichoderma* sp. F2L41, *B. bassiana* ICIPE 35(4), *B. bassiana* ICIPE 35(15) endophytically-colonised host plants. Our results concur with findings by Dash et al. (2018) who also found a reduction in the survival of adult spider mites whose larvae fed on endophytically-colonised bean plants. Akello et al. (2008) reported an antagonistic activity mediated by the endophytic fungus *B. bassiana* towards the banana weevil adult, *Cosmopolites sordidus* (Coleoptera: Curculionidae). However, we did not record any sign of fungal infection on the dead insects which suggests that a probable mechanism of systemic resistance or feeding deterrence would be the factor responsible for the adverse effect of the inoculated plants on adult survivorship. Such deterrence exhibited by inoculated plants is related to the production of secondary metabolites by some fungi which may be an interesting exploitable feature for their sustainable use against agricultural insect pests of economic importance (Golo et al., 2014).

3.5.Conclusion

In this study, we have identified *T. asperellum* M2RT4, *B. bassiana* ICIPE 706 and *H. lixii* F3ST1 as the most potent endophytic fungal isolates mediating improvement of tomato and nightshade anti-herbivore defense against *P. absoluta* through the reduction of adult oviposition, leafmining, pupation and adult emergence as compared to other treatments. *Trichoderma asperellum* M2RT4, *B. bassiana* ICIPE 706 and *H. lixii* F3ST1 could therefore be considered the best candidates for development of endophytic-based biopesticide and

could be integrated as a component in a sustainable integrated *P. absoluta* management strategy for tomato and nightshade production systems. However, further studies are required to clearly understand the underlying mechanisms by which the presence of endophytic fungi within tomato and nightshade host plants affect *P. absoluta* as well as validate the findings under field conditions.

References

Aigbedion-Atalor, P. O., Hill, M. P., Zalucki, M. P., Obala, F., Idriss, G. E., Midingoyi, S.-K., Sunday, E., and Mohamed, S. (2019). The South America tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae), spreads its wings in Eastern Africa: distribution and socioeconomic impacts. *J. Econ. Entomol.* 112, 2797–2807. doi:10.1093/jee/toz220.

Aigbedion-Atalor, P. O., Mohamed, S. A., Hill, M. P., Zalucki, M. P., Azrag, A. G. A., Srinivasan, R., and Sunday E. (2020). Host stage preference and performance of *Dolichogenidea gelechiidivoris* (Hymenoptera: Braconidae), a candidate for classical biological control of *Tuta absoluta* in Africa. *Biol. Control* 144, 1–8. doi:10.1016/j.biocontrol.2020.104215.

Akello, J., Dubois, T., Coyne, D., and Kyamanywa, S. (2008). Endophytic *Beauveria bassiana* in banana (*Musa* spp.) reduces banana weevil (*Cosmopolites sordidus*) fitness and damage. *Crop Prot.* 27, 1437–1441. doi:10.1016/j.cropro.2008.07.003.

Akello, J., Dubois, T., Gold, C. S., Coyne, D., Nakavuma, J., and Paparu, P. (2007). *Beauveria bassiana* (Balsamo) Vuillemin as an endophyte in tissue culture banana (*Musa* spp.). *J. Invertebr. Pathol.* 96, 34–42. doi:10.1016/j.jip.2007.02.004.

Akello, J., and Sikora, R. (2012). Systemic acropedal influence of endophyte seed treatment on *Acyrthosiphon pisum* and *Aphis fabae* offspring development and reproductive fitness. *Biol. Control* 61, 215–221. doi:10.1016/j.biocontrol.2012.02.007.

Akutse, K. S., Khamis, F. M., Ekesi, S., Wekesa, S., and Subramanian, S. (2019a). Effect of endophytically-colonized tomato and nightshade host plants on life-history parameters of *Tuta absoluta* (Lepidoptera: Gelechiidae). (Valencia: International Congress on Invertebrate Pathology and Microbial Control & 52nd Annual Meeting of the Society for Invertebrate Pathology & 17th Meeting of the IOBC-WPRS Working Group "Microbial and Nematode Control of Invertebrate Pests"), 73.

Akutse, K. S., Kimemia, J. W., Ekesi, S., Khamis, F. M., Ombura, O. L., and Subramanian,

S. (2019b). Ovicidal effects of entomopathogenic fungal isolates on the invasive Fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *J. Appl. Entomol.* 143, 626–634. doi:10.1111/jen.12634.

Akutse, K. S., Maniania, N. K., Fiaboe, K. K. M., Van den Berg, J., and Ekesi, S. (2013). Endophytic colonization of *Vicia faba* and *Phaseolus vulgaris* (Fabaceae) by fungal pathogens and their effects on the life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Fungal Ecol.* 6, 293–301. doi:10.1016/j.funeco.2013.01.003.

Akutse, K. S., Subramanian, S., Khamis, F. M., Ekesi, S., and Mohamed, S. A. (2020). Entomopathogenic fungus isolates for adult *Tuta absoluta* (Lepidoptera: Gelechiidae) management and their compatibility with *Tuta* pheromone. *J. Appl. Entomol.*, 1–11. doi:10.1111/jen.12812.

Allegrucci, N., Velazquez, M. S., Russo, M. L., Perez, E., and Scorsetti, A. C. (2017). Endophytic colonisation of tomato by the entomopathogenic fungus *Beauveria bassiana*: the use of different inoculation techniques and their effects on the tomato leafminer *Tuta absoluta* (Lepidoptera : Gelechiidae). *J. Plant Prot. Res.* 54, 331–337. doi:10.1515/jppr-2017-0045.

Barelli, L., Moonjely, S., Behie, S. W., and Bidochka, M. J. (2016). Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi. *Plant Mol. Biol.* 90, 657–664. doi:10.1007/s11103-015-0413-z.

Barelli, L., Moreira, C. C., and Bidochka, M. J. (2018). Initial stages of endophytic colonization by *Metarhizium* involves rhizoplane colonization. *Microbiology* 164, 1531–1540. doi:10.1099/mic.0.000729.

Barta, M. (2018). In planta bioassay on the effects of endophytic *Beauveria* strains against larvae of horse-chestnut leaf miner (*Cameraria ohridella*). *Biol. Control* 121, 88–98. doi:10.1016/j.biocontrol.2018.02.013.

Behie, S. W., and Bidochka, M. J. (2014). Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi : an additional branch of the soil nitrogen cycle. *Appl. Environ. Microbiol.* 80, 1553–1560. doi:10.1128/AEM.03338-13.

Behie, S. W., Jones, S. J., Bidochka, M. J., and Hyde, K. (2015). Plant tissue localization of the endophytic insect pathogenic fungi *Metarhizium* and *Beauveria*. *Fungal Ecol.* 13, 112–119. doi:10.1016/j.funeco.2014.08.001.

Bing, L. A., and Lewis, L. C. (1991). Suppression of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin.

Environ. Entomol. 20, 1207–1211. doi:10.1093/ee/20.4.1207.

Bing, L. A., and Lewis, L. C. (1992). Endophytic *Beauveria bassiana* (Balsamo) Vuillemin in corn: The influence of the plant growth stage and *Ostrinia nubilalis* (Hubner). *Biocontrol Sci. Technol.* 2, 39–47. doi:10.1080/09583159209355216.

Biondi, A., Guedes, R. N. C., Wan, F.-H., and Desneux, N. (2018). Ecology, worldwide spread, and management of the invasive South American tomato pinworm, *Tuta absoluta*: past, present, and future. *Annu. Rev. Entomol.* 63, 239–258. doi:10.1146/annurev-ento-031616-034933.

Bogner, C. W., Kariuki, G. M., Elashry, A., Sichtermann, G., Buch, A. K., Mishra, B., and Schouten, A. (2016). Fungal root endophytes of tomato from Kenya and their nematode biocontrol potential. *Mycol. Prog.* 15, 1–17. doi:10.1007/s11557-016-1169-9.

Branine, M., Bazzicalupo, A., and Branco, S. (2019). Biology and applications of endophytic insect-pathogenic fungi. *PLOS Pathog.* 15, 1–7. doi:10.1371/journal.ppat.1007831.

Card, S., Johnson, L., Teasdale, S., and Caradus, J. (2016). Deciphering endophyte behaviour : the link between endophyte biology and efficacious biological control agents. *FEMS Microbiol. Ecol.* 92, 1–19. doi:10.1093/femsec/fiw114.

Carroll, G. (1988). Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbiont. *Ecology* 69, 2–9. doi:10.2307/1943154.

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Del-Val, E., and Larsen, J. (2016). Ecological functions of *Trichoderma* spp. and their secondary metabolites in the rhizosphere: interactions with plants. *FEMS Microbiol. Ecol.* 92, 1–17. doi:10.1093/femsec/fiw036.

Contreras-cornejo, H. A., Macías-rodríguez, L., and Larsen, J. (2017). The root endophytic fungus *Trichoderma atroviride* induces foliar herbivory resistance in maize plants. *Appl. Soil Ecol.* 124, 45–53. doi:10.1016/j.apsoil.2017.10.004.

Coppola, M., Cascone, P., Chiusano, M. L., Colantuono, C., Lorito, M., Pennacchio, F., Gurreirio, E., and Digilio, M. C. (2017). *Trichoderma harzianum* enhances tomato indirect defense against aphids. *Insect Sci.* 24, 1025–1033. doi:10.1111/1744-7917.12475.

Crawley, M. J. (2007). *The R Book*. John Wiley & Sons, Ltd. doi:10.1002/9780470515075.

Dash, C. K., Bamisile, B. S., Keppanan, R., Qasim, M., Lin, Y., Islam, S. U., and Wang, L. (2018). Endophytic entomopathogenic fungi enhance the growth of *Phaseolus vulgaris* L. (Fabaceae) and negatively affect the development and reproduction of *Tetranychus*

urticae Koch (Acari: Tetranychidae). *Microb. Pathog.* 125, 385–392. doi:10.1016/j.micpath.2018.09.044.

Davis, T. S., Crippen, T. L., Hofstetter, R. W., and Tomberlin, J. K. (2013). Microbial volatile emissions as insect semiochemicals. *J. Chem. Ecol.* 39, 840–859. doi:10.1007/s10886-013-0306-z.

De Mendiburu, F. (2020). *agricolae: statistical procedures for agricultural research. R package version 1.3–2 https://CRAN.R-project.org/ package=agricolae*.

Demers, J. E., Gugino, B. K., and Jiménez-Gasco, M. del M. (2015). Highly diverse endophytic and soil *Fusarium oxysporum* populations associated with field-grown tomato plants. *Appl. Environ. Microbiol.* 81, 81–90. doi:10.1128/AEM.02590-14.

Desneux, N., Luna, M. G., Guillemaud, T., and Urbaneja, A. (2011). The invasive South American tomato pinworm, *Tuta absoluta*, continues to spread in Afro-Eurasia and beyond: The new threat to tomato world production. *J. Pest Sci.* 84, 403–408. doi:10.1007/s10340-011-0398-6.

Desneux, N., Wajnberg, E., Wyckhuys, K. A. G., Burgio, G., Arpaia, S., Narváez-Vasquez, C. A., Cabello, T., and Urbaneja, A. (2010). Biological invasion of European tomato crops by *Tuta absolut*a: ecology, geographic expansion and prospects for biological control. *J. Pest Sci.* 83, 197–215. doi:10.1007/s10340-010-0321-6.

Dimbi, S., Maniania, N. K., and Ekesi, S. (2013). Horizontal transmission of *Metarhizium anisopliae* in fruit flies and effect of fungal infection on egg laying and fertility. *Insects* 4, 206–216. doi:10.3390/insects4020206.

Ekesi, S., Chabi-Olaye, A., Subramanian, S., and Borgemeister, C. (2011). Horticultural pest management and the African economy: Successes, challenges and opportunities in a changing global environment. *Acta Hortic.* 911, 165–183. doi:10.17660/ActaHortic.2011.911.17.

Fadiji, A. E., and Babalola, O. O. (2020). Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects. *Front. Bioeng. Biotechnol.* 8, 00467. doi:10.3389/fbioe.2020.00467.

Gathage, J. W., Lagat, Z. O., Fiaboe, K. K. M., Akutse, K. S., Ekesi, S., and Maniania, N. K. (2016). Prospects of fungal endophytes in the control of *Liriomyza leafminer* flies in common bean Phaseolus vulgaris under field conditions. *BioControl* 61, 741–753. doi:10.1007/s10526-016-9761-0.

Goettel, M. S., and Inglis, D. G. (1997). *Fungi: Hyphomycetes*. doi:10.1016/B978-

012432555-5/50013-0.

Golo, P. S., Gardner, D. R., Grilley, M. M., Takemoto, J. Y., Krasnoff, S. B., Pires, M. S., et al. (2014). Production of destruxins from *Metarhizium* spp. fungi in artificial medium and in endophytically colonized cowpea plants. *PLoS One* 9, 1–9. doi:10.1371/journal.pone.0104946.

Greenfield, M., Gómez-Jiménez, M. I., Ortiz, V., Vega, F. E., Kramer, M., and Parsa, S. (2016). *Beauveria bassiana* and *Metarhizium anisopliae* endophytically colonize cassava roots following soil drench inoculation. *Biol. Control* 95, 40–48. doi:10.1016/j.biocontrol.2016.01.002.

Guedes, R. N. C. C., Roditakis, E., Campos, M. R., Haddi, K., Bielza, P., Siqueira, H. A. A. A., and Nauen, R. (2019). Insecticide resistance in the tomato pinworm *Tuta absoluta*: patterns, spread, mechanisms, management and outlook. *J. Pest Sci.* 92, 1329–1342. doi:10.1007/s10340-019-01086-9.

Hardoim, P. R., Overbeek, L. S. Van, Berg, G., Pirttilä, M., Compant, S., Campisano, A., and Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.* 79, 293–320. doi:10.1128/mmbr.00050-14.

Idriss, G. E. A., du Plessis, H., Khamis, F. M., Ekesi, S., Tanga, C. M., and Mohamed, S. A. (2020). Host range and effects of plant species on preference and fitness of *Tuta absoluta* (Lepidoptera: Gelechiidae). *J. Econ. Entomol.* 113, 1279–1289. doi:10.1093/jee/toaa002.

Inglis, G. D., Enkerli, J., and Goettel, M. S. (2012). *Laboratory techniques used for entomopathogenic fungi. Hypocreales.* Second Edi. Elsevier Ltd doi:10.1016/B978-0-12-386899-2.00007-5.

Inglis, G. D., Goettel, M. S., Butt, T. M., and Strasser, H. (2001). "Use of hyphomycetous fungi for managing insect pests.," in *Fungi as biocontrol agents: progress, problems and potential.*, eds. T. M. Butt and M. Magan, 23–69. doi:10.1079/9780851993560.0023.

Jaber, L. R., and Vidal, S. (2010). Fungal endophyte negative effects on herbivory are enhanced on intact plants and maintained in a subsequent generation. *Ecol. Entomol.* 35, 25–36. doi:10.1111/j.1365-2311.2009.01152.x.

Jallow, M. F. A., Dugassa-Gobena, D., and Vidal, S. (2008). Influence of an endophytic fungus on host plant selection by a polyphagous moth via volatile spectrum changes.

Arthropod. Plant. Interact. 2, 53–62. doi:10.1007/s11829-008-9033-8.

Jensen, R. E., Enkegaard, A., and Steenberg, T. (2019). Increased fecundity of *Aphis fabae* on *Vicia faba* plants following seed or leaf inoculation with the entomopathogenic fungus *Beauveria bassiana*. *PLoS One* 14, 1–12. doi:10.1371/journal.pone.0223616.

Klieber, J., and Reineke, A. (2016). The entomopathogen *Beauveria bassiana* has epiphytic and endophytic activity against the tomato leaf miner *Tuta absoluta*. *J. Appl. Entomol.* 140, 580–589. doi:10.1111/jen.12287.

Lahrmann, U., Ding, Y., Banhara, A., Rath, M., Hajirezaei, M. R., Döhlemann, S., and Zuccaro, A. (2013). Host-related metabolic cues affect colonization strategies of a root endophyte. *Proc. Natl. Acad. Sci. U. S. A.* 110, 13965–13970. doi:10.1073/pnas.1301653110.

Landa, B. B., López-Díaz, C., Jiménez-Fernández, D., Montes-Borrego, M., Muñoz-Ledesma, F. J., Ortiz-Urquiza, A., and Queseda-Moraga, E. (2013). In-planta detection and monitorization of endophytic colonization by a *Beauveria bassiana* strain using a new-developed nested and quantitative PCR-based assay and confocal laser scanning microscopy. *J. Invertebr. Pathol.* 114, 128–138. doi:10.1016/j.jip.2013.06.007.

Latz, M. A. C., Jensen, B., Collinge, D. B., and Jørgensen, H. J. L. (2018). Endophytic fungi as biocontrol agents: elucidating mechanisms in disease suppression. *Plant Ecol. Divers.* 11, 555–567. doi:10.1080/17550874.2018.1534146.

Lewis, L. C., Berry, E. C., Obrycki, J. J., and Bing, L. A. (1996). Aptness of insecticides (*Bacillus thuringiensis* and carbofuran) with endophytic *Beauveria bassiana*, in suppressing larval populations of the European corn borer. *Agric. Ecosyst. Environ.* 57, 27–34.

Maniania, N. K., Ekesi, S., and Dolinski, C. (2016). *Entomopathogens Routinely Used in Pest Control Strategies: Orchards in Tropical Climate*. Elsevier Inc. doi:10.1016/B978-0-12-803527-6.00018-4.

Martin, J. T. (1964). Role of cuticle in the defense against plant disease. *Annu. Rev. Phytopathol.* 2, 81–100.

Meera, M. S., Shivanna, M. B., Kageyama, K., and Hyakumachi, M. (1995). Persistence of induced systemic resistance in cucumber in relation to root colonization by plant growth promoting fungal isolates. *Crop Prot.* 14, 123–130. doi:10.1016/0261-2194(95)92866-L.

Mutune, B., Ekesi, S., Niassy, S., Matiru, V., Bii, C., and Maniania, N. K. (2016). Fungal

endophytes as promising tools for the management of bean stem maggot *Ophiomyia phaseoli* on beans Phaseolus vulgaris. *J. Pest Sci.* 89, 993–1001. doi:10.1007/s10340-015-0725-4.

Muvea, A. M., Meyhöfer, R., Maniania, N. K., Poehling, H. M., Ekesi, S., and Subramanian, S. (2015). Behavioral responses of *Thrips tabaci* Lindeman to endophyte-inoculated onion plants. *J. Pest Sci.* 88, 555–562. doi:10.1007/s10340-015-0645-3.

Muvea, A. M., Meyhöfer, R., Subramanian, S., Poehling, H. M., Ekesi, S., and Maniania, N. K. (2014). Colonization of onions by endophytic fungi and their impacts on the biology of *Thrips tabaci*. *PLoS One* 9, 1–7. doi:10.1371/journal.pone.0108242.

Mweke, A., Ulrichs, C., Nana, P., Akutse, K. S., Fiaboe, K. K. M., Maniania, N. K., and Sunday, E. (2018). Evaluation of the entomopathogenic fungi *Metarhizium anisopliae*, *Beauveria bassiana* and *Isaria* sp. for the management of *Aphis craccivora* (Hemiptera: Aphididdae). *J. Econ. Entomol.* 111, 1587–1594. doi:10.1093/jee/toy135.

Oliveira, F. A., da Silva, D. J. H., Leite, G. L. D., Jham, G. N., and Picanço, M. (2009). Resistance of 57 greenhouse-grown accessions of *Lycopersicon esculentum* and three cultivars to *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Sci. Hortic.* 119, 182–187. doi:10.1016/j.scienta.2008.07.012.

Ownley, B. H., Griffin, M. R., Klingeman, W. E., Gwinn, K. D., Moulton, J. K., and Pereira, R. M. (2008). *Beauveria bassiana*: Endophytic colonization and plant disease control. *J. Invertebr. Pathol.* 98, 267–270. doi:10.1016/j.jip.2008.01.010.

Pereyra, P. C., and Sánchez, N. E. (2006). Effect of two solanaceous plants on developmental and population parameters of the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Neotrop. Entomol.* 35, 671–676. doi:10.1590/s1519-566x2006000500016.

Petrini, O., and Fisher, P. J. (1986). Fungal endophytes in *Salicornia perennis*. *Trans. - Br. Mycol. Soc.* 87, 647–651. doi:10.1016/S0007-1536(86)80109-7.

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and Putten, W. H. Van Der (2013). Going back to the roots : the microbial ecology of the rhizosphere. *Nat. Publ. Gr.* 11, 789–799. doi:10.1038/nrmicro3109.

Posada, F., Aime, M. C., Peterson, S. W., Rehner, S. A., and Vega, F. E. (2007). Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). *Mycol. Res.* 111, 748–757. doi:10.1016/j.mycres.2007.03.006.

Posada, F. J., and Vega, F. E. (2005). A new method to evaluate the biocontrol potential of

single spore isolates of fungal entomopathogens. *J. Insect Sci.* 5, 1–10. doi:10.1093/jis/5.1.37.

Powell, W. A., Klingeman, W. E., Ownley, B. H., and Gwinn, K. D. (2009). Evidence of endophytic *Beauveria bassiana* in seed-treated tomato plants acting as a systemic entomopathogen to larval *Helicoverpa zea* (Lepidoptera: Noctuidae). *J. Entomol. Sci.* 44, 391–396. doi:10.18474/0749-8004-44.4.391.

Pratt, C. F., Constantine, K. L., and Murphy, S. T. (2017). Economic impacts of invasive alien species on African smallholder livelihoods. *Glob. Food Sec.* 14, 31–37. doi:10.1016/j.gfs.2017.01.011.

Qayyum, M. A., Wakil, W., Arif, M. J., Sahi, S. T., and Dunlap, C. A. (2015). Infection of *Helicoverpa armigera* by endophytic *Beauveria bassiana* colonizing tomato plants. *Biol. Control* 90, 200–207. doi:10.1016/j.biocontrol.2015.04.005.

Quesada-Moraga, E., Muñoz-Ledesma, F. J., and Santiago-Álvarez, C. (2009). Systemic protection of *Papaver somniferum* L. against *Iraella luteipes* (Hymenoptera: Cynipidae) by an endophytic strain of *Beauveria bassiana* (Ascomycota: Hypocreales). *Environ. Entomol.* 38, 723–730. doi:10.1603/022.038.0324.

R Core Team (2019). R: A language and environment for statistical computing R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org/.

Resquín-romero, G., Garrido-jurado, I., Delso, C., Ríos-moreno, A., and Quesada-moraga, E. (2016). Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. *J. Invertebr. Pathol.* 136, 23–31. doi:10.1016/j.jip.2016.03.003.

Russo, M. L., Pelizza, S. A., Vianna, M. F., Allegrucci, N., Cabello, M. N., Toledo, A. V., and Scorsetti, A. C. (2018). Effect of endophytic entomopathogenic fungi on soybean *Glycine max* (L.) Merr. growth and yield. *J. King Saud Univ. - Sci.* 31, 728–736. doi:10.1016/j.jksus.2018.04.008.

Russo, M. L., Scorsetti, A. C., Vianna, M. F., Cabello, M., Ferreri, N., and Pelizza, S. (2019). Endophytic effects of *Beauveria bassiana* on Corn (*Zea mays*) and its herbivore, *Rachiplusia nu* (Lepidoptera: Noctuidae). *Insects* 10, 2–9. doi:10.3390/insects10040110.

Schulz, B., Guske, S., Dammann, U., and Boyle, C. (1998). Endophyte-host interactions. II. Defining symbiosis of the endophyte-host interaction. *Symbiosis* 25, 213–227.

Shapiro, S. S., and Wilk, M. B. (1965). An analysis of variance test for normality (complete

samples). *Biometrika* 52, 591–611.

Silva, D. B., Bueno, V. H. P., Lins, J. C., and Van Lenteren, J. C. (2015). Life history data and population growth of *Tuta absoluta* at constant and alternating temperatures on two tomato lines. *Bull. Insectology* 68, 223–232.

Slansky Jr., F. (1982). Insect nutrition : An adaptationist's perspective. *Florida Entomol.* 65, 45–71.

Therneau, T. (2020). A Package for Survival Analysis in R. R package version 3.1-12, https://CRAN.R-project.org/package=survival.

Tumuhaise, V., Ekesi, S., Mohamed, S. A., Ndegwa, P. N., Irungu, L. W., Srinivasan, R., and Maniania, N. K. (2015). Pathogenicity and performance of two candidate isolates of *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales: Clavicipitaceae) in four liquid culture media for the management of the legume pod borer *Maruca vitrata* (Lepidoptera: Crambidae). *Int. J. Trop. Insect Sci.* 35, 34–47. doi:10.1017/S1742758414000605.

Wilson, D. (1995). Endophyte : The evolution of a term, and clarification of its use and definition. *Oikos* 73, 274–276.

Wyrebek, M., Huber, C., Sasan, R. K., and Bidochka, M. J. (2011). Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. *Microbiology* 157, 2904–2911. doi:10.1099/mic.0.051102-0.

CHAPTER FOUR

The endophyte *Trichoderma asperellum* M2RT4 induces the systemic release of methyl salicylate and (*Z*)-jasmone in tomato plant affecting host location and herbivory of *Phthorimaea absoluta*

This chapter has been published as:

Agbessenou, A., Akutse, K. S., Yusuf, A. & Khamis, F. M. (2022). The endophyte *Trichoderma asperellum* M2RT4 induces the systemic release of methyl salicylate and (*Z*)-jasmone in tomato plant affecting host location and herbivory of *Tuta absoluta*. *Frontiers in Plant Science*, 13: 860309. https://doi.org10.3389/fpls.2022.860309

Abstract

The use of endophytic fungi has dramatically increased plant performance through the enhancement of plant protection against abiotic and biotic stressors. We previously demonstrated that the endophytic fungus *Trichoderma asperellum* M2RT4 improves tomato defenses against the South American tomato pinworm *Phthorimaea absoluta* through the reduction of oviposition, leafmining, pupation and adult emergence. However, the underlying mechanism by which the presence of this endophytic fungus within tomato host plant affects *P. absoluta* host selection and life-history traits is unknown. We tested the behavioural responses of *P. absoluta* in Y-tube olfactometer bioassays and found that female moths preferred non-inoculated tomato plants against those inoculated by endophytes. Additionally, female moths were not attracted to non-inoculated infested nor to inoculated-infested tomato plants. Chemical analysis revealed the emission of methyl salicylate in inoculated tomato plant and an increase in the amounts of monoterpenes emitted from non-inoculated infested plants. Additionally, we found that upon herbivory, *T. asperellum* M2RT4 modulates tomato plant chemistry through the production of (*Z*)-jasmone thus activating both salicylic and jasmonic acid defense pathways. Further, *P. absoluta* female moths were attracted to monoterpernes including α-pinene, 2-carene and β-phellandrene but repelled by methyl salicylate. Methyl salicylate could therefore be considered as a good semiochemical-based candidate for sustainable *P. absoluta* management using a "push-pull" approach. However, in dose-response bioassays, of *P. absoluta* female moths did not show any preference to the four component-blend (α-pinene, 2-carene, β-phellandrene, and methyl salicylate). (*Z*)-jasmone-treated tomato leaflets significantly reduced the leafmining activity of the pest at the concentration of 10 ng/µL and causing the highest larval mortality rate (83%) with the shortest LT_{50} (1.73 days) seven days post-treatment. *Trichoderma asperellum* M2RT4 effect on herbivore performance was then (*Z*)-jasmone-mediated. These findings expand our understanding of how the endophytic fungus *T. asperellum* M2RT4 could mediate chemical interactions between *P. absoluta* and its host plant which are potentially important for development of environmentally friendly *P. absoluta* management programs.

4.1.Introduction

Plants represent a rich source of nutrients for insects and are known to emit a complex of chemical cues which are used by herbivorous insects to locate their host and find suitable oviposition sites (Bruce et al., 2005). Additionally, plants are closely associated with a diversity of beneficial microorganisms living within their tissues, some of which offer protection against herbivorous insects (Barelli et al., 2016). Upon herbivory, plants respond by releasing defense compounds termed herbivore-induced plant volatiles (HIPVs) which have been reported to often vary quantitatively and qualitatively (Rostás et al., 2006; Shrivastava et al., 2015). Herbivore-induced plant volatiles are explored for use in pest management programs, particularly for improving biological control (Ayelo et al., 2021; Lin et al., 2017).

The South American tomato pinworm *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae) which is native to South America, has become an invasive and serious economic pest of tomato and several crops belonging to the Solanaceae family in Africa (Desneux et al., 2010; Idriss et al., 2020). Female moths primarily rely on host plant volatiles to locate and select suitable oviposition sites (Proffit et al., 2011) where eggs are laid on the upper surface of the leaves. Upon hatching, the larvae mine the leaves voraciously and fruits of the crop, interfering with photosynthetic activity, nutrient transport and eventually creates avenues for entry of opportunistic microorganisms (Desneux et al., 2010). It is reported that the tomato leafminer causes up to 100% crop losses estimated at USD 1.1 billion worldwide annually (Pratt et al., 2017). Synthetic chemical insecticides remain the primary control strategy used against *P. absoluta* in Africa, but the growing threat of insecticide resistance coupled with public concerns over non-target effects on beneficial organisms associated with their indiscriminate use (Guedes et al., 2019) prompted the urgent need to develop alternative, eco-friendly and sustainable control tools (Agbessenou et al., 2020). Antagonists such as endophytic fungi are considered a safer alternative to synthetic insecticides due to their reduced toxicity and lower chances of resistance (La Spada et al., 2020). Endophytic fungi are ubiquitous microorganisms that live within their host plants (Poveda, 2021a; Vega, 2008) and have been reported to provide *ab initio* protection which represents a state of heightened defense throughout the plant (Wei et al., 2020; Wu et al., 2017). It has been shown that the presence of endophytes in host plants often elicit induced systemic resistance probably as a result of the modification of the plant's physiology and biochemistry which

subsequently enhances defense against herbivorous insects (Pineda et al., 2013; Rostás et al., 2006).

Trichoderma asperellum is an opportunistic, asymptomatic microbial endophytic fungus that lives within a wide range of host plants (Poveda, 2021a; Siddaiah et al., 2017), which can successfully colonise tomato and nightshade plant tissues (Agbessenou et al., 2020). *Trichoderma* spp. have been reported to increase host plants resistance against below- and above-ground biotic stressors such as the root-knot nematode, *Meloidogyne incognita* and the southern green stink bug, *Nezara viridula* (Hemiptera: Pentatomidae) (Affokpon et al., 2011; Alınç et al., 2021; Pineda et al., 2010; Poveda, 2021b). In addition, *Trichoderma* species produce secondary metabolites with biological activity against herbivores (Poveda, 2021b; Vinale et al., 2012) and could influence plant defense chemistry, including the constitutive and induced expression of phytohormones such as jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) which are known to regulate plant defense response to herbivory (Davis et al., 2013; Jallow et al., 2008; Kottb et al., 2015; Poveda et al., 2020). Among these metabolites, JA and SA play an important role in the regulation of cellular immune responses in plants (Singh et al., 2019). Jasmonic acid belongs to a collective group of cyclopentanone plant hormones (jasmonates) that are known to regulate a variety of processes in plant development and inducing insecticidal activities in plants (Black et al., 2003; Degenhardt et al., 2010). On the other hand, SA regulates defense systems against pathogens and herbivores with piercing-sucking mouthparts, such as aphids (Homoptera: Aphididae) (Loake and Grant, 2007). In chapter three, we reported that, when present in both tomato and nightshade plants, *T. asperellum* M2RT4 was one of the three most potent endophytic isolates that triggered the systemic protection of both host plants against *P. absoluta* through the reduction of adult oviposition and leafmining activity (Agbessenou et al., 2020). However, the underlying mechanisms mediating these interactions between endophytic fungi and how they prime plant defense systems especially in the tomato-*P. absoluta* model is not yet elucidated. Besides, the selection of *T. asperellum* M2RT4 isolate for the study of the chemical interaction in this chapter, was based on the antagonistic activity it has against several pests of economic importance including the greenhouse whitefly, *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) (Paradza et al., 2021), the bean stem maggot, *Ophiomyia phaseoli* (Diptera: Agromyzidae) (Mutune et al., 2016) and the pea leafminer, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) (Akutse et al., 2013); which will help to fast-track the registration and

commercialisation process of *T. asperellum* M2RT4 isolate. Therefore, this study aims at identifying volatile compounds (including phytohormones) released by inoculated tomato plant that prime defense responses against attack of both adult and immature stages of *P. absoluta*. We hypothesised that *T. asperellum* inoculated tomato plants trigger the production of phytohormones (JA and SA) which could reduce the attractiveness to *P. absoluta* female moths while negatively affecting leafmining activity of the pest. To test this hypothesis, we investigated the behavioural responses of *P. absoluta* female moths to non-inoculated, inoculated, non-inoculated infested and inoculated infested tomato plant volatiles. We then identified the odours associated with non-inoculated and inoculated tomato plants under *P. absoluta* attack. Thereafter we tested the behavioural responses of *P. absoluta* female moths to synthetic compounds of the volatile organic compounds (VOCs), and lastly assessed the ability of (*Z*)-jasmone to protect tomato plant from *P. absoluta* herbivory.

4.2.Materials and methods

Fungal culture

Trichoderma asperellum M2RT4 obtained from *icipe* Nairobi, Kenya's Arthropod Pathology Unit Germplasm was cultured on potato dextrose agar (PDA) (OXOID CM0139, Oxoid Ltd., Basingstoke, UK), and maintained at 25 ± 2 °C in complete darkness. Conidia were harvested by scraping the surface of two to three-week-old sporulated cultures using a sterile spatula. The harvested conidia were then suspended in 10 mL sterile distilled water containing 0.05% Triton X-100 (MERCK KGaA, Darmstadt, Germany) and vortexed for five minutes at about 700 rpm to break conidial clumps and ensure a homogenous suspension (Agbessenou et al., 2020; Akutse et al., 2013). Conidial concentrations were quantified using an improved Neubauer hemocytometer under a light microscope (Goettel and Inglis, 1997). The conidial suspension was adjusted to a concentration of 1×10^8 conidia mL^{-1} through serial dilution prior to inoculating the tomato seeds.

Prior to commencement of the bioassays, spore viability was determined by plating 0.1 mL of 3×10^6 conidia mL^{-1} evenly onto a 9-cm Petri dishes containing PDA. Three sterile microscope cover slips (2 × 2 cm) were placed on the surface of the inoculated plates. Plates were then sealed with Parafilm and incubated in complete darkness at 25 ± 2 °C and were examined after 16–20 h. Germination rate (%) of conidia was determined from 100 conidia that were randomly selected on the surface area covered by each cover slip under a light

microscope (×400) using the method described by Goettel and Inglis (1997). Conidia were considered to have germinated when the length of the germ tube was at least twice the diameter of the conidium. All treatments were replicated four times.

Inoculation of seeds and assessment of colonisation by the endophyte *Trichoderma asperellum* M2RT4

The tomato *Solanum lycopersicum* cultivar Moneymaker, used in the present study was purchased locally from Simlaw Seeds Company Ltd., Nairobi, Kenya. Seeds were surface-sterilised by washing them successively in 70% ethanol for two minutes followed by 1.5% sodium hypochlorite for another three minutes and finally rinsed three times in sterile distilled water. Thereafter the seeds were placed on sterile filter paper in a cabinet to remove any residual water. Effectiveness of the surface sterilisation technique was confirmed by plating out 0.1 mL of the last rinse water onto potato dextrose agar and also imprinting of surface sterilised seeds onto PDA (tissue imprint) supplemented with 100 mgL^{-1} Streptomycin and plates were incubated at 25 °C for 14 days (Schulz et al., 1998). Seeds were then soaked overnight for 12 h in conidial suspensions titrated at 1×10^8 conidia mL^{-1}. Sterilised seeds soaked overnight for 12 h in sterile distilled titrated (0.05% Triton X-100) water were used as the control (Agbessenou et al., 2020). Seeds were then planted in plastic pots (8 cm diameter × 7.5 cm high) containing the planting substrate (mixture of 0.5 Kg of peat compost and soil 1:5) (Mea Ltd., Nairobi, Kenya) with a volume of 0.5 L. The substrate was sterilised in an autoclave for 2 h at 121 °C and allowed to cool for 72 h prior to planting. Five seeds were sowed per pot and maintained at room temperature (25 ± 2 °C, 60% RH and 12:12 L:D photoperiod). Pots were transferred immediately after germination to the screen house (2.8 m length × 1.8 m width × 2.2 m height) at 25 ± 2 °C, 55% RH and 12:12 L:D photoperiod for four to five weeks. After germination, seedlings were thinned to two per pot and watered twice per day (morning and evening). No additional fertiliser was added to the planting substrate. Plants of 4–5-weeks-old were used for the various experiments.

To confirm endophytic colonisation before the experiments, tomato plants were carefully uprooted from the pots four to five weeks post-inoculation and washed under running tap water to remove any soil attached to the plants. Seedlings (ca. 30 cm in height) were divided into three different sections (ca. 5 cm long): leaves, stems and root using a sterile scalpel (Agbessenou et al., 2020; Akutse et al., 2013). Five sections each from the leaf; stem and root of each plant were randomly selected and surface -sterilised as previously described for

seeds. The different plant parts were then aseptically cut under a laminar flow hood into 1 × 1 cm pieces and placed four cm apart from each other on PDA plates supplemented with a 0.05% solution of antibiotic (streptomycin sulphate salt) (Akutse et al., 2013). Plates were incubated at 25 ± 1 °C for 10 days, after which the presence of endophyte was determined. In addition to the last rinse water that was plated, plate imprinting was also conducted to assess the effectiveness of surface sterilisation of plant materials (Inglis et al., 2012). The colonisation of the different plant parts was recorded by counting the number of pieces of plant parts that showed the presence of inoculated fungal growth/mycelia according to Koch's postulates (Petrini and Fisher, 1986). Only the presence of endophyte that was inoculated was scored from each incubated plate. Fungal isolate was identified morphologically using slides which were prepared from the mother plates. Treatments (non-inoculated and inoculated plants) were arranged in a randomised complete block design (RCBD) with four replicates. The success rate of fungal endophyte colonisation (%) of tomato host plant parts was calculated as follows:

$$\text{Colonisation (\%)} = \frac{\text{Number of pieces exhibiting fungal outgrowth}}{\text{Total number of pieces plated out}} \times 100 \qquad (1)$$

Insects

A colony of *P. absoluta* was established from wild moths and larvae collected from infested tomato leaves and fruits in Mwea (0° 36′ 31.3″ S 037° 22′ 29.7″ E), Kenya in June 2019. The moths were kept in ventilated, sleeved Perspex cages (40 × 40 × 45 cm) and were fed *ad libitum* with 10% honey solution placed on the top side of each cage as food source (Agbessenou et al., 2020). *Phthorimaea absoluta* were reared on non-inoculated tomato plants which were grown under screen house conditions at 25 ± 2 °C, 65 ± 10% RH at *icipe*. Tomato nurseries were established by sowing seeds on a mixture of 5:1 soil: manure (i.e. 10 g of peat compost) in a seed raising plastic tray. Three weeks later, tomato seedlings were transplanted on a 5:1 soil: manure mixture (i.e. 0.5 Kg of peat compost) in plastic pots (8 cm diameter × 7.5 cm high) at a density of two plants per pot and watered as needed. Three weeks after transplanting, four potted non-inoculated tomato plants were placed in the rearing cages for oviposition. The plants were removed 24 h post-exposure and transferred to separate wooden cages (50 × 50 × 60 cm) ventilated with netting material at the sides and on the top until the eggs hatched. Leaves with larvae were removed from these plants, three

days after hatching and placed into clean sterile plastic containers (21 cm long × 15 cm wide × 8 cm high) lined with paper towel to absorb excess moisture and fine netting infused lid for ventilation. The larvae were supplied daily with fresh tomato leaves (free from endophytes) as food until they pupated. The pupae were collected from the plastic containers using a fine camel hair-brush and placed inside a clean plastic container for adult emergence. The colony was rejuvenated every three months through infusion, with infested tomato leaves collected from the field to reduce inbreeding (Agbessenou et al., 2020; Akutse et al., 2020). Insects were maintained under a controlled rearing condition of 28 ± 2 °C, 48% RH and 12:12 L:D photoperiod in the laboratory at the Animal Rearing and Quarantine Unit (ARQU) of *icipe* for five generations prior to bioassays.

Behavioural responses of *Phthorimaea absoluta* female moths to inoculated tomato plant

Y-tube olfactometer bioassays

The responses of *P. absoluta* female moths to volatiles of non-inoculated, inoculated, non-inoculated infested and inoculated infested tomato plants were tested in dual-choice assays using a vertically-oriented Y-tube olfactometer (3 cm internal diameter; 15 cm stem; two 10 cm side arms) (Sigma Scientific, Gainesville, FL, USA) connected with Teflon tubing to the test odor sources which were headspace of tomato plant volatiles. The control arm was an empty oven bag (25 cm × 380 cm, Baco & BacoFoil, Wrap- Film Systems Ltd., United Kingdom). Depending on the experiments, each arm of the olfactometer is connected to either the tomato plant volatiles or an empty oven bag with Teflon tubing. A vacuum pump (Cole-Parmer Instrument Co., Chicago, IL 60648) was used to generate the air, which was filtered by an active charcoal filter and passed through each odor source tied in an oven baked bag at a constant flow rate of 150 mL min^{-1}. Infested plants were obtained by infesting a four-week-old plant with 20 *P. absoluta* second instar larvae for seven days. Potted plants were wrapped in aluminum foil to avoid contamination by volatiles from the pot and soil. To eliminate visual cues from the bioassay arena, the olfactometer was illuminated using 20 W red fluorescent tubes placed at a height of 0.5 m above the device (Sokame et al., 2019). Individual insects were released into the stem of the olfactometer and allowed five minutes to settle and another five minutes to make a choice. An insect was considered to have made a choice when it walked and reached the end of a given arm and remained there for at least 20 s. Insects which did not make a choice within five (5) minutes were considered as non-

respondent and were subsequently excluded from the statistical analysis. After every five tests, the odor sources were interchanged to eliminate positional bias. After every 10 replicates, a Y-tube and new test odor were used. Y-tubes were cleaned with Teepol odorless detergent (Sudi Chemical Industries Ltd, Nairobi, Kenya) rinsed with distilled water and dried in an oven overnight at 80 °C before use. All experiments were conducted at 26 ± 1 °C and 48-60% RH during the scotophase 1800–2000 h in sync with the behaviour and activity of *P. absoluta* which has been shown to be active during this time (Ataide et al., 2017; Proffit et al., 2011). Responses of two-day-old mated gravid *P. absoluta* female moths towards the different odor sources are presented in Table 4.1. Forty insects were tested per odor combination and a total of 440 insects used in the whole experiment.

Table 4.1. Odour sources tested in the Y-tube olfactometer bioassays

Experiment	Odour sources
A	air vs. air (control)
B	air vs. non-inoculated plant
C	air vs. inoculated plant
D	air vs. non-inoculated infested plant
E	air vs. inoculated infested plant
F	non-inoculated plant vs. inoculated plant
G	inoculated infested plant vs. inoculated infested plant
H	inoculated plant vs. non-inoculated infested plants
I	inoculated plant vs. inoculated infested plants
J	non-inoculated plant vs. inoculated infested plants
K	inoculated plant vs. non-inoculated infested plant

Collection of volatiles

Headspace volatiles were trapped from four-week-old tomato plants using a push-pull entrainment system (Analytical Research System, Gainesville, Florida, USA) at night for 12 h onto a preconditioned Super-Q adsorbent (30 mg, Analytical Research System, Gainesville, Florida, USA) by passing charcoal-purified air through tomato leaves tied in an oven baked bag at 350 mL/min. Volatiles were collected from non-inoculated, inoculated, non-inoculated infested and inoculated infested tomato plants, as well as from an empty oven bag with no plant (control). This was repeated three times with two plants per replicate. Volatiles were eluted into a two mL sample vial using 150 µL of dichloromethane (Analytical grade, Sigma-Aldrich, St, Louis, MO) and stored at -80 °C until required for chemical analysis.

Analysis of volatiles

One µl aliquot of each volatile extract was injected onto a gas chromatograph coupled mass spectrometer (GC-MS) in a splitless mode. The GC was equipped with a non-polar HP-5 MSI ultra-inert column (30 m × 0.25 mm i.d., 0.25 µm film thickness) (J&W, Folsom, CA, USA) with helium as the carrier gas at a flow rate of 1.2 mL min^{-1}. The oven temperature was held at 35 °C for 5 minutes, then programmed to increase at 10 °C min^{-1} to 280 °C and was maintained at this temperature for 10.5 minutes. The massselective detector was maintained at ion source temperature of 230 °C and a MS quadrapole temperature of 150 °C. Spectra were recorded at 70 eV in the electron impact (EI) ionisation mode. Fragment ions was analysed over 40-550 *m/z* mass range in the full scan mode. Compounds were identified by comparing their mass spectra with those from the libraries Adams2, Chemecol and NIST11 search program (v. 2.0) and NIST Chemistry Webbook and retention indices using retention times of a mixture of n-alkanes (C_8-C_{31}). Where available, the identities of compounds were confirmed by co-injection with commercially available authentic standards. Quantification was achieved using external calibration curves made from 1000 ng/µL stock solutions of the monoterpene β-pinene and the sesquiterpenes (*E*)-β-caryophyllene in a range of concentrations from 0.1 to 1000 ng/µL. Concentration of compounds were computed by extrapolating the peak area of the unknown against those of the known concentration and expressed in ng/plant/h.

Chemicals

Authentic standards of (*E*)-2-hexenal, *p*-xylene, α-pinene, β-pinene, trans-isolimonene, β-myrcene, 2-carene, α-phellandrene, 3-carene, α-terpinene, *p*-cymene, β-phellandrene, (*Z*)-β-ocimene, (*E*)-β-ocimene, γ-terpinene, terpinolene, n-nonanal, allo-ocimene, methyl salicylate, β-elemene, (*Z*)-jasmone, (*E*)-β-caryophyllene and α-humulene (>95% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Dichloromethane (99.9% purity) was purchased from Merck (Germany).

Bioassays with synthetic compounds

Of the volatiles identified from inoculated tomato plant: α-pinene, β-phellandrene, 2-carene, (*E*)-β-caryophyllene were selected and tested based on results obtained from a similarity percentage (SIMPER) analysis. Methyl salicylate was also tested because it has previously been reported to elicit activity in antennae of *P. absoluta* female moths (Anastasaki et al.,

2018). The behavioural responses of *P. absoluta* female moths were tested using a Y-tube olfactometer similar to the one used for initial behavioural bioassays. Three concentrations of each compound were tested in their natural release rate (ng/plant/h) from inoculated tomato plant, then by doubling and halving the amounts of each individual compound. Based on the results, three compounds (α-pinene, 2-carene, and β-phellandrene) were found attractive to *P. absoluta* female moths and were further tested in three-component blend at three concentrations against the control and methyl salicylate which was repellent to the females (2.17 ng/µL): (1.86 ng/µL α-pinene, 53.94 ng/µL 2-carene, 74.87 ng/µL β-phellandrene) (blend B1) which was subsequently doubled (blend B2) and diluted to one-half (blend B3) (Kihika et al., 2020; Njuguna et al., 2018). In another set of experiment, methyl salicylate (2.17 ng/µL) was tested against non-inoculated tomato plant. Thereafter, a four-component blend (B4) comprised of optimal attractant/repellent concentrations of the individual compound (1.86 ng/µL α-pinene, 53.94 ng/µL 2-carene, 74.87 ng/µL β-phellandrene and 2.17 ng/µL methyl salicylate) was formulated and tested against the control. Blend B4 was subsequently doubled (blend B5) and diluted to one-half (blend B6) (Table 4.2). Each individual compound and blends were diluted in dichloromethane and a 50 µL aliquot of the test solution was applied on a filter paper (3 × 3 cm) (Whatman, UK) and tested against the control (i.e., filter paper loaded with 50 µL dichloromethane). The solvent was allowed to evapourate at room temperature for 30 seconds prior to the bioassays. Thereafter, the impregnated filter papers were placed at the edge of the olfactometer arms and renewed for every insect. Forty insects were tested per choice and a total of 1,000 insects at the end.

Table 4.2. Summary of blends tested in the Y-tube olfactometer assays

Blend type	Blend composition
Blend B1	α-pinene (1.86) + 2-carene (53.94) + β-phellandrene (74.87)
Blend B2	α-pinene (3.73) + 2-carene (107.88) + β-phellandrene (149.74)
Blend B3	α-pinene (1) + 2-carene (26.97) + β-phellandrene (37.44)
Blend B4	α-pinene (1.86) + 2-carene (53.94) + β-phellandrene (74.87) + methyl salicylate (2.17)
Blend B5	α-pinene (3.73) + 2-carene (107.88) + β-phellandrene (149.74) + methyl salicylate (4.34)
Blend B6	α-pinene (1) + 2-carene (26.97) + β-phellandrene (37.44) + methyl salicylate (1.09)

Numbers in parenthesis indicate concentration of each compound in ng/µL.

Herbivory feeding bioassay with (*Z*)-jasmone

Tomato leaflet bioassay using first instar *P. absoluta* larvae was conducted to assess the herbivore response. Fresh tomato leaflets were treated with aqueous (*Z*)-jasmone solutions

at three different concentrations (1, 10 and 100 ng/µL) using a Potter Precision Laboratory Spray Tower (Burkard Manufacturing Co., Rickmansworth, UK) at constant air pressure of 10 PSI. Control leaflets were treated with sterile distilled water. First instar larvae (n = 10/treatment) were individually weighed and transferred to plastic Petri dishes (9 cm diameter) with fresh treated or untreated tomato leaflets. The petioles were wrapped in cotton cloth to maintain turgidity. The Petri dishes were sealed with Parafilm and incubated at 25 ± 2 °C and 45 ± 1% RH. Every 3 days, leaflets were replaced to ensure fresh food for the larvae. Larval mortality was recorded daily for seven days. The experiment consisted of 10 insects per treatment and was replicated four times.

Statistical analyses

All statistical analyses were performed using R statistical software, version 3.6.3 (R Core Team, 2019) and PAST, version 4.02. *Phthorimaea absoluta* female moths preference for odors in the Y-tube olfactometer was assessed by comparing the recorded frequencies of choice of either of the olfactometer arms using a chi-square (χ^2) test. Concentrations of volatile compounds between the four treatments including non-inoculated, inoculated, non-inoculated infested, and inoculated infested tomato plants were analysed using the non-parametric Kruskal-Wallis test because the data were not normally distributed. Whenever there was a significant difference, a post-hoc Dunn's test was performed for mean separation with Bonferroni's adjustment. Principal components analysis (PCA) and non-metric multi-dimensional scaling (NMDS) were used to visualise the profile of identified headspace volatiles. The headspace chemical profiles from the four treatments were compared using one-way ANOSIM with the Bray–Curtis dissimilarity index. Similarity percentage (SIMPER) analysis was performed on peak areas of volatile compounds to determine the relative contribution of different compounds to the dissimilarity among volatiles of the different treatments. Cumulative mortality was corrected using Abbott's formula (Abbott, 1925). Mortality data were analysed using logistic regression in a GLM for a binomial distribution. Lethal time (LT_{50}) was estimated by probit analysis using the package "ecotox"(Wheeler et al., 2006).

4.3.Results

Endophytic colonisation of tomato plant by *Trichoderma asperellum* M2RT4

Trichoderma asperellum M2RT4 successfully colonised 95, 90 and 85% of roots, stems and leaves of tomato plant, respectively (Figure 4.1). Additionally, no fungal outgrowth was observed in the non-inoculated plant (Figure 4.1).

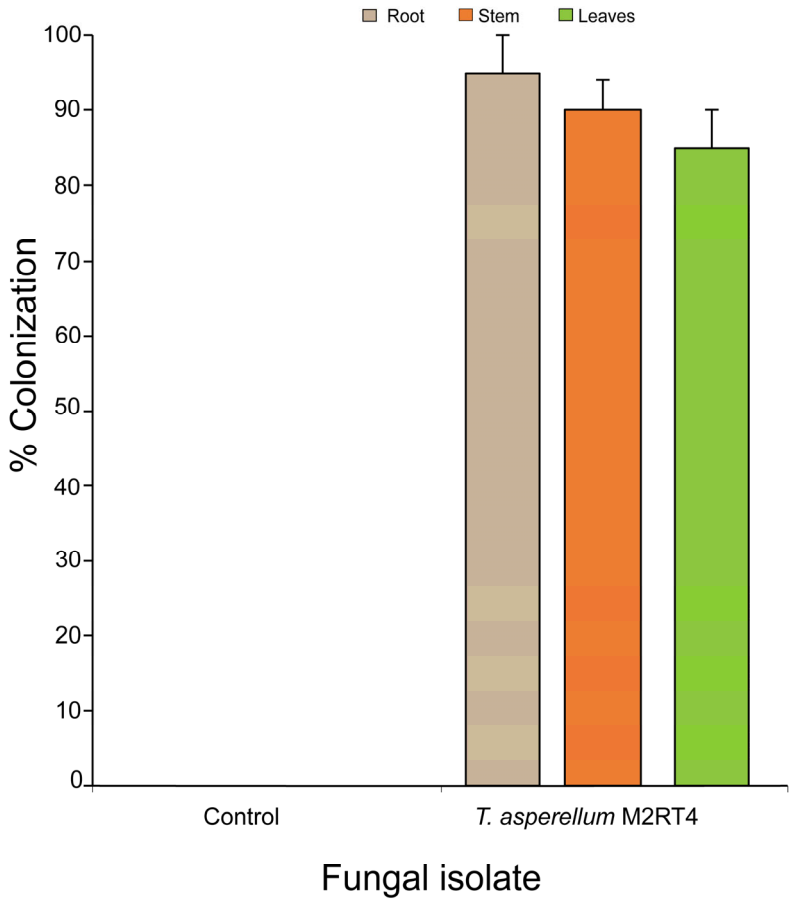

Figure 4.1. Endophytic colonisation of tomato *Solanum lycopersicum* host plant parts by *Trichoderma asperellum* M2RT4 at 4-5 weeks post-inoculation. Bar chart represents means ± SE (standard error) at 95% CI ($P < 0.05$; $n = 4$)

Olfactory response of *Phthorimaea absoluta* female moths to tomato plant volatiles

Phthorimaea absoluta female moths were significantly attracted to volatiles from non-inoculated tomato plants compared to clean air (control) ($\chi^2 = 27.22$, df = 1, $P < 0.0001$) (Figure 4.2). In contrast, *P. absoluta* female moths significantly avoided inoculated tomato plant volatiles ($\chi^2 = 8.30$, df = 1, $P < 0.001$) (Figure 4.2). On the other hand, of *P. absoluta*

female moths were not attracted to non-inoculated infested ($\chi^2 = 0.02$, df = 1, $P = 0.86$) or inoculated infested tomato plants ($\chi^2 = 0.43$, df = 1, $P = 0.51$) when compared to clean air (Figure 4.2). In paired assays, *P. absoluta* female moths were more attracted to the odor of non-inoculated tomato plant than to those of inoculated plants ($\chi^2 = 6.56$, df = 1, $P < 0.01$) (Figure 4.2). Similarly, *P. absoluta* female moths significantly preferred odors from non-inoculated plants compared to non-inoculated infested plants ($\chi^2 = 21.02$, df = 1, $P < 0.001$) and to inoculated plants ($\chi^2 = 24.02$, df = 1, $P < 0.001$) (Figure 4.2). However, *P. absoluta* female moths did not show any preference between non-inoculated infested and inoculated infested plants ($\chi^2 = 0.78$, df = 1, $P = 0.18$), neither between inoculated and inoculated infested plants ($\chi^2 = 1.34$, df = 1, $P = 0.84$), nor between inoculated and non-inoculated infested plants ($\chi^2 = 2.34$, df = 1, $P = 0.49$) (Figure 4.2).

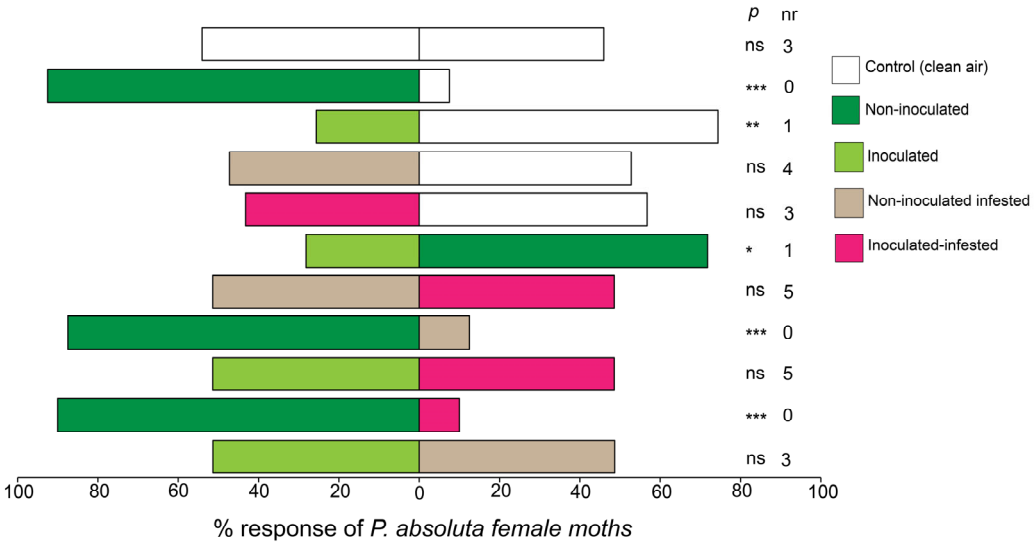

Figure 4.2. Responses (%) of *Phthorimaea absoluta* female moths to volatiles from non-inoculated, inoculated, non-inoculated infested and inoculated infested tomato plants in a Y-tube olfactometer choice test. nr = number of non-respondent insects (i.e. no choice). *P* stands for statistical significance levels with ns = no significant difference ($P > 0.05$); *, **, *** = significant differences at $P < 0.05$; $P < 0.01$; and $P < 0.001$, respectively, from χ^2 test at $\alpha = 0.05$

Analysis of volatiles

A bouquet of 31 compounds representing eight chemical classes: monoterpenes (16), sesquiterpenes (6), aldehydes (4), alcohol (1), benzoid (1), homoterpene (1), ester (1) and ketone (1) were detected, identified and quantified in the volatile profiles of non-inoculated, inoculated, non-inoculated infested and inoculated infested plants (Table 4.3, Figure 4.3).

Quantitative and qualitative differences in volatile emission were observed between non-inoculated, inoculated, non-inoculated -infested and inoculated infested plants (Table 4.3, Figures 4.3A and 4.3B). The monoterpenes 2-carene and β-phellandrene were the most abundant VOCs identified in the four treatments. The concentrations of α-pinene, 2-carene, α-phellandrene and β-phellandrene were significantly higher in non-inoculated infested plants than in non-inoculated, inoculated and inoculated-infested plants. One compound (benzaldehyde) was only found in both non-inoculated and inoculated infested plants while three compounds (sabinene, δ- and γ-elemene) were only emitted by non-inoculated infested plants (Table 4.3). Additionally, methyl salicylate emission was 2-fold more abundant in inoculated plant than in non-inoculated infested and inoculated infested plants (Table 4.3). Further, (*Z*)-jasmone was only detected from inoculated infested tomato plants (Table 4.3). Compounds that did not differ significantly in the emission rate among the four treatments included hexanal, (*E*)-2-hexenal, *p*-xylene, β-myrcene, 3-carene, (*E*)-β-ocimene, γ-terpinene, terpinolene, n-nonanal, allo-ocimene, α-cedrene, (*E*)-β-caryophyllene and α-humulene (Table 3). The NMDS clustered the four treatments successfully into four groups based on their VOC profiles (ANOSIM, $P < 0.01$) with five compounds [β-phellandrene (53.93%), 2-carene (19.91%), α-phellandrene (3.81%), (*E*)-β-caryophyllene (2.59%) and α-pinene (2%)] contributing the most to explaining the variation (Figures 4.4A and 4.4B). Furthermore, principal component analysis (PCA) of the VOCs showed that the first two components accounted for 68.9 (PC1), and 15.4% (PC2) of the total variation between the four treatments (Figure 4.4C). Dimension 1 was correlated with α-pinene, 2-carene, (*E*)-β-caryophyllene, α-phellandrene and β-phellandrene while dimension 2 was correlated with benzaldehyde.

Table 4.3. Mean amount (ng/plant/h) of volatile compounds identified in the headspace of non-inoculated, inoculated, non-inoculated infested, and inoculated infested tomato plants (n = 3)

Peak No.	RT	RI_{alk}[1]	RI_{lit}[2]	Compound[3]	Chemical class	non-inoculated plant	inoculated plant	non-inoculated infested plant	inoculated infested plant	*P*-value[4]
1	6.64	807	801	Hexanal	Aldehyde	15.96 ± 6.91	15.22 ± 7.13	16.42 ± 2.16	8.56 ± 1.12	0.361
2	8.03	860	856	(*E*)-2-hexenal*	Aldehyde	7.46 ± 0.46	7.81 ± 0.41	9.47 ± 1.02	6.71 ± 0.33	0.098
3	8.11	863	860	(*Z*)-3-hexenol	Alcohol	7.67 ± 0.28 b	8.5 ± 0.34 b	17.22 ± 6.89 a	6.51 ± 0.08 b	**0.018**
4	8.37	873	865	*p*-xylene*	Benzoid	8.55 ± 0.69	6.52 ± 0.27	7.01 ± 0.06	6.44 ± 0.09	0.06
5	9.83	936	934	α-pinene*	Monoterpene	35.67 ± 10.3 ab	23.31 ± 3.11 ab	63.33 ± 27.42 a	12.74 ± 1.76 b	**0.033**
6	10.42	963	963	Benzaldehyde	Aldehyde	8.79 ± 0.62	nd	nd	6.51 ± 0.06	**0.014**
7	10.61	972	972	3,7,7-trimethyl-1,3,5-cycloheptatriene	Homoterpene	25.69 ± 9.6 ab	18.75 ± 3.07 ab	52.7 ± 26.24 a	11.56 ± 1.69 b	**0.043**
8	10.67	975	974	Sabinene	Monoterperne	Nd	nd	9.76 ± 1.54	nd	-
9	10.73	978	978	β-pinene*	Monoterperne	14.69 ± 1.48 a	7.96 ± 0.18 ab	9.46 ± 1.34 ab	6.77 ± 0.1 b	**0.027**
10	10.83	983	983	trans-isolimonene*	Monoterperne	Nd	8.4 ± 0.29 ab	10.46 ± 1.76 b	6.78 ± 0.08 ab	**0.017**
11	11.037	992	992	β-myrcene*	Monoterperne	12.12 ± 2.54	12.92 ± 0.45	18.75 ± 4.72	7.15 ± 0.13	0.057
12	11.21	1001	1001	2-carene*	Monoterperne	253.84 ± 123.79 ab	168.57 ± 10.2 ab	480.06 ± 204.04 a	58.38 ± 10.27 b	**0.043**
13	11.28	1005	1005	α-phellandrene*	Monoterperne	52.67 ± 23.81 ab	36.81 ± 1.72 ab	97.96 ± 41.45 a	15.87 ± 1.49 b	**0.043**
14	11.41	1011	1011	3-carene*	Monoterperne	9.59 ± 1.51	8.65 ± 0.63	10.59 ± 1.75	6.99 ± 0.12	0.086
15	11.52	1018	1018	α-terpinene*	Monoterperne	26.84 ± 10.3 ab	19.96 ± 0.8 ab	48.23 ± 19.04 a	9.96 ± 0.74 b	**0.043**
16	11.67	1026	1026	*p*-cymene*	Monoterperne	13.52 ± .92 a	8.93 ± 0.18 ab	12.7 ± 3.31 ab	7.29 ± 0.09 b	**0.038**
17	11.75	1031	1032	β-phellandrene*	Monoterperne	698.16 ± 306.67 ab	467.93 ± 20.98 ab	1071.57 ± 385.23 a	158.65 ± 19.51 b	**0.044**
18	11.90	1039	1039	(*Z*)-β-ocimene*	Monoterperne	7.17 ± 0.88 ab	7.35 ± 7.35 ab	9.17 ± 0.93 a	nd	**0.041**
19	12.09	1050	1050	(*E*)-β-ocimene*	Monoterperne	10.71 ± 1.4	9.57 ± 0.41	13.82 ± 3.0	7.6 ± 0.24	0.063
20	12.29	1060	1060	γ-terpinene*	Monoterperne	11.87 ± 1.87	9 ± 0.22	12.7 ± 2.58	7.49 ± 0.52	0.061
21	12.80	1089	1090	Terpinolene*	Monoterperne	13.91 ± 4.26	11.92 ± 0.38	17.17 ± 5.59	6.75 ± 0.06	0.086
22	13.06	1104	1102	n-nonanal*	Aldehyde	12.78 ± 3.15	12.05 ± 1.9	8.31 ± 1.48	6.66 ± 0.08	0.055
23	13.33	1121	1121	Allo-ocimene*	Monoterperne	8.85 ± 1.64	8.85 ± 1.64	10.93 ± 2.27	6.39 ± 0.02	0.064
24	14.55	1198	1199	Methyl salicylate*	ar-Ester	Nd	13.54 ± 4.83 b	7.66 ± 0.83 a	6.4 ± 0.04 a	**0.018**
25	16.63	1344	1342	δ-elemene	Sesquiterpene	12.27 ± 2.77	17.35 ± 1.36	20.83 ± 6.2	8.16 ± 0.7	0.082
26	17.37	1398	13.97	β-elemene*	Sesquiterpene	Nd	nd	9.96 ± 1.5	nd	-

27	17.49	1407	1403	(*Z*)-jasmone*	Ketone	Nd	nd	nd	6.47 ± 0.06	-
28	17.72	1426	1424	α-cedrene	Sesquiterpene	10.3 ± 2.08	9.62 ± 2.92	8.19 ± 0.32	6.62 ± 0.28	0.218
29	17.79	1432	1430	(*E*)-β-caryophyllene*	Sesquiterpene	23.75±7.6	30.29±4.38	47.54±18	13.94 ± 2.35	0.121
30	17.91	1441	1441	γ-elemene	Sesquiterpene	Nd	nd	7.53 ± 0.46	nd	-
31	18.23	1467	1465	α-humulene*	Sesquiterpene	11.63 ± 2.56	13.26 ± 2.19	16.69 ± 4.07	7.93 ± 0.5	0.075

[1]Retention index relative to C8-C23 n-alkanes of an Inert Cap 5MS/NP capillary column
[2]Retention index obtained from the literature (Khan et al., 2012)
[3]Identification of compounds based on the retention time (RT) and comparison of mass spectra with published mass spectral library data from NIST11 and Wiley9. * indicates compounds confirmed with authentic standards
[4]*P*-value of the non-parametric Kruskal Wallis test for comparison of volatile compounds between non-inoculated, inoculated, non-inoculated infested and inoculated infested tomato plants. Significant values are highlighted in bold, where means (± SE) followed by the same letters within rows are not significantly different
nd = not detected

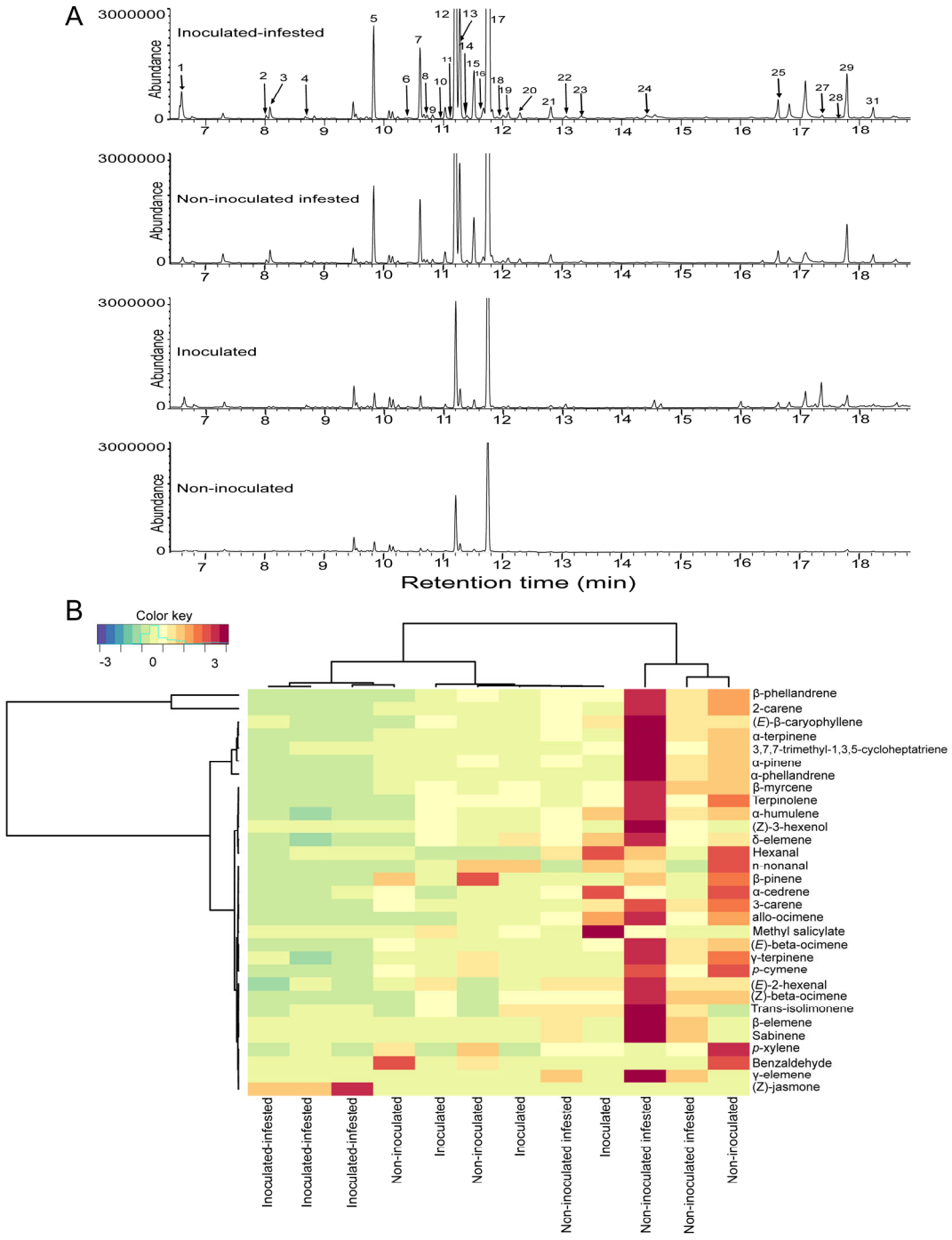

Figure 4.3. Variations in volatile organic compounds across non-inoculated, inoculated, non-inoculated infested and inoculated infested tomato plants (A) Representative total ion chromatogram of non-inoculated, inoculated, non-inoculated infested and inoculated infested tomato plants. (B) Heatmap clustering showing the abundance (in decreasing color

intensity) of the volatile compounds across replicates of the four treatments

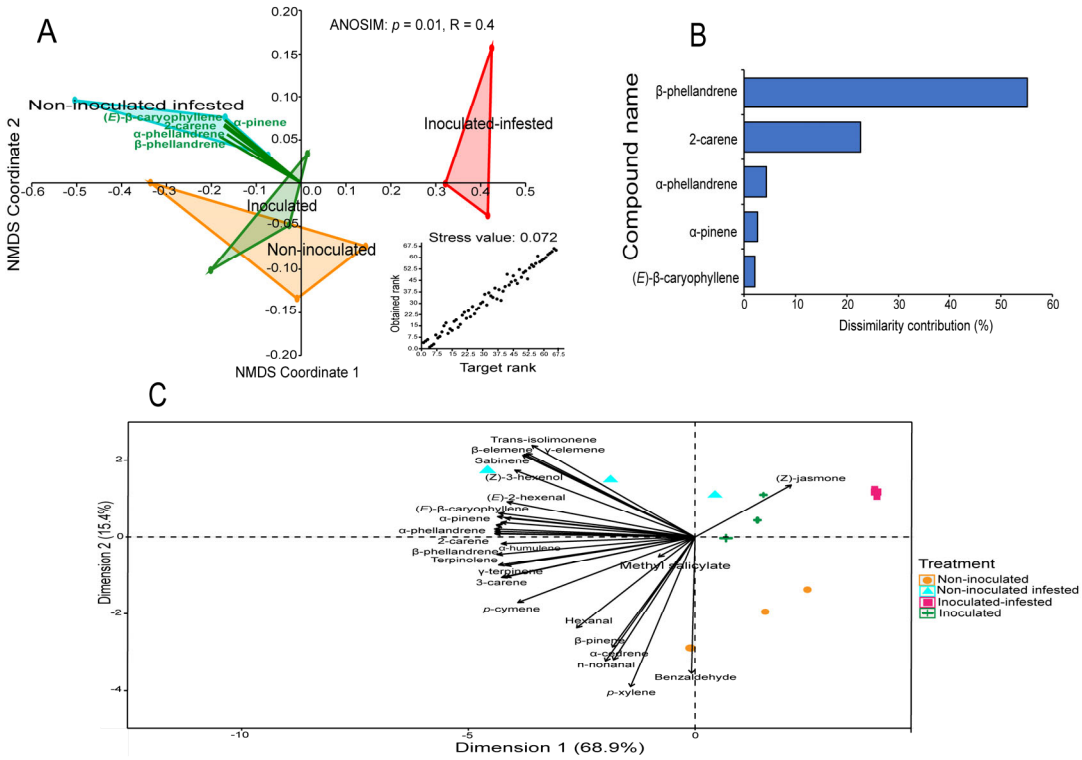

Figure 4.4. Variations in volatile organic compounds across the four treatments (A) Non-metric multidimensional scaling analysis (NMDS) of the volatile pattern of non-inoculated, inoculated, non-inoculated infested and inoculated infested tomato plants showing the variation in volatiles. (B) Similarity percentage analysis and the percent contribution of the predominant compounds for the dissimilarity between the four treatments. (C) Principal component analysis (PCA) of the volatile profiles produced from non-inoculated, inoculated, non-inoculated infested and inoculated infested tomato plants

Behavioural response to synthetic compounds

Phthorimaea absoluta female moths responded differently to the various concentrations of synthetic compounds tested individually compared to the control (Figure 4.5). The females significantly preferred α-pinene at 1.86 ng/µL (χ^2 = 10.02, df = 1, P < 0.001, Figure 4.5A), 2-carene at 53.94 ng/µL (χ^2 = 4.36, df = 1, P = 0.03, Figure 4.5B) and β-phellandrene at 74.87 ng/µL (χ^2 = 6.61, df = 1, P < 0.01, Figure 4.5C) compared to the control. In contrast, (*E*)-β-caryophyllene was not attractive to the females at any of the tested concentrations (Figure 4.5D) while methyl salicylate repelled them at a concentration of 2.17 ng/µL (χ^2 = 4.36, df = 1, P = 0.03) (Figure 4.5E). Hence, this concentration was used in the subsequent experiments.

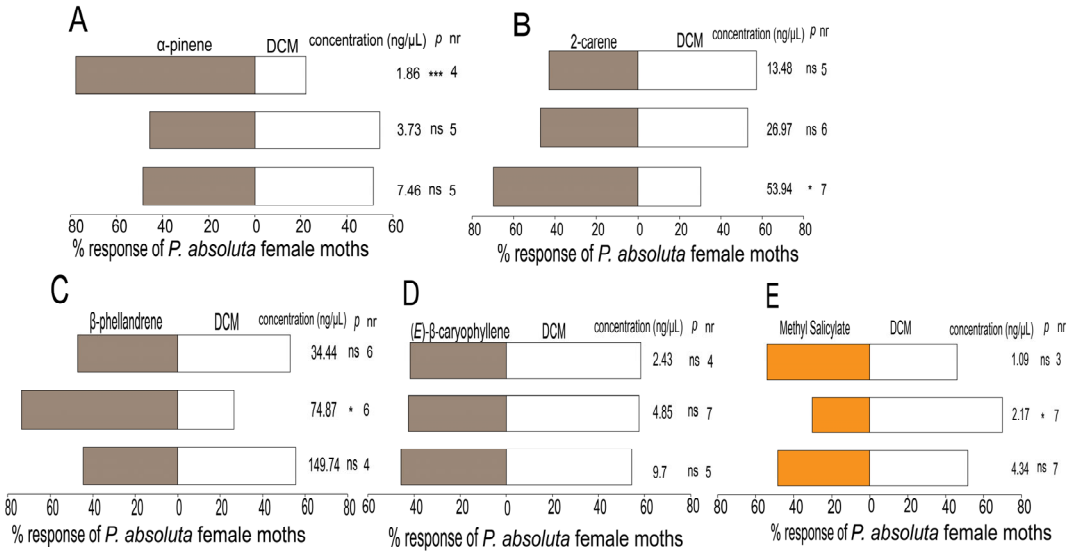

Figure 4.5. Behavioural responses of *Phthorimaea absoluta* female moths to odor components tested at three concentrations (A) α-pinene, (B) 2-carene, (C) β-phellandrene, (D) (*E*)-β-caryophyllene and (E) methyl salicylate, all against DCM = dichloromethane (control solvent) in a Y-tube olfactometer. Each pair of evaluation used a total of n = 40 of *Phthorimaea absoluta* female moths for each compound concentration released singly per choice. nr = number of non-respondent insects (i.e. no choice). *P* stands for statistical significance levels with ns = no significant difference ($P > 0.05$); *, *** = significant differences at $P < 0.05$ and $P < 0.001$, respectively, from χ^2 test at $\alpha = 0.05$

Phthorimaea absoluta female moths did not show any preference to the blend of the three attractants (α-pinene, 2-carene, β-phellandrene) mixed at their attractive concentrations (blend B1) ($\chi^2 = 0.28$, df = 1, $P = 0.59$) or when the concentrations were doubled (blend B2) ($\chi^2 = 0.33$, df = 1, $P = 0.87$) compared to the control (Figure 4.6A). However, the blend B3 composed of one-half of the blend B1 concentration was relatively attractive to the females ($\chi^2 = 4.03$, df = 1, $P = 0.04$) (Figure 4.6A). Interestingly, *P. absoluta* female moths preferred the blend of the three attractants (α-pinene, 2-carene, β-phellandrene) mixed at their attractive concentrations (blend B1) ($\chi^2 = 5.60$, df = 1, $P < 0.01$) or when doubled (blend B2) ($\chi^2 = 4.97$, df = 1, $P = 0.02$) compared to methyl salicylate (Figure 4.6B). Surprisingly, *P. absoluta* female moths were not attracted to the blend B3 composed of one-half of the blend B1 concentration compared to methyl salicylate ($\chi^2 = 3.78$, df = 1, $P = 0.06$) (Figure 4.6B). Also, *P. absoluta* female moths preferred volatiles of non-inoculated tomato plant ($\chi^2 = 9.25$, df = 1, $P < 0.01$) compared to methyl salicylate (Figure 4.6B). In addition, *P. absoluta* female moths did not show any preference for the four-component blend (B4) comprised of optimal

attractant/repellent concentrations of the individual compound ($\chi^2 = 1.93$, df = 1, $P = 0.16$), or when the concentrations were doubled (blend B5) ($\chi^2 = 0.03$, df = 1, $P = 0.86$) or halved (blend B6) ($\chi^2 = 0.03$, df = 1, $P = 0.87$) compared to the control (Figure 4.6C).

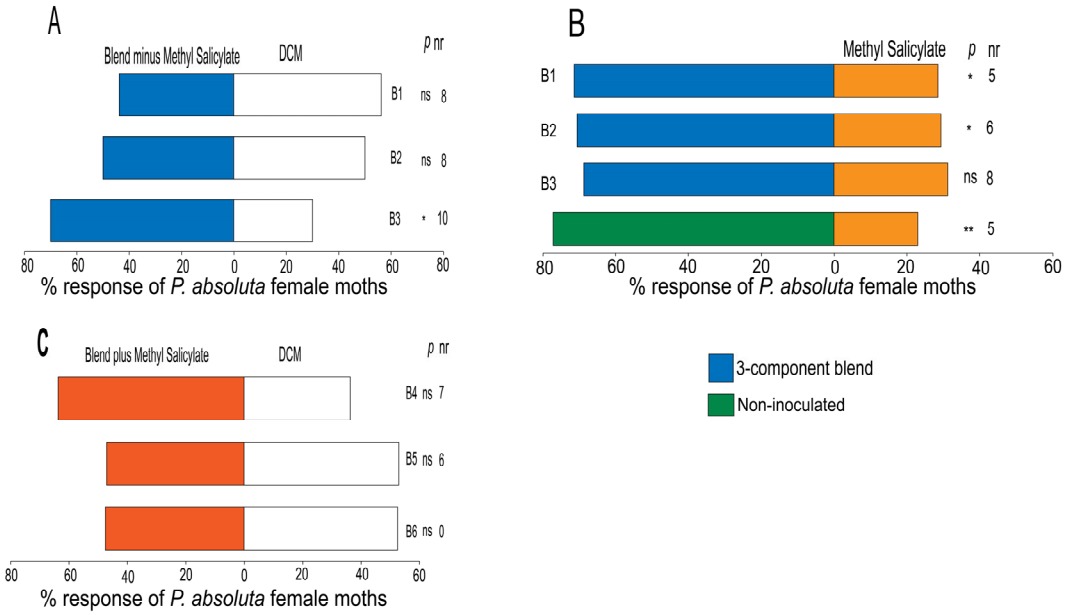

Figure 4.6. Behavioural responses of *Phthorimaea absoluta* female moths to (A) the three-component blend consisting of the most attractive compounds, α-pinene, 2-carene, β-phellandrene, all against DCM = dichloromethane (control solvent). (B) % responses of *P. absoluta* female moths to three-component blend and non-inoculated tomato plant all against methyl salicylate (positive control). (C) % responses of *P. absoluta* female moths to the four-component blend containing α-pinene, 2-carene, β-phellandrene, and methyl salicylate in a Y-tube olfactometer. nr = number of non-respondent insects (i.e. no choice). *P* stands for statistical significance levels with ns = no significant difference ($P > 0.05$); *, ** = significant differences at $P < 0.05$; and $P < 0.01$, respectively, from χ^2 test at $\alpha = 0.05$

Herbivory feeding bioassay with (*Z*)-jasmone

Larval mortality varied significantly among the three concentrations ($\chi^2 = 40.58$, df = 3, $P < 0.001$) seven days post-treatment (Figure 4.7). The concentration (10 ng/µL) had the highest (83%) larval mortality rate followed by the concentration at 1 ng/µL (67.5%) and at 100 ng/µL (64.24%) (Figure 4.7). Additionally, the concentration at 10 ng/µL had the shortest LT_{50} (1.73 days) (Table 4.4). Overall, (*Z*)-jasmone-treated tomato leaflets significantly reduced *P. absoluta* leafmining activity as compared to the control (Figure 4.7).

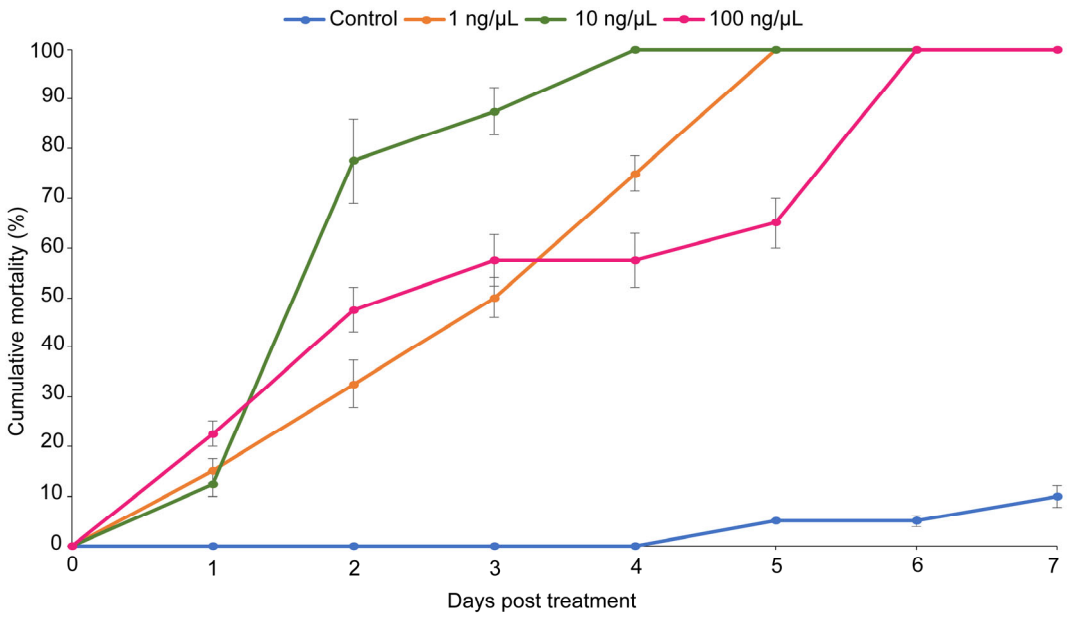

Figure 4.7. Cumulative larval mortality of *Phthorimaea absoluta* induced by three concentrations (1, 10 and 100 ng/µL) of (Z)-jasmone

Table 4.4. LT_{50} values seven days post-treatment of *Phthorimaea absoluta* larvae exposed to different concentrations of (*Z*)-jasmone

(*Z*)-jasmone concentration (ng/µL)	LT_{50} (days)[a]
1	2.72 (2.49-2.94)
10	1.73 (1.53-1.91)
100	2.79 (1.95-3.42)

[a]Values in the bracket represent 95 % Fiducial Limits (FL)

4.4.Discussion

Our results indicate that *in planta* colonisation of tomato plant tissues by *T. asperellum* M2RT4 modifies the chemical response of the host plant affecting the abilities of *P. absoluta* female moths to locate their host. Specifically, we demonstrated that the endophytic fungus *T. asperellum* quantitatively and qualitatively alters tomato leaf volatiles composition by enhancing both the SA and JA plant defense pathways.

In the Y-tube olfactometer assays, we observed that *P. absoluta* female moths avoided inoculated plants. This indicates that attraction of ovipositing females likely depends on the perception of *bona fide* semio-chemicals, therefore orienting their movement towards odors released by suitable host plants. Previously, Ataide et al. (2017) reported that healthy tomato plant volatiles play an important role in modifying both flight and oviposition behaviour of mated *P. absoluta* female moths. Clearly, plant volatiles of inoculated tomato plants are far

less attractive to ovipositing females, as reflected in reduced oviposition on the inoculated plants (Agbessenou et al., 2020). Additionally, the preference of *P. absoluta* female moths towards non-inoculated plants in dual-choice assay suggests that they might perceive odors from inoculated plants which could signal unsuitable hosts leading to their avoidance and this is considered as a crucial step for the reproductive success of the pest. Our results also showed that *P. absoluta* female moths were not attracted either to non-inoculated infested or to inoculated infested tomato plants. This could be explained by the fact that *P. absoluta* female moths perceive the infested plant through chemical cues as poor sources for the development of their offspring. This is in agreement with De Moraes et al. (2001) who reported that *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) female moths exploit volatile signals from infested plants to avoid oviposition on previously damaged plants. However, this contrasts with Shiojiri and Takabayashi (2003) who demonstrated that the female diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) prefer to oviposit on cabbage plants damaged by conspecifics than on plants not damaged by conspecifics.

Our findings reveal the emission of a bouquet of VOCs largely dominated by monoterpenes and sesquiterpenes. Terpenes are known to play an important ecological role in plant-insect interactions, including serving as attractants or repellents to herbivores, parasitoids and predators (Helms et al., 2017; von Mérey et al., 2013). For example, α-pinene, α-phellandrene and β-phellandrene are shown to be attractants to the predator *Nesidiocoris tenuis* (Hemiptera: Miridae) (Ayelo et al., 2021). Notably, we found an increase in the emission of terpenoids by infested plants upon herbivory by *P. absoluta*, with 2-carene and β-phellandrene as the most abundant VOCs similar to findings by Silva et al. (2018). Earlier on, Turlings et al. (1995) also reported an increase in the emission of terpenoids (α-pinene and β-phellandrene) in cotton and corn plants upon herbivory by the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae). Turlings et al. (1995) speculated that the terpenoids are stored in the glands located in the leaves of cotton, the host plant which may serve as toxins to directly discourage herbivores from feeding on the leaves. Similarly, Gouinguené et al. (2003) reported an increase in terpenoid emissions in *Zea mays* which was elicited by oral secretions of *Spodoptera littoralis* (Lepidoptera: Noctuidae). Interestingly, we found that the quantitative variation in the emission of volatiles was only evident in the monoterpenes 2-carene, α-pinene, α-phellandrene, β-phellandrene, but not in sesquiterpenes such as δ-elemene, α-cedrene, (*E*)-β-caryophyllene and α-humulene. Similar findings on increase in the production of monoterpenes were also reported in below ground interactions

between the tomato root-knot nematode *Meloidogyne javanica* and tomato plants (Kihika et al. (2020). It can therefore be speculated that increases in the release of monoterpenes by plants is triggered by low infestations which could serve as priming signals for activating plant defense systems/mechanisms. When present in tomato plants, *T. asperellum* alter the composition of volatile emissions, thereby inducing a quantitative difference between non-inoculated and inoculated plants. Specifically, we found that inoculated tomato plants elicited a reduction in the emission of several compounds including α-pinene, β-pinene, 2-carene, α-phellandrene, β-phellandrene compared to non-inoculated plant. This is in contrast with Shrivastava et al. (2015) who reported an increase in the concentration of the monoterpenes (2-carene and sabinene) in *Beauveria bassiana*-inoculated tomato plants compared to the control. Conversely, we observed an increase even though non-significant in the emission of (*E*)-β-caryophyllene in inoculated plants compared to non-inoculated plants. This change in volatile profile was previously reported by Battaglia et al. (2013) who also observed an enhanced release of (*E*)-β-caryophyllene by *Trichoderma longibrachiatum* MK1-colonised tomato plants compared to the control. Interestingly, we found a significant reduction in the emission of most monoterpenes (α-pinene, 2-carene, α-phellandrene, α-terpinene, and β-phellandrene) by inoculated-infested plants compared to non-inoculated infested treatment.

The colonisation of tomato plant by *T. asperellum* M2RT4 induces the release of trans-isolimonene and methyl salicylate which could probably explain the avoidance of inoculated plants by *P. absoluta* female moths. This is quite interesting as microbial infection is known to provide long term resistance to herbivore/pathogen attacks through the activation of the SA pathway (Shoresh et al., 2010). Although *T. asperellum* M2RT4 could have an intrinsic ability to colonise tomato host plant, its presence does not cause any harmful/negative effects to its host (Paradza et al., 2021). Therefore, its colonisation of tomato triggered the defense mechanism of the plant leading to the emission of methyl salicylate. Conversely, previous studies have reported that the presence of endophytic fungi within host plants often leads to the inactivation of the plant salicylic acid pathway (Bastías et al., 2018). The emission of methyl salicylate by inoculated plants is suggestive of molecular mechanism mediating interaction between the endophytes, insect and its host plants, calling for further studies. It is worth noting that one essential benefit this endophyte provides is that of a source of bioactive alkaloids and nitrogen-based compounds that protect host plants against herbivores (Bastías et al., 2018; Poveda, 2021b). The release of methyl salicylate by inoculated tomato

plant indicates that the metabolic machinery of the plant is prepared for subsequent defense upon herbivory. Because of the high energy costs and nutrient requirements associated with the production and maintenance of defensive metabolites, the presence of endophytes helps the plant for protection against the pest without spending energy for self-defense or have energy to allocate to other functions (Thaler, 1999).

In this study, we observed that methyl salicylate is also released from infested plants supporting the suggestion that this compound could function as a signal associated with damage or induced defense (Hardie et al., 1994). Indeed, plants exhibit self-defense strategies against herbivores either directly, through negative effects on herbivore performance, or indirectly, by recruiting natural enemies of herbivores through the release of HIPVs (Moujahed et al., 2014). Previously, Silva et al. (2018) reported the emission of methyl salicylic upon *P. absoluta* herbivory. Salicylic acid is a stress-related hormone that has been reported to play an important role in the orchestration of plant defenses against herbivores (Shi et al., 2016). Interestingly, we observed that the level of methyl salicylate is higher in inoculated plants than in non-inoculated infested tomato plants. This protection mediated by methyl salicylate is a promising control strategy as the plants predominantly exhibit sessile-life style which exposes their vulnerability to biotic and abiotic stressors. Therefore, induction of resistance at the early stage (seed stage) may enhance the protection of tomato plant and suppress the population buildup of multivoltine pests like *P. absoluta* (Strapasson et al., 2014; Thaler et al., 2012).

We observed an emission of the key defense phytohormone (*Z*)-jasmone from inoculated infested plant. In contrast, Caparros Megido et al. (2014) and De Backer et al. (2015) reported the emission of (*Z*)-jasmone by non-inoculated infested tomato plants which we didn't observe in this study. This could be attributed to its suppression through elicitors present in the oral regurgitant or saliva of *P. absoluta* immature stages. Several authors have previously reported the chemical response of tomato plant to *P. absoluta* herbivory and the signaling pathways involved (Ayelo et al., 2021; De Backer et al., 2017; Silva et al., 2018). Besides, we observed a significant reduction in the abundance of monoterpenes as well as in the quantity of methyl salicylate in inoculated infested tomato plants compared to other treatments. This could be attributed to the activation of (*Z*)-jasmone signaling-pathway where tomato host plant responded to herbivore damage by producing (*Z*)-jasmone while reducing the quantity of methyl salicylate. Consistently, JA and SA have been reported as the dominant plant-signaling compounds that trigger induced resistance against herbivorous

insects which most of the time render plants less susceptible to subsequent attack by pests (Cui et al., 2012). This supports results of our olfactometer bioassays where of *P. absoluta* female moths did not show any preference for infested plants. This confirms the observation that jasmonate synthesis is triggered by feeding damage from chewing herbivores (Cooper and Goggin, 2005).

The results of the herbivory feeding bioassay showed that (*Z*)-jasmone-treated tomato leaflets significantly reduced *P. absoluta* leafmining activity as compared to the control. Previously, Strapasson et al. (2014) reported that treatment of tomato seeds with methyl jasmonate enhances plant resistance at the seed stage which subsequently result in the reduction of the performance of *P. absoluta*. Apart from *P. absoluta* larvae, previous studies have shown the efficacy of (*Z*)-jasmone against immature stages of other insect pests. For instance, Sobhy et al. (2020) reported that priming potato plant with (*Z*)-jasmone negatively impacts the performance traits of aphids *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae). Similarly, Black et al. (2003) reported the inhibitory effect of JA and this can be used as a vaccine against the leafminers (Diptera: Agromyzidae). The central role played by (*Z*)-jasmone in systemic feeding damage signaling in tomato plants underlines the relevance of treating tomato seeds with fungal inoculum so as to sustainably manage *P. absoluta*. Induction of (*Z*)-jasmone is known to attract parasitoids and predators of caterpillars (Turlings et al., 1990). However, these two defensive pathways (SA and JA) are not independent, and there could be antagonistic cross-talk between SA and JA pathways (Filgueiras et al., 2016). Therefore, it would be interesting to unravel the molecular mechanism underlying such interactions between endophyte and tomato plants against *P. absoluta* leading to activation of both SA and JA defense pathways and the production of methyl salicylate and (*Z*)-jasmone.

The response of an insect to a given compound is known to occur in a dose-dependent manner (Kihika et al., 2017; Njuguna et al., 2018). In this study, we showed that *P. absoluta* female moths responded differently to the concentrations of the individual compounds and the blend with and without methyl salicylate against the control. Individually, α-pinene, 2-carene and β-phellandrene were attractive to *P. absoluta* female moths at 1.86 ng/µL, 53.94 ng/µL and 149.74 ng/µL, respectively, while (*E*)-β-caryophyllene did not elicit attraction of female moths at the three different concentrations tested. Interestingly, methyl salicylate was the only compound that elicited repellence behaviour to *P. absoluta*. In addition, methyl salicylate's significance in *P. absoluta* host location was evidenced when it was removed

from the 3-component blend (α-pinene, 2-carene and β-phellandrene) which resulted in the attraction of the moths. The repellent effect of methyl salicylate has previously been reported in aphids and the glasshouse whitefly (Xu et al., 2018; Conboy et al., 2020). Methyl salicylate has also been shown to induce defenses against *P. absoluta* (Pérez-Hedo et al., 2021). However, methyl salicylate has been shown to be an oviposition attractant for the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Yactayo-Chang et al., 2021).

In conclusion, our findings have shed light on the role of the endophyte *T. asperellum* M2RT4 in the activation of plant defense mechanisms in tomato plant through both the SA and JA pathways. Based on our results, we demonstrated that *T. asperellum* M2RT4 primes tomato plant defense in two ways. On the one hand, we found that colonisation of tomato plant by *T. asperellum* M2RT4 induces the systemic release of methyl salicylate which has a repellent effect on the host location behaviour of *P. absoluta* female moths. On the other hand, we also showed that *T. asperellum* M2RT4 triggered the release of (*Z*)-jasmone in inoculated infested tomato plants reducing the performance of the larval activity of the pest. This study has therefore laid the groundwork for future studies aimed at elucidating the underlying molecular mechanism that mediate endophyte-induced plant resistance against *P. absoluta*.

References

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18, 265–267.

Affokpon, A., Coyne, D. L., Htay, C. C., Agbèdè, R. D., Lawouin, L., and Coosemans, J. (2011). Biocontrol potential of native *Trichoderma* isolates against root-knot nematodes in West African vegetable production systems. *Soil Biol. Biochem.* 43, 600–608. doi:10.1016/j.soilbio.2010.11.029.

Agbessenou, A., Akutse, K. S., Yusuf, A. A., Ekesi, S., Subramanian, S., and Khamis, F. M. (2020). Endophytic fungi protect tomato and nightshade plants against *Tuta absoluta* (Lepidoptera: Gelechiidae) through a hidden friendship and cryptic battle. *Sci. Rep.* 10, 22195. doi:10.1038/s41598-020-78898-8.

Akutse, K. S., Maniania, N. K., Fiaboe, K. K. M., Van den Berg, J., and Ekesi, S. (2013). Endophytic colonization of *Vicia faba* and *Phaseolus vulgaris* (Fabaceae) by fungal pathogens and their effects on the life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Fungal Ecol.* 6, 293–301. doi:10.1016/j.funeco.2013.01.003.

Akutse, K. S., Subramanian, S., Khamis, F. M., Ekesi, S., and Mohamed, S. A. (2020). Entomopathogenic fungus isolates for adult *Tuta absoluta* (Lepidoptera: Gelechiidae) management and their compatibility with *Tuta* pheromone. *J. Appl. Entomol.*, 1–11. doi:10.1111/jen.12812.

Alınç, T., Cusumano, A., Peri, E., Torta, L., and Colazza, S. (2021). *Trichoderma harzianum* strain T22 modulates direct defense of tomato plants in response to *Nezara viridula* feeding activity. *J. Chem. Ecol.* 47, 455–462. doi:10.1007/s10886-021-01260-3.

Anastasaki, E., Drizou, F., and Milonas, P. G. (2018). Electrophysiological and oviposition responses of *Tuta absoluta* females to herbivore-induced volatiles in tomato plants. *J. Chem. Ecol.* 44, 288–298. doi:10.1007/s10886-018-0929-1.

Ataide, L. M. S., Arce, C. C. M., Curtinhas, J. N., da Silva, D. J. H., DeSouza, O., and Lima, E. (2017). Flight behavior and oviposition of *Tuta absoluta* on susceptible and resistant genotypes of *Solanum lycopersicum*. *Arthropod. Plant. Interact.* 11, 567–575. doi:10.1007/s11829-017-9500-1.

Ayelo, P. M., Yusuf, A. A., Pirk, C. W. W., Chailleux, A., Mohamed, S. A., and Deletre, E. (2021a). Terpenes from herbivore-induced tomato plant volatiles attract *Nesidiocoris tenuis* (Hemiptera: Miridae), a predator of major tomato pests. *Pest Manag. Sci.* 77, 5255–5267. doi:10.1002/ps.6568.

Ayelo, P. M., Yusuf, A. A., Pirk, C. W. W., Mohamed, S. A., Chailleux, A., and Deletre, E. (2021b). The role of *Trialeurodes vaporariorum*-infested tomato plant volatiles in the attraction of *Encarsia formosa* (Hymenoptera: Aphelinidae). *J. Chem. Ecol.* 47, 192–203. doi:10.1007/s10886-021-01245-2.

Barelli, L., Moonjely, S., Behie, S. W., and Bidochka, M. J. (2016). Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi. *Plant Mol. Biol.* 90, 657–664. doi:10.1007/s11103-015-0413-z.

Bastías, D. A., Alejandra Martínez-Ghersa, M., Newman, J. A., Card, S. D., Mace, W. J., and Gundel, P. E. (2018). The plant hormone salicylic acid interacts with the mechanism of anti-herbivory conferred by fungal endophytes in grasses. *Plant Cell Environ.* 41, 395–405. doi:10.1111/pce.13102.

Battaglia, D., Bossi, S., Cascone, P., Digilio, M. C., Prieto, J. D., Fanti, P., Lorito, M., Massa, N., Sasso, R., and Trotta, T. (2013). Tomato below ground–above ground interactions: *Trichoderma longibrachiatum* affects the performance of *Macrosiphum euphorbiae* and its natural antagonists. *Mol. Plant-Microbe Interact.* 26, 1249–1256.

doi:10.1094/mpmi-02-13-0059-r.

Black, C. A., Karban, R., Godfrey, L. D., Granett, J., and Chaney, W. E. (2003). Jasmonic acid: A vaccine against leafminers (Diptera: Agromyzidae) in celery. *Environ. Entomol.* 32, 1196–1202. doi:10.1093/ee/32.5.1196.

Bruce, T. J. A., Wadhams, L. J., and Woodcock, C. M. (2005). Insect host location: A volatile situation. *Trends Plant Sci.* 10, 269–274. doi:10.1016/j.tplants.2005.04.003.

Caparros Megido, R., De Backer, L., Ettaïb, R., Brostaux, Y., Fauconnier, M. L., Delaplace, P., Francis, F., and Verheggen, F. J. (2014). Role of larval host plant experience and solanaceous plant volatile emissions in *Tuta absoluta* (Lepidoptera: Gelechiidae) host finding behavior. *Arthropod. Plant. Interact.* 8, 293–304. doi:10.1007/s11829-014-9315-2.

Conboy, N. J. A., McDaniel, T., George, D., Ormerod, A., Edwards, M., Donohoe, P., and Tosh, C. R. (2020). Volatile organic compounds as insect repellents and plant elicitors: an integrated pest management (IPM) strategy for glasshouse whitefly (*Trialeurodes vaporariorum*). *J. Chem. Ecol.* 46, 1090–1104. doi:10.1007/s10886-020-01229-8.

Cooper, W. R., and Goggin, F. L. (2005). Effects of jasmonate-induced defenses in tomato on the potato aphid, *Macrosiphum euphorbiae*. *Entomol. Exp. Appl.* 115, 107–115. doi:10.1111/j.1570-7458.2005.00289.x.

Cui, H., Sun, Y., Su, J., and Li, C. (2012). Reduction in the fitness of *Bemisia tabaci* fed on three previously infested tomato genotypes differing in the jasmonic acid pathway. *Environ. Entomol.* 41, 1443–1453.

Davis, T. S., Crippen, T. L., Hofstetter, R. W., and Tomberlin, J. K. (2013). Microbial volatile emissions as insect semiochemicals. *J. Chem. Ecol.* 39, 840–859. doi:10.1007/s10886-013-0306-z.

De Backer, L., Bawin, T., Schott, M., Gillard, L., Markó, I. E., Francis, F., and Verheggen, F. (2017). Betraying its presence: identification of the chemical signal released by *Tuta absoluta*-infested tomato plants that guide generalist predators toward their prey. *Arthropod. Plant. Interact.* 11, 111–120. doi:10.1007/s11829-016-9471-7.

De Backer, L., Megido, R. C., Fauconnier, M. L., Brostaux, Y., Francis, F., and Verheggen, F. (2015). *Tuta absoluta*-induced plant volatiles: attractiveness towards the generalist predator *Macrolophus pygmaeus*. *Arthropod. Plant. Interact.* 9, 465–476. doi:10.1007/s11829-015-9388-6.

De Moraes, C. M., Mescher, M. C., and Tumlinson, J. H. (2001). Caterpillar-induced

nocturnal plant volatiles repel conspecific females. *Nature* 410, 577–579. doi:10.1038/35069058.

Degenhardt, D. C., Refi-Hind, S., Stratmann, J. W., and Lincoln, D. E. (2010). Systemin and jasmonic acid regulate constitutive and herbivore-induced systemic volatile emissions in tomato, *Solanum lycopersicum*. *Phytochemistry* 71, 2024–2037. doi:10.1016/j.phytochem.2010.09.010.

Desneux, N., Wajnberg, E., Wyckhuys, K. A. G., Burgio, G., Arpaia, S., Narváez-Vasquez, C. A., Cabello, T., and Urbaneja, A. (2010). Biological invasion of European tomato crops by *Tuta absoluta*: ecology, geographic expansion and prospects for biological control. *J. Pest Sci.* 83, 197–215. doi:10.1007/s10340-010-0321-6.

Filgueiras, C. C., Willett, D. S., Pereira, R. V., Moino Junior, A., Pareja, M., and Duncan, L. W. (2016). Eliciting maize defense pathways aboveground attracts belowground biocontrol agents. *Sci. Rep.* 6, 36484. doi:10.1038/srep36484.

Goettel, M. S., and Inglis, D. G. (1997). *Fungi: Hyphomycetes*. doi:10.1016/B978-012432555-5/50013-0.

Gouinguené, S., Alborn, H., and Turlings, T. C. J. (2003). Induction of volatile emissions in maize by different larval instars of *Spodoptera littoralis*. *J. Chem. Ecol.* 29, 145–162. doi:10.1023/A:1021984715420.

Guedes, R. N. C. C., Roditakis, E., Campos, M. R., Haddi, K., Bielza, P., Siqueira, H. A. A. A., and Nauen, R. (2019). Insecticide resistance in the tomato pinworm *Tuta absoluta*: patterns, spread, mechanisms, management and outlook. *J. Pest Sci.* 92, 1329–1342. doi:10.1007/s10340-019-01086-9.

Hardie, J., Isaacs, R., Pickett, J. A., Wadhams, L. J., and Woodcock, C. M. (1994). Methyl salicylate and (-)-(1 R,5 S)-myrtenal are plant-derived repellents for black bean aphid, *Aphis fabae* Scop. (Homoptera: Aphididae). *J. Chem. Ecol.* 20, 2847–2855. doi:10.1007/BF02098393.

Helms, A. M., De Moraes, C. M., Tröger, A., Alborn, H. T., Francke, W., Tooker, J. F., and Mescher, M. C. (2017). Identification of an insect-produced olfactory cue that primes plant defenses. *Nat. Commun.* 8, 337. doi:10.1038/s41467-017-00335-8.

Idriss, G. E. A., du Plessis, H., Khamis, F. M., Ekesi, S., Tanga, C. M., and Mohamed, S. A. (2020). Host range and effects of plant species on preference and fitness of *Tuta absoluta* (Lepidoptera: Gelechiidae). *J. Econ. Entomol.* 113, 1279–1289. doi:10.1093/jee/toaa002.

Inglis, G. D., Enkerli, J., and Goettel, M. S. (2012). *Laboratory techniques used for entomopathogenic fungi. Hypocreales.* Second Edi. Elsevier Ltd doi:10.1016/B978-0-12-386899-2.00007-5.

Jallow, M. F. A., Dugassa-Gobena, D., and Vidal, S. (2008). Influence of an endophytic fungus on host plant selection by a polyphagous moth via volatile spectrum changes. *Arthropod. Plant. Interact.* 2, 53–62. doi:10.1007/s11829-008-9033-8.

Khan, M., Mousa, A. A., Syamasundar, K. V., and Alkhathlan, H. Z. (2012). Determination of chemical constituents of leaf and stem essential oils of *Artemisia monosperma* from central Saudi Arabia. *Nat. Prod. Commun.* 7, 1079–1082. doi:10.1177/1934578x1200700829.

Kihika, R., Murungi, L. K., Coyne, D., Ng, M., Hassanali, A., Teal, P. E. A., and Torto, B. (2017). Parasitic nematode *Meloidogyne incognita* interactions with different *Capsicum annum* cultivars reveal the chemical constituents modulating root herbivory. *Sci. Rep.* 7, 2903. doi:10.1038/s41598-017-02379-8.

Kihika, R., Tchouassi, D. P., Ng'Ang'A, M. M., Hall, D. R., Beck, J. J., and Torto, B. (2020). Compounds associated with infection by the root-Knot nematode, *Meloidogyne javanica*, influence the ability of infective juveniles to recognize host plants. *J. Agric. Food Chem.* 68, 9100–9109. doi:10.1021/acs.jafc.0c03386.

Kottb, M., Gigolashvili, T., Großkinsky, D. K., and Piechulla, B. (2015). *Trichoderma* volatiles effecting *Arabidopsis*: from inhibition to protection against phytopathogenic fungi. *Front. Microbiol.* 6, 995. doi:10.3389/fmicb.2015.00995.

La Spada, F., Stracquadanio, C., Riolo, M., Pane, A., and Cacciola, S. O. (2020). *Trichoderma* counteracts the challenge of *Phytophthora nicotianae* infections on tomato by modulating plant defense mechanisms and the expression of crinkler, necrosis-inducing *Phytophthora* protein 1, and cellulose-binding elicitor lectin pathogenic effecto. *Front. Plant Sci.* 11, 583539. doi:10.3389/fpls.2020.583539.

Lin, Y., Qasim, M., Hussain, M., Akutse, K. S., Avery, P. B., Dash, C. K., et al. (2017). The herbivore-induced plant volatiles methyl salicylate and menthol positively affect growth and pathogenicity of entomopathogenic fungi. *Sci. Rep.* 7, 40494. doi:10.1038/srep40494.

Loake, G., and Grant, M. (2007). Salicylic acid in plant defence-the players and protagonists. *Curr. Opin. Plant Biol.* 10, 466–472. doi:10.1016/j.pbi.2007.08.008.

Moujahed, R., Frati, F., Cusumano, A., Salerno, G., Conti, E., Peri, E., and Colazza, S.

(2014). Egg parasitoid attraction toward induced plant volatiles is disrupted by a non-host herbivore attacking above or belowground plant organs. *Front. Plant Sci.* 5, 601. doi:10.3389/fpls.2014.00601.

Mutune, B., Ekesi, S., Niassy, S., Matiru, V., Bii, C., and Maniania, N. K. (2016). Fungal endophytes as promising tools for the management of bean stem maggot *Ophiomyia phaseoli* on beans *Phaseolus vulgaris*. *J. Pest Sci.* 89, 993–1001. doi:10.1007/s10340-015-0725-4.

Njuguna, P. K., Murungi, L. K., Fombong, A., Teal, P. E. A., Beck, J. J., and Torto, B. (2018). Cucumber and tomato volatiles: Influence on attraction in the melon fly *Zeugodacus cucurbitate* (Diptera: Tephritidae). *J. Agric. Food Chem.* 66, 8504–8513. doi:10.1021/acs.jafc.8b03452.

Paradza, V. M., Khamis, F. M., Yusuf, A. A., Subramanian, S., Ekesi, S., and Akutse, K. S. (2021). Endophytic colonisation of *Solanum lycopersicum* and *Phaseolus vulgaris* by fungal endophytes promotes seedlings growth and hampers the reproductive traits, development, and survival of the greenhouse whitefly, *Trialeurodes vaporariorum*. *Front. Plant Sci.* 12, 771534. doi:10.3389/fpls.2021.771534.

Pérez-Hedo, M., Alonso-Valiente, M., Vacas, S., Gallego, C., Rambla, J. L., Navarro-Llopis, V., and Urbaneja, A. (2021). Eliciting tomato plant defenses by exposure to herbivore induced plant volatiles. *Entomol. Gen.* 41, 209–218. doi:10.1127/entomologia/2021/1196.

Petrini, O., and Fisher, P. J. (1986). Fungal endophytes in *Salicornia perennis*. *Trans. - Br. Mycol. Soc.* 87, 647–651. doi:10.1016/S0007-1536(86)80109-7.

Pineda, A., Soler, R., Weldegergis, B. T., Shimwela, M. M., Van Loon, J. J. A., and Dicke, M. (2013). Non-pathogenic rhizobacteria interfere with the attraction of parasitoids to aphid-induced plant volatiles via jasmonic acid signalling. *Plant, Cell Environ.* 36, 393–404. doi:10.1111/j.1365-3040.2012.02581.x.

Pineda, A., Zheng, S. J., van Loon, J. J. A., Pieterse, C. M. J., and Dicke, M. (2010). Helping plants to deal with insects: The role of beneficial soil-borne microbes. *Trends Plant Sci.* 15, 507–514. doi:10.1016/j.tplants.2010.05.007.

Poveda, J. (2021a). Glucosinolates profile of *Arabidopsis thaliana* modified root colonization of *Trichoderma* species. *Biol. Control* 155, 104522. doi:10.1016/j.biocontrol.2020.104522.

Poveda, J. (2021b). *Trichoderma* as biocontrol agent against pests: New uses for a

mycoparasite. *Biol. Control* 159, 104634. doi:10.1016/j.biocontrol.2021.104634.

Poveda, J., Abril-Urias, P., and Escobar, C. (2020). Biological control of plant-parasitic nematodes by filamentous fungi inducers of resistance: *Trichoderma*, mycorrhizal and endophytic fungi. *Front. Microbiol.* 11, 992. doi:10.3389/fmicb.2020.00992.

Pratt, C. F., Constantine, K. L., and Murphy, S. T. (2017). Economic impacts of invasive alien species on African smallholder livelihoods. *Glob. Food Sec.* 14, 31–37. doi:10.1016/j.gfs.2017.01.011.

Proffit, M., Birgersson, G., Bengtsson, M., Reis, R., Witzgall, P., and Lima, E. (2011). Attraction and oviposition of *Tuta absoluta* females in response to tomato leaf volatiles. *J. Chem. Ecol.* 37, 565–74. doi:10.1007/s10886-011-9961-0.

R Core Team (2019). R: A language and environment for statistical computing R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org/.

Rostás, M., Ton, J., Mauch-Mani, B., and Turlings, T. C. J. (2006). Fungal infection reduces herbivore-induced plant volatiles of maize but does not affect naïve parasitoids. *J. Chem. Ecol.* 32, 1897–1909. doi:10.1007/s10886-006-9147-3.

Schulz, B., Guske, S., Dammann, U., and Boyle, C. (1998). Endophyte-host interactions. II. Defining symbiosis of the endophyte-host interaction. *Symbiosis* 25, 213–227.

Shi, X., Chen, G., Tian, L., Peng, Z., Xie, W., Wu, Q., et al. (2016). The salicylic acid-mediated release of plant volatiles affects the host choice of *Bemisia tabaci*. *Int. J. Mol. Sci.* 17, 1048. doi:10.3390/ijms17071048.

Shiojiri, K., and Takabayashi, J. (2003). Effects of specialist parasitoids on oviposition preference of phytophagous insects: encounter–dilution effects in a tritrophic interaction. *Ecol. Entomol. 28*, 573-578.

Shoresh, M., Harman, G. E., and Mastouri, F. (2010). Induced Systemic Resistance and Plant Responses to Fungal Biocontrol Agents. *Annu. Rev. Phytopathol.* 48, 21–43. doi:10.1146/annurev-phyto-073009-114450.

Shrivastava, G., Ownley, B. H., Augé, R. M., Toler, H., Dee, M., Vu, A., and Chen, F. (2015). Colonization by arbuscular mycorrhizal and endophytic fungi enhanced terpene production in tomato plants and their defense against a herbivorous insect. *Symbiosis* 65, 65–74. doi:10.1007/s13199-015-0319-1.

Siddaiah, C. N., Satyanarayana, N. R., Mudili, V., Kumar Gupta, V., Gurunathan, S., Rangappa, S., and Srivastava, R. K. (2017). Elicitation of resistance and associated defense responses in *Trichoderma hamatum* induced protection against pearl millet

downy mildew pathogen. *Sci. Rep.* 7, 1–18. doi:10.1038/srep43991.

Silva, D. B., Bueno, V. H. P., Loon, J. J. A. Van, Peñaflor, M. F. G. V, Bento, J. M. S., and van Lenteren, J. C. (2018). Attraction of three mirid predators to tomato infested by both the tomato leaf mining moth *Tuta absoluta* and the whitefly *Bemisia tabaci*. *J. Chem. Ecol.* 44, 29–39. doi:10.1007/s10886-017-0909-x.

Singh, U. B., Malviya, D., Singh, S., Kumar, M., Sahu, P. K., Singh, H. V., and Saxena, A. K. (2019). *Trichoderma harzianum*- and methyl jasmonate-induced resistance to bipolaris sorokiniana through enhanced phenylpropanoid activities in bread wheat (*Triticum aestivum* L.). *Front. Microbiol.* 10, 1697. doi:10.3389/fmicb.2019.01697.

Sobhy, I. S., Caulfield, J. C., Pickett, J. A., and Birkett, M. A. (2020). Sensing the danger signals: *cis*-Jasmone reduces aphid performance on potato and modulates the magnitude of released volatiles. *Front. Ecol. Evol.* 7, 1–14. doi:10.3389/fevo.2019.00499.

Sokame, B. M., Ntiri, E. S., Ahuya, P., Torto, B., Le Ru, B. P., Kilalo, D. C., and Calatayaud, P. A. (2019). Caterpillar-induced plant volatiles attract conspecific and heterospecific adults for oviposition within a community of lepidopteran stemborers on maize plant. *Chemoecology* 29, 89–101. doi:10.1007/s00049-019-00279-z.

Strapasson, P., Pinto-zevallos, D. M., and Zarbin, P. H. G. (2014). Enhancing plant resistance at the seed stage: low concentrations of methyl jasmonate reduce the performance of the leaf miner *Tuta absoluta* but do not alter the behavior of its predator *Chrysoperla externa*. *J. Chem. Ecol.* 40, 1090–1098. doi:10.1007/s10886-014-0503-4.

Thaler, J. S. (1999). Induced resistance in agricultural crops: Effects of jasmonic acid on herbivory and yield in tomato plants. *Environ. Entomol.* 28, 30–37. doi:10.1093/ee/28.1.30.

Thaler, J. S., Humphrey, P. T., and Whiteman, N. K. (2012). Evolution of jasmonate and salicylate signal crosstalk. *Trends Plant Sci.* 17, 260–270. doi:10.1016/j.tplants.2012.02.010.

Turlings, T. C. J., Loughrin, J. H., Mccall, P. J., Röse, U. S. R., Lewis, W. J., and Tumlinson, J. H. (1995). How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. U. S. A.* 92, 4169–4174. doi:10.1073/pnas.92.10.4169.

Turlings, T. C. J., Tumlinson, J. H., and Lewis, W. J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250, 1251–1253.

Vega, F. E. (2008). Insect pathology and fungal endophytes. *J. Invertebr. Pathol.* 98, 277–

279. doi:10.1016/j.jip.2008.01.008.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Ruocco, M., Woo, S., and Lorito, M. (2012). *Trichoderma* secondary metabolites that affect plant metabolism. *Nat. Prod. Commun.* 7, 1545–1550. doi:10.1177/1934578x1200701133.

von Mérey, G. E., Veyrat, N., D'Alessandro, M., and Turlings, T. C. J. (2013). Herbivore-induced maize leaf volatiles affect attraction and feeding behavior of *Spodoptera littoralis* caterpillars. *Front. Plant Sci.* 4, 209. doi:10.3389/fpls.2013.00209.

Wei, Q. Y., Li, Y. Y., Xu, C., Wu, Y. X., Zhang, Y. R., and Liu, H. (2020). Endophytic colonization by *Beauveria bassiana* increases the resistance of tomatoes against *Bemisia tabaci*. *Arthropod. Plant. Interact.* 14, 289–300. doi:10.1007/s11829-020-09746-9.

Wheeler, M. W., Park, R. M., and Bailer, A. J. (2006). Comparing median lethal concentration values using confidence interval overlap or ratio tests. *Environ. Toxicol. Chem.* 25, 1441–1444. doi:10.1897/05-320R.1.

Wu, Q., Zhang, L., Xia, H., Yu, C., Dou, K., Li, Y., and Chen, J. (2017). Omics for understanding synergistic action of validamycin A and *Trichoderma asperellum* GDFS1009 against maize sheath blight pathogen. *Sci. Rep.* 7, 1–11. doi:10.1038/srep40140.

Xu, Q., Hatt, S., Lopes, T., Zhang, Y., Bodson, B., Chen, J., and Francis, F. (2018). A push–pull strategy to control aphids combines intercropping with semiochemical releases. *J. Pest Sci.* 91, 93–103. doi:10.1007/s10340-017-0888-2.

Yactayo-Chang, J. P., Mendoza, J., Willms, S. D., Rering, C. C., Beck, J. J., and Block, A. K. (2021). *Zea mays* volatiles that influence oviposition and feeding behaviors of *Spodoptera frugiperda*. *J. Chem. Ecol.* 47, 799–809. doi:10.1007/s10886-021-01302-w.

CHAPTER FIVE

Transcriptional profiles reveal up-regulation of genes involved in the activation of tomato plant defense pathways mediated by the endophyte *Trichoderma asperellum* M2RT4 against *Phthorimaea absoluta*

This chapter is formatted as a manuscript for submission to Journal of Experimental Botany as:

Agbessenou, A., Akutse, K. S., Yusuf, A. & Khamis, F. M. (2022). Transcriptional analysis reveals up-regulation of genes involved in the activation of tomato plant defense pathways mediated by the endophyte *Trichoderma asperellum* M2RT4 against *Phthorimaea absoluta.*

Abstract

Endophytic fungi are microbial and asymptomatic microorganisms that live within host plant tissues, and are known to provide beneficial effects to their hosts. We previously established that the endophytic fungus *Trichoderma asperellum* M2RT4 protects tomato plants against the South American pinworm, *Phthorimaea absoluta* through the reduction of oviposition, leafmining, pupation and adult emergence. We further demonstrated that *T. asperellum* M2RT4 modulates tomato plant chemistry and primes both salicylic and jasmonic acid defense pathways in tomato plants. However, the underlying molecular mechanism mediating interaction between the endophyte, *P. absoluta* and the tomato host plant remains unknown. Here, we use whole-transcriptome sequencing to characterise and compare transcriptional changes involved in colonised, colonised-infested, and non-colonised infested tomato plants. We identified nine differentially expressed genes (DEGs) common to both colonised and colonised-infested plants while 14 DEGs were expressed in non-colonised infested plants. Specifically, we found that *N*-methyltransferase ATXR7 and the indeterminate-domain 5 protein were up-regulated in colonised tomato plant while the transcription repressor OFP6 was down-regulated. One DEG (XRI1-like protein) was shared between colonised-infested and non-colonised infested plants while the remaining genes showed diverging expression pattern. This study enhanced our understanding of the gene regulatory networks governing endophyte-tomato-*P. absoluta* interactions.

5.1.Introduction

Plants harbour diverse microbes that can be beneficial (Poveda, 2021a). Endophytic fungi are naturally occurring microbes that inhabit plants without causing any apparent symptoms to them. They are known to promote nutrients uptake, elicit production of metabolites, and trigger resistance against biotic and abiotic stressors (Paradza et al., 2021; Pieterse et al., 2014). To counteract the actions of attacking pests, plants must initiate an immune response that is timely, accurate, and effective (Moore et al., 2011). Plant cells have sophisticated gene transcription factors that help the plants to regulate their development, communication, and response to environmental stressors (Moore et al., 2011). Endophytic fungi are known to modify plant physiology and biochemistry by mediating expression of defensive genes, and production of secondary metabolites leading to dramatic transcriptional reprogramming to protect host plant against biotic stressors (Raad et al., 2019).

An appropriate defense response to biotic attacks requires initial recognition (Artico et al., 2014). Herbivores or beneficial organisms are recognised when conserved patterns of molecules, known as herbivore- or microbe-associated molecular patterns (HAMP or MAMP) (Artico et al., 2014; Morán Diez et al., 2009) are detected by pattern recognition receptors (PRR) on the surface of the host plant cell, leading to HAMP-triggered immunity (HTI) (Artico et al., 2014). Following recognition of the beneficial organism or the attacker, plants use different signalling cascades to reprogram their phenotypes. This transcriptional reprogramming is regulated by a network of signalling pathways in which phytohormones such as salicylic acid (SA) and jasmonic acid (JA) play a central role (Pieterse 2009). For example, Coppola et al. (2019) reported that the endophyte *T. harzianum* T22 induces transcriptional changes in colonised tomato plants which display overexpression of transcripts encoding several families of defense-related transcription factors. Moreover, some *Trichoderma* species are known to produce microbe-associated molecular patterns (MAMPs) that induce plant defense responses including the production of elicitors in tomato (Morán Diez et al., 2009).

The South American tomato pinworm, *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the most devastating insect pests of tomato and other solanaceous crops in Africa, causing huge yield losses owing to its leafmining activity (Aigbedion-Atalor et al., 2019). Currently, *P. absoluta* is managed through indiscriminate use of synthetic insecticides, but mostly are not effective due to the cryptic feeding behaviour of the immature stages and the development of resistance against different classes of synthetic

insecticides (Desneux et al., 2021). Additionally, this insecticide control method has unprecedented effects on human, environmental, animal and soil health (Akutse et al., 2020). This therefore resulted in the need to search for alternatives to chemical control to manage *P. absoluta* infestations with a great interest in developing biological control approaches using natural enemies (predators, parasitoids or microbials). Endophytic fungi have been reported to be effective and promising control methods in managing both the adult and immature stages of the pest (Agbessenou et al., 2020). *Trichoderma asperellum* M2RT4 has been proved to be an efficient/potent endophytic fungus that could successfully colonise tomato host plant tissues (Agbessenou et al., 2020). The presence of this beneficial fungus within tomato host plant has been reported to provide systemic protection against the destructive immature and potential damage from oviposition by *P. absoluta* female moths (Agbessenou et al., 2020). Recently, we demonstrated that upon host plant colonisation, *T. asperellum* M2RT4 primes tomato plant defense pathways (JA and SA) which negatively affect *P. absoluta* oviposition and herbivory (Agbessenou et al., 2022). However, the underlying genetic regulatory mechanisms mediating these interactions between endophytic fungi, tomato, and *P. absoluta* and how endophytic fungi prime plant defense systems in the tomato-*P. absoluta* model remains to be elucidated.

Next generation sequencing (NGS) technologies are increasingly used to study the molecular mechanisms involved in plant-microbe-pest interactions (Chen et al., 2022). For example, transcriptomics studies using RNA-sequencing have been instrumental to improve the understanding of gene expression changes that occur within host plants upon colonisation by endophytes (Doni et al., 2019; Oshiquiri et al., 2020). Therefore, this chapter aimed at investigating the transcriptional regulation of gene expression in endophyte-tomato-*P. absoluta* interactions.

5.2.Materials and methods

Fungal culture

Trichoderma asperellum M2RT4 isolate obtained from *icipe*'s Arthropod Pathology Unit Germplasm was cultured on potato dextrose agar (PDA) (OXOID CM0139, Oxoid Ltd., Basingstoke, UK), and maintained at 25 ± 2 °C in complete darkness. Conidia were harvested by scraping the surface of two to three-week-old sporulated cultures using a sterile spatula. The harvested conidia were then suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 (MERCK KGaA, Darmstadt, Germany) and vortexed for

five minutes at about 700 rpm to break conidial clumps and ensure a homogenous suspension (Agbessenou et al., 2020; Akutse et al., 2013). Conidial concentrations were quantified using an improved Neubauer hemocytometer under a light microscope (Goettel and Inglis, 1997). The conidial suspension was adjusted to a concentration of 1×10^8 conidia mL^{-1} through serial dilutions before inoculating the tomato seeds.

Prior to commencement of the bioassays, spore viability was determined by plating evenly 0.1 mL of 3×10^6 conidia mL^{-1} onto 9-cm Petri dishes containing PDA. Three sterile microscope cover slips (2 × 2 cm) were placed randomly on the surface of the inoculated plates. Plates were sealed with Parafilm and incubated in complete darkness at 25 ± 2 °C and were examined after 16–20 h. The germination rate of conidia was determined from 100 randomly selected conidia on the surface area covered by each cover slip under a light microscope (×400) using the method described by Goettel aand Inglis (1997). Conidia were considered to have germinated when the length of the germ tube was at least twice the diameter of the conidium (Goettel and Inglis, 1997). Four replicates were used for this experiment.

Seed inoculation and colonisation assessment of the endophyte *Trichoderma asperellum* M2RT4

The *Solanum lycopersicum* Moneymaker cultivar, used in the present study was purchased locally from Simlaw Seeds Company Ltd., Nairobi, Kenya. Seeds were surface-sterilised by washing successively in 70% ethanol for two minutes followed by 1.5% sodium hypochlorite for three minutes and finally rinsed thrice in sterile distilled water. The seeds were then placed on sterile filter paper on a clean working surface under a cabinet until the residual water evapourated. Effectiveness of the surface sterilisation technique was confirmed by plating out 0.1 mL of the last rinse water onto potato dextrose agar (PDA) supplemented with 100 mg/L Streptomycin and also imprinting of surface sterilised seeds onto PDA (tissue imprint) and plates were incubated at 25 °C for 14 days (Schulz et al., 1998). Seeds were then soaked overnight for 12 h in conidial suspensions titrated at 1×10^8 conidia mL^{-1}. For the control, sterilised seeds were soaked overnight for 12 h in sterile distilled titrated (0.05% Triton X-100) water (Agbessenou et al., 2020). Seeds were then transferred into plastic pots (8 cm diameter × 7.5 cm high) containing the planting substrate with a volume of 0.5 L (mixture of manure and soil 1:5). The substrate was sterilised in an autoclave for 2 h at 121 °C and allowed to cool for 72 h prior to planting. Five seeds were sowed per pot and

maintained at room temperature (25 ± 2 °C, 60% RH and 12:12 L:D photoperiod). Pots were transferred immediately after germination to the screen house (2.8 m length × 1.8 m width × 2.2 m height) at 25 ± 2 °C, 55% RH and 12:12 L:D photoperiod for 4–5 weeks. After germination, seedlings were thinned to two per pot and watered twice (~ 150 cm^3) per day (morning and evening). No additional fertiliser was added to the planting substrate. Three to four week-old plants after transplanting were used for the experiments.

To confirm endophytic colonisation, tomato plants were carefully uprooted from the pots four to five weeks after inoculation and washed under running tap water to remove any soil attached to the plants. Seedlings (ca. 30 cm in height) were divided into three different sections (ca. 5 cm long): leaves, stems and root sections using a sterile scalpel (Agbessenou et al., 2020; Akutse et al., 2013). Five randomly selected leaf, stem and root sections from each plant were surface-steriliszed as described above. The different plant parts were then aseptically cut under a laminar flow hood into 1 × 1 cm pieces before placing them at 4 cm apart on PDA plates supplemented with a 0.05% solution of antibiotic (streptomycin sulphate salt) (Akutse et al., 2013). Plates were incubated at 25 ± 1 °C for 10 days, after which the presence of endophytes was determined. The last rinse water was also plated to assess the effectiveness of the surface sterilisation procedure as described earlier. Plate imprinting was also conducted to assess effective surface sterilisation of plant materials (Inglis et al., 2012). The colonisation of the different plant parts was recorded by counting the number of pieces of the different plant parts that showed the presence or outgrowth of the inoculated fungal growth/mycelia according to Koch's postulates (Petrini and Fisher, 1986). Only the presence of the endophyte (*T. asperellum* M2RT4) that was inoculated was scored. Fungal isolate was identified morphologically using slides which were prepared from the mother plates. The experiment was replicated four times.

Insects

A colony of *P. absoluta* was established from wild moths and larvae collected from infested tomato leaves and fruits in Mwea (0° 36′ 31.3″ S 037° 22′ 29.7″ E), Kenya in June 2019. The moths were kept in ventilated, sleeved Perspex cages (40 × 40 × 45 cm) and were fed *ad libitum* with 10% honey solution placed on the top side of each cage as food source (Agbessenou et al., 2020). Four potted tomato plants were placed in the cages for oviposition. The plants were removed 24 h post-exposure to female insects and transferred to separate wooden cages (50 × 50 × 60 cm) ventilated with netting material at the sides and on the top

until the eggs hatched. Leaves with larvae were removed from these plants, three days after the larvae hatched and placed into a clean sterile plastic container (21 cm long × 15 cm wide × 8 cm high) lined with paper towel to absorb excess moisture and fine netting infused lid for ventilation. The larvae were supplied daily with fresh tomato leaves as food until they pupated. The pupae were collected from the plastic containers using a fine camel hair-brush and placed inside clean plastic containers for adult emergence. The colony was rejuvenated every three months through infusion, with infested tomato leaves collected from the field to reduce inbreeding (Agbessenou et al., 2020; Akutse et al., 2020). Insects were maintained under a rearing condition of 28 ± 2 °C, 48% RH and 12:12 L:D photoperiod at the Animal Rearing and Quarantine Unit (ARQU) of *icipe* for five generations prior to bioassays.

RNA extraction, sequencing, and library preparation

Total RNA was extracted from non-colonised, colonised, non-colonised infested and colonised infested tomato plants of six independent biological replicates per treatment. Four-week-old plant were infested with 20 *P. absoluta* second instar larvae for seven days. Tomato leaves were then harvested and frozen immediately in liquid nitrogen where they were mechanically ground into a fine powder, and total RNA extracted using the Isolate II RNA Plant Mini Kit (Bioline, London, UK), following the manufacturer's instructions. The RNA was suspended in 60 µL RNase-free water, and RNA yield was determined using a Nanodrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Preparation of libraries for RNA sequencing using nanopores

Messenger RNA (mRNA) was isolated and Nanopore libraries were prepared using the nanopore direct cDNA native barcoding kit (SQK-DCS109 with EXP-NBD104, Oxford Nanopore Technologies) according to manufacturer's instructions. First-strand cDNA was synthesised by SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific) using random hexamers. The RNA–cDNA hybrid was then purified using Agencourt RNAClean XP magnetic beads (Beckman Coulter). Libraries were loaded onto R9.4 SpotON Flow Cells (Oxford Nanopore Technologies) and sequenced using a 6 h run time. Six biological replicates were performed for each treatment.

Processing of nanopore data, reads alignment and transcripts quantification

Nanopore libraries were sequenced using a FLO-MIN106 flow cells. The data were generated using the MinKNOW 20.10.3 and basecalled with Guppy (version 4.2.2) fast model. Base-called data were trimmed using Porechop (v_0.2.4, no further parameters set). Long reads were mapped to the *Solanum lycopersicum* IGT3 genome using Minimap2 (version 2.24) (Li and Durbin, 2010). Sequence Alignment/Map (SAM) and BAM file manipulations were performed using Samtools (version 1.9).

Statistical analyses

Differential expression (DE) analysis was carried out with the DESeq2 (Galaxy Version 2.11.40.7) (Love et al., 2014). Benjamini-Hochberg procedure was used for multiple testing corrections. Genes with log2(fold-change) > 0.5 and q < 0.05 were considered as differentially expressed. These thresholds were used to select for relevant and robust differentially expressed genes.

5.3.Results

Transcriptomic reprogramming upon *Trichoderma asperellum* M2RT4 colonisation and *Phthorimaea absoluta* infestation

Colonisation of the tomato plant with the endophyte *T. asperellum* M2RT4 induced differential expression of nine genes. Of these nine genes, four were up-regulated while five were down-regulated (Table 5.1, Figure 5.1). *N*-methyltransferase ATXR7 and the chloroplastic-like protein indeterminate-domain 5 were up-regulated while the transcription repressor OFP6 was downregulated in the colonised tomato plant. Furthermore, the transcription profiles revealed nine and 14 DEGs in colonised-infested and non-colonised infested tomato plants, respectively (Table 5.1, Figure 5.1). One DEG (XRI1-like protein) was shared between colonised-infested and non-colonised infested plants while the remaining showed diverging expression patterns (Table 5.1, Figure 5.1).

Table 5.1. Significantly differentially expressed genes in endophytically-colonised, colonised-infested, and non-colonised infested tomato plants

Treatment	Gene ID	Log_2FC	*P*-value	Gene name	Gene product
Colonised	MSTRG.1057	-2.75	6E-3	-	Transcription repressor OFP6
	MSTRG.1284	-1.90	6E-3	-	Hypothetical protein
	MSTRG.630	-2.79	2E-3	-	Hypothetical protein
	MSTRG.860	0.90	3E-3	-	Histone-lysine N-methyltransferase ATXR7
	MSTRG.1285	0.76	3E-3	-	Hypothetical protein
	MSTRG.267	-1.70	3E-3	-	Hypothetical protein
	MSTRG.596	-0.92	3E-3	-	Hypothetical protein
	MSTRG.1017	0.95	3E-3	-	Hypothetical protein
	MSTRG.646	0.83	3E-3	-	Chloroplastic-like protein indeterminate-domain 5
Colonised-infested	MSTRG.2231	7.75	8E-4	-	XRI1-like protein
	MSTRG.781	5.77	2E-3	-	Hypothetical protein
	MSTRG.602	6.76	3E-3	-	Hypothetical protein
	MSTRG.1009	-6.49	5E-3	-	Hypothetical protein
	MSTRG.1133	5.83	5E-3	-	Hypothetical protein
	MSTRG.1747	-5.94	4E-3	-	U1 spliceosomal RNA
	Solyc10g052880.1	-5.76	2E-2	-	Hypothetical protein
	MSTRG.62	5.99	3E-2	-	Hypothetical protein
	MSTRG.498	4.62	3E-2	-	Hypothetical protein
Non-colonised infested	MSTRG.491	-9.00	2E-10	-	Hypothetical protein
	MSTRG.577	-9.85	2E-09	-	Hypothetical protein
	MSTRG.2231	-7.48	3E-4	-	XRI1-like protein
	MSTRG.602	-6.58	1E-4	-	Hypothetical protein
	MSTRG.1491	3.76	4E-3	-	Hypothetical protein
	MSTRG.1094	-4.40	2E-2	-	Hypothetical protein
	MSTRG.362	-4.62	2E-2	-	Hypothetical protein
	MSTRG.62	-5.76	3E-2	-	Hypothetical protein
	MSTRG.2289	3.39	3E-2	-	Hypothetical protein
	MSTRG.522	2.63	4E-2	-	Hypothetical protein
	MSTRG.781	-3.34	4E-2	-	Hypothetical protein
	MSTRG.1615	-4.36	4E-2	-	Hypothetical protein
	MSTRG.2324	-4.74	4E-2	-	Hypothetical protein
	MSTRG.83	-4.71	4E-2	-	Hypothetical protein

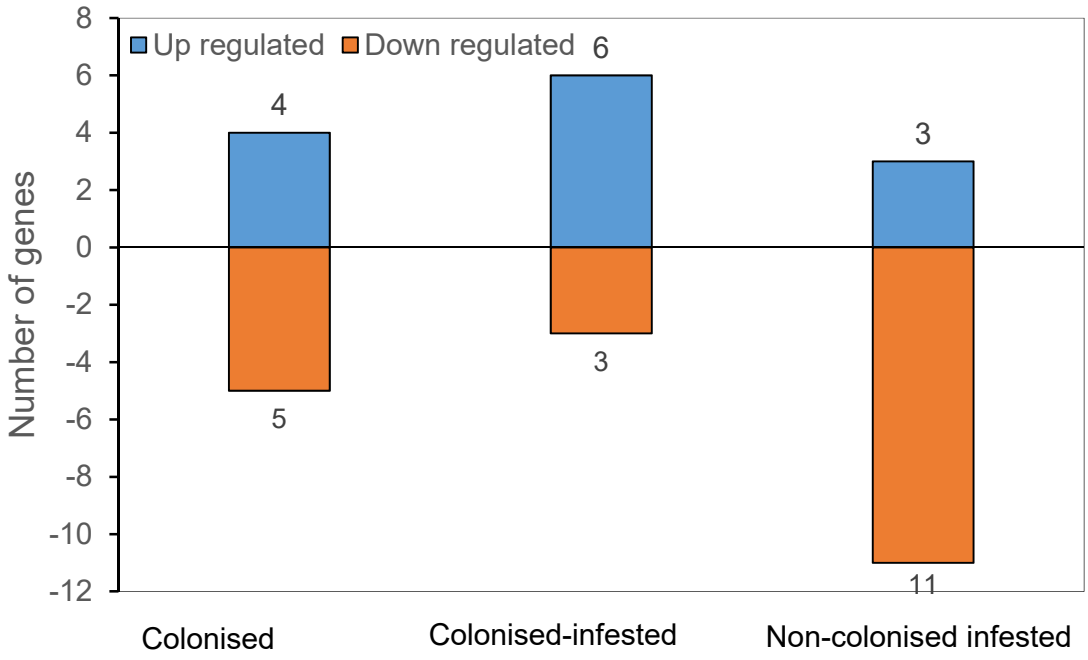

Figure 5.1. Bar graph showing the number of significantly differentially expressed genes in endophytically-colonised, colonised-infested, and non-colonised infested tomato plants.

5.4.Discussion

In Chapter 4, we showed that *T. asperellum* M2RT4 primes tomato plant defense pathways (JA and SA) which negatively affect *P. absoluta* host location and herbivory. Here, our results reveal important transcriptomic reprogramming upon *T. asperellum* M2RT4 colonisation and *P. absoluta* herbivory, providing insight into their role of systemic resistance provided to tomato host plant. Chapters 4 and 5 have therefore significantly improved our understanding of the underlying chemical and molecular mechanisms by which *T. asperellum* M2RT4 affects *P. absoluta* within tomato host plant.

In this study, we showed that, in the interaction between *Trichoderma*-tomato plant, few genes involved in defense responses as well as in growth and development were differentially expressed. This is the case of the transcription repressor OFP6, which was down-regulated. Previous studies have shown that most of OFP genes are involved in a range of biological processes (plant growth and development), and their functions are often found to be associated with plant hormones and environmental stresses (Wang et al., 2021). Notably, Yu et al. (2015) reported that OFP proteins are predominantly localised in the rice and may act as transcriptional regulators during seed development.

We found that upon colonisation of tomato plant, *T. asperellum* M2RT4 induces up-regulation of *N*-methyltransferase ATXR7. It was previously reported that members of methyltransferase family play a significant role in modulating host plants response against biotic and abiotic stressors through the induction of systemic acquired resistance (SAR) (Chen et al., 2003). This was evidenced by the chemical modulation of tomato plant where the presence of *T. asperellum* M2RT4 within the host plant induces the systemic release of methyl salicylate (Agbessenou et al., 2022). Methyl salicylate (MeSA) is a plant hormone that is synthesised *in planta* via a reaction catalysed by methyltransferases whereby a methyl group is transferred from the donour molecule S-adenosine-L-methionine (SAM) to the carboxyl group of salicylic acid (SA) (Chen et al., 2003). It is worth noting that salicylic acid is a crucial signal molecule for the activation of plant defense responses. Therefore, this finding suggests that MeSA production is triggered by the presence of *T. asperellum* M2RT4 within the tomato host plant.

We also found an up-regulation of the indeterminate-domain 5 protein in colonised tomato plants. Indeterminate-domain proteins (IDDs) belong to a diverse plant-specific of transcriptional regulators that coordinate distinct functions during plant growth and development (Völz et al., 2019). Notably, indeterminate-domain 5 protein was reported to serve as transcriptional scaffold and enable transactivation activity of the gibberellin-inhibitor DELLA/RGA proteins (Yoshida et al., 2014). It is therefore worth noting that gibberellins are diterpene phytohormones that regulate many cellular and developmental events such as cell elongation, leaf expansion, flowering, pollen maturation, and the transition from vegetative growth to flowering (Yoshida et al., 2014). Consequently, this could explain the growth promotion observed in the *T. asperellum* M2RT4-endophytically colonised tomato seedlings.

In this study, we have shown that more DEGs were mostly down-regulated in response to feeding by *P. absoluta* larvae. These results are supported by previous studies reporting that infestations of host plants by insect pests drive transcriptional suppression over induction (Dubey et al., 2013). This is in contrast with Liu et al. (2016) who reported that more DEGs were up-regulated than down-regulated in rice plant in response to feeding by *Chilo suppressalis* (Lepidoptera: Crambidae) larvae. However, these results are in agreement with Li et al. (2016) who showed that more DEGs were down-regulated than up-regulated, when cotton plants were infested with the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae). Immature stages of *P. absoluta* feed on the leaves of their host plants by creating irregular

mines while *B. tabaci* is a sap-sucking insect. This confirms the hypothesis that damages caused by herbivorous pests exhibiting different feeding behaviour might lead to similar host plant defense response (Huang et al., 2022). Interestingly, we found that the transcriptome reprogramming in response to *P. absoluta* infestation revealed the up-regulation of XRI1-like protein in colonised infested plants while it was down-regulated in non-colonised infested plants. *X-ray induced 1* (XRI1) was previously reported to be an important gene responsible for plant fertility where it plays a crucial role in meiosis and male gametogenesis (Dean et al., 2009).

In conclusion, the transcriptional analysis has enhanced our understanding on the gene regulatory network(s) governing endophyte-tomato-*P. absoluta* interactions demonstrated in Chapter 4. These findings show the possibility that some defense-related proteins have a role in signaling in response to *P. absoluta* attack or upon colonisation by the endophyte *T. asperellum* M2RT4. Further studies using selected genes for validation should be conducted through real-time quantitative polymerase chain reaction (RT-qPCR).

References

Agbessenou, A., Akutse, K. S., Yusuf, A. A., Ekesi, S., Subramanian, S., and Khamis, F. M. (2020). Endophytic fungi protect tomato and nightshade plants against *Tuta absoluta* (Lepidoptera: Gelechiidae) through a hidden friendship and cryptic battle. *Sci. Rep.* 10, 22195. doi:10.1038/s41598-020-78898-8.

Agbessenou, A., Akutse, K. S., Yusuf, A. A., and Khamis, F. M. (2022). The endophyte *Trichoderma asperellum* M2RT4 induces the systemic release of methyl salicylate and (Z)-jasmone in tomato plant affecting host location and herbivory of *Tuta absoluta*. *Front. Plant Sci.* 13, 860309. doi:10.3389/fpls.2022.860309.

Aigbedion-Atalor, P. O., Hill, M. P., Zalucki, M. P., Obala, F., Idriss, G. E., Midingoyi, S.-K., Sunday, E., and Mohamed, S. (2019). The South America tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae), spreads its wings in Eastern Africa: distribution and socioeconomic impacts. *J. Econ. Entomol.* 112, 2797–2807. doi:10.1093/jee/toz220.

Akutse, K. S., Maniania, N. K., Fiaboe, K. K. M., Van den Berg, J., and Ekesi, S. (2013). Endophytic colonization of *Vicia faba* and *Phaseolus vulgaris* (Fabaceae) by fungal pathogens and their effects on the life-history parameters of *Liriomyza huidobrensis*

(Diptera: Agromyzidae). *Fungal Ecol.* 6, 293–301. doi:10.1016/j.funeco.2013.01.003.

Akutse, K. S., Subramanian, S., Khamis, F. M., Ekesi, S., and Mohamed, S. A. (2020). Entomopathogenic fungus isolates for adult *Tuta absoluta* (Lepidoptera: Gelechiidae) management and their compatibility with *Tuta* pheromone. *J. Appl. Entomol.*, 1–11. doi:10.1111/jen.12812.

Artico, S., Ribeiro-Alves, M., Oliveira-Neto, O. B., de Macedo, L. L. P., Silveira, S., Grossi-de-Sa, M. F., and Alves-Ferreira, M. (2014). Transcriptome analysis of *Gossypium hirsutum* flower buds infested by cotton boll weevil (*Anthonomus grandis*) larvae. *BMC Genomics* 15, 1–24. doi:10.1186/1471-2164-15-854.

Chen, F., D'Auria, J. C., Tholl, D., Ross, J. R., Gershenzon, J., Noel, J. P., and Pichersky, E. (2003). An *Arabidopsis thaliana* gene for methyl salicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J.* 36, 577–588. doi:10.1046/j.1365-313X.2003.01902.x.

Chen, X., Sun, M., Chong, S., Si, J., and Wu, L. (2022). Transcriptomic and metabolomic approaches deepen our knowledge of plant–endophyte interactions. *Front. Plant Sci.* 12, 700200. doi:10.3389/fpls.2021.700200.

Coppola, M., Diretto, G., Digilio, M. C., Woo, S. L., Giuliano, G., Molisso, D., Lorito, M., and Rao, R. (2019). Transcriptome and metabolome reprogramming in tomato plants by *Trichoderma harzianum* strain T22 primes and enhances defense responses against aphids. *Front. Physiol.* 10, 745. doi:10.3389/fphys.2019.00745.

Dean, P. J., Siwiec, T., Waterworth, W. M., Schlögelhofer, P., Armstrong, S. J., and West, C. E. (2009). A novel ATM-dependent X-ray-inducible gene is essential for both plant meiosis and gametogenesis. *Plant J.* 58, 791–802. doi:10.1111/j.1365-313X.2009.03814.x.

Desneux, N., Han, P., Mansour, R., Arnó, J., Brévault, T., Campos, M. R., Chailleux, A., Guedes, R. N., Karimi, J., Perez-Hedo, M., Urbaneja, A., Verheggen, F. J., Zappalà, L., Abbes, K., Bayram, Y., Cantor, F., Cuthbertson, A. G., Firake, D. M., Ismoilov, K., Messelink, G., Nofemela, R., Oke, A., Ramos, C., Ricupero, M., Roditakis, E., Zhang, Y-B., and Biondi, A. (2021). Integrated pest management of *Tuta absoluta*: practical implementations across different world regions. *J. Pest Sci.* 1–23. doi:10.1007/s10340-021-01442-8.

Doni, F., Muhamad, F. F., Mispan, S., Suhaimi, N. S. M., Yusoff, W. M. W., and Uphoff, N. (2019). Transcriptomic profiling of rice seedlings inoculated with the symbiotic

fungus *Trichoderma asperellum* SL2. *J. Plant Growth Regul.* 38, 1507–1515. doi:10.1007/s00344-019-09952-7.

Dubey, N. K., Goel, R., Ranjan, A., Idris, A., Singh, S. K., Bag, S. K., Singh, P. K., and Sawant, S. V. (2013). Comparative transcriptome analysis of *Gossypium hirsutum* L. in response to sap sucking insects: Aphid and whitefly. *BMC Genomics* 14, 1–20. doi:10.1186/1471-2164-14-241.

Goettel, M. S., and Inglis, D. G. (1997). *Fungi: Hyphomycetes*. doi:10.1016/B978-012432555-5/50013-0.

Huang, X., Kou, J., Jing, W., Han, X., Liu, D., Ghasemzadeh, S., Shi, W., and Zhang, Y. (2022). Transcriptomic and metabolomic reprogramming in cotton after *Apolygus lucorum* feeding implicated in enhancing recruitment of the parasitoid *Peristenus spretus*. *J. Pest Sci.* 95, 249–262. doi:10.1007/s10340-021-01369-0.

Inglis, G. D., Enkerli, J., and Goettel, M. S. (2012). *Laboratory techniques used for entomopathogenic fungi. Hypocreales.* Second Edi. Elsevier Ltd doi:10.1016/B978-0-12-386899-2.00007-5.

Li, H., and Durbin, R. (2010). Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 26, 589–595. doi:10.1093/bioinformatics/btp698.

Li, J., Zhu, L., Hull, J. J., Liang, S., Daniell, H., Jin, S., et al. (2016). Transcriptome analysis reveals a comprehensive insect resistance response mechanism in cotton to infestation by the phloem feeding insect *Bemisia tabaci* (Whitefly). *Plant Biotechnol. J.*, 1956–1975. doi:10.1111/pbi.12554.

Liu, Q., Wang, X., Tzin, V., Romeis, J., Peng, Y., and Li, Y. (2016). Combined transcriptome and metabolome analyses to understand the dynamic responses of rice plants to attack by the rice stem borer *Chilo suppressalis* (Lepidoptera: Crambidae). *BMC Plant Biol.* 16, 259. doi:10.1186/s12870-016-0946-6.

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 1–21. doi:10.1186/s13059-014-0550-8.

Moore, J. W., Loake, G. J., and Spoel, S. H. (2011). Transcription dynamics in plant immunity. *Plant Cell* 23, 2809–2820. doi:10.1105/tpc.111.087346.

Morán Diez, E., Hermosa, R., Ambrosino, P., Cardoza, R. E., Gutiérrez, S., Lorito, M., and Monte, E. (2009). The ThPG1 endopolygalacturonase is required for the *Trichoderma harzianum*-plant beneficial interaction. *Mol. Plant-Microbe Interact.* 22, 1021–1031.

doi:10.1094/MPMI-22-8-1021.

Oshiquiri, L. H., dos Santos, K. R. A., Ferreira Junior, S. A., Steindorff, A. S., Barbosa Filho, J. R., Mota, T. M., and Georg, R. C. (2020). *Trichoderma harzianum* transcriptome in response to cadmium exposure. *Fungal Genet. Biol.* 134, 103281. doi:10.1016/j.fgb.2019.103281.

Paradza, V. M., Khamis, F. M., Yusuf, A. A., Subramanian, S., Ekesi, S., and Akutse, K. S. (2021). Endophytic colonisation of *Solanum lycopersicum* and *Phaseolus vulgaris* by fungal endophytes promotes seedlings growth and hampers the reproductive traits, development, and survival of the greenhouse whitefly, *Trialeurodes vaporariorum*. *Front. Plant Sci.* 12, 771534. doi:10.3389/fpls.2021.771534.

Petrini, O., and Fisher, P. J. (1986). Fungal endophytes in *Salicornia perennis*. *Trans. - Br. Mycol. Soc.* 87, 647–651. doi:10.1016/S0007-1536(86)80109-7.

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M., and Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* 52, 347–375. doi:10.1146/annurev-phyto-082712-102340.

Poveda, J. (2021). Trichoderma as biocontrol agent against pests: New uses for a mycoparasite. *Biol. Control* 159, 104634. doi:10.1016/j.biocontrol.2021.104634.

Raad, M., Glare, T. R., Brochero, H. L., Müller, C., and Rostás, M. (2019). Transcriptional reprogramming of *Arabidopsis thaliana* defence pathways by the entomopathogen *Beauveria bassiana* correlates with resistance against a fungal pathogen but not against insects. *Front. Microbiol.* 10, 3389. doi:10.3389/fmicb.2019.00615.

Schulz, B., Guske, S., Dammann, U., and Boyle, C. (1998). Endophyte-host interactions. II. Defining symbiosis of the endophyte-host interaction. *Symbiosis* 25, 213–227.

Völz, R., Rayapuram, N., and Hirt, H. (2019). Phosphorylation regulates the activity of INDETERMINATE-DOMAIN (IDD/BIRD) proteins in response to diverse environmental conditions. *Plant Signal. Behav.* 14, e1642037. doi:10.1080/15592324.2019.1642037.

Wang, H., Chen, J. G., and Chang, Y. (2021). Identification, expression, and interaction analysis of ovate family proteins in Populus trichocarpa reveals a role of PtOFP1 regulating drought stress response. *Front. Plant Sci.* 12, 650109. doi:10.3389/fpls.2021.650109.

Yoshida, H., Hirano, K., Sato, T., Mitsuda, N., Nomoto, M., Maeo, K., Kawamura, M., Tada, T., Ohme-Takagi, M., Matsuoka, M., and Ueguchi-Tanaka, M. (2014). DELLA protein

functions as a transcriptional activator through the DNA binding of the INDETERMINATE DOMAIN family proteins. *Proc. Natl. Acad. Sci. U. S. A.* 111, 7861–7866. doi:10.1073/pnas.1321669111.

Yu, H., Jiang, W., Liu, Q., Zhang, H., Piao, M., Chen, Z., and Bian, M. (2015). Expression pattern and Subcellular localization of the ovate protein family in rice. *PLoS One* 10, 1–19. doi:10.1371/journal.pone.0118966.

CHAPTER SIX

General discussion, conclusion, and recommendations

6.1 General discussion

The main challenge of agriculture in this century is to produce enough food that is nutritious for the growing world population in a sustainable manner while protecting human, environmental and animal health (Rubio et al., 2020). The outbreak and spread of insect pests has been highly facilitated by human activities, travel, and worldwide transport of agricultural products, but also climate change, thus resulting in the invasion of new pests across unaffected regions (Biganski et al., 2021). In about a decade, the South American tomato pinworm, *P. absoluta*, has become one of the most damaging insect pests of tomato and other solanaceous crops in Africa (Aigbedion-Atalor et al., 2019). Entomopathogenic fungi (EPF) have been reported to offer an effective and viable alternative to synthetic insecticides, as they cause significant epizootics in the target hosts through an inundative approach (Akutse et al., 2020a), and they can also be used as plant endophytes whose presence within host plants are beneficial (Agbessenou et al., 2020; Paradza et al., 2021). Therefore, this research assessed the efficacy of candidate entomopathogenic- and endophytic-fungal isolates with the aim of integrating them into a sustainable management program to control *P. absoluta*.

In Chapter two, this study examined the thermotolerance and virulence of three candidate entomopathogenic fungal isolates against *P. absoluta* moths under different temperature regimes. In Chapter three, the endophytic properties of 15 fungal isolates on both tomato and nightshade host plants were evaluated and their insecticidal activity against both adult and immature stages of *P. absoluta* was investigated. Chapters four and five unravelled the underlying chemical and molecular mechanisms by which the presence of the endophyte *Trichoderma asperellum* M2RT4 within tomato host plant affects *P. absoluta* host selection and herbivory, respectively. The selection of *T. asperellum* M2RT4 isolate to study the chemical and molecular interactions in this research, was based on the antagonistic activity it has against several pests of economic importance including greenhouse whitefly, *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) (Paradza et al., 2021), the bean stem maggot, *Ophiomyia phaseoli* (Diptera: Agromyzidae) (Mutune et al., 2016) and the pea leafminer, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) (Akutse et

al., 2013). This study will therefore help fast-track the registration and commercialisation of *T. asperellum* M2RT4 isolate. This chapter highlights the synthesis of the key findings from each of the preceding chapters by bringing together temperature-dependent modelling studies, chemical ecology, and transcriptomics of plant-insect-microbe interactions and highlighting areas for future research.

Fungal virulence, accurate spatial prediction, and mass production potential are among the important parameters required for selecting potent and or effective candidate isolate(s) for biopesticide development and deployment (Bayissa et al., 2017; Tumuhaise et al., 2018; Wraight et al., 2007). Temperature plays an important role in the success of entomopathogenic fungi as microbial agents in the field due to its potential deleterious effect on fungal growth, sporulation, and arthropod infections (Bayissa et al., 2017; Dimbi et al., 2004; Ekesi et al., 1999; Tumuhaise et al., 2018). Results from the present study revealed that the optimum temperatures for conidial germination were observed at 20 °C, 25 °C, and 30 °C for all the three tested *M. anisopliae* isolates (ICIPE 18, ICIPE 20, and ICIPE 655) (Chapter 2). Previous studies estimated the temperature range for growth and sporulation of most *M. anisopliae* isolates between 15 °C and 35 °C (Bayissa et al., 2017; Dimbi et al., 2004; Tumuhaise et al., 2018). In addition to the *in vitro* germination and growth, the highest level of virulence against *P. absoluta* moths was observed at 25 and 30 °C for all three fungal isolates. Rapid germination is an important attribute of virulent strains; facilitating infection of target pests when climatic conditions are favorable (Ekesi et al., 1999; Onsongo et al., 2019). Subsequently, spatial prediction studies using entomopathogenic fungi application (EPFA) software for simulating the performance of the most potent isolates (ICIPE 18 and ICIPE 20) against *P. absoluta* moths indicate that environmental conditions appeared to be conducive to the pathogens' virulence across the three countries (Kenya, Tanzania, and Uganda). These two fungal isolates are also known to be highly virulent against several insect pests of economic importance occurring in Africa (Akutse et al., 2020b; Migiro et al., 2010). Although these candidate biopesticides hold considerable promise to manage *P. absoluta*, unpredictable weather conditions in the field could seriously undermine their performance leading to poor delivery. The main challenge of predicting the spatial virulence of fungal pathogens lies in the high variability of the environmental factors. It is therefore important to assess the combined impact of temperature and relative humidity on the

performance of the candidate biopesticides in future studies. From a commercial standpoint, these isolates provide great advantage since fungi with a broader spectrum of activity are more attractive if they provide control of more than one pest with a single application (Mweke et al., 2018; Srinivasan et al., 2019). Therefore, a prerequisite for their registration and commercialisation requires multilocation field validation trials before their deployment.

Considering the versatility of microbial agents, the use of endophytes for sustainable management of insect pests and diseases has also become a new prominent area of interest. Among the 15 fungal isolates tested in this study, 12 were endophytic to both tomato and nightshade host plants with varying colonisation rates while *M. anisopliae* isolates failed to colonise both host plants (Chapter 3). Previously, the failure of *M. anisopliae* isolates to establish as endophyte in French bean and Faba bean has also been demonstrated by Akutse et al. (2013) and Mutune et al. (2016). This might be due to innate characteristics of the fungal isolate (Posada et al., 2007) or host plant genetics (Hardoim et al., 2015). Interestingly, irrespective of the host plant, fungal isolates belonging to the genera *Fusarium* (*F. proliferatum* F2S51), *Trichoderma* (*T. asperellum* M2RT4, *T. atroviride* F2S21 and *Trichoderma* sp. F2L41) and *Hypocrea* (*H. lixii* F3ST1) have demonstrated high colonisation rates of all tomato and nightshade plant tissues. It is also important to note that, competition with other endophytes naturally occurring within host plants could lead to differential colonisation rates of plants by some fungal isolates (Jaber and Ownley, 2018). After overcoming the two host plants' physical barriers, the endophytic fungi spread throughout the different cell layers of their host. In addition to the colonisation property as endophytes, we observed that *T. asperellum* M2RT4, *B. bassiana* ICIPE 706 and *H. lixii* F3ST1 not only endophytically-colonised the host plants, but also outperformed the other isolates in conferring systemic resistance against *P. absoluta* immature and adult stages (Chapter 3). Typically, the number of eggs laid on endophytically-colonised tomato and nightshade plants was very low, and the level of damage was significantly reduced as compared to the control (Chapter 3). Even though there was strong evidence of mortality of insects feeding on endophytically-colonised plants, there was no signs of fungal outgrowth on the cadavers indicating that the cause of death of the insects remains elusive (Chapter 3). However, it has been hypothesised that negative effects on herbivorous insects could be as a result of antibiosis and feeding deterrence mediated by the presence of the endophytes *in*

planta, triggering the production of secondary metabolites (Golo et al., 2014; Jaber and Vidal, 2009). Thus, both host plants and endophytic fungal isolates exhibited symbiotic relationship which allows both partners to evolve strategies such as chemical and molecular mechanisms for mutual adaptation following *P. absoluta* herbivory. The results obtained corroborate the hypothesis that resistance could be enhanced against *P. absoluta* upon endophytic plant colonisation. The enhanced protection in tomato and nightshade plants could therefore most likely due to the activation of different signaling pathways triggered by the presence of endophytic fungal isolates. Even though *T. asperellum* M2RT4, *B. bassiana* ICIPE 706 and *H. lixii* F3ST1 have shown high potential to be developed as endophytic-fungal-based biopesticide for the management of *P. absoluta*, further studies are warranted to validate the findings under field conditions. In addition, considering the integrated pest and pollinator management (IPPM) paradigm, further studies aimed at assessing the effect of endophytically-colonised plants on the performance of insect pollinators should be investigated.

Plants release chemical signals which are known to play an important role in the location and selection of suitable oviposition sites (Turlings et al., 1990). In the Y-tube olfactometer assays, we observed that *P. absoluta* female moths preferred non-colonised tomato plants against those colonised by endophytes. The results showed that *P. absoluta* female moths were not attracted neither to non-colonised infested nor to colonised-infested tomato plants (Chapter 4). This preference for non-colonised tomato plant is a result of the ability of females to select sites that are most favourable for the survival of their offspring (Silva et al., 2021). Further, the findings revealed the emission of a bouquet of VOCs largely dominated by monoterpenes and sesquiterpenes. Interestingly, we found that the quantitative variation in the emission of these volatiles across the four treatments was only evident in the monoterpenes including 2-carene, α-pinene, α-phellandrene, β-phellandrene, but not in sesquiterpenes such as δ-elemene, α-cedrene, (*E*)-β-caryophyllene and α-humulene (Chapter 4). Upon colonisation of tomato plant by *T. asperellum* M2RT4, we observed that the endophyte triggers a cascade of defense mechanisms in tomato plant providing protection against both the adult and immature stages of *P. absoluta* (Chapters 3 and 4). This has been evidenced by the production of phytohormones (methyl salicylate and (*Z*)-jasmone) by tomato plant which we reported to be responsible for the reduction of the pest oviposition and leafmining activity (Chapters 3 and 4). The emission of methyl salicylate by colonised

tomato plants is known as systemic acquired resistance (SAR) (Pieterse et al., 2014; Shoresh et al., 2010). Systemic acquired resistance, commonly triggered by local infection, can provide long-term resistance throughout the plant to subsequent attack by biotic stressors (Shoresh et al., 2005). This *Trichoderma*-induced priming comes at a cost for the tomato plant as it reduces volatile emissions of some key compounds (Chapter 4). When present in tomato plants, *T. asperellum* alters the composition of volatile emissions by inducing a quantitative difference between non-colonised and colonised plants. Specifically, we found that endophytically-colonised tomato plants elicited a reduction in the emission of several compounds including α-pinene, β-pinene, 2-carene, α-phellandrene, β-phellandrene as compared to non-colonised plants. When assessing the behavioural responses of *P. absoluta* female moths towards the synthetic compounds, it was found that some monoterpenes (α-pinene, and β-phellandrene) attracted the moths while methyl salicylate repelled them (Chapter 4) (Conboy et al., 2020; Xu et al., 2018). Therefore, further studies should be conducted under field conditions to assess the attractiveness/repellence of the semiochemicals α-pinene, 2-carene and β-phellandrene and methyl salicylate for sustainable management of *P. absoluta*.

Furthermore, the findings showed that induced systemic resistance (ISR) is triggered upon local recognition of elicitors such as volatile organic compounds (Chapter 4) and microbe-associated molecular patterns (MAMPs) (Chapter 5) and then cascade into a systemic response by the host plant. It is worth mentioning that the phytohormone (*Z*)-jasmone released by endophytically-colonised infested tomato plants accounted for the poor performance of the larval stage of *P. absoluta* (Chapter 5). This demonstrates that the mechanisms underlying *T. asperellum* M2RT4 effect on *P. absoluta* control within tomato host plant are diverse. This systemic resistance mechanism is likely the result of the modulation of the plant defense network that may translate *Trichoderma*-induced early signaling events into a more efficient activation of defense responses (Martinez-Medina et al., 2013). This clear potential of endophytic fungi for use as part of sustainable crop protection is already being upscaled among private sector actors and smallholder farmers across several countries in Africa (Akutse et al., 2020b).

This study demonstrated that, among the genes that are activated in tomato host plant in response to endophytic colonisation, some are involved in transcriptional regulation and

defense (Chapter 5). For example, the findings established that upon colonisation of tomato plant, *T. asperellum* M2RT4 induces up-regulation of *N*-methyltransferase ATXR7. Interestingly, it was previously reported that members of methyltransferase family play a significant role in modulating host plants response against biotic and abiotic stressors through the induction of systemic acquired resistance. The results also showed that more DEGs were down-regulated than up-regulated in response to feeding by *P. absoluta* larvae. However, further studies using selected genes for validation should be conducted through real-time quantitative polymerase chain reaction (RT-qPCR) which will help to develop effective strategies for sustainable management of *P. absoluta*. Also, since no silver-bullet has been found so far to sustainably manage the pest, there is an urgent need to develop a comprehensive IPM strategy with the aim to assess the compatibility of the most promising control tactics (EPF, endophytes, parasitoids, RNA interference technology) within the context of an integrated *P. absoluta* management.

6.2 Conclusion

Metarhizium anisopliae isolates ICIPE 18 and ICIPE 20 were found to be effective against *P. absoluta* female moths and could be developed as biopesticides based on their efficacy across a broad range of temperature regimes (germination, growth and sporulation), speed of kill and virulence against the pest. In addition, both isolates can successfully be mass-produced on rice using a simple, fast, and cost-effective mass-production technique (especially for private sector for business incubation) suitable for deployment in the field.

Twelve fungal isolates successfully established as endophytes in tomato and nightshade host plants through seed inoculation and could also potentially be explored for their systemic resistance effects against other solanaceous pests such as aphids and whitefly as well as diseases.

Trichoderma asperellum M2RT4, *B. bassiana* ICIPE 706 and *H. lixii* F3ST1 were identified as the most potent endophytic fungal isolates improving tomato and nightshade defenses against *P. absoluta* through the reduction of adult oviposition, leafmining, pupation and adult emergence as compared to other treatments. This finding would significantly contribute to the improvement of tomato productivity when integrated into the management of the pest under tomato and other solanaceous cropping systems.

In planta colonisation of tomato plant tissues by *T. asperellum* M2RT4 modifies the chemical profile and response of the host plant affecting the ability of *P. absoluta* female moths to locate their host.

The endophytic fungus *T. asperellum* M2RT4 quantitatively and qualitatively alters tomato plant volatiles composition by activating both the SA and JA plant defense pathways. This finding provides a clear understanding of the mechanism behind the detrimental effects of the endophytically-colonised tomato plant to the pest location and herbivory.

The transcriptome analysis showed that *in planta* colonisation of tomato plant by *T. asperellum* M2RT4 activates tomato plant defense pathways through the expression of *N*-methyltransferase ATXR7 protein.

6.3 Recommendations

1. Field validation trials using the most potent *M. anisopliae* isolates (ICIPE 18 and ICIPE 20) should be conducted under different agroecological zones based on the model results targeting *P. absoluta* moths using an "attract-and-infect" strategy. This could be achieved using an auto-dissemination device in the field where baited-Delta traps will be inoculated with conidia powder, which will serve to attract and infect *P. absoluta* male moths. The trapped insects will be infected with the fungal inoculum and will subsequently disseminate the pathogen to conspecific insects once they return to the environment.

2. Further studies are needed to clearly assess the effect of the potent endophytic fungal isolates *T. asperellum* M2RT4, *B. bassiana* ICIPE 706 and *H. lixii* F3ST1 against *P. absoluta* under field conditions. A possible way to do this is to conduct multilocational field trials to assess the effect of endophytically-colonised tomato and nightshade plants on the life history parameters of *P. absoluta*. It is also important to further assess the potential of these endophytes on major tomato diseases management.

3. Neither the entomopathogenic fungi nor the endophytic fungi identified in this study can be used as a stand-alone control measure for *P. absoluta*, therefore their incorporation into a comprehensive IPM strategy would be more efficient rather than promoting them individually. Such study can be performed under field conditions by combining different control tactics (EPF, endophytes, parasitoids) with the aim to assess their compatibility within the context of an integrated *P. absoluta* management.

4. Field trials should be conducted to assess the attractiveness/repellence of the semiochemicals α-pinene, 2-carene and β-phellandrene and methyl salicylate for sustainable management of *P. absoluta*. In addition, these key volatiles should also be tested against the major *P. absoluta* associated natural enemies (parasitoids and predators) for effective integrative management and suppression of the pest population under different cropping systems.

5. Further studies using selected genes for validation should be conducted through real-time quantitative polymerase chain reaction (RT-qPCR) which will help to design effective strategies for sustainable management of *P. absoluta*. In addition, further studies are warranted to explore the Actin-depolymerizing factors (ADFs) and Toll-like receptors (TLRs) gene family, and their relative expression rates in the endophytically-colonised tomato host plant.

6. Proteomic and metabolomic studies should be investigated to understand the underlying mechanisms that *T. asperellum* M2RT4 mediates resistance in tomato plant against *P. absoluta*.

References

Agbessenou, A., Akutse, K. S., Yusuf, A. A., Ekesi, S., Subramanian, S., and Khamis, F. M. (2020). Endophytic fungi protect tomato and nightshade plants against *Tuta absoluta* (Lepidoptera: Gelechiidae) through a hidden friendship and cryptic battle. *Sci. Rep.* 10, 22195. doi:10.1038/s41598-020-78898-8.

Aigbedion-Atalor, P. O., Hill, M. P., Zalucki, M. P., Obala, F., Idriss, G. E., Midingoyi, S.-K., Sunday, E., and Mohamed, S. (2019). The South America tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae), spreads its wings in Eastern Africa: distribution and socioeconomic impacts. *J. Econ. Entomol.* 112, 2797–2807. doi:10.1093/jee/toz220.

Akutse, K. S., Maniania, N. K., Fiaboe, K. K. M., Van den Berg, J., and Ekesi, S. (2013). Endophytic colonization of *Vicia faba* and *Phaseolus vulgaris* (Fabaceae) by fungal pathogens and their effects on the life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Fungal Ecol.* 6, 293–301. doi:10.1016/j.funeco.2013.01.003.

Akutse, K. S., Subramanian, S., Khamis, F. M., Ekesi, S., and Mohamed, S. A. (2020a). Entomopathogenic fungus isolates for adult *Tuta absoluta* (Lepidoptera: Gelechiidae)

management and their compatibility with *Tuta* pheromone. *J. Appl. Entomol.*, 1–11. doi:10.1111/jen.12812.

Akutse, K. S., Subramanian, S., Maniania, N. K., Dubois, T., and Ekesi, S. (2020b). Biopesticide research and product development in Africa for sustainable agriculture and food security – Experiences from the International Centre of Insect Physiology and Ecology (icipe). *Front. Sustain. Food Syst.* 4, 563016. doi:10.3389/fsufs.2020.563016.

Bayissa, W., Ekesi, S., Mohamed, S. A., Kaaya, G. P., Wagacha, J. M., Hanna, R., and Maniania, N. K. (2017). Selection of fungal isolates for virulence against three aphid pest species of crucifers and okra. *J. Pest Sci.* 90, 355–368. doi:10.1007/s10340-016-0781-4.

Biganski, S., Fückel, S., Jehle, J. A., and Kleespies, R. G. (2021). Infection effects of the new microsporidian species *Tubulinosema suzukii* on its host *Drosophila suzukii*. *Sci. Rep.* 11, 10151. doi:10.1038/s41598-021-89583-9.

Conboy, N. J. A., McDaniel, T., George, D., Ormerod, A., Edwards, M., Donohoe, P., and Tosh, C. R. (2020). Volatile organic compounds as insect repellents and plant elicitors: an integrated pest management (IPM) strategy for glasshouse whitefly (*Trialeurodes vaporariorum*). *J. Chem. Ecol.* 46, 1090–1104. doi:10.1007/s10886-020-01229-8.

Dimbi, S., Maniania, N. K., Lux, S. A., and Mueke, J. M. (2004). Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. *BioControl* 49, 83–94. doi:10.1023/B:BICO.0000009397.84153.79.

Ekesi, S., Maniania, N. K., and Ampong-Nyarko, K. (1999). Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Sci. Technol.* 9, 177–185. doi:10.1080/09583159929767.

Golo, P. S., Gardner, D. R., Grilley, M. M., Takemoto, J. Y., Krasnoff, S. B., Pires, M. S., Bittencourt, V. R. E. P. and Roberts, D. W. (2014). Production of destruxins from *Metarhizium* spp. fungi in artificial medium and in endophytically colonized cowpea plants. *PLoS One* 9, 1–9. doi:10.1371/journal.pone.0104946.

Hardoim, P. R., Overbeek, L. S. Van, Berg, G., Pirttilä, M., Compant, S., Campisano, A., and Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol.*

Rev. 79, 293–320. doi:10.1128/mmbr.00050-14.

Jaber, L. R., and Ownley, B. H. (2018). Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? *Biol. Control* 116, 36–45. doi:10.1016/j.biocontrol.2017.01.018.

Jaber, L. R., and Vidal, S. (2009). Interactions between an endophytic fungus, aphids and extrafloral nectaries: Do endophytes induce extrafloral-mediated defences in *Vicia faba*? *Funct. Ecol.* 23, 707–714. doi:10.1111/j.1365-2435.2009.01554.x.

Martinez-Medina, A., Fernandez, I., Sanchez-Guzman, M. J., Jung, S. C., Pascual, J. A., and Pozo, M. J. (2013). Deciphering the hormonal signaling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. *Front. Pl* 4, 1–12. doi:10.3389/fpls.2013.00206.

Migiro, L. N., Maniania, N. K., Chabi-Olaye, A., and Vandenberg, J. (2010). Pathogenicity of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales: Clavicipitaceae) isolates to the adult pea leafminer (Diptera: Agromyzidae) and prospects of an autoinoculation device for infection in the field. *Environ. Entomol.* 39, 468–475. doi:10.1603/en09359.

Mutune, B., Ekesi, S., Niassy, S., Matiru, V., Bii, C., and Maniania, N. K. (2016). Fungal endophytes as promising tools for the management of bean stem maggot *Ophiomyia phaseoli* on beans *Phaseolus vulgaris*. *J. Pest Sci. (2004).* 89, 993–1001. doi:10.1007/s10340-015-0725-4.

Mweke, A., Ulrichs, C., Nana, P., Akutse, K. S., Fiaboe, K. K. M., Maniania, N. K., and Sunday, E. (2018). Evaluation of the entomopathogenic fungi *Metarhizium anisopliae*, *Beauveria bassiana* and *Isaria* sp. for the management of *Aphis craccivora* (Hemiptera: Aphididdae). *J. Econ. Entomol.* 111, 1587–1594. doi:10.1093/jee/toy135.

Onsongo, S. K., Gichimu, B. M., Akutse, K. S., Dubois, T., and Mohamed, S. A. (2019). Performance of three isolates of *Metarhizium anisopliae* and their virulence against *Zeugodacus cucurbitae* under different temperature regimes, with global extrapolation of their efficiency. *Insects* 10, 1–13. doi:10.3390/insects10090270.

Paradza, V. M., Khamis, F. M., Yusuf, A. A., Subramanian, S., Ekesi, S., and Akutse, K. S. (2021). Endophytic colonisation of *Solanum lycopersicum* and *Phaseolus vulgaris* by fungal endophytes promotes seedlings growth and hampers the reproductive traits, development, and survival of the greenhouse whitefly, *Trialeurodes vaporariorum*.

Front. Plant Sci. 12, 771534. doi:10.3389/fpls.2021.771534.

Pieterse, M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Wees, S. C. M. Van, Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* 52, 347–375. doi:10.1146/annurev-phyto-082712-102340.

Posada, F., Aime, M. C., Peterson, S. W., Rehner, S. A., and Vega, F. E. (2007). Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). *Mycol. Res.* 111, 748–757. doi:10.1016/j.mycres.2007.03.006.

Rubio, L., Galipienso, L., and Ferriol, I. (2020). Detection of plant viruses and disease management: Relevance of genetic diversity and evolution. *Front. Plant Sci.* 11, 1–23. doi:10.3389/fpls.2020.01092.

Shoresh, M., Harman, G. E., and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48, 21–43. doi:10.1146/annurev-phyto-073009-114450.

Shoresh, M., Yedidia, I., and Chet, I. (2005). Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. *Phytopathology* 95, 76–84. doi:10.1094/PHYTO-95-0076.

Silva, G. A., Queiroz, E. A., Arcanjo, L. P., Lopes, M. C., Araújo, T. A., Galdino, T. S. V., and Picanço, M. (2021). Biological performance and oviposition preference of tomato pinworm *Tuta absoluta* when offered a range of Solanaceous host plants. *Sci. Rep.* 11, 1153. doi:10.1038/s41598-020-80434-7.

Srinivasan, R., Sevgan, S., Ekesi, S., and Tamò, M. (2019). Biopesticide based sustainable pest management for safer production of vegetable legumes and brassicas in Asia and Africa. *Pest Manag. Sci.* 886, 2446–2454. doi:10.1002/ps.5480.

Tumuhaise, V., Ekesi, S., Maniania, N. K. K., Tonnang, H. E. Z. E. Z., Tanga, C. M. M., Ndegwa, P. N. N., Srinivasan, R., and Mohamed, S. (2018). Temperature-dependent growth and virulence, and mass production potential of two candidate isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin for managing *Maruca vitrata* Fabricius (Lepidoptera: Crambidae) on cowpea. *African Entomol.* 26, 73–83. doi:10.4001/003.026.0073.

Turlings, T. C. J., Tumlinson, J. H., and Lewis, W. J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250, 1251–1253.

Wraight, S. P., Sporleder, M., and Lacey, L. A. (2007). Chapter VII-1 Application and

evaluation of entomopathogens in potato. *Techniques*, 329–359.

Xu, Q., Hatt, S., Lopes, T., Zhang, Y., Bodson, B., Chen, J., and Francis, F. (2018). A push–pull strategy to control aphids combines intercropping with semiochemical releases. *J. Pest Sci.* 91, 93–103. doi:10.1007/s10340-017-0888-2.