Antagonistic microbes for the management of *Cosmopolites sordidus* and *Radopholus similis* in East African bananas

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Thesis accepted in fulfilment of the requirements for the degree *Doctor of Philosophy in Science with Zoology* at the North-West University

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Graduation May 2023

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DEDICATION

To my beloved wife: Sophie Nassimbwa, our children: Abraham Lubwama, Benjamin Ssebagala, Sarah Nanteza and Hannah Nabwami, my parents: Mr. James Mpanga and Mrs. Eseza Mpanga for their love, perseverance, sacrifice, encouragement and prayers.

ACKNOWLEDGEMENTS

I wish to express my gratitude to the International Centre of Insect Physiology and Ecology (*icipe*) for providing me the chance to conduct my PhD research activities, and the North-West University as my host University. I gratefully acknowledge the German Academic Exchange Services (DAAD) for providing my university fees, stipend and part of the research funds through *icipe*'s African Regional Postgraduate Programme in Insect Science (ARPPIS) programme. I appreciate the support provided by *icipe*'s Capacity Building and Institutional Development (CB & ID) programme.

I acknowledge the European Union, through its Horizon 2020 research and innovation funding programme, for providing research funds to implement the "Microbial uptakes for sustainable management of major banana pests and diseases" (MUSA) project: grant number, 727624, under which I conducted my research activities.

I am extremely grateful to my supervisors: Prof. H Fourie, Dr. S Haukeland, Dr. L Cortada and Dr. D Coyne. Thank you for your invaluable mentorship and constant guidance though my PhD journey.

Special gratitude to all the members of *icipe*/IITA Nematology lab (NemAfrica), thank you for the continuous social and technical support whenever I needed it. A special thanks to members of the *icipe*'s Arthropod Pathology Unit (APU). I am particularly indebted to J Kimemia, P Wafula, L Ombura and M Adhiambo for the technical support offered while I conducted my lab work in the APU lab.

I'm greatly indebted to the IITA-Uganda team where I conducted objective 2 of my PhD work, special thanks to J Kisitu, S Nakimera and S Okello, your commitment to taking care of plants in the green house, setting up of the field experiment, dedication to data collection on a weekly basis and assistance in nematode counting have been a great contribution to my PhD research and progress, thank you.

I highly acknowledge my fellow postgraduate scholars; you've been a great support system through this journey. Finally, sincere appreciation to my wife who has been a strong pillar and graciously took on my obligations during my prolonged absence, and my children who have a been a point of encouragement.

ABSTRACT

The East African highland banana (EAHB, *Musa* spp.) is a key staple in East and Central Africa (ECA), where they provide up to 60% of total calorie consumed per capita. Banana production has declined since the 1970s, with the current production being a fraction of its potential. The banana weevil (BW), *Cosmopolites sordidus*, and a complex of plant-parasitic nematodes (PPN) are among the key biotic constraints to banana production. *Radopholus similis* is the most damaging nematode parasite of banana in ECA, often appearing in a complex with *Helicotylenchus multicinctus*, *Pratylenchus* spp. and *Meloidogyne* spp. Thus, developing proper and effective management strategies against both BW and PPN is crucial for the improvement and restoration of banana production in ECA. Therefore, this PhD study aimed at: 1) assessing the efficacy of indigenous entomopathogenic fungi (EPF) against BW, 2) evaluating the potential of local endophytic fungal isolates for the management of *R. similis* infection of banana and 3) testing the potential of multiple endophytic isolates for the management of *R. similis* infection of banana, 4) studying the possible mode of action of fungal endophytes against PPN infection of banana and 5) assessing the field performance of endophyte enhanced banana tissue culture plantlets with respect to suppression of PPN and BW infection, persistence of the endophytic fungus in the banana roots and impact on yield and longevity of the banana field. Amongst the 20 EPF isolates screened (15 *Beauveria bassiana*, 4 *Metarhizium anisopliae* and 1 *Isaria fumosorosea*), 8 *B. bassiana* isolates caused >50% mortality of adult BW, while *Metarhizium* and *Isaria* isolates were less pathogenic. *Beauveria bassiana* isolates ICIPE 273, ICIPE 648 and ICIPE 660 were the most pathogenic compared to other isolates, killing ≥80% of adult BW in the shortest time ($LT_{50} \leq 11.1$ days) and at the lowest spore concentration ($LC_{50} \leq 5.49 \times 10^7$ spores ml^{-1}). Furthermore, the three *Beauveria* isolates, ICIPE 273, ICIPE 648 and ICIPE 660 were able to sporulate on BW cadavers, producing $\geq 3.6 \times 10^7$ spores per BW cadaver after 14 days of incubation, an indication of the potential of these fungal isolates for self-dissemination.

On the other hand, the non-pathogenic *Fusarium oxysporum* (isolate V5w2) and the *B. bassiana* (isolate WA) were endophytically introduced into tissue cultured (TC) plants of the cooking banana *cv.* Mbwazirume and the dessert banana *cv.* Grande Naine which were then grown in the field for two cropping cycles. The fungal endophytes were inoculated separately and in combination. Colonisation of the banana plants by the fungal endophytes reduced from 45% root colonisation at field establishment to <3% colonisation at 6 months post field establishment, while no root colonisation was recorded beyond 6 months. Inoculation of the

banana plants with V5w2 alone significantly reduced nematode (*R. similis* and *H. multicinctus*) infection in the first cropping cycle by 35%, compared to 13% and 36% reductions when the plants were inoculated with WA alone or a combination (V5w2 and WA), respectively. Similarly, plants inoculated with V5w2 alone or a combination (V5w2 and WA) had higher yield (≥11 t ha^{-1}) compared to those inoculated with WA alone (8.6 t ha^{-1}) or the uninoculated plants (7.9 t ha^{-1}). This revealed that inoculation of the TC plants with V5w2 alone is ideal for nematode control and improvement of crop yield demonstrating the benefits of using fungal endophytes in improving the yield of both cooking and dessert banana through nematode suppression. However, these benefits were not observed in the ratoon crop, an indication that repeat in-field application of the fungal isolate is required to boost the endophyte inoculum in successive crop cycles.

To understand the mode of action of fungal endophytes in suppression of *R. similis* infection of banana, the EAHB *cv.* Ng'ombe was inoculated with the fungal endophytes *Trichoderma asperellum* (ICIPE 700) and *Hypocrea lixii* (ICIPE 697) individually and in combination. Inoculation of banana TC with the fungal endophytes significantly suppressed *R. similis* infection by >81% and led to enhanced upregulation of the defence-related gene *PR-1*, the cell wall strengthening gene *β-1,3-glucan synthase* and the cell signalling gene *calmodulin-*Ca^{2+}, an indication that defence genes play a critical role in the suppression of *R. similis* infection of endophyte-inoculated banana plants.

Keywords: *Musa* spp., banana weevil, nematodes, endophytes, entomopathogens, biological control, bio-enhanced plants

PREFACE

This work was carried out by J Kisaakye under the supervision of Prof. H Fourie, IPM sub-program, Unit for Environmental Sciences and Management, North-West University; Dr. S Haukeland, Plant Health Theme, International Centre of Insect Physiology and Ecology (*icipe*); and Dr. L Cortada, Nematology Unit, Department of Biology, Ghent University.

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Chapter 1 – Introduction and thesis structure (**NWU Harvard)**

Chapter 2 – Literature review (**NWU Harvard**)

Chapter 3 – Article 1 (published): **Agriculture (MDPI)**

Chapter 4 – Article 2 (published): **Agriculture (MDPI)**

Chapter 5 – Article 3 (prepared): **Nematology**

Chapter 6 – Conclusions and recommendations (**NWU Harvard)**

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Table A: Author contributions and consent for use of article / manuscript for this thesis.

Author	Article/manuscript	Contribution	Consent*
J Kisaakye	1, 2 and 3	Principal investigator: Designed and conducted experiments, acquired data, performed analysis and interpretation, and wrote the manuscript and thesis.	
H Fourie	1, 2 and 3	Promoter: Supervised the design and execution of the study, provided intellectual input on interpretation of findings as well as guidance during writing of the articles and thesis.	
S Haukeland	1, 2 and 3	Co-promoter: Supervised the design and execution of the study, provided intellectual input on interpretation of findings as well as guidance during writing of the articles and thesis. Secured funding for the study.	
L Cortada	1, 2 and 3	Co-promoter: Supervised the design and execution of the study, provided intellectual input on interpretation of findings as well as guidance during writing of the articles and thesis.	
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J Kisitu	1	Provided insights about experiment design, assisted with data acquisition and writing of the article.	
S Nakimera	1	Assisted with data acquisition, synthesis and writing of the article.	
S Masinde	2 and 3	Assisted with data acquisition, synthesis and writing of the articles.	
F M Khamis	3	Provided technical guidance, interpretation of results and writing of the manuscript.	

*I declare that the stated contributions are accurate and I consent to use of these article(s)/manuscript as part of the thesis of Mr. J. Kisaakye

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CHAPTER 1

General introduction

1.1 Introduction

Banana (*Musa* spp.) is an important food and cash crop globally, it is the 12th most important food crop in terms of production (4th in Africa) and the world's most important fruit (FAOSTAT, 2020). Banana is an important crop in the Great Lakes region of Africa both in terms of income and food security and is a staple food in East and Central Africa (ECA). The all-year-round fruiting habit of the crop ensures continuous supply of food. The ECA region, comprising of Burundi, Democratic Republic of Congo, Kenya, Rwanda, Tanzania and Uganda accounts for about 53% of the banana produced in sub-Saharan Africa and approximately one third of the global *Musa* production, which is equivalent to US$ 4.3 billion annually. The average daily per capita consumption of bananas in the ECA region ranges from 0.61 to over 1.6 kg, one of the highest daily consumption globally and providing up to 22% of the total calorie intake per capita (FAOSTAT, 2020).

The most common type of banana cultivated in the East African region are; the East African highland cooking bananas (EAHB; AAA-EA) commonly known as 'Matooke' and Mchare, with the dessert and plantain type accounting for <10% of the production (Gold *et al.*, 2002; Karamura *et al.*, 1998). Banana cultivation in this region mostly relies on small-scale farming, predominantly characterized by low inputs, high pest and disease incidences and thus reduced production (Van Asten *et al.*, 2010).

Banana production in East Africa is constrained by both abiotic and biotic factors. Nutrient deficiencies coupled with water stress are key primary abiotic constraints (Wairegi *et al.*, 2010), with nitrogen and potassium deficiencies accounting for up to 68% of the banana yield gap (Nyombi *et al.*, 2010; Taulya, 2013). The primary biotic constraints are pests, notably the banana weevil (BW; *Cosmopolites sordidus*) and a multifaceted array of plant-parasitic nematodes (PPN), which include the burrowing nematode *Radopholus similis*, the root-lesion nematode *Pratylenchus* spp., the spiral nematode *Helicotylenchus multicinctus* and the root knot nematodes *Meloidogyne* spp. (Murongo *et al.*, 2019; Nyang'au *et al.*, 2021; Sikora *et al.*, 2018; Ssango *et al.*, 2004). Additionally, several fungal, viral and bacterial diseases also form a complex of biotic constraints that hamper banana production (Kimunye *et al.*, 2020; Nakato *et al.*, 2018; Viljoen *et al.*, 2020; Vuylsteke *et al.*, 1993). The impacts of these constraints are

worsened by poor agronomic management (Wairegi *et al.*, 2010). Several strategies have been studied for the management BW and PPN, these include use of clean planting materials, treatment of planting materials with pesticides/nematicides, use of resistant varieties and use of eco-friendly biocontrol agents.

1.2 Problem statement

East African highland bananas are a key staple crop throughout the Great Lakes region of Eastern Africa. Recent yield declines of cooking banana in the region have been associated with increased occurrences of BW and PPN infections.

The BW and PPN are key below ground pests of banana. The weevil larvae primarily tunnels through the banana corm weakening plant stability and obstructing water and nutrient uptake. This damage results in plant loss through snapping, stunted plant growth and premature mortality before bunches are fully developed, reduced bunch weights, increased ratooning, mat disappearance and shortened plantation life (Gold *et al.*, 2001). Yield losses of up to 100% have been attributed to this pest (Sengooba, 1986). Moreover, recent studies have demonstrated the potential of BW as a vector for *Xanthomonas campestris pv. Musacearum*, the causal agent of banana bacterial wilt (Meldrum *et al.*, 2013) and *Fusarium oxysporum f. sp. cubense* tropical race 4, the causal agent of Fusarium wilt (Were *et al.*, 2015).

The migratory endo-parasitic nematodes; *R. similis*, *H. multicinctus* and *Pratylenchus* spp. are the most damaging PPN of banana. Nematode damage to banana roots results in reduced plant anchorage and thus plant toppling. The BW and PPN often co-exist in EAHB (Speijer *et al.*, 1994).

Preventive measures against these pests have been advocated and these include the following: use of tissue culture (TC) plantlets or pared suckers treated with hot (52°C for 20 minutes) or boiling (boiling for 30 seconds) water (Hauser, 2007). Treatment of planting materials with synthetic chemical, use of resistant varieties and practicing of cultural methods, such as fallowing the land for 5 - 12 months, intercropping and crop rotation, mulching and use of fertilizer have also been recommended. Traditionally, the management of BW and PPN has relied on synthetic chemical pesticides. However, most farmers in the region cultivate bananas without practicing any pest management strategies, while a limited number of farmers use synthetic agrochemicals. However, agrochemicals not only pose a great threat to the environment and public, but also their continued use renders them inactive as pests become resistant. This has created a need for development of alternative user- and environment-friendly

management strategies. Bio-control agents (BCAs), in addition to being environmentally friendly, work through a complex system of specific and non-specific interactions, hence the possibility of pests developing resistance against them is very minimal.

The use of TC banana plants provides clean planting material compared to the conventional planting material such as field suckers (Gold *et al.*, 2001). However TC plants are more susceptible to pests and diseases, because they are not only free of pests, but are also free of the natural antagonists (Blomme *et al.*, 2004). The potential application of antagonistic microorganisms as biological control agents of BW and PPN has been studied under controlled (screen house) conditions, with limited studies conducted in the field. Enhancing TC plantlets with mutualistic fungal pest antagonists that can be applied in or around the plant's roots during the deflasking stage or hardening of the plantlets may allow them sufficient time to colonise the root tissues before they are planted into the field (Dubois *et al.*, 2006).

1.3 Justification

A range of natural antagonists to PPN are present in naturally existing PPN suppressive soils, attacking their nematode hosts at different stages of their life cycle, i.e. eggs, juveniles, sedentary females, etc. (Kerry, 1990). In such systems, each antagonist may kill relatively few nematodes (or nematode stages), but the pooled effect of several antagonists generally results in the suppressive characteristic of such soils (Kerry, 1990). A treatment combination of the nematophagus fungi *Verticillium chlamydosporium* and the bacterial parasite *Pasteuria penetrans* was found to be more effective against the root knot nematode *M. incognita* in tomato compared to when the antagonists were used in isolation (De Leij *et al.*, 1992). A study by Zum Felde *et al*. (2006) showed that inoculation of banana TC plantlets with multiple fungal endophytes reduced *R. similis* population in the root system compared to single endophyte inoculated plants. Similarly, (Hasyim *et al.*, 2009) established that 'natural' control of BW in banana fields was linked to a complex of antagonists. These findings suggest that multiple microbial antagonists may create a more stable biocontrol system.

So far, no studies have been performed to assess the antagonist potential of multiple fungal isolates against multiple pests in banana. Understanding the interaction and effectiveness of multiple combinations of microbial antagonists against banana pests and nematode constraints is central to the development of potential control strategies. This study aims at not only establishing new potential antagonistic agents but also assessing and understanding the

tripartite interaction between combinations of fungal antagonists against the BW and the burrowing nematode, *R. similis* in EAHB, with a focus on pest infection, endophyte biological activity, mode of action and persistence of the fungi in the plant under field conditions.

1.4 Objectives

The overall objective of this study was to evaluate the efficacy of local candidate fungal antagonists against the banana nematode, *R. similis*, and the BW, *C. sordidus*, in East African highland bananas. The specific objectives are:

1) Evaluate the efficacy of selected indigenous entomopathogenic fungal isolates (EPF) against BW and assess antagonistic efficacy in the field.

2) Evaluate the efficacy of selected local fungal endophytic isolates against *R. similis* and assess antagonistic efficacy in banana TC plants.

3) Test the potential of multiple endophytic isolates for the management of *R. similis* infection of banana.

4) Assess the possible mode of action of selected endophytic fungal isolates for nematode management in banana.

5) Assess the field performance of endophyte enhanced banana TC plantlets with respect to suppression of PPN and BW infection, persistence of the endophytic fungus in the banana roots and impact on yield and longevity of the banana field.

1.5 Research hypotheses

The following hypotheses were tested:

1) Indigenous EPF isolates previously isolated from the plant's rhizosphere or endorhiza possess varying levels of antagonistic activity against BW.

2) Endophytic fungi previously isolated from the plant's rhizosphere or endorhiza possess antagonistic activity against *R. similis* infection of banana.

3) The antagonistic potential of endophytic fungus against *R. similis* infection of banana is enhanced when multiple fungal isolates are used as opposed to a single isolate.

4) Mode of action of endophytic fungus against *R. similis* infection is mediated by the up- or down regulation of defence-related genes in the banana roots.

5) Endophyte enhanced bananas are more resilient against *R. similis* infection in the field, yield more and last longer.

1.6 Thesis structure

This thesis is subdivided into the following chapters:

1) **General introduction**: this formulates the basis of the study, provides the justification, objectives, and hypotheses

2) **Literature review**: this provides an assessment of the previous research, identifying the gaps and linking it to the current study

3) **Article 1**: here we investigated the potential of using indigenous EPF for the management of BW. A total of 20 EPF isolates: 15 *Beauveria*, 4 *Metarhizium* and 1 *Isaria* were assessed *in vitro* for their antagonistic potential against BW. Emphasis was placed on BW mortality, the lethal time to 50% mortality (LT_{50}), the lethal concentration to 50% mortality (LC_{50}), and number of spores produced per BW cadaver. Pathogenicity of the three most promising isolates was tested in the field. Furthermore, an assessment of loss of spore viability upon exposure to field conditions was performed. In the conclusion, suggestions were made that would help maintain high spore viability over a long period of time in the field, thus sustain EPF effectiveness in the field.

4) **Article 2:** two fungal isolates: a *B. bassiana* isolate WA, and a non-pathogenic *F. oxysporum* isolate V5w2 were studied individually and in combination for the management of *R. similis* infection of banana in the field. In this chapter we established that one time inoculation with the fungal endophyte would only be effective in the mother crop with no cross-over effect to the subsequent ratoon crops, hence we established that in-field application of the endophytes is crucial for prolonged efficacy.

5) **Article 3:** this investigated the potential of indigenous endophytic fungal isolates to manage *R. similis* infection in EAHB. The study focused on the effect of endophytic fungal isolates on nematode reproduction and plant growth. Furthermore, the two most

promising isolates were selected and studies on dose response vs single and combined inoculation of selected fungal isolates were performed. Lastly, the expression of selected defence-related genes was assessed in endophyte enhanced plants challenged with or without *R. similis.*

6) **Conclusion and recommendation:** this summarises the key findings of the PhD study by discussing whether the hypotheses for each objective were met or discarded and provides guidance for follow-up studies.

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CHAPTER 2

Literature review

2.1 Banana crop

Banana is a giant perennial monocotyledonous herb grown in the warm and humid tropical and sub-tropical regions of Asia, America, Africa and Australia. The crop's primary centre of origin is in Southeast Asia and Indochina (Janssens *et al.*, 2016; Simmonds, 1962) then spread to other places by explorers and commercial planters. Banana (*Musa* spp.) belongs to the family Musaceae under order Zingiberales (Karamura, 1998). The cultivated edible bananas originated from several inter- and intra-specific hybridizations of two wild seeded progenitors, *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) (Heuzé & Tran, 2016; Simmonds, 1962).

Globally, banana is produced in more than 130 countries, primarily by small-holder farmers. Currently, global banana cultivation occupies approximately 11.7 million hectares of arable land, with a total production estimate of approximately 167 million metric tons and feeding more than 400 million people (FAOSTAT, 2020). In 2016, banana was estimated at an international trade value of US$ 11.5 billion (Evans *et al.*, 2020), with Africa contributing approximately 42.6% of the global banana production (FAOSTAT, 2020). More than 70 million people in 15 sub-Saharan Africa countries depend on banana for their livelihood and food supply (FAO, 2010). More than 36 metric tons of banana are produced in the ECA region annually (FAOSTAT, 2020), the majority of which are EAHB (Ocimati *et al.*, 2016) and are produced for local consumption.

The EAHB, a starchy staple in the ECA region provide more than 60% of the required calorie intake in the region. In addition to being a food source, EAHB are a key source of income to rural households, mostly sold to local markets and consumers. Bananas have become an important component of mixed production systems, especially in areas with high population density and limited arable and grazing land. The banana peels, pseudostem and leaves are used as fodder for zero-grazed animals, in turn, the animals provide composite manure for the farm (Karamura *et al.*, 1998). Additionally, the banana pseudostem and leaves are used as a source of mulch, helping to preserve soil moisture and reduce weeds, especially during the prolonged drought, while the dried fibres are used in the production of handicrafts, ropes for tethering

small animals, for making play items and as raw material for house construction (Kamira *et al.*, 2015).

2.2 Banana production constraints

Despite the significance of the EAHB in the ECA region, crop production is threatened by several biotic and abiotic constraints. The key abiotic factors hampering banana production include; declining soil fertility, coupled with a reduction in available arable land due to land fragmentation, which is attributed to the increasing human population, lack of funds to expand and/or properly maintain the available plantations and persistent drought. Furthermore, the generally ageing population of farmers as youths move to other sectors of the economy has greatly contributed to the reduction in banana production (Van Asten *et al.*, 2010; Mwangi & Mbaka, 2010; Wairegi *et al.*, 2010).

Banana pests and diseases are the key biotic constraints to banana production and sustainability. The most important banana diseases include, Fusarium wilt caused by *F. oxysporum* f. Sp. *Cubense* (Foc), a complex of yellow and black sigatoka caused by *Pseudocercospora musicola* and *P. Fijiensis*, respectively (Viljoen *et al.*, 2017), banana bacterial wilt caused by *Xanthomonas campestris* pv. *Musacearum* (Tripathi *et al.*, 2009; Tushemereirwe *et al.*, 2004). Traditionally, the soil-borne fungus *F. oxysporum* f. sp. *cubense* was known to have variants: race 1, 2 and 4, affecting bananas mostly in the subtropics, however, since the 90s, a more devastating strain of the Foc known as Foc Tropical Race 4 (TR4) was reported in Asia. This strain was reported as the most devastating of all Foc strains (Ploetz, 2006). In Africa, TR4 was first reported in Mozambique in 2013 (Viljoen *et al.*, 2020) from where it spread to the ECA region (Viljoen *et al.*, 2017) and is responsible for significant reductions in banana yield in the ECA region. The BW and PPN are key pests of banana occurring in the tropics and subtropics, where banana is cultivated and often occur simultaneously in banana plantations.

2.3 Pest problems of banana

The two most common pests hindering banana production in the East African region are the banana weevil (BW), *C. sordidus* (Gold *et al.*, 2002; Gold *et al.*, 2004; Gold *et al.*, 1999) and the plant-parasitic nematodes (PPN), *R. similis*, *H. multicinctus* and *P. goodeyi* (Nyang'au *et al.*, 2021; Speijer *et al.*, 1994). These pests often occur simultaneously in banana fields. The

combined loss in yield attributed to these two pests amounts to more than 80%, which increases with successive seasons (Musabyimana *et al.*, 2000)

2.3.1 Banana weevil

The BW, a native of Southeast Asia is an important pest problem of banana globally. It has been found to occur in the banana growing areas of East and Central Africa, Central America, Brazil, Eastern Australia, the West Indies, and on many Indian and Pacific Ocean islands (Gold *et al.*, 2001; Treverrow, 2003).

The adult BW are nocturnal and during the day, they usually shelter in or around the corms, between the leaf sheaths (Treverrow, 2003), or in soil around the base of the banana mat or in close association with crop residues (Gold *et al.*, 2004). They are relatively sedentary, rarely dispersing more than 25 m in 6 months. Banana weevils are very sensitive to desiccation and can die within 2 days if kept on dry substrate. The BW can live for more than 1 year, with the females producing 1-4 eggs per week. On standing plants the most preferred laying site is between the leaf sheath scars on the crown of the corm, just above the ground, while on toppled plants there is often massive oviposition at the point where the corm and stem meet, on the side of the plant in contact with the soil (Treverrow, 2003). The eggs are singly deposited in cavities that the female makes in the plant with her mouthparts. The cavities provide shelter to the eggs and the newly hatched larvae, protecting them from natural enemies. The eggs hatch in about 8 days and the young larvae easily gain access into the corm or occasionally the pseudostem by tunnelling their way through as they feed and grow. The larvae exhibit developmental polymorphism going through five to seven instars (Gold *et al.*, 1999) and the larval stage lasts about 20 - 30 days. The fully fed larva works towards the surface of the corm and pupates in an oval chamber. In warm weather the pupal stage lasts about 8 days, then the adult weevil emerges, with the complete life cycle (Figure 1) lasting between 35 - 49 days (Treverrow, 2003; Viljoen *et al.*, 2017).

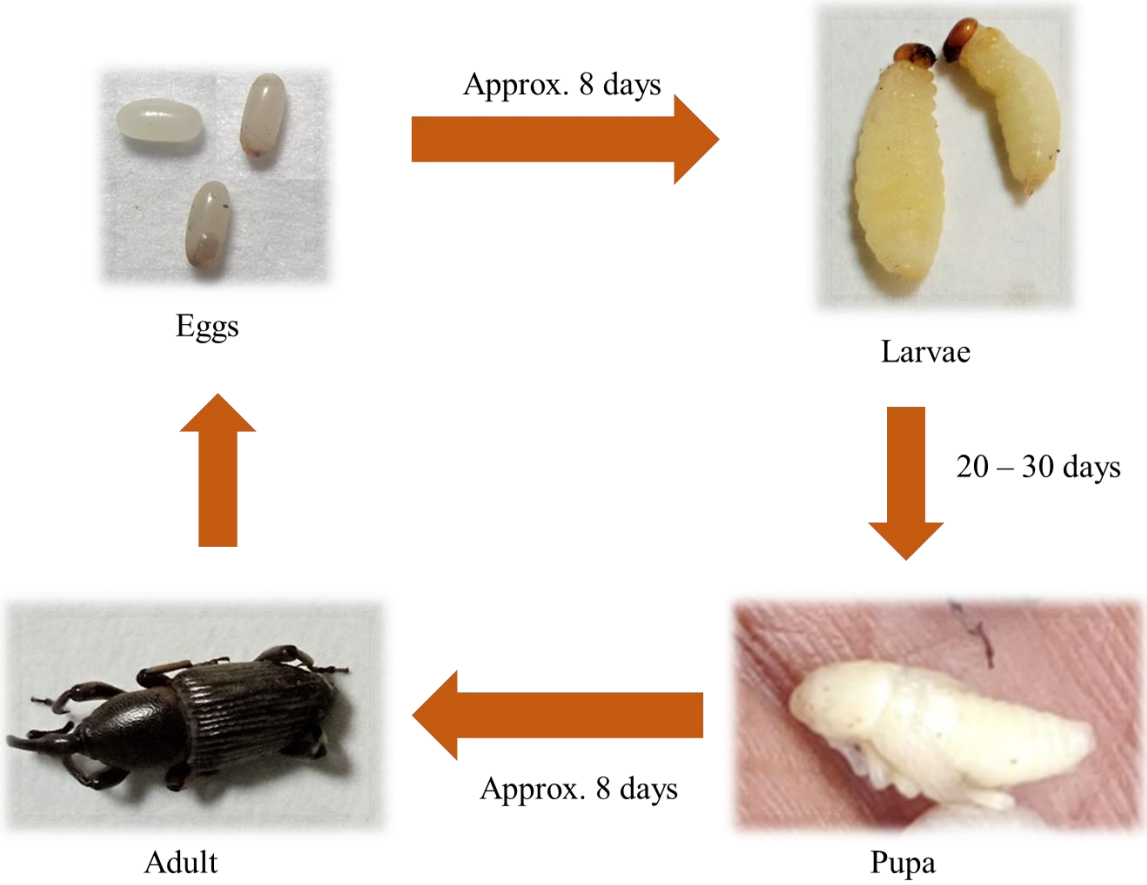

Figure 1. Life cycle of the banana weevil, *Cosmopolites sordidus* (photos: J. Kisaakye)

Severe plant damage is mainly caused by the larvae, which tunnel extensively into the corm and pseudostem. Larval tunnelling weakens the corm-pseudostem junction, which eventually results into snapping of mostly the fruit bearing plants (Figure 2). Infestation of young fragile plants damages the root and vascular system, disrupting nutrient and water uptake. Stress in the plant and poor maintenance of banana fields lead to elevated levels of BW infestation (Rukazambuga *et al.*, 1998). Banana weevil attack has been shown to prevent crop establishment and cause death of suckers and young plants (Abera *et al.*, 1999), leads to an increase in the production cycle and reduces yields (Gold & Messiaen, 2000). Resistant *Musa* varieties have been shown to reduce BW damage by only reducing the rate at which the larvae develop, however survival of the larvae is not influenced by plant resistance (Night *et al.*, 2010a). Unfortunately, it appears that the majority of the EAHB are susceptible to BW (Kiggundu *et al.*, 2003).

Figure 2. Snapped banana plant following banana weevil larvae tunnelling and weakening of the corm-pseudostem junction (photo: J. Kisaakye)

Infestations by BW results in over 40% yield loss, primarily due to plant snapping and reduced banana plantation life (Gold *et al.*, 2004; Rukazambuga *et al.*, 1998). Furthermore, damage and yield reduction increase over time and tend to be higher in ratoon crops. Infestations by BW are most severe in plantains, ensete and EAHB. Weevil infestation has greatly contributed to the reduction and disappearance of EAHB in some parts of East Africa, making this pest of great economic importance (Gold *et al.*, 1999; Gold & Messiaen, 2000). In fact, Gold *et al.* (1999) reported that infestation of EAHB by BW was one of the key reasons for the drastic decline in the cultivation of bananas in Central Uganda, and this led to a geographic shift in

banana cultivation from Central to Western Uganda. However, this was disputed by Price (2006) who argued that the introduction of *R. similis* was the primary cause and that this was overlooked by the more visibly obvious BW. Gold *et al.* (2000) reported BW pest status varies across sites, farms, and other groups of bananas especially in commercial cavendish plantations where the pest is reported relatively unimportant. Usually, BW population densities are often very low in newly established fields, but the numbers build-up slowly and intensify in ratoon crops (Gold & Messiaen, 2000). Banana weevil dispersion is primarily through movement of infested planting materials containing the immature stages of the pest and occasionally adults (Gold *et al.*, 2001; Yadav *et al.*, 2017). Studies have reported genetic variation in BW found at the same geographic location in comparison to different locations (Twesigye *et al.*, 2018; Yadav *et al.*, 2017), which further corroborates the passive role played by humans in the dispersion of this banana pest between different geographic locations.

2.3.2 Plant-parasitic nematodes

Plant-parasitic nematodes pose a serious threat to sustainable banana production. The PPN invade the banana roots primarily through the root tip, they feed, multiply and migrate with-in the banana root system (Speijer, 1993) and the corm tissue, which results in necrotic lesions and poor root development, with root damage reducing water and nutrient uptake. Nematode destruction of the plant root system affects plant anchorage which results in plant toppling (Figure 3) especially during bunch filling (Sarah *et al.*, 1996; Sikora *et al.*, 2018; Stover & Simmonds, 1987). Reduction in nutrient and water uptake leads to stunted growth, delayed flowering and ratooning, reduced bunch size and yield (Speijer *et al.*, 1994; Stoffelen *et al.*, 2000).

A complex of PPN affect banana in the East Africa region, and these primarily include; the root-lesion nematode *P. goodeyi*, the burrowing nematode *R. similis* and the spiral nematode *H. multicinctus* (Coyne & Kidane, 2018; Gowen *et al.*, 2005; Nyang'au *et al.*, 2021). All vermiform stages (male, female and juvenile) of both *H. multicinctus* and *P. goodeyi* are infective, while only the females and juvenile of *R. similis* are infective. These nematodes migrate inter- and intracellularly, feeding on the cytoplasm of cortical cells; this leads to collapsing of cell walls and causes cavities and tunnels which evolve as small spots or lesions of necrotic tissue and may extend over the whole cortex. These are observed as red-black lesions in the cortex. Sporadically, the nematodes can also migrate towards the corm, causing

red-black lesions. As the nematodes infect and move through the plant roots, the damaged areas of the roots act as entry points for secondary root rot fungal and bacterial infections (Shehabu *et al.*, 2010).

In Africa, close to 70% yield loss in plantains and cooking banana has been attributed to nematode parasites. *Radopholus similis* is the most destructive nematode parasite of banana, it causes higher root necrosis, more plant toppling and lower bunch weight compared to *H. multicinctus* (De Waele & Alsen, 2009), especially at altitudes below 1400 m.a.s.l (Kashaija *et al.*, 1994) and *P. goodeyi* (Talwana *et al.*, 2003) at altitudes above 1400 m.a.s.l (Kashaija *et al.*, 1994; Nyang'au *et al.*, 2021). The EAHB were traditionally grown in Central Uganda, however, this gradually declined since the 1970s. Due to this decline, cultivation of the traditional EAHB was replaced by exotic cultivars and annual food crops. At the same time, cultivation of the crop shifted to the south-western part of the country. *Radopholus similis* was identified as the principal cause leading to this decline (Price, 2006). In Kenya, *P. goodeyi* was found to be more prevalent in the banana (*Musa* AAA, Matooke group and AAA & ABB, exotic groups) growing regions across the country (Nyang'au *et al.*, 2021; Reddy *et al.*, 2007). Furthermore, *P. goodeyi* populations from 1300 m.a.s.l and above were found to be more pathogenic compared to those obtained from below 1300 m.a.s.l.

Figure 3: Toppled banana plant due to severe nematode damage to root system (photo: J. Kisaakye)

2.4 Management strategies against the banana weevil and plant-parasitic nematodes

For an operational and economically viable BW and PPN management program, there needs to be integration of a range of methods, tuned to the local conditions and continually monitored so that modifications are made as needed. There is a range of recommended options for the control and management of BW and PPN in banana, these can be employed at plantation establishment and/or during the plantation cycle. However, most of these management strategies are labour intensive, require special safety precautions or are environmentally unfriendly. Management strategies that exist or are being researched against BW and PPN include chemical, physical, cultural and biological control options.

2.4.1 Cultural control

Several labour-intensive cultural control methods have been advocated, most of these are centred on cleaning, protecting planting material, trapping and crop sanitation. Paring and hot water treatment of banana suckers is recommended to generate clean planting material and has been shown to suppress several growth stages of both the BW and PPN (Gold, Night, Abera, *et al.*, 1998). The use of clean planting materials reduces initial infection levels of both BW and PPN and, due to the BW's low fecundity and slow population growth the pest level build-up is retarded. Trapping of the adult weevils using pseudo stem traps has been used to control BW (Queiroz *et al.*, 2017) and this can be modified by incorporation of pheromone lures (Tinzaara *et al.*, 2005). However, farmers' adoption of BW trapping method is constrained by the unavailability of enough trapping materials (Gold *et al.*, 2002) and the laborious nature of the strategy, as farmers need to manually capture and kill the trapped BWs daily, otherwise the traps become breeding grounds. Banana corms and pseudo stem residues act as shelters and breeding sites for BW, thus removal and destruction of these plant remains is an essential component of the pest-cultural control method (Masanza *et al*., 2005).

Cutting the post-harvest banana stumps and covering them with soil has been shown to reduce BW oviposition during the wet season (Masanza, *et al.*, 2005). However, this method is less effective during the dry season as it has been demonstrated that covering stumps with soil enhances BW oviposition around the soil-covered stumps during the dry season (Masanza, *et al.*, 2005). Furthermore, this method is labour intensive, thus its applicability and efficiency are constrained by the luck of required labour. In central Uganda, the BW problem was exacerbated due to a reduction in the available labour to practice proper crop sanitation and BW trapping, which are key components of the cultural control strategy (Gold *et al.*, 2004).

Maintaining a weed-free banana field is key in the management of PPN in banana. Weeds act as reservoirs or transition hosts for PPN (Thomas *et al.*, 2005). During a survey to establish the diversity and abundance of the PPN associated with weeds in banana plantations in the Caribbean Island of Martinique, Quénéhervé *et al.* (2006) established that 24 weed species were hosts to *R. similis*, 23 were hosts to *Helicotylenchus* spp., 13 were hosts to *Pratylenchus* spp., while 29 were hosts to *Meloidogyne* spp., among others. The densities of PPN recovered from weed roots were similar to, or greater than those recovered from banana roots.

2.4.2 Clean planting materials

It is with no doubt that use of pest and disease-free planting materials is key in managing the BW and PPN problem in banana and in reducing their spread. Removing roots from suckers and paring of the banana corm prior to field establishment has been used to disinfect planting materials. Paring suckers prior to planting helps eliminate a majority of PPN and BW eggs and larvae, and adult PPN. Then the pared suckers can further be disinfected by soaking in hot water. The nematodes: *R. similis, H. multicinctus*, *Pratylenchus* spp. and *Meloidogyne* spp. have been successfully managed by dipping pared suckers in hot water (53 - 55℃) for 15 - 20 minutes. Similarly, dipping pared suckers in hot water (43℃) for 3 h resulted in > 94% mortality of BW eggs and larvae (Gold *et al.*, 1998). While the hot water treatment technique can work effectively in commercial settings, the requirement for precise temperature and strict time of dipping renders it tricky for implementation by subsistence farmers. Consequently, a simplified, boiling-water treatment was adapted, in which pared suckers are dipped in boiling (100℃) water for 30 seconds (Coyne *et al.*, 2010; Hauser, 2007).

Macropropagation has been used to generate PPN and BW free planting materials, especially for subsistence farmers. The technique relies on the use of pared healthy banana corms, which are treated with hot/boiling water, often split into 2 or 4 portions before incubation in a sterile moist media, such as saw dust, in a humid, polythene covered chamber. The pared corms often start to sprout within 3 - 4 weeks and the emerging plantlets can be removed and transferred into pots for acclimatisation in the green house before field establishment (Ntamwira *et al.*, 2017).

In vitro micropropagation is used for mass production of tissue cultured planting materials in a small space over a short period. The tissue culture technique is based on the shoot tip propagation method (Vuylsteke, 1998) to generate disease and pest-free banana planting

materials. In addition to being disease and pest free, TC plants have the advantage of uniform growth and higher yields. Most commercial banana plantations now use TC plants for field establishment and there is increasing uptake and use by small-holder farmers in the East African region (Dubois *et al.*, 2013)

The use of clean planting materials helps reduce initial PPN and BW infestations and helps retard pest build-up over several crop cycles (Gold *et al.*, 1998; Speijer *et al.*, 1995). However, these clean planting materials are prone to reinfestation. The period of protection offered by the clean planting materials is primarily dependent on farming practices: planting in isolated sites with no recent history of banana production will lead to a prolonged period of protection against the BW, while planting in infested sites or adjacent to infested sites will significantly reduce the period of protection (Gold *et al.*, 2001).

2.4.3 Breeding for resistance

Breeding for host plant resistance is a commonly studied tool in the management of both the BW (Arinaitwe *et al.*, 2015; Kiggundu, 2000; Kiggundu *et al.*, 2003) and PPN (Dochez *et al.*, 2012, 2005). Conventional banana breeding remains a technically challenging approach given the low reproductive fertility, polyploidy nature, time and space needed, all of which impede the rapid breeding progress (Ortiz, 2013). Although some of these can be alleviated by screening *Musa* germplasm for fertility, manipulation of ploidy and the intensive application of tissue culture techniques (Vuylsteke *et al.*, 1997) and the introduction of rapid pest and disease screening techniques. Standardization of cultivar screening methods is necessary, as well as further research to identify resistant and/or tolerant reference genotypes (Gold & Messiaen, 2000). It is also critical to study the mechanisms of resistance that can define selection criteria, applied earlier than the harvest stage if breeding work is to be made less time consuming (Gold & Messiaen, 2000). In a study to assess the response of banana hybrids to BW infection, Arinaitwe *et al*. (2014) reported some level of resistance in the triploid (AAA) NARITA hybrids against BW. Results from a related study by Kiggundu *et al.* (2003) demonstrated existence of partial resistance/tolerance in some cultivars belonging to the genome groups AAA (Yangambi Km5, Gros Michel and Cavendish), ABB (Kayinja), AAB (Ndiizi), and AB (Kisubi) against BW population build-up and damage. The same study also reported cultivars with an AABB genome (FHIA-03) and AA genome (TMB2X8075-7 and TMB2X6142-1) showing resistance to BW attack.

In a study to establish response of EAHB cultivars to *R. similis* infection, Dochez *et al.* (2005) established that except *cv*. Muvubo, all the other 18 EAHB *cvs*. screened were susceptible to *R. similis*. In another study to establish the pathogenicity of *R. similis* populations from different regions of Uganda, it was established that *R. similis* population exhibited varying levels of pathogenicity (Dochez *et al.*, 2012; Speijer *et al.*, 2013), with the population from Mbarara, western Uganda, found to be more pathogenic and managed to break the resistance of the diploid banana Pisang Jari Buaya, a known source of resistance worldwide (Dochez *et al.*, 2012). This indicates how complex it can be to generate a new cultivar with resistance (or tolerance) to *R. similis*, let alone all banana PPN.

On the other hand, most of the commonly resistant (or tolerant) banana cultivars are not cooking types, this presents a challenge to the breeders as these are not staple to the consumers. Conventionally, breeding bananas for resistance or tolerance to PPN or BW is a laborious and time-consuming process. Current research is exploring genetic improvement through biotechnology techniques including molecular breeding (https://breedingbetterbananas.org/).

Although ovipositing adult female BW tend not to discriminate between resistant and susceptible banana cultivars, plants of resistant cultivars tend to suppress weevil larvae growth and thus sustain less damage in the field. Night *et al.* (2010b) suggested existence of a toxin in the resistant banana cultivar Yangambi Km5, which distorts the feeding ability of the BW larvae thus reducing the damage potential of this pest. Targeting and harnessing such factors during the banana breeding schemes can be a possibility. However, it is possible that this toxin tends to break down after fruit harvest (Night *et al.*, 2010b) and as such, plant remains of the resistant cultivars can act as breeding grounds.

Most mechanisms of resistance to the BW have been attributed to factors that affect larval performance (antibiosis), rather than larval attraction (antixenosis). Pavis & Minest (1993) found no correlation between BW attraction towards pseudostem and infestation. Furthermore, Pavis & Minest (1993) revealed that BW were equally attracted to resistant as they were to the susceptible varieties, hence ruling out non-preference (antixenosis) as a mechanism for BW resistance in bananas.

2.4.4 Pesticides, nematicides and botanicals

The use of chemical pesticides to control BW and PPN has been well documented (Bakaze *et al.*, 2022; Coyne & Kidane, 2018; Sikora *et al.*, 2018), especially in commercial banana

plantations. However, this comes with a substantial negative impact to human, animal and environment health. For example, use of the organochlorine pesticide chlordecone for management of BW in banana plantations between 1972 and 1973 was linked to the persistent cancer cases in the French West Indies (Devault *et al.*, 2018; Joachim *et al.*, 2019). Furthermore, the synthetically derived systemic pesticide, carbofuran is reported to reduce both PPN and BW infection of banana (Bujulu *et al.*, 1983).

Usually nematicides are fast acting and efficient when applied close to the banana corm (Gold & Messiaen, 2000). However, these petro-chemical pesticides are not only expensive, but their residues contaminate the environment and have detrimental effects on animal, human and environmental health (Bortoluzzi *et al.*, 2013; Gallegos-Avila *et al.*, 2010; Satar *et al.*, 2005). Consequently the use of most chemical pesticides is discouraged or banned (WHO, 2010). On the other hand, the development of both BW and PPN resistance to chemical pesticides has been documented (Bujulu *et al.*, 1983; Collins *et al.*, 1991).

Development of human and environment-friendly integrated pest management (IPM) strategies is advocated. As a result, botanical compounds are being developed and promoted to act as substitutes for chemical pesticides. In a pot experiment, Yang *et al*. (2015) demonstrated the possibility of using *Camellia* seed cake to control the root knot nematode *M. javanica* infection in banana plants, however, the potential of applying this plant extract under field conditions is yet to be proven. Furthermore, dipping of banana suckers in a 20% neem *(Azadirachta indica)* seed solution prior to planting is known to protect young banana plants from BW attack due to its repellent effect on adult weevils (Gold & Messiaen, 2000). However, some botanicals, such as nicotine are not only phytotoxic, but can also be harmful to mammals, birds as well as hazardous to environmental health (Guleria & Tiku, 2009). Hence a thorough assessment needs to be taken in the development, testing and promotion of botanicals for pest control.

2.4.5 Biological control

The use of natural enemies to suppress pest populations to lower densities has been a commonly studied subject in the recent years. The BW is an important pest problem of banana and plantain in most regions in the world. However, this pest has a low prevalence in Southeast Asia, the presumed area of origin of this pest (Hasyim *et al.*, 2009). In a survey to ascertain the possible existence of natural enemies against this pest in Southeast Asia, Hasyim *et al.* (2009) revealed the presence of a complex of predators that co-existed with this pest. The most important of

these predators was the Jepson's beetle, *Plaesius javanus.* Under controlled laboratory conditions, the same author demonstrated that both adults and larvae of *P. javanus* predated on BW larvae and pupae. In the same survey, several predatory ants were found to be in association with the banana plants and residues, notably, adults of the ant *Myopopone castanea* Smith (Ponerinae) were observed directly attacking BW larvae in the crop residues.

Bacteria and fungi are the most abundant microorganisms in the soil and these have great potential as microbial antagonists against the PPN and BW. Some of the studied microorganisms against PPN and BW include fungal endophytes (Akello *et al.*, 2008a; Paparu *et al.*, 2009) and EPF (Nankinga & Moore, 2000), respectively.

2.4.5.1 Entomopathogenic fungi in the management of banana weevil

The potential of EPF to control BW has been studied, with the most studied EPF belonging to the genera *Beauveria* and *Metarhizium* (Hasyim *et al.*, 2009; Kaaya *et al.*, 1993; Khun *et al.*, 2020; Membang *et al.*, 2020; Omukoko *et al.*, 2014). Studies have used either exotic or indigenous EPF for control of BW. Most studies conducted so far have focused on establishing the most effective EPF isolate(s), with the majority of tests conducted under *in vitro* laboratory conditions and few under field conditions. In fact, while BW mortality has been achieved in the laboratory, more studies are required to achieve economically impactful BW control in the field (Khun *et al.*, 2020). This is because mortality of BW due to EPF takes more than 15 days, while the targeted adults continue to lay eggs in the leaf sheaths, the hatching larvae tunnel into the corm, hidden away from the EPF conidia, and continue causing damage to the banana plant.

Efficacy tests have been conducted using either dry spores (Nankinga & Moore, 2000), aqueous (Kaaya *et al.*, 1993; Membang *et al.*, 2020; Omukoko *et al.*, 2014) or oil (Nankinga & Moore, 2000) formulated spore suspensions. With the EPF applied as dry spores around the banana stem (Nankinga & Moore, 2000; Negrete González *et al.*, 2018) or on a pseudostem trap or bait (Schoeman & Botha, 2003; Tinzaara *et al.*, 2015, 2005)

Towards improving the field management of BW, combinations of EPF with attractants such as pheromones have been studied. Tinzaara *et al.* (2007) explored the possibility of using aggregation pheromone to improve efficacy of *B. bassiana* isolate G41 against BW in the field; results indicated that BW mortality could be increased by >3-fold when the EPF isolate was used in combination with the aggregation pheromone compared to when it was used alone.

Studies containing multiple species isolates against BW have demonstrated that *Beauveria* isolates perform better than *Metarhizium* isolates (Kaaya *et al.*, 1993; Krauss *et al.*, 2004), while some studies produced similar level of efficacy between *Metarhizium* and *Beauveria* (Membang *et al.*, 2020; Negrete González *et al.*, 2018). While the majority of studies conducted have focused on testing the efficacy of indigenous fungal isolates, often isolated from local sources (Membang *et al.*, 2020; Nankinga, 1994; Omukoko *et al.*, 2014), some studies have compared the efficacy of indigenous and exotic isolates. Indigenous isolates often produce comparable (or better) efficacy than exotic isolates against BW (Kaaya *et al.*, 1993; Lopes *et al.*, 2013). Using indigenous isolates provides an advantage due to absence of phytosanitary restrictions and they don't require permits for introduction, testing and registration. Additionally, indigenous isolates are often more adapted to local environmental conditions.

2.4.5.2 Pathogenicity of entomopathogenic fungi

In general, the infection process of the target host insect by a suitable EPF isolate involves adhesion of fungal spores to the insect body through hydrophobic mechanisms, followed by germination of the fungal spores under favourable conditions and subsequent penetration of the host insect by the growing hyphae through the integuments (Inglis *et al.*, 1996; Ortiz-Urquiza & Keyhani, 2013). Penetration of the germ tube through the insect's cuticle is aided by the action of enzymes such as chitinases, lipases, proteases, peptidases and other bioactive metabolites (Fang *et al.*, 2005; Sánchez-Pérez *et al.*, 2014). Additionally, environmental factors such as humidity, pH and temperature play an important role in spore adhesion, germination and penetration into the host cuticle (Inglis *et al.*, 1996). Upon penetration of the host insect cuticle, the fungus develops as hyphal bodies, disseminating through the haemocoel, and invade the muscle tissue, malpighian tubules, fatty bodies, mitochondria and haemocytes. As the fungus multiplies in the insect's body cavity, it produces toxins such as beauvericins, bassianolide beauverolides, destruxins and other metabolites which are toxic to the host insect (Islam *et al.*, 2021; Maina *et al.*, 2018; Sánchez-Pérez *et al.*, 2014). The insect host dies within 3 to 14 days after infection due to either nutrient depletion, suffocation or poisoning, or a combination of these factors. Following death, the insect cadaver continues to act as a source of nourishment to the blastophores and vegetative hyphae until the fugus breaks open the integument and forms aerial mycelia and spores on the insect cadaver, which disseminate to infect new hosts (Maina *et al.*, 2018).

2.4.5.3 Endophytic fungi in the management of plant-parasitic nematodes of banana

Beneficial endophytic fungi colonise inner plant tissue without causing pathogenic symptoms to the host (Wilson, 1995). Several of such fungal endophytes appear to be mutualistic and are often numerous in plant tissue (Saikkonen *et al.*, 1998). However, the functions of most endophytic fungi are not well understood. While some have been shown to provide beneficial effects on their host plants, such as improvement of plant growth (Saikkonen *et al.*, 1998) and resistance against plant pathogens and PPN (Kiarie *et al.*, 2020; Sikora *et al.*, 2008). The most studied fungal endophytes for disease and PPN control belong to the *Fusarium* and *Trichoderma* genera.

The antagonistic potential of fungal endophytes has been demonstrated under *in vitro* conditions by expressing their nematicidal effect against PPN. Exposure of *R. similis, P. goodeyi* and *H. multicinctus* to culture filtrates of *F. oxysporum* resulted in reduced juvenile hatchability, increased paralysis and mortality of juveniles after 24 h of exposure (Athman *et al.*, 2006; Van Dessel *et al.*, 2011; Mwaura *et al.*, 2010). Similar, results were obtained when eggs and juveniles of *M. incognita* were exposed to filtrates of *F. oxysporum* (Hallmann & Sikora, 1996) and *Trichoderma* spp. (Dababat *et al.*, 2006).

The potential of fungal endophytes to express its antagonistic capability *in vivo* largely depends on the ability of the candidate isolate to colonise the host plant. Colonisation of banana plants by endophytic fungi has been demonstrated in the green house and the field (Akello *et al.*, 2009; Kato, 2013; Paparu *et al.*, 2004, 2006a), with resultant control of PPN (Waweru *et al.*, 2014, 2013) and BW (Akello *et al.*, 2008a,b). The reestablishment of fungal endophytes into host plants can be achieved through root dip, soil drench, foliar spray or stem injection with a fungal spore suspension or solid substrate (Akello *et al.*, 2007; Muvea *et al.*, 2014; Paparu *et al.*, 2004, 2006a), while seed soaking (Akello *et al.*, 2017; Akutse *et al.*, 2013; Kiarie *et al.*, 2020; Muvea *et al.*, 2014) or coating (Cortés-Rojas *et al.*, 2021) can be used for grain or cereal crops prior to sowing. In banana, soil drenching (Hillnhütter, 2007; Ting *et al.*, 2008), root and corm dip (Paparu *et al.*, 2004, 2006a) have been shown to cause substantial fungal colonisation of TC plants. However, improved root development prior to fungal inoculation is key for successful colonisation, as the roots act as attachment surfaces and entry points for the germinated fungal spores into the host plant tissue (Paparu *et al.*, 2006a). Prior root improvement can be achieved by first growing the TC plants in a high nutrient liquid media or appropriate nutrient-rich solid substrate.

Inoculation with fungal endophytes has been shown to boost plant growth and development (Akello *et al.*, 2017; Baron & Rigobelo, 2022; Morsy *et al.*, 2020; Paradza *et al.*, 2021) with improved resilience to pests and pathogens. Studies conducted in pots demonstrated the potential of fungal endophytes in the management of PPN infection of banana (Kumar & Dara, 2021; Paparu *et al.*, 2010; Waweru *et al.*, 2013). Similarly, a combined inoculation of multiple fungal isolates provides better management of PPN (Paparu *et al.*, 2009).

Fungal endophytes have been shown to persist in banana corms and root for up to 8 months after field establishment (Paparu *et al.*, 2008). However, contradicting information is available on how endophyte inoculation influences PPN infection in the field and the subsequent effect on yield. For example, Waweru *et al.* (2014) reported up to 45% reduction in PPN infection and up to 35% increment in yield of mother crop upon inoculation of the banana plants with endophytic *F. oxysporum* prior to field establishment. On the other hand, Kato (2013) observed no effect of fungal endophyte inoculation on PPN infection or yield. Additionally, no information is available on how application of fungal endophytes influences PPN infection and yield of successive cropping cycles, and how this impacts yield. Furthermore, limited knowledge is available on the interaction of multiple endophytic isolates towards PPN control in the field and banana yield.

2.4.5.4 Mode of action of fungal endophytes

Fungal endophytes employ several mechanisms to protect the host plant from infection by PPN, thus contribute towards the improvement of plant health. These mechanisms can be grouped into direct and indirect as described below.

2.4.5.4.1 Direct mechanisms

Endophytic fungi have been observed to directly attack, immobilize or kill PPN. Furthermore, endophytes can repel or confuse PPN as they locate their host, interfere with nurse cell development, compete for resources, or employ a combination of those options. Fungal endophytes produce metabolites such as phenols, flavonoids, quinones, peptides, alkaloids, steroids, terpenoids, pyrones polyketones (Dutta *et al.*, 2014; Khan *et al.*, 2019; Toghueo, 2020) or lytic enzymes such as cellulases, chitinases, proteinases, pectinases and 1,3-glucanases (Fadiji & Babalola, 2020; Schouten, 2016). These compounds play a vital role in blocking host location, inhibiting infection, growth and development of PPN through paralysis and antibiosis (Poveda *et al.*, 2020). For example, *in vitro* tests demonstrated the direct

paralysis and mortality caused by non-volatile extracts of *F. oxysporum* isolates on *R. similis* (Athman *et al.*, 2006) and *P. goodeyi* (Mwaura *et al.*, 2010). Furthermore endophytes colonise plant tissues, which acts as a source of carbon and other nourishment, and thus protect their niche by inhibiting any other competitors, including PPN through niche competition or competitive displacement (Schouten, 2016).

Once they infect a host plant, the sedentary cyst- and root-knot nematodes modify the host plant cells into giant feeding cells into which nutrients from the phloem sink (Hofmann *et al.*, 2009) and act as a source of nourishment for the nematodes. The nutrient-rich giant cells could be an important target for endophyte nourishment (Martinuz *et al.*, 2013). Moreover, Martinuz *et al.* (2013) observed a delay in *M. incognita* development and reduced fecundity in tomato plants inoculated with *F. oxysporum* strain Fo162 compared to control plants, which was attributed to the direct competition for sugars between the nematode and endophyte in the giant cell.

2.4.5.4.2 Indirect mechanisms

Endophytes indirectly antagonise PPN through upregulation of genes that produce various phytohormones, volatile organic compounds, phytoalexin, pathogenesis-related proteins, and trigger jasmonic acid, salicylic acid, and ethylene pathways that protect plants from PPN infection (Gond *et al.*, 2015; Kisaakye *et al.*, 2014; Paparu *et al.*, 2007). Some of these phytohormones have been shown to promote plant growth and vigour (Bamisile *et al.*, 2020; Baron & Rigobelo, 2022) which help compensate for the damage caused by PPN infection, and hence help maintain optimal nutrient uptake by the plant. Likewise, endophytes have been shown to influence root exudate production and composition that can indirectly impact PPN infection. For example, root exudates from tomato plants inoculated with *F. oxysporum* strain Fo162 repelled *M. incognita* compared to exudates from control plants (Dababat, 2006; Dababat & Sikora, 2007). A similar trend was observed for *R. similis* with respect to banana plants inoculated with (and without) *F. oxysporum* strains Fo162 and V5w2 (Vu *et al.*, 2006; Vu, 2005) and *M. graminicola* in rice plants inoculated with (and without) *F. moniliforme* strain Fe14 (Le *et al.*, 2016).

Whereas these insights show how far research has developed with regards to providing alternatives and an understanding of management of the BW and PPN problem in banana. to date, most farmers in East Africa continue to grow bananas without employing any BW or PPN

management strategies. While a negligible number of commercial farmers use synthetic, environmentally unfriendly chemicals for control of BW and PPN in their fields, with no (or low) usage of microbial-based products. Failure of farmers to embrace the use of microbial-based products (MBP) in banana fields can partly be attributed to lack of awareness, a slightly higher cost of MBP and slow-acting nature of MBP against the target pest in comparison to the synthetic chemical alternatives. Consequently, there is need for more research towards development of MBP, especially focussing on identifying more virulent microbial isolates against the BW and PPN.

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CHAPTER 3: Article 1

Evaluation of the entomopathogenic potential of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* for management of *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae)

How to cite this article:

Kisaakye, J.; Fourie, H.; Coyne, D.; Cortada, L.; Masinde, S.; Subramanian, S.; Haukeland, S. Evaluation of the Entomopathogenic Potential of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* for management of *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae). *Agriculture* 2021, 11, 1290. https://doi.org/10.3390/agriculture11121290

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Evaluation of the Entomopathogenic Potential of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* for Management of *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae)

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Citation: Kisaakye, J.; Fourie, H.; Coyne, D.; Cortada, L.; Masinde, S.; Subramanian, S.; Haukeland, S. Evaluation of the Entomopathogenic Potential of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* for Management of *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae). *Agriculture* **2021**, *11*, 1290. https://doi.org/10.3390/agriculture11121290

Academic Editor: Alessandro Vitale

Received: 18 November 2021
Accepted: 14 December 2021
Published: 18 December 2021

Abstract: The banana weevil (BW), *Cosmopolites sordidus*, is the main coleopteran pest of banana, causing up to 100% yield loss. In this study, we screened 20 isolates of entomopathogenic fungi (EPF) for the management of BW. In the lab, eight *Beauveria bassiana* isolates caused >50% mortality of the adult BW, whereas *Metarhizium anisopliae* and *Isaria fumosorosea* isolates were less pathogenic. *B. bassiana* isolates ICIPE 648, ICIPE 660 and ICIPE 273 were the most pathogenic, killing ≥80% of adult BW. *B. bassiana* isolate ICIPE 622 yielded the highest spores per BW cadaver (1.84×10^8 spores), followed by ICIPE 660, ICIPE 273 and ICIPE 648—1.17×10^8, 3.8×10^7 and 3.6×10^7 spores, respectively. ICIPE 273 had the shortest LT_{50} (5.3 days) followed by ICIPE 648 (9.8 days) and 660 (11.1 days). Similarly, the LC_{50} values for the three isolates were 5.18×10^7, 5.49×10^7 and 5.2×10^7 spores mL^{-1}, respectively. In the field, ICIPE 273 and ICIPE 648 had the highest (31.3%) and lowest (20.8%) pathogenicity, respectively. This study indicates that the *B. bassiana* isolates ICIPE 273, ICIPE 648 and ICIPE 660 are potential candidates for the environmentally sustainable management of BW.

Keywords: banana weevil; spore production; pathogenicity; biological control; banana

1. Introduction

Banana (*Musa* spp.) is an important food and cash crop feeding more than 400 million people, with an annual global production estimated at approx. 167 million metric tons [1]. The estimated international trade value for banana in 2016 was US$ 11.5 billion [2]. In 2018, approximately 10% of the global banana production was produced in the East African region [1] with most of the production coming from small plots and backyard gardens. For some countries in East Africa, the annual per capita consumption exceeds 200 kg of banana. Nonetheless, banana production in this region has declined over the years. Biotic and abiotic constraints have been reported as major impediments to banana production in the region [3,4].

The banana weevil (BW), *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae), is the most important insect pest hindering banana production in the major banana growing areas [5,6]. The adult BW are cryptic and nocturnal, sheltering in or around the banana corms and between leaf sheaths. The female adult lays between one to four eggs per week, which it deposits in cavities that it makes with its rostrum in the banana corm and

lower pseudostem. The newly hatched larvae tunnel their way into the corm as they feed and grow, making it the most destructive stage of the insect. The BW is disseminated primarily as eggs or larvae in infested planting materials. Banana weevil infestation can deter crop establishment or shorten plantation life [5,7–10]. In the 1980s, BW infestation was implicated as a primary factor that contributed to the shift in cultivation of East African highland banana (*Musa* spp., genome group AAA-EA, EAHB) from its traditional central region of Uganda to the South-Western part of the country [11]. In on-station trials, yield losses of more than 40% have been attributed to BW infestation [5,6], while up to 100% yield loss has been observed in severely infested farmer's fields [12]. Moreover, recent studies have demonstrated the potential of BW as a vector for several banana diseases, including *Fusarium oxysporum* f. sp. *cubense* tropical race 4 [13,14]. The design and development of new and sustainable management options for this pest is, therefore, of vital importance.

Control and management of BW in smallholder farming systems has relied on the use of pseudostem traps and maintenance of a clean, healthy banana plantation [7,10,15], with other cultural, chemical and biological strategies practiced to a lesser extent [16–18]. Most farmers in the East African region grow banana without practicing any control measures against BW infestation. Currently, breeding for resistance to BW is one of the main targets of banana breeding activities in the region [17,19–21]. Due to the cryptic nature of the adults and given that other life stages grow within and are protected by the banana corm, the use of systemic synthetic pesticides would be recommended. However, the increase in the global ban on most of the synthetic pesticides coupled with the high cost of the pesticides leaves banana farmers with few alternative management options. The possibility of using entomopathogenic fungi (EPF) in the management of BW has been studied in vitro [22–25] and in the field [26,27].

The possibility to identify and develop new potential fungal isolates against BW is dependent on continued bioprospecting and screening of EPFs. Using local and indigenous isolates provides a further advantage, as these are not subjected to phytosanitary restrictions and require no permit for introduction into the country; in addition, they are adapted to local environmental conditions. The Arthropod Pathology Unit (APU) of the International Centre of Insect Physiology and Ecology (*icipe*) has a collection of more than 300 EPFs classified into *Beauveria*, *Metarhizium* and *Isaria*, among others [28]. Efficacy of these EPFs has been tested against a number of plant pests including leaf miners: *Liriomyza sativae* (Blanchard) and *L. trifolii* (Burgess) [29,30], *L. huidobrensis* (Blanchard) [31], *Spoladea recurvalis* (Fabricius) [32], stem borers: *Chilo partellus* (Swinhoe) and *Busseola fusca* (Fuller) [29,33,34], pod borers: *Maruca vitrata* (Fabricius) [35], sap-sucking: *Megalurothrips sjostedti* (Trybom) [36], *Aphis craccivora* (Koch) [37] and fruit flies: *Ceratitis capitata* (Weidemann), *C. rosa* (Karsch), *C. cosyra* (Walker) [38] and *Zeugodacus cucurbitae* (Coquillett) [39], among others. Similarly, Real IPM Ltd., Thika, Kenya, has a collection of several potential fungal isolates isolated from various locations within Kenya. However, the potential of Kenyan isolates of EPF for the management of BW is not well determined.

This study was, therefore, conducted to assess the efficacy of selected *Beauveria*, *Metarhizium* and *Isaria* EPF isolates from the *icipe* and Real IPM fungal repositories against adults of the BW in the laboratory, and to identify the most effective isolate(s) based on mortality rate, lowest effective dosage and highest spore production on BW cadavers. The selected effective isolate(s) from the pathogenicity screening in the laboratory were further tested for efficacy under the field and these would be recommended to be developed into mycoinsecticides.

2. Materials and Methods

2.1. Trapping and Maintenance of the Adult Banana Weevil

Adult BW of indeterminate age were obtained from a naturally infested EAHB field at the Industrial Crops Research Institute (ICRI) of the Kenya Agricultural and Livestock Research Organization (KALRO), Mwea, Kenya. The BW were trapped using the split banana pseudostem trap [40]. Pieces of banana pseudostems (~30 cm long) were split and

placed around banana mats in the field. The BW were collected from the sliced part of the pseudostems facing the ground after 1–2 days. Identity of the collected BW was made based on the description provided by Treverrow [10] and Viljoen et al. [40]. Collected BW were maintained in 10 L buckets with screened lids. A banana corm piece (~400 g) from a susceptible EAHB cultivar (Ngombe) was provided for BW to feed on and was changed weekly; the BW were maintained in the laboratory (25 ± 2 °C) for at least two weeks before they were used in bioassays.

2.2. Fungal Isolates

A total of 20 fungal isolates, 15 *B. bassiana*, 4 *M. anisopliae* and 1 *I. fumosorosea* were screened for their entomopathogenic potential against the adult BW. Fungal cultures were obtained from the *icipe* and Real IPM fungal germplasm collections. Origin, year of isolation and primary host are presented in Table 1. The identities of these fungal isolates were previously established based on morphological features as described by Goettel et al. [41] and Humber [42], and molecular tools based on the conserved internal transcribed spacer (ITS) regions of the respective EPF isolate DNA. *Beauveria bassiana* and *I. fumosorosea* isolates were sub-cultured on potato dextrose agar (PDA) medium (39 g/L distilled water), while *M. anisopliae* isolates were sub-cultured on Sabouraud dextrose agar (SDA) medium (65 g/L distilled water). All isolates were cultured in sterile 90 mm plastic Petri dishes and maintained in the incubator (25 ± 2 °C) in the dark for 3–4 weeks until sporulation.

Table 1. Identity of selected entomopathogenic fungal isolates and germination of their respective spores on Sabouraud dextrose agar (SDA) media after 18 h incubation at 25 ± 2 °C.

Fungal Species	Isolate	Year of Isolation	Isolation Host/Source	Locality (Country) of Isolation	Repository	% Viability (Mean ± SE)
Beauveria bassiana	ICIPE 273	2006	Soil	Mbita (Kenya)	ICIPE, Kenya	94.2 ± 1.36 ab
	ICIPE 279	2005	Coleopteran larvae	Kericho (Kenya)	ICIPE, Kenya	92.2 ± 1.22 b
	ICIPE 281	2005	Soil	Mauritius	ICIPE, Kenya	94.1 ± 0.10 ab
	ICIPE 284	2005	Soil	Mauritius	ICIPE, Kenya	94.5 ± 1.07 ab
	ICIPE 603	2007	Hymenoptera	Taita hills (Kenya)	ICIPE, Kenya	92.6 ± 1.15 ab
	ICIPE 609	2008	Soil	Meru (Kenya)	ICIPE, Kenya	93.9 ± 0.92 ab
	ICIPE 621	2008	Soil	Kericho (Kenya)	ICIPE, Kenya	93.5 ± 1.07 ab
	ICIPE 622	2008	Soil	Kericho (Kenya)	ICIPE, Kenya	94.0 ± 1.79 ab
	ICIPE 644	2007	Soil	Mauritius	ICIPE, Kenya	96.1 ± 1.44 ab
	ICIPE 647	2005	Soil	Mauritius	ICIPE, Kenya	94.4 ± 0.02 ab
	ICIPE 648	2007	Soil	Kericho (Kenya)	ICIPE, Kenya	95.5 ± 1.00 ab
	ICIPE 660	2008	Soil	Kemokock (Kenya)	ICIPE, Kenya	93.0 ± 1.36 ab
	ICIPE 662	2008	Soil	Mariakani (Kenya)	ICIPE, Kenya	94.9 ± 0.68 ab
	SD 229-Bb01	2008	Soil	Thika, Kenya	Real IPM, Kenya	96.3 ± 1.62 ab
	SD 277-Bb02	2018	White scale insect	Thika, Kenya	Real IPM, Kenya	93.8 ± 0.48 ab
Metarhizium anisopliae	ICIPE 18	1989	Soil	Mbita (Kenya)	ICIPE, Kenya	96.9 ± 0.48 ab
	ICIPE 62	1990	Soil	Matete (DRC)	ICIPE, Kenya	95.8 ± 0.74 ab
	ICIPE 69	1990	Soil	Matete (DRC)	ICIPE, Kenya	97.9 ± 0.37 a
	ICIPE 78	1990	*Temnoschoita nigroplagiata*	Ungoe (Kenya)	ICIPE, Kenya	98.0 ± 0.59 a
Isaria fumosorosea	ICIPE 682	2015	Soil	Masai Mara (Kenya)	ICIPE, Kenya	92.6 ± 0.32 ab

Means within same column followed by same letter(s) are not significantly different by Student-Newman-Keuls (SNK) test at $p < 0.05$.

For each individual fungal isolate, a spore suspension was prepared by washing the plates containing the spores with 10 mL of a sterile aqueous solution of 0.01% (*v/v*) Triton X-100 and spores scraped off using a sterile metal spatula. Individual suspensions were each collected into a sterile 30 mL universal bottle containing 3 mm diameter glass beads; this formed the stock suspensions.

2.3. Spore Viability

For each isolate, the fungal spores were quantified using a Neubauer hemocytometer under a light microscope (LEICA DMLS, Leica Microsystems GmbH, Wetzlar, Germany) at 400× magnification; the spore concentrations for each isolate were adjusted to 3.0×10^6 spores mL^{-1}. For each isolate a 100 µL spore suspension was spread plated onto SDA media in individual 90 mm Petri dishes using a drigalski spatula. Four replicate plates were used for each isolate. The plates were sealed with parafilm and maintained in an incubator (25 ± 1 °C) for 16–18 h. Spore germination was halted by spreading ~1 mL lactophenol cotton blue solution on the agar surface in each Petri dish. Four sterile glass cover slips were then placed on the agar surface of each Petri dish. The percentage spore viability was determined by selecting ~100 spores under each coverslip and both the germinated and non-germinated spores counted using a light microscope (400× magnification). Spores were considered as germinated if their germ tubes were two times longer than the propagule diameter [41].

2.4. Bioassays

2.4.1. Pathogenicity against Adult *Cosmopolites sordidus* in the Laboratory

Prior to use in any bioassay, the adult BW were placed on a kitchen sieve, washed with sterile distilled water and then blotted dry with a paper towel. The BW were further subjected to a 'fitness test' in which they were placed on one side of a rectangular plastic container (20 × 12 × 5 cm, L × W × H) and a piece of banana corm (~100 g) placed on the opposite side and then left to stand for 1 h. Only BW that moved towards the banana corm were considered as fit to be used in a bioassay and were selected for further use.

To screen out the most effective isolates among the 20 under study, 10 fit adult BW were placed on a plastic 90 mm Petri dish lined with sterile filter paper and sprayed (treated) with a 10 mL suspension of 1.0×10^8 spores mL^{-1} of respective fungal isolates [25,32,37]. Ten untreated control BW were sprayed with 10 mL of sterile 0.01% (*v*/*v*) Triton X-100. The spraying was performed using a potter precision laboratory spray tower (Burkard Scientific, London, UK) [43]. Rotation of the spray tower from the base ensured a homogeneous distribution of the designated spore suspension onto the plate containing the test BW. The treated BW remained in the Petri dish for 24 h, before transferring into plastic containers (20 × 12 × 5 cm, L × W × H) with screened lids (~1 mm mesh) using sterile forceps [44], along with ~150 g banana corm as food, which was changed every three days. All the treatments were maintained in a dark room at 25 ± 2 °C. Each treatment was replicated four times and the treatments were arranged in a completely randomized design; the experiment was repeated once in time.

Observation of dead BW was conducted at three-day intervals for 35 days [22,23]. Since disturbed adult BW display thanatosis (feigning death) [45], a 'confirmation of death' test was performed before dead BW were removed from the containers. The BW were separated from the banana corm, placed on one side of the container and fresh banana corm placed on the opposite side. The containers were left to stand in darkness for 1 h. Only those BW that did not move towards the banana corm after 1 h were considered as dead. Dead BW were disinfected with 1% sodium hypochlorite and 70% ethanol, followed by three rinses in sterile distilled water for ~1 min. The disinfected BW were placed in a plastic sterile Petri dish lined with moist sterile filter paper; Petri dishes were sealed with parafilm and incubated (25 ± 2 °C) for 14 days to monitor for mycosis [46].

2.4.2. Assessment of Spore Production on Banana Weevil Cadavers

Eight fungal isolates that displayed >50% BW mortality were selected for determination of spore production levels on BW cadavers. Treatment and spraying of BW with the fungal isolates was performed as described for the pathogenicity assay above. For each isolate, the setup was maintained until three cadavers were picked from each replicate, thus, a total of 12 cadavers were assessed for each isolate. The cadavers were incubated in the dark (25 ± 2 °C) for 14 days in sterile plastic Petri dishes lined with sterile moist

filter paper. Each mycosed BW cadaver was individually vortexed in 5 mL of 0.01% sterile Triton X-100 contained in 30 mL universal bottles. Spore production was quantified using a Neubauer counting chamber [46] under a light microscope at 400× magnification.

2.4.3. Dose-Mortality Bioassay

Three fungal isolates were selected for dose-mortality bioassays based on: (1) High mortality potential, (2) low LT_{50} and (3) high spore production on BW cadavers. Based on these criteria, three *B. bassiana* isolates: ICIPE 273, ICIPE 648 and ICIPE 660, were selected for dose-mortality assessments. Ten fit adult BW were sprayed with 10 mL of each test isolate at five levels of concentrations: 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, 1.0×10^8 spores mL^{-1}. Untreated control BW were sprayed with 10 mL

Sterile 0.01% Triton X-100. Each treatment was replicated four times and the treatments were arranged in a completely randomized design; the experiment was repeated once in time. As described in the pathogenicity assay above, a 'confirmation of death' test was performed prior to recording of weevil mortality. Mortality data was recorded every two days for 34 days. Dead BW were disinfected in a 1% sodium hypochlorite and 70% ethanol solution, followed by three rinses in sterile distilled water. The BW cadavers were placed in sterile plastic Petri dishes lined with moist sterile filter paper and incubated (25 ± 2 °C) in the dark for mycosis.

2.4.4. Efficacy of EPFs under Field Conditions

The field trial was conducted at the *icipe* campus, Nairobi, Kenya. The banana field contained 20 mats spaced at 2.5 × 2.5 m, and previously infested with BW.

Three fungal isolates (ICIPE 273, ICIPE 660 and ICIPE 648) were selected to test for pathogenicity against BW in the field. Fungal spores for each EPF isolate were produced on PDA as described in Section 2.2 above and a spore suspension of 1.0×10^8 spore mL^{-1} was prepared for each isolate. Banana pseudostem traps were prepared from freshly harvested pseudostems of EAHB plant as described by Viljoen et al. [40]. The banana pseudostems were cut into ~30 cm pieces and longitudinally split into two halves. Prior to setting up the weevil traps, each trap was dosed with a 10 mL suspension of 1.0×10^8 spore mL^{-1} of the respective EPF isolate. The EPF spore suspension was applied on the sliced part of the trap and the traps were left to stand for 15–20 min to soak the spore suspension. Control traps were applied with 10 mL of 0.01% (*v/v*) Triton X-100. Four weevil traps were used for each fungal isolate.

The traps were randomly placed on individual banana mats close to the base of the plant with the sliced part facing the ground. Traps were set up in the evening and the trapped weevils were collected from each trap daily (between 8–9 am) for seven consecutive days. For each day, weevils collected from each trap were separately placed in a rectangular plastic container (20 × 12 × 5 cm, L × W × H) and supplied with ~100 g piece of banana corm as food. The weevils were maintained in the laboratory at ambient temperature (25 ± 2 °C) for 21 days. Weevil mortality was checked at 3-day intervals for 21 days.

2.4.5. Viability of Fungal Spores after Exposure to Field Conditions

To assess viability of fungal spores in the field, ~1 g of plant tissue was scraped from each pseudostem trap. The plant tissue samples were collected at 3-day intervals for 9 days. The plant tissue was scraped from the part of the pseudostem where the spore suspension was applied. Plant tissue collected from each trap was placed in a separate sterile 15 mL Falcon tube and ~2 mL of sterile 0.01% Triton X-100 was added. The contents of the Falcon tube were vortexed to separate the fungal spores from the plant tissue. The fungal spores were quantified using a Neubauer hemocytometer and the spore concentration for each isolate was adjusted to 3.0×10^6 spores mL^{-1}. Spore viability was determined as described before. However, the SDA media used was supplemented with the antibiotics: streptomycin sulphate (0.2 g L^{-1}), penicillin G (0.1 g L^{-1}) and chlortetracycline (0.05 g L^{-1}).

2.5. Statistical Analysis

Percentage spore viability and percent mortality of the adult BW following exposure to the EPF were subjected to analysis of variance (ANOVA) and the means separated using the Student-Newman-Keuls (SNK) test from the package 'agricolae' [47]. Prior to analysis, percent mortality data were first corrected for natural mortality [48] then angular transformed to conform to the requirement of normality of variances [49]. Time- and concentration to mortality data were analyzed using the generalized linear model (GLM) with binomial distribution to generate the slope and intercept of the regression curves. Lethal time and lethal concentration to 50% mortality (LT50 and LC50) values were estimated using the 'dose.p' function of the package 'MASS' [50]. LT50 was evaluated only for isolates that yielded >50% mortality of adult BW. GLM analysis with binomial distribution was run for each replication to determine the lethal concentration to 50% mortality. For each EPF test isolate, insect mortality between doses was analyzed using the Kaplan–Meier survival analysis (log-rank method) using the R package 'survival' [51]. Data on production of spores on BW cadavers were checked for normality [49], then fitted to GLM analysis using negative binomial regression analysis of the package 'MASS' [50] and group means were separated using SNK test from the package 'agricolae' [47] at $p = 0.05$. Analysis of variance was performed on the number of captured weevils from the traps fitted with a linear mixed effects model using the 'lmer' function of the package 'lme4' [52]. EPF treatment and number of days to weevil capture were specified as the fixed variables, while trap was specified as the random variable. Computation of least square means was performed using the 'lsmeans' function of the package 'emmeans' [53] and group means were separated using the adjusted Tukey's method executed using the 'cld' function from the package 'multicomp' [54]. All data analyses were performed using R (Version 4.0.2) statistical software [55].

3. Results

3.1. Spore Viability and Pathogenicity of Fungal Isolates to Adults of Cosmopolites sordidus in the Laboratory

Spore viability differed significantly among the 20 EPF isolates ($F = 2.469$, $df = 19$, $p = 0.008$). Generally, spores of all the isolates were viable. However, viability of ICIPE 69 and 79 was significantly higher than that of ICIPE 279 (Table 1).

There was no significant effect between the repeat experiments on pathogenicity of the fungal isolates ($F = 1.67$, $df = 1$, $p = 0.76$), thus data from both experiments were pooled for analysis. Among the 20 isolates tested, 18 were pathogenic to adults of BW at 35 days post exposure (DPE). BW mortality differed significantly among the EPF isolates. The *B. bassiana* isolates ICIPE 660, 648, 273, 284, 622, 644, SD-229-Bb01 and SD-277-Bb02 caused significantly higher BW mortality in comparison to other tested isolates ($F = 12.53$, $df = 19$, $p < 0.001$). Among the eight highly pathogenic *B. bassiana* isolates, ICIPE 660, 648 and 273 resulted in the highest BW mortality ($\geq$ 80%). EPF isolates ICIPE 69 and 281 were non-pathogenic to adults of BW (0%) (Table 2). All four *Metarhizium* isolates: ICIPE 18, 62, 69 and 78 caused low mortality from 0% to 7.5%. The *Isaria fumosorosea* isolate ICIPE 682, also caused low BW adult mortality (2.5%). Only the *Beauveria* isolates caused >50% mortality of BW. The lethal time to 50% mortality (LT_{50}) was calculated for the eight *Beauveria* isolates that yielded >50% mortality 35 DPE. Among the eight isolates, ICIPE 273 exhibited the highest mortality rate, yielding the least LT_{50} value (5.3 days), while the Real IPM isolate SD-277-Bb02 exhibited the lowest rate of mortality, consequently yielding the highest LT_{50} value (26.9 days) (Table 2).

Table 2. Pathogenicity of entomopathogenic fungal isolates against adults of *Cosmopolites sordidus*.

Fungal Species	Isolate	% Weevil Mortality (Mean ± SE)	LT_{50} (Days) (95% FL)	Regression Line	
				Slope (± SE)	Intercept
Beauveria bassiana	ICIPE 273	80 ± 14.1 a	5.3 (1.9–8.8)	0.04 ± 0.008	−0.21
	ICIPE 279	7.8 ± 2.6 c	na	na	na
	ICIPE 281	0 ± 0 c	na	na	na
	ICIPE 284	62.5 ± 16.5 ab	18.3 (16.2–20.4)	0.037 ± 0.008	−0.68
	ICIPE 603	2.5 ± 2.5 c	na	na	na
	ICIPE 609	30 ± 17.8 bc	na	na	na
	ICIPE 621	2.5 ± 2.5 c	na	na	na
	ICIPE 622	53.6 ± 8.5 ab	24.2 (21.7–26.7)	0.033 ± 0.008	−0.79
	ICIPE 644	62.5 ± 9.5 ab	17.4 (15.2–19.7)	0.035 ± 0.008	−0.61
	ICIPE 647	20 ± 4.1 bc	na	na	na
	ICIPE 648	82.5 ± 8.5 a	9.8 (7.9–11.7)	0.057 ± 0.009	−0.56
	ICIPE 660	82.5 ± 6.3 a	11.1 (9.4–12.8)	0.061 ± 0.009	−0.68
	ICIPE 662	27.5 ± 2.5 bc	na	na	na
	SD-229-Bb01	62.5 ± 4.8 ab	14.7 (11.5–18)	0.026 ± 0.008	−0.39
	SD-277-Bb02	50.8 ± 10.3 ab	26.9 (24.3–29.6)	0.035 ± 0.008	−0.95
Metarhizium anisopliae	ICIPE 18	6.7 ± 3.5 c	na	na	na
	ICIPE 62	7.5 ± 7.5 c	na	na	na
	ICIPE 78	7.5 ± 4.8 c	na	na	na
	ICIPE 69	0 ± 0 c	na	na	na
Isaria fumosorosea	ICIPE 682	2.5 ± 2.5 c	na	na	na

Means within same column followed by same letter(s) are not significantly different by Student-Newman-Keuls (SNK) test at $p < 0.05$. [na] values not estimated (<50% mortality achieved). FL: 95% fiducial limits.

3.2. Spore Production per Cosmopolites Sordidus Cadaver

After 14 days of incubation, production of spores on the BW cadavers varied significantly between the eight selected *Beauveria* EPF isolates ($\chi^2 = 116.21$, $df = 7$, $p < 0.001$). The isolates ICIPE 622, ICIPE 660, ICIPE 273 and ICIPE 648 produced 1.8×10^8, 1.2×10^8, 3.8×10^7 and 3.3×10^7 spores per cadaver, respectively, which was significantly higher than spores produced by isolates SD-277-Bb02 (4.4×10^6) and ICIPE 644 (2.8×10^6), respectively (Figure 1).

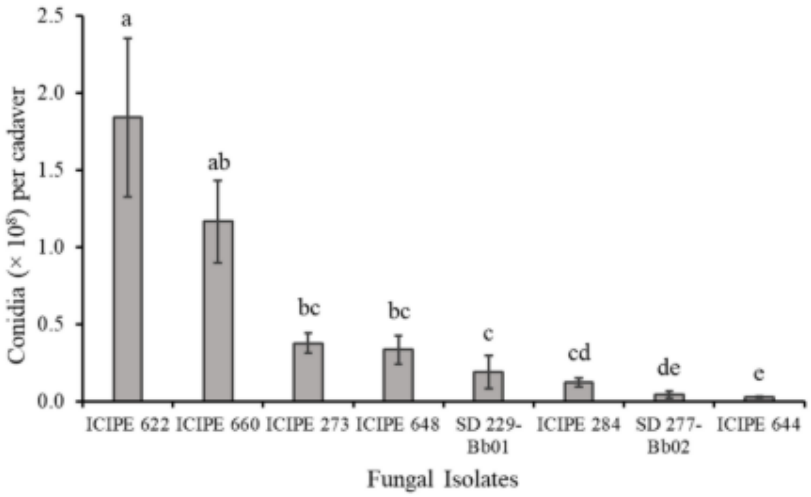

Figure 1. Spore production on individual *Cosmopolites sordidus* cadavers after 14 days of incubation at 25 ± 2 °C. Bars denote mean ± SE, bars with same lower-case letter(s) are not significantly different (Student-Newman-Keuls (SNK) test) at $p < 0.05$.

3.3. *Dose Response*

At 34 days post treatment, the lethal concentration to 50% mortality (LC_{50}) did not vary between the three EPF isolates ($p > 0.05$). The isolates ICIPE 273, ICIPE 660 and ICIPE 648 exhibited LC_{50} of 5.18×10^7, 5.2×10^7 and 5.49×10^7 spores mL^{-1}, respectively.

Survival analysis indicated that an increase in spore concentration significantly reduced the survival rate of the BW following exposure to ICIPE 273 (log-rank test, $\chi^2 = 69.2$; $df = 4$; $p < 0.0001$), ICIPE 648 (long-rank test, $\chi^2 = 92$; $df = 4$; $p < 0.0001$) and ICIPE 660 (log-rank test, $\chi^2 = 69.7$; $df = 4$; $p < 0.0001$). The isolates ICIPE 273, ICIPE 660 and ICIPE 648 attained a cumulative BW mortality of 76%, 80% and 80% at spore concentration 1.0×10^8 spore mL^{-1}, respectively (Figure 2).

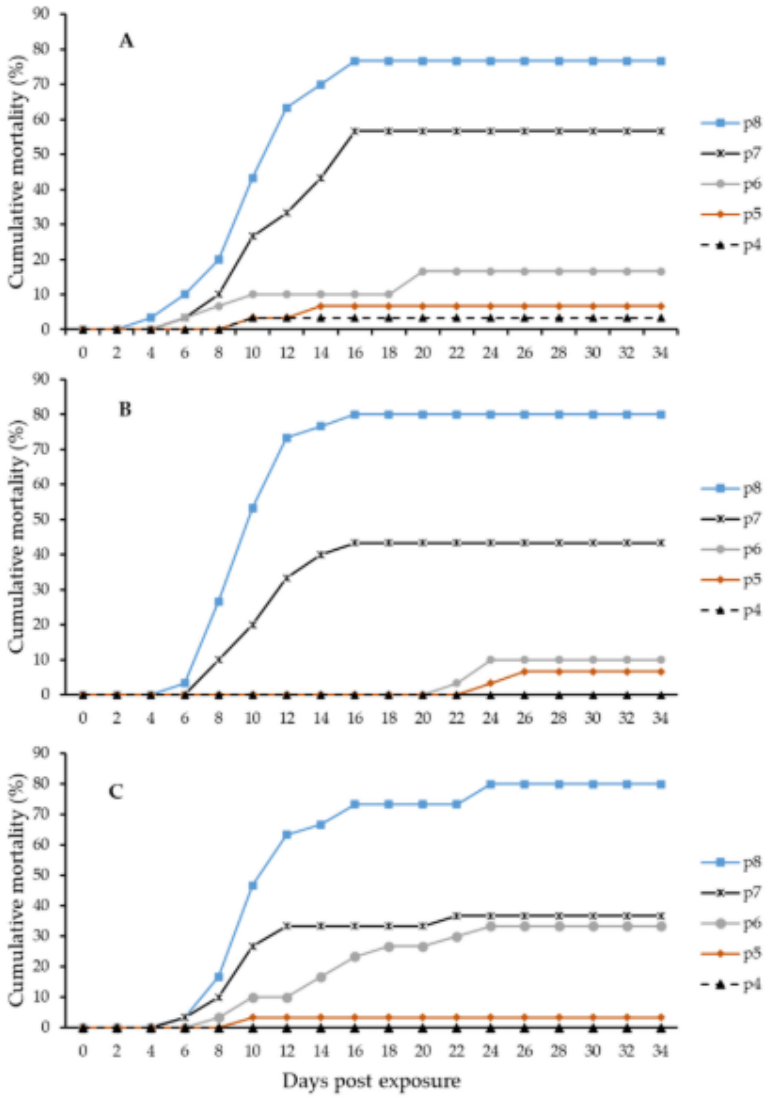

Figure 2. Dose-dependent cumulative mortality of adult *Cosmopolites sordidus* weevils induced by exposure to ICIPE 273 (**A**), ICIPE 648 (**B**) and ICIPE 660 (**C**) at spore concentrations of 1.0×10^4 (p4), 1.0×10^5 (p5), 1.0×10^6 (p6), 1.0×10^7 (p7) and 1.0×10^8 (p8) spores mL^{-1}.

3.4. Number of Captured Weevils and Pathogenicity of EPFs in the Field

There was no effect of EPF treatment on the number of weevils captured per trap per day during the assessment period ($F = 0.86$, $df = 3$, $p = 0.46$). However, there was a significant effect of time of capture on number of captured weevils ($F = 13.91$, $df = 6$, $p < 0.001$), and there was no interaction effect between EPF treatment and time of capture ($F = 0.45$, $df = 18$, $p = 0.97$). Overall, the number of weevils captured per trap during the seven-day period gradually reduced from >three weevils per trap on day one to <one weevil per trap on day seven (Table 3).

Table 3. Number of weevils captured per trap during a seven-day period following set up of the traps in the field.

Treatment	Days Post Trap Set Up in the Field						
	1	2	3	4	5	6	7
Control	3.8 ± 0.9 aA	2.8 ± 1.5 abA	1.8 ± 0.5 abA	2.0 ± 0.0 abA	1.0 ± 0.4 bA	1 ± 0.4 bA	0.3 ± 0.3 bA
ICIPE 273	3.3 ± 0.3 abA	3.8 ± 0.6 aA	2.3 ± 0.9 abcA	1.3 ± 0.6 abcA	0.8 ± 0.3 bcA	0.8 ± 0.3 bcA	0.3 ± 0.3 cA
ICIPE 648	4.3 ± 0.6 aA	3.0 ± 1.2 abA	2.0 ± 0.7 abA	1.8 ± 0.6 abA	1.8 ± 0.6 abA	1.3 ± 0.6 bA	1.0 ± 0.4 bA
ICPE 660	3.3 ± 0.3 abA	2.0 ± 0.4 abA	2.0 ± 0.4 abA	1.8 ± 0.5 abA	1.3 ± 0.6 abA	1.0 ± 0.0 abA	0.5 ± 0.3 aA

Values represent means ± standard error. At each day, means followed by the same upper-case letter indicate no treatment difference. While for each treatment, means with the same lower-case letter(s) indicate no difference between days (Tukey HSD test) at $p < 0.05$.

Mortality of the captured weevils varied significantly between EPF treatment ($\chi^2 = 12.61$, $df = 3$, $p < 0.006$) and time of capture ($\chi^2 = 19.57$, $df = 6$, $p = 0.003$); however, there was no interaction effect ($\chi^2 = 2.89$, $df = 18$, $p = 1$). While none of the captured weevils from the control treatment died during the 21 days of incubation in the laboratory, mortality of weevils captured from traps sprayed with fungal isolates ICIPE 273 and ICIPE 684 was significantly higher than weevils captured from the control treatment on day one. There was no mortality amongst the weevils captured from the traps after three days following set up of the weevil traps (Table 4).

Table 4. Weevil mortality (%) at three weeks of incubation in the laboratory after capture from the EPF-treated traps in the field.

Treatment	Days post EPF Application and Trap Set Up in the Field			
	1	2	3	4 *
Control	0 ± 0 aB	0 ± 0 aA	0 ± 0 aA	0 ± 0 aA
ICIPE 273	31.3 ± 2.1 aA	17.5 ± 6.0 aA	8.3 ± 8.3 abA	0 ± 0 bA
ICIPE 648	20.8 ± 7.2 aA	8.3 ± 8.3 abA	0 ± 0 bA	0 ± 0 bA
ICIPE 660	22.9 ± 7.9 aAB	8.3 ± 8.3 abA	8.3 ± 8.3 abA	0 ± 0 bA

Values represent means ± standard error. At each day, means followed by the same upper-case letter(s) indicate no treatment difference. While for each treatment, means with the same lower-case letter(s) indicate no difference between days (Tukey HSD test) at $p < 0.05$. * No mortality for weevils captured on days 4, 5, 6 and 7 post EPF application.

3.5. Spore Viability after Exposure to Field Conditions

Viability of fungal spores recovered from the weevil traps was significantly different between EPF isolates ($\chi^2 = 51.63$, $df = 2$, $p < 0.001$), time of exposure ($\chi^2 = 2228$, $df = 3$, $p < 0.001$), and there was a significant interaction ($\chi^2 = 16.96$, $df = 6$, $p = 0.009$). Prolonged exposure of fungal spores to field conditions significantly reduced spore viability of all the three EPF isolates. Viability of fungal spores at day zero (90.3–92.3%) did not vary significantly between the three EPF isolates. However, at day three, viability of spores of ICIPE 273 (40.9%) remained significantly higher compared to ICIPE 648 (21.6%) and ICIPE 660 (16.2%). Conversely, spore viability did not differ significantly between the three EPF isolates at days six and nine, and this had considerably reduced to <10% by day nine post field application (Figure 3).

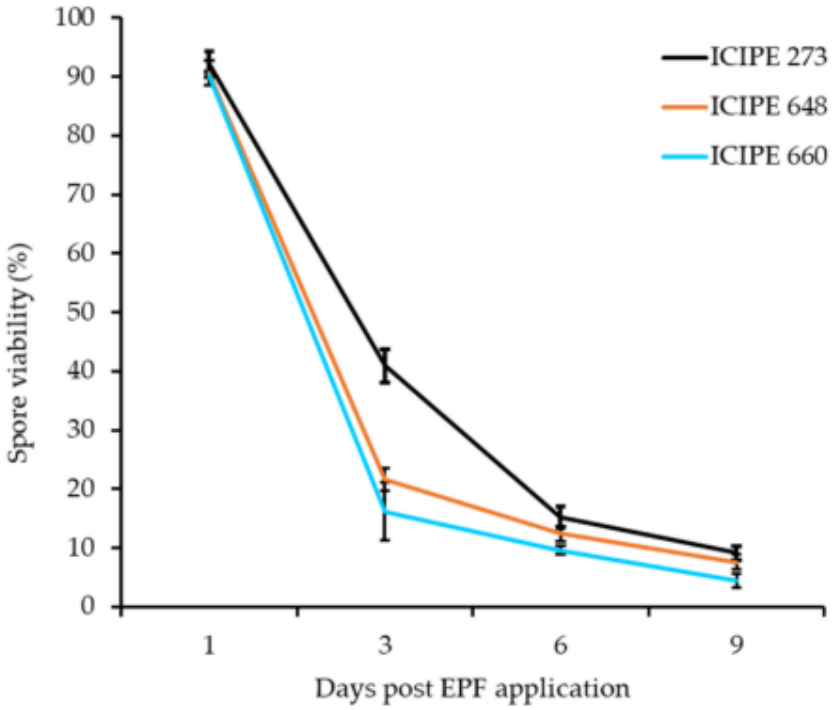

Figure 3. Viability of fungal spores collected from the pseudostem traps at zero, three, six and nine days post entomopathogenic fungal isolate application in the field.

4. Discussion

Spore viability is a vital component to determine prior to screening of any EPF isolate for use as a biological control agent, as spore viability can influence the pathogenicity of EPF isolates [56]. The viability of the EPF isolates used in our study varied between 92.5% and 98%, ranges that were considered sufficiently high for all isolates to be deemed fit for further studies. Whereas spore viability can be influenced by various factors, including temperature, humidity, culture media, age of the culture [57,58], among others, these were all uniform during the in vitro culture of the isolates used in this study, which may explain the minimal variation in spore viability values of the test isolates prior to exposure to environmental conditions.

However, exposure of the spores of the fungal isolates ICIPE 273, ICIPE 648 and ICIPE 660 to environmental conditions in the field led to a substantial reduction in spore viability for all the three EPF isolates tested. While the three selected EPF isolates were originally isolated from soil (Table 1), and it would be assumed that they would withstand environmental conditions and naturally survive in soil, their continued maintenance on artificial culture media prior to use in our study could have negatively affected their viability upon re-exposure to environmental conditions. In fact, while Wang et al. [59] demonstrated that fungal conidia synthesize and store messenger RNA (mRNA) transcripts for future use in the production of enzymes and metabolites needed for conidial germination and virulence, they also established that the type of mRNA transcripts generated is dependent on the state of the environment in which the conidia are exposed to at the time. Consequently, while the spore conidia were formed on synthetic PDA media, exposure of the spores to the soil and pseudostem trap conditions in the field could have rendered the transcripts less effective in modulating spore viability, hence the observed considerable reduction in spore viability from >90% on day zero to ≤41% on day three with prolonged time of exposure. Furthermore, corms and pseudostems of freshly cut banana plants have been

shown to emit volatile compounds with an attractive effect on the BW [60–62]. Whether these biomolecules and compounds have any effect on fungal spore viability, or how they affect the viability is not fully understood.

Our study in the laboratory revealed variable pathogenicity between the EPF isolates, as depicted by the mean mortality (20–82.5%) and LT_{50} values (5.3 days–∞). Of the 15 *B. bassiana* isolates tested in the lab, eight were highly pathogenic to adults of BW (mortality >50%), while seven were less pathogenic (mortality <50%). Pathogenicity of *B. bassiana* isolates against the adult BW has been studied previously in the lab, with Lopes et al. [44] reporting mortality levels of 6–96%, while Omukoko et al. [25] reported 4–51%, Membang et al. [24] 34.8–96.3% and Kaaya et al. [22] 63–97%. Our range of values for adult BW mortality following exposure to *B. bassiana* reflect the findings of these studies. Similar to our methods, Omukoko et al. [25] and Membang et al. [24] used spore suspensions, while Kaaya et al. [22] used dry spores. This indicates that *B. bassiana* isolates can be developed as either a liquid or powder formulation and still be able to achieve the same effect. On the other hand, all isolates of *M. anisopliae* and *I. fumosorosea* used in our study were less pathogenic to adult BW (mortality $\leq$ 7.5%). Kaaya et al. [22] also reported low mortality levels (11.7%) of the adult BW following exposure of BW to spores of *M. anisopliae*. In contrast, Lopes et al. [63] and Membang et al. [24] reported mortality rates of 24–79.5% and 7.4–92.6% following exposure of the adult BW to isolates of *M. anisopliae*, respectively. The low pathogenicity of *M. anisopliae* isolates exhibited in our study could be attributed to inter-species variation in virulence of the fungus.

Efficacy of an EPF depends on its ability to attach onto the insect host body (cuticle), germinate, invade and proliferate in its hemocoel. Host death results from nutrient depletion, physical obstruction and/or toxinosis [41]. The insect cuticle is the primary physical barrier that EPFs encounter prior to establishment of an epizootic in the host insect population. Studies have shown EPFs to produce proteases, lipases, chitinases and other bioactive metabolites [64,65]. These enzymes play an important role in the pathogenesis and physiological processes, and form part of the initial host infection process. While testing the efficacy of three *Cordyceps fumosoroseus* (Wize) isolates against nymphs of the whitefly *Trialeurodes vaporariorum* (Westwood) (Homoptera: Aleyrodidae), Castellanos-Moguel et al. [66] established that efficacy was proportional to the amount of protease enzyme produced by the respective isolates. Similarly, while testing the efficacy of six *B. bassiana* isolates against larvae of the cabbage pest *Pieris brassicae* (L.), Dhawan and Joshi [67], established that there was variation in the amount and type of enzyme (chitinase, protease and lipases) produced by *B. bassiana* isolates. Furthermore, efficacy of each isolate was proportional to the total amount of enzymes produced by the respective *B. bassiana* isolate [67]. Consequently, EPF isolates belonging to the same (or different) species express variations in the level (and type) of bioactive metabolite(s). Thus, the observed inter- and intra-species mortality variations in our study could be attributed to variations in the type and level of bioactive metabolites produced by the different EPF isolates. However, the type and levels of metabolites produced by each isolate used in our study are yet to be established.

Short kill time and low effective dose are critical factors to consider in the selection and development of an effective mycoinsecticide, which positively impacts on the subsequent selection for commercial production [68]. One of the major reasons why farmers continue to use synthetic chemicals is due to their rapid knock-down effect, while microbial alternatives tend to have a slow and more prolonged killing time. Thus, selecting EPF isolates with a shorter kill time can help facilitate adoption and positively impact the acceptance of these mycoinsecticides by the banana farmers as alternatives to synthetic chemical pesticides. In our in vitro pathogenicity study, the three *B. bassiana* isolates—ICIPE 273, ICIPE 648 and ICIPE 660—exhibited the shortest kill time ($LT_{50} \leq 11.1$ days) and lowest effective dose ($LC_{50} \leq 5.3 \times 10^7$ spores mL^{-1}), thus presenting them as promising isolates for development into mycoinsecticides. Furthermore, the commercial production of microbial insecticides is largely dependent on cost-effective production systems that can produce

high yields of highly infective fungal spores [69]. Consequently, achieving high efficacy at a low spore dose and high degree of sporulation are key aspects in the selection and potential commercialization of a given microbial insecticide.

Pathogenicity of ICIPE 273 has been studied previously; among the nine *B. bassiana* isolates screened, Omukoko et al. [25] established that ICIPE 273 was the most pathogenic causing 51% mortality of adult BW, which is a relatively low mortality level compared to the 80% observed in our study. The differences observed between the two studies in BW mortality following exposure to ICIPE 273 could be attributed to the difference in method of application used. In our study, the adult BW were sprayed with the fungal suspension and left in the Petri dish lined with sterile filter paper for 24 h, while Omukoko et al. [25], dipped the BW in a spore suspension for 11 s followed by draining off the excess suspension. Spore viability is humidity depended, the moist, EPF-soaked filter papers used in our study could not only have provided a prolonged conducive environment for spore germination, but also helped maintain a relatively moist BW exoskeleton thus facilitating the infection process. On the other hand, the immediate draining off of the excess EPF suspension from the BW body by Omukoko et al. [25] meant a gradual loss of moisture, hence could have rendered the environment suboptimal for EPF spore germination on the BW body, and hence the observed difference in the infection levels of ICIPE 273 between the two studies.

Efficacy of the selected three *B. bassiana* isolates (ICIPE 273, ICIPE 648 and ICIPE 660) in the field reduced to $\leq$31% BW mortality on day one, with no weevil mortality reported after three days post field application. The observed variation in the laboratory versus field BW mortality levels across the three EPF isolates could be attributed to the sudden reduction in spore viability upon application in the field. Efficacy of an EPF can be improved by manipulating the formulation or mode of delivery of the fungal spores [28]. Generally, oil-based formulations of the mycoinsecticides are reported to be more effective than aqueous, granular or dry spore formulations, as the oil improves the shelf life of the mycoinsecticide, improves the thermotolerance, protects the propagules against the UV radiation, enhances attachment of the conidia onto the insects body and maintains spore viability for a longer time [70–74].

The potential of a mycoinsecticide to produce spores on insect cadavers is beneficial as this acts as a source of inoculum for secondary infections and secondary control, thus increasing the probability of spreading the epizooty among the pest population [75]. Furthermore, the potential of the candidate EPF isolates to sporulate on BW cadavers results in a reduction in the number of applications needed in the field, as initial culture can be maintained through self-proliferation on cadavers, requiring only a small booster dose over time. This would ultimately result in a reduction in cost to the farmer, due to the reduction in frequency of application. In our study, *B. bassiana* isolates: ICIPE 273, ICIPE 648 and ICIPE 660 were able to produce high spore concentrations ($> 3.3 \times 10^7$ spores) in BW cadavers. In addition to host behavior, secondary infection can be aided by environmental conditions. Rain has been reported to aid dispersal of fungal spores over greater distances through run off water, the splashing droplets and vibration made by the impact of rain droplets [75]. In addition, wind can support both short- and long-distance dispersal of fungal spores. While BW numbers are reported to surge during the wet and humid season [76], fungal spore production and efficacy are also aided by high humidity [77]. Thus, field application of fungal-based microbial insecticides during the rainy season has the potential of counteracting the surge in BW numbers. This can further be aided by the spreading of the cadaver-generated fungal spores by the splashing of droplets and by runoff water, in addition to the humid environment being favorable for fungal sporulation, spore germination and epizooty [77].

5. Conclusions

The *B. bassiana* isolates ICIPE 273, ICIPE 648 and ICIPE 660 exhibited high mortality levels and low median lethal time against the BW in the lab. In addition, the isolates were able to sporulate efficiently on BW cadavers. Furthermore, the three EPF caused BW

mortality when applied in the field. Thus, these three isolates can be regarded as potential candidates for the management and control of BW. However, different formulations of these isolates need to be assessed across a range of environmental conditions. In addition, due to the genetic variability in BW from the different banana growing regions, it is crucial that the candidate isolates be tested against BW from different geographical locations. Furthermore, testing compatibility and performance of these candidate isolates with already existing environmentally friendly management strategies will help develop a strong IPM strategy against the BW. Since *B. bassiana* isolates are known to have a wide host range, including non-target species, testing of the selected pathogenic isolates against non-target organisms is recommended. This will help establish their direct effect on the environment and thus guide decisions on developing them into environmentally friendly biopesticides. In addition, the endophytic potential of the fungal isolates for the management of BW in banana would be useful to study. This will ultimately help to reduce the reliance on synthetic agrochemical-chemical pesticides, towards better protection of ecological services, the environment and human life. In turn, this will boost the use of climate and environment smart management strategies against the BW.

Author Contributions: Conceptualization, J.K., H.F., D.C., L.C., S.M., S.S. and S.H.; methodology, J.K., H.F., D.C., S.M., S.S., S.H.; software, J.K.; validation, J.K., H.F., D.C., S.S. and S.H.; formal analysis, J.K.; investigation, J.K., S.M., D.C. and S.H.; resources, J.K., H.F., S.S., L.C., D.C. and S.H.; data curation, J.K. and S.M.; writing—original draft preparation, J.K.; writing—review and editing, J.K., H.F., D.C., L.C., S.M., S.S. and S.H.; visualization, J.K., H.F., D.C., S.S. and S.H.; supervision, H.F., D.C., L.C., S.S. and S.H.; project administration, S.H., S.S. and D.C.; funding acquisition, D.C., H.F., L.C., S.S. and S.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by H2020 European Union under the project Microbial Uptakes for Sustainable management of major banana pests and diseases (MUSA): project number, 727624. J.K. was supported by the German Academic Exchange Service (DAAD) through the African Regional Postgraduate Programme in Insect Science (ARPPIS).

Data Availability Statement: The raw data supporting the conclusions of this study will be made available by the authors, without undue reservation.

Acknowledgments: We gratefully acknowledge the financial support for this research by the following organizations and agencies: UK's Foreign, Commonwealth and Development Office (FCDO); the Swedish International Development Cooperation Agency (Sida); the Swiss Agency for Development and Cooperation (SDC); the Federal Democratic Republic of Ethiopia; and the Government of the Republic of Kenya. The Real IPM company Ltd. is acknowledged for supply of two of the fungal isolates used in this study. Appreciation is extended to colleagues in the Arthropod Pathology Unit (APU) and the NemAfrica team for their guidance and assistance in experimental setup and data collection. We also appreciate the Plant Biotechnology and Breeding (Plant B+B) International Thematic Network supported by Ghent University for the financial support towards Article Processing Charge (APC).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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CHAPTER 4: Article 2

Endophytic non-pathogenic *Fusarium oxysporum*-derived dual benefit for nematode management and improved banana (*Musa* spp.) productivity

How to cite this article:

Kisaakye, J.; Fourie, H.; Haukeland, S.; Kisitu, J.; Nakimera, S.; Cortada, L.; Subramanian, S.; Coyne, D. Endophytic non-pathogenic *Fusarium oxysporum*-derived dual benefit for nematode management and improved banana (*Musa* spp.) productivity. *Agriculture* 2022, 12, 125. https://doi.org/10.3390/agriculture12020125

agriculture

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Article

Endophytic Non-Pathogenic *Fusarium oxysporum*-Derived Dual Benefit for Nematode Management and Improved Banana (*Musa* spp.) Productivity

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Abstract: The banana weevil (*Cosmopolites sordidus*) and the burrowing nematode *Radopholus similis* represent two of the most important pests of bananas. Previously, colonization of banana plants by the non-pathogenic *Fusarium oxysporum* (isolate V5w2) and the entomopathogenic *Beauveria bassiana* (isolate WA) have been shown to increase host resistance to various banana pests and diseases. However, there is limited data on how the combined inoculation of these isolates would affect field performance of bananas. In this study, the fungal endophytes were inoculated separately and in combination. Tissue cultured plantlets of cooking banana cultivar Mbwazirume and dessert banana cultivar Grande Naine were inoculated by root drenching with a suspension of 1.0×10^7 spores mL^{-1} of the endophytes on three occasions, separated 4 weeks apart, before transplanting into the field. Each plantlet was further inoculated with 1800 nematodes, composed primarily of *R. similis*. Inoculation of banana plants with the fungal endophytes significantly reduced nematode densities by >34%. Similarly, plant toppling was lower in the endophyte-enhanced plants (<16.5%) compared with the control (23.3%). We also observed improved yield of the first crop cycle in the endophyte-enhanced plants, which yielded >11 t ha^{-1} $year^{-1}$ versus 9 t ha^{-1} $year^{-1}$ achieved in the non-inoculated plants. These findings demonstrate the benefits of fungal endophytes in improving the yield of both cooking and dessert bananas via suppression of nematode densities and nematode-related damage.

Keywords: banana nematodes; biological control; endophytes; microbial antagonists; bio-enhanced plants; *Cosmopolites sordidus*

Citation: Kisaakye, J.; Fourie, H.; Haukeland, S.; Kisitu, J.; Nakimera, S.; Cortada, L.; Subramanian, S.; Coyne, D. Endophytic Non-Pathogenic *Fusarium oxysporum*-Derived Dual Benefit for Nematode Management and Improved Banana (*Musa* spp.) Productivity. *Agriculture* **2022**, *12*, 125. https://doi.org/10.3390/agriculture12020125

Academic Editor: Oscar E. Liburd

Received: 13 December 2021
Accepted: 7 January 2022
Published: 18 January 2022

1. Introduction

Banana (*Musa* spp.) is a major food and cash crop in more than 135 countries and territories across the tropics and subtropics. The crop ranks second in fruit production with a global production estimated at approximately 167 million metric tons (t) [1] feeding more than 400 million people. Banana is mainly cultivated by smallholder farmers. The cooking bananas are produced primarily for home consumption, local and regional markets, with over 85% consumed in-country. On the other hand, dessert bananas are majorly cultivated for commercial purposes, and sold both on the local and international markets. In 2016, the estimated trade value for bananas was US$11.5 billion [2], with Africa responsible for approximately 33% of the global banana production. In 2018, approximately 10% of the

global banana production was produced in the East African region [1]. In some East African countries, banana provides up to 60% of the daily calorie intake. Uganda has the highest consumption at >0.5 kg person^{-1} day^{-1}.

There has been a global decline in banana production for the past 40 years [1] with the current yield being a fraction of its potential [3,4]. The banana weevil (*Cosmopolites sordidus*) and a multifaceted array of plant-parasitic nematodes (PPNs) are among the critical biotic constraints to banana production [5]. *Radopholus similis* is generally viewed as the most damaging nematode pest of bananas [6–8], but in East Africa a complex of species, comprising *R. similis, Helicotylenchus multicinctus*, *Pratylenchus* spp. and *Meloidogyne* spp. create a combined community that affects bananas [9–11]. The community composition varies by locality, climate and banana genotype [10]. Most of the developmental stages of the weevil (eggs, larvae and pupa) are found in the corms of infested plants, while the adults are found in crop residues, sheltering in or around the corms, and between the leaf sheaths in close association with the banana corms. In contrast, all development stages of the nematodes reside in the corm and roots of infested plants [12]. Despite the free movement of adult weevils and their ability to infest new fields, the dissemination of both weevils and nematodes is primarily through contaminated planting materials. Most banana farms in the region are plagued with both weevils and PPNs, usually in combination. Heavy infestation of banana fields by both pests can result in up to 100% yield losses [13].

The physical damage caused by weevils and nematodes is also a crucial entry point for banana pathogens, while weevils are also known to act as a vector for some banana diseases [14,15]. A key strategy to improve banana production therefore relies on developing appropriate and effective management options against both the weevil and the PPNs.

The use of fungal endophytes in the management of both the weevil and nematodes has previously been shown to be effective. The non-pathogenic *Fusarium oxysporum* isolate V5w2 effectively colonized banana plants without penalty to the host, and even improved plant growth [16–18]. Similarly, the entomopathogen *Beauveria bassiana* isolate WA effectively colonized banana plants without hampering plant vigor or development [19]. Endophytic colonization of *F. oxysporum* also led to reduced nematode damage and infection levels [17,20–22], while colonization by *B. bassiana* reduced weevil density and damage [23,24]. The majority of studies, however, have been undertaken in pots in the screenhouse, although some limited field work has been conducted [25]. Field studies in Uganda demonstrated that following inoculation with *F. oxysporum*, colonization persisted for up to 6 months [26], and that bunch weight and yield during the first crop cycle was higher when enhanced with *F. oxysporum* compared with uninoculated controls [25]. There is limited information about the dual inoculation of *F. oxysporum* and *B. bassiana* in banana and the potential effect on plant pest and disease resistance and effect on yield. The current study was consequently designed to assess the performance of single and combined inoculation of *F. oxysporum* and *B. bassiana* into tissue-cultured banana plants to determine endophyte colonization persistence, effect on weevil damage and PPN infection levels, plant growth and yield under field conditions, simulating smallholder farming conditions in Uganda.

2. Materials and Methods

2.1. Fungal Cultures

Fusarium oxysporum isolate V5w2, previously isolated from roots of healthy banana plants in central Uganda [27], and the *B. bassiana* isolate WA, previously isolated from a mycosed banana weevil from Mbarara, Western Uganda [28,29] were sourced from the Stellenbosch University, South Africa and the microbial culture collection unit at the Centre for Agriculture and Bioscience International (CABI), United Kingdom, respectively. The two isolates were sub-cultured from cultures on synthetic nutrient agar (SNA) media by placing SNA media plugs containing the actively growing mycelia onto freshly prepared half-strength potato dextrose agar (PDA) media in 90 mm glass Petri dishes. Cultures

were maintained in the laboratory at ambient temperature (approximately 25 °C) and a natural photo period (12:12 h, light: dark) for 3 weeks until sporulation. After sporulation, 3–4 media blocks (approximately 0.5 cm^3) from each isolate were placed into 300 mL Erlenmeyer flasks containing 150 mL of sterile potato dextrose broth (PDB) media, and was incubated for 3 days on a rotary shaker (300 rpm) [30,31].

2.2. Mass Production of Fungal Spores and Inoculum Preparation

Long grain rice (*Oryza sativa*) (2 kg) was soaked in boiling water for 30 min, placed on a 1 mm pore sieve to drain off excess water and then rinsed under running tap water to eliminate all the starch. The washed rice was transferred into self-aerating Milner bags (600 mm × 350 mm) and autoclaved at 121 °C for 1 h, then left overnight to cool down. Each bag was individually inoculated with 25 mL of the 3-day old PDB mycelial culture of the fungal isolates. The bags were aseptically sealed and incubated at ambient temperature (25 ± 2 °C) for 2–3 weeks with daily massaging to enhance uniform fungal growth and spore formation [31].

Following sporulation, the rice substrate was placed on an 850 µm-aperture pore sieve, the spores gently washed off the grains using sterile distilled water and were collected in a 1 L beaker placed below the sieve. The viability of the fungal spores was determined as described by Inglis et al. [32] and the fungal inoculum was prepped by adjusting the spore concentration of each individual isolate to 1.5×10^7 spores mL^{-1} using a haemocytometer under a compound microscope. Additionally, an inoculum containing spores of both fungal isolates was prepared by mixing equal volumes of the *F. oxysporum* isolate V5w2 and *B. bassiana* isolate WA, to result in a spore concentration of 7.5×10^6 spores mL^{-1} per isolates and a combined spore concentration of 1.5×10^7 spores mL^{-1}.

2.3. Tissue Cultured Banana Plants

The East African Highland cooking banana cultivar Mbwazirume (EA-AAA) and the dessert banana cultivar Grande Naine (AAA) were used for the study. The banana cultivars Mbwazirume and Grande Naine are among the nematode susceptible cultivars grown in the Great Lakes region of Africa, primarily for cooking and dessert purposes, respectively. Tissue-cultured (TC) plants at the deflasking stage were sourced from two separate commercial tissue culture laboratories. Plants of cultivar Mbwazirume were sourced from Agro-Genetic Technologies Ltd. (AGT), Buloba, Uganda, while plants of cultivar Grande Naine were sourced from Agromax Uganda Ltd., Kampala, Uganda. The plant roots were washed free of rooting media with tap water and planted in 66 multi-cell plug plastic propagation trays containing a steam sterilized potting mixture of forest soil: composted manure (2:1, *v*/*v*). Plants were maintained in a humidity chamber (relative humidity > 80%, Temp = 25 ± 2 °C) for 5 weeks, before transferring into 2 L plastic potting bags filled with the same steam sterilized potting mixture. The plants were placed in the greenhouse for acclimatisation and watered daily for 10 weeks before transplanting into the field.

2.4. Inoculation of Tissue Cultured Plants

Plants were inoculated with the fungal endophytes on three occasions: (1) at the deflasking stage, (2) at 4 weeks after deflasking, (3) and at 4 weeks after transfer into the 2 L potting bags (8 weeks old plants). For each inoculation, the plant roots were drenched with approximately 10% (*v*/*v*) of the fungal suspension, which was equivalent to~4 mL per plant in the seedling trays and ~200 mL per plant in the potting bags. The volume used for plant drenching was based on the maximum volume of suspension that the potting substrate could absorb without leaking. The control plants were drenched with distilled water drained through rice grains not inoculated with the fungal isolates.

2.5. Field Site and Experimental Design

The field trial was established in November 2018 at the International Institute of Tropical Agriculture (IITA) research station, Namulonge, Uganda (00°31′49.6″ N, 32°36′42.2″ E) at 1128 m above sea level.

The field trial was conceived as a two-factorial experiment, where each banana cultivar (cultivar Mbwazirume and cultivar Grande Naine) was assessed vis-à-vis each endophyte treatment. Treatments were arranged in a randomized complete block design (RCBD) comprising four blocks with eight experimental plots per block, each plot corresponding to a different experimental treatment. Each experimental plot contained 16 plants with a 3 × 3 m inter-plant spacing, and a 5 m inter-plot and inter-block spacing. A border row containing an untreated mixture of banana cultivars was planted around the experimental area, spaced 4 m from the trial plots.

2.6. Nematode Inoculation, Field Establishment and Maintenance

Prior to the transplant of the banana plants in the field, nematode assessment was undertaken from two soil samples per plot. Soil per plot were bulked and nematodes extracted using 100 mL sub-samples to determine nematode presence and density [33]. No PPNs associated with banana were recovered from the trial area and so the plants were each inoculated with 1800 nematodes 8 weeks after transfer into the 2 L potting bags, and 2 weeks before transplanting to the field. The inoculated nematodes included, 1000 pure *R. similis* cultured on carrot discs [34], which was supplemented with 800 individuals (males, females, second-stage juveniles) of a mixed population (*R. similis* (62%), *H. multicinctus* (36%) and *Meloidogyne* spp. (2%)) using 11 g of chopped, infected fresh banana roots collected from a neighbouring field, after assessing density using a modified Baermann method [33,35]. This was undertaken to resemble the multi-species distribution frequently observed in naturally infested fields. To inoculate plantlets, a layer of topsoil was removed from the potted plantlets exposing the topmost banana roots. Four holes were created around each plantlet's roots with a pencil and the *R. similis* pipetted into the holes, and the chopped banana roots placed around the banana roots before replacing the topsoil over the roots.

The banana plants were planted into planting holes measuring approximately 60 × 60 × 60 cm (L × W × H) filled with 20 kg of composted manure mixed with top-loam soil [36,37]. After transplanting, no supplementary irrigation was provided during the first month as it was the wet season. From December onwards, during the dry season, each plant received 10 L of water twice a week until February 2019, and dry grass was additionally applied as mulch around each plant to reduce evaporation. Plants that died due to transplant shock in the first month following transplanting were replaced with plants of the same treatment. The field was weeded regularly, and the trial was maintained through two cropping cycles (~24 months).

2.7. Data Collection

2.7.1. Plant Growth and Yield Data

Plant growth parameters were recorded for plant height (from the soil level to the point of the youngest leaf emergence), pseudostem girth (measured at the soil level and 100 cm above ground) and the number of functional leaves were recorded for each plant at transplanting (0-), 3-, 6- and 9-months post transplanting (MPT), at flowering and at harvest stages. In addition, root samples were collected at the same time points to assess fungal colonization and nematode damage and density estimations.

The trial was monitored 3 times a week and dead, snapped (broken at the pseudostem-corm junction) and toppled (uprooted) plants were recorded. Plant loss was attributed to weevils if plants were dead with characteristic weevil symptoms, i.e., stunted plant growth with a dead central cylinder [38], or snapped, or attributed to nematodes if toppled.

Bunches were harvested upon the ripening of the first finger and the weight recorded. The date was recorded and the number of days from planting to harvest and flowering to maturity calculated.

2.7.2. Banana Weevil Damage

Banana weevil assessment was undertaken for all plants at harvest or when snapped, toppled or recorded as dead according to Viljoen et al. [39]. Cross-sections were cut through the corm at the collar to assess damage to the pseudostem and at 10 cm below the collar to assess damage to the corm. The cortex and the central cylinder were divided into 4 equal-sized portions at each cross-section, each representing 25% of the surface area and the percentage of tissue consumed by the weevil larvae (i.e., galleries) was scored out of 25% for each quarter. The total damage to the pseudostem and corm was calculated as the sum from all 4 quarters.

2.7.3. Nematode Infection and Endophyte Colonization from Root Samples

At three-month intervals (3-, 6- and 9-MPT), 4 plants per treatment per block were randomly selected for assessment of PPN densities and damage, and endophyte colonization. At field establishment, 5 randomly selected plants were sampled from each banana cultivar × endophyte treatment to assess for endophytic root colonization. For each sampling, 10 roots were randomly collected from a hole (5 × 5 × 5 cm) excavated at the base of the mat. Five roots were used for assessment for nematode damage and densities, and five roots for endophyte colonization. Furthermore, 5 roots were sampled from each dead, snapped and toppled plant, and for each plant at flowering and harvest of the mother crop (R0) and ratoon crop (R1) for nematode damage and densities.

To assess nematode damage each root was cut to 10 cm length and sliced lengthwise. One half of the sliced root was scored for necrotic root damage, each root damage was scored out of 20% according to Speijer et al. [40], and the total percentage of root necrotic damage calculated as the sum of the five root scores. These roots were then chopped into ca. 0.5 cm pieces and thoroughly mixed, and a 5 g sample used to extract nematodes over a 48-h period using the modified Baermann technique [26,28]. Nematode suspensions were reduced to 25 mL and nematode densities quantified from 3 × 2 mL aliquots using a compound microscope (×20 magnification) (Leica Microsystems, Wetzlar, Germany).

The roots for endophyte colonization were thoroughly washed under running tap water, then blotted dry with tissue paper. In the laminar flow cabinet, roots were disinfected by dipping in 400 mL of 15% (v/v) commercial bleach (3.85% m/v sodium hypochlorite) for 1 min, followed by dipping in 400 mL of 75% ethanol for 1 min before rinsing thrice in 400 mL sterile distilled water and blotting dry with sterile tissue paper. Two pieces approximately 0.5 cm long were cut from the tip, middle and base of each root. The 6 root pieces per root were inserted halfway into half-strength PDA media supplemented with streptomycin sulphate (0.2 g L^{-1}), penicillin G (0.1 g L^{-1}) and chlortetracycline (0.05 g L^{-1}) in 90 mm diameter sterile Petri dishes. These were incubated at ambient temperature (25 ± 2 °C) for 10–14 days. Regrowth of the characteristic fungal isolate from plated root piece(s) signified colonization by the respective fungal isolate(s), the root pieces were monitored and those exhibiting growth of the fungal isolate(s) were quantified and used to calculate percentage fungal colonization (Equation (1)). Identification of the fungal isolates was conducted based on the morphometric characteristics of the fungal colony, whereas a white aerial mycelia tinged with purple to reddish-brown color, short microconidia produced on false heads was assigned to *F. oxysporum* [41], and the white mycelia with a creamy to powdery appearance and rounded to single ovoid conidia was attributed to *B. bassiana* [42].

$$\text{Percentage fungal colonization per plant} = \frac{\text{A}}{\text{B}} \times 100\% \quad (1)$$

where:

A = Total number of root pieces exhibiting characteristic growth of the fungal isolate per plant;

B = Total number of root pieces plated per plant.

2.8. Data Analysis

Due to the binary nature of the endophyte colonization data: colonized vs. non-colonized, nematode damage: necrotized vs. non-necrotized root tissue, weevil damage: damaged vs. non-damaged banana corm tissue, plant survival at harvest: snapped or toppled vs. healthy plants, the data were fitted using generalized linear models (GLM) with binomial distribution [43] to check the effect of endophyte inoculation on each of the variables. The significance of the model was established using an analysis of deviance (Wald chi-square test) followed by computation of least-square means using the 'emmeans' package [44], and group mean separation was performed by Tukey multiple comparisons.

Analysis of variance was performed on nematode, plant growth, bunch weight and plant yield data fitted with linear mixed-effects models using 'lmer' function of the package 'lme4' [45]. Cropping cycle and/or banana cultivar and/or endophyte treatment were specified as fixed variables, while the block was specified as the random variable. Model significance was established using an analysis of deviance (Wald chi-square test). When significant differences were observed between group means of the explanatory variable(s), computation of least-square means was performed using the 'lsmeans' function of the package 'emmeans' and group means were separated using the adjusted Tukey's method executed using the 'cld' function from the 'multicomp' package.

All data analyses were performed using R, Version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria) statistical software [46].

3. Results

3.1. Endophyte Colonization of Plant Roots

No difference in root colonization was observed between banana cultivars for the fungal isolates V5w2 ($\chi^2 = 0.24$, $p = 0.62$) or WA ($\chi^2 = 0.15$, $p \geq 0.59$) and, therefore, data were pooled across banana cultivars for analysis.

Isolation of fungal isolates from banana roots prior to transplanting confirmed the successful colonization of both fungal isolates V5w2 (>30%) and WA (11%). Differences in fungal colonization of roots at the sampling occasions were observed for V5w2 ($\chi^2 = 299.4$, $df = 2$, $p < 0.001$) and WA ($\chi^2 = 199.6$, $df = 2$, $p < 0.001$), with the colonization effect reducing with plant age (Figure 1).

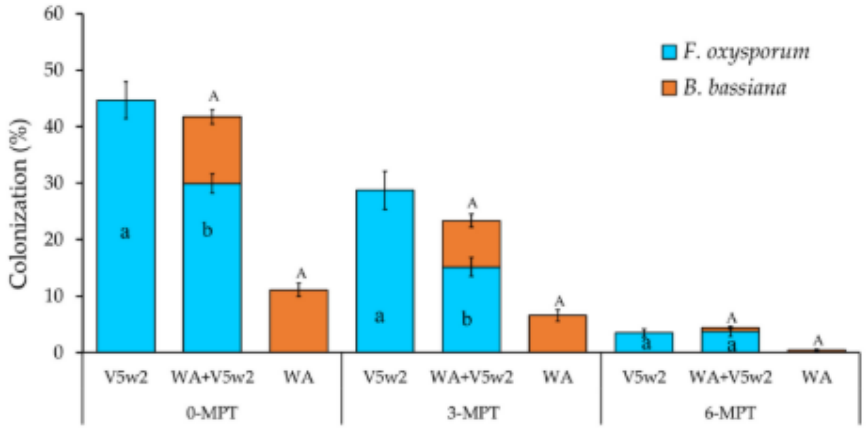

Figure 1. Colonization of banana root tissue of cultivars Mbwazirume and Grande Naine (data pooled) by the fungal endophytes *Fusarium oxysporum* isolate V5w2 and *Beauveria bassiana* isolate WA at 0-, 3-, and 6-months post transplanting. V5w2: plants singly inoculated with *F. oxysporum* isolate V5w2, WA: plants singly inoculated with *B. bassiana* isolate WA, WA + V5w2: plants inoculated with a combination of the two fungal isolates. For each time point, bars followed by different letter(s) indicate significant difference in colonization by *F. oxysporum* (lower case letters) and *B. bassiana* (upper case letter) between treatments. MPT: months post-transplanting.

There was a significant effect of the fungal treatment on the root colonization by the isolate V5w2 at transplanting (χ^2 = 16.94, $p < 0.001$) and 3 months post transplanting (χ^2 = 14.62, $p < 0.001$). For both sampling occasions, the root colonization was significantly higher in plants where the V5w2 isolate was inoculated singly, compared to plants in which the two fungal isolates (WA and V5w2) were simultaneously inoculated. However, there was no effect of fungal treatment on V5w2 colonization of plant roots at 6-months post transplanting in the field (χ^2 = 0.06, p = 0.80). On the contrary, there was no treatment effect on root colonization by isolate WA at 0-, 3- and 6-MPT ($\chi^2 \leq 1.05$, $p \geq 0.30$) (Figure 1). None of the fungal isolates were recovered from the banana roots from either cultivar at 9-MPT.

3.2. Plant Growth, Survival, Toppling and Snapping

Plant height, girth at the base of banana pseudostem, girth at 100 cm above pseudostem base, and the number of functional leaves did not differ between endophyte treatments across all growth stages ($p \geq 0.11$). However, these growth parameters differed between banana cultivars ($p < 0.001$). Except for the number of functional leaves, which was significantly higher in cultivar Grande Naine, plant height, girth at the pseudostem base, and girth at 100 cm above pseudostem base were significantly higher for cultivar Mbwazirume compared to cultivar Grande Naine (Table 1).

Table 1. Effect of single and combined inoculation of *Fusarium oxysporum* isolate V5w2 and *Beauveria bassiana* isolate WA on banana plant development in the field at six growth stages.

Banana Cultivar	Treatment	Banana Growth Stage					
		0-MPT	3-MPT	6-MPT	9-MPT	Mother Crop (Harvest)	Ratoon Crop (Harvest)
Plant height (cm)							
Grande Naine	Control	13.1 ± 0.3 bB	57.6 ± 1.7 aB	103.3 ± 3.0 aB	162.0 ± 3.5 aB	181.2 ± 3.9 aB	207.9 ± 4.4 aB
	V5W2	18.0 ± 0.4 aB	58.7 ± 2.2 aB	106.3 ± 3.1 aB	161.3 ± 3.2 aB	175.8 ± 3.3 aB	202.9 ± 4.3 aB
	WA	14.7 ± 0.5 bB	58.4 ± 2.1 aB	101.1 ± 3.2 aB	158.4 ± 3.6 aB	177.1 ± 4.2 aB	197.3 ± 3.9 aB
	WA + V5w2	16.6 ± 0.3 aB	59.5 ± 1.9 aB	103.8 ± 3.2 aB	166.2 ± 3.1 aB	183.4 ± 2.8 aB	214.9 ± 4.4 aB
Mbwazirume	Control	24.0 ± 0.6 abA	78.9 ± 1.8 aA	132.9 ± 2.7 aA	217.2 ± 3.7 aA	260.9 ± 3.2 aA	284.7 ± 4.7 aA
	V5W2	24.2 ± 0.6 abA	77.8 ± 2.1 aA	133.9 ± 3.3 aA	212.3 ± 4.3 aA	257.8 ± 3.3 aA	284.3 ± 3.6 aA
	WA	22.6 ± 0.6 bA	79.6 ± 2.2 aA	135.4 ± 3.2 aA	219.9 ± 4.6 aA	263.4 ± 2.0 aA	291.7 ± 3.5 aA
	WA + V5w2	24.8 ± 0.5 aA	79.1 ± 1.6 aA	132.2 ± 2.8 aA	217.7 ± 4.3 aA	256.5 ± 3.4 aA	279.9 ± 5.3 aA
Girth (cm) at pseudostem base							
Grande Naine	Control	na	24.4 ± 0.8 aB	42.2 ± 1.2 aB	63.8 ± 1.3 aB	63.3 ± 0.9 abB	69.7 ± 1.2 abA
	V5W2	na	25.2 ± 0.8 aB	44.4 ± 1.2 aB	63.5 ± 1.0 aB	60.2 ± 0.6 bB	67.3 ± 1.2 bB
	WA	na	24.8 ± 0.9 aB	41.0 ± 1.2 aB	61.6 ± 1.1 aB	60.8 ± 0.8 abB	65.5 ± 1.0 bB
	WA + V5w2	na	24.8 ± 0.8 aB	42.3 ± 1.3 aB	64.7 ± 1.1 aB	63.5 ± 0.9 aB	73.7 ± 1.0 aA
Mbwazirume	Control	na	27.7 ± 0.7 aA	49.7 ± 1.0 aA	69.4 ± 0.9 aA	67.0 ± 1.1 aA	71.5 ± 1.3 aA
	V5W2	na	27.7 ± 0.9 aA	48.4 ± 1.3 aA	68.8 ± 1.1 aA	66.6 ± 1.0 aA	72.0 ± 1.0 aA
	WA	na	27.8 ± 0.8 aA	48.5 ± 1.1 aA	69.8 ± 0.9 aA	69.0 ± 0.9 aA	75.7 ± 1.2 aA
	WA + V5w2	na	26.8 ± 0.5 aA	47.7 ± 1.0 aA	67.0 ± 1.1 aA	66.7 ± 0.9 aA	72.7 ± 1.2 aA
Girth (cm) at 100 cm above pseudostem base							
Grande Naine	Control	na	na	36.4 ± 0.6 aA	45.8 ± 0.9 aA	46.2 ± 0.8 aB	56.9 ± 1.1 bA
	V5W2	na	na	35.3 ± 0.6 aA	44.8 ± 0.6 aA	44.8 ± 0.7 aB	54.6 ± 1.0 bA
	WA	Na	na	35.0 ± 0.9 aA	43.7 ± 0.7 aB	45.6 ± 0.7 aB	53.8 ± 0.8 bB
	WA + V5w2	na	na	35.3 ± 0.6 aA	45.1 ± 0.8 aA	46.8 ± 0.8 aB	61.1 ± 1.0 aA
Mbwazirume	Control	na	na	34.2 ± 0.5 aB	47.1 ± 0.8 abA	48.8 ± 0.8 aA	53.5 ± 1.3 bA
	V5W2	na	na	34.1 ± 0.6 aA	46.4 ± 0.9 abA	47.9 ± 0.8 aA	54.5 ± 1.0 abA
	WA	na	na	35.0 ± 0.5 aA	48.7 ± 1.0 aA	50.1 ± 0.8 aA	57.7 ± 1.1 aA
	WA + V5w2	na	na	33.5 ± 0.6 aA	45.1 ± 1.0 bA	47.6 ± 0.7 aA	53.5 ± 1.1 bB
Number of functional leaves							
Grande Naine	Control	6.4 ± 0.1 aA	8.7 ± 0.2 aA	11.8 ± 0.3 aA	8.4 ± 0.2 abA	0.1 ± 0.1 aB	0.6 ± 0.2 aB
	V5W2	6.5 ± 0.1 aA	8.9 ± 0.3 aA	12.4 ± 0.2 aA	8.0 ± 0.2 bcA	0.4 ± 0.1 aB	0.4 ± 0.2 aB
	WA	6.0 ± 0.1 bA	8.6 ± 0.2 aA	12.0 ± 0.2 aA	7.9 ± 0.2 cA	0.3 ± 0.1 aB	0.6 ± 0.2 aB
	WA + V5w2	5.6 ± 0.1 bA	8.4 ± 0.2 aA	12.4 ± 0.2 aA	8.6 ± 0.1 aA	0.3 ± 0.1 aB	0.6 ± 0.2 aB
Mbwazirume	Control	5.3 ± 0.1 aB	7.2 ± 0.1 aB	10.9 ± 0.1 aB	7.5 ± 0.1 aB	2.9 ± 0.2 aA	1.1 ± 0.3 aA
	V5W2	5.4 ± 0.1 aB	7.0 ± 0.2 aB	10.6 ± 0.2 aB	7.4 ± 0.1 aB	3.2 ± 0.2 aA	0.9 ± 0.2 aA
	WA	5.2 ± 0.1 aB	7.0 ± 0.2 aB	10.3 ± 0.2 aB	7.5 ± 0.1 aA	3.0 ± 0.2 aA	1.7 ± 0.2 aA
	WA + V5w2	4.5 ± 0.1 bB	6.8 ± 0.1 aB	10.6 ± 0.2 aB	7.4 ± 0.1 aB	3.4 ± 0.2 aA	1.0 ± 0.3 aA

Values represent means ± standard error. At each growth stage and per each banana cultivar, means followed by the same lower-case letter(s) indicate no treatment difference (per column). While for each endophyte treatment, means with the same upper-case letter(s) indicate no difference between banana cultivars at p = 0.05. na: data not applicable to the specified stage of plant growth. MPT: months post transplanting (per row).

The proportion of flowered plants did not differ between cropping cycles ($\chi^2 = 1.85$, $df = 1$, $p = 0.17$), banana cultivar ($\chi^2 = 0.12$, $df = 1$, $p = 0.72$) and endophyte treatment ($\chi^2 = 7.39$, $df = 3$, $p = 0.06$). Similarly, there was no interaction effect of cropping cycle, banana cultivar and endophyte treatment on the proportion of flowered plants ($\chi^2 = 81.28$, $df = 3$, $p = 0.73$) (Table 2). However, the proportion of harvested plants was significantly different between cropping cycle ($\chi^2 = 23.60$, $df = 1$, $p < 0.001$) and endophyte treatment ($\chi^2 = 17.83$, $df = 3$, $p < 001$). Similarly, there was a significant interaction effect of the cropping cycle and endophyte treatment on the proportion of harvested plants ($\chi^2 = 10.74$, $df = 3$, $p = 0.01$). In the mother crop, inoculation of the banana plants, with either the single *F. oxysporum* isolate V5w2 or a combination of V5w2 and WA, resulted in more harvested plants ($\geq$89.1%) compared to the non-inoculated plants (73.4%) (Table 2).

Table 2. The effect of single and combined inoculation of *Fusarium oxysporum* isolate V5w2 and *Beauveria bassiana* isolate WA on the proportion of flowered, harvested, toppled and snapped banana cultivars Mbwazirume and Grande Naine (data pooled) along two cropping cycles.

Crop Cycle	Treatment	Flowered Plants (%)	Harvested Plants (%)	Toppled Plants (%)	Snapped Plants (%)
Mother crop (harvest)	Control	90.6 ± 3.1 aA	73.4 ± 3.0 cA	23.3 ± 3.2 aA	3.3 ± 1.2 aB
	V5w2	95.3 ± 2.0 aA	93.6 ± 1.7 aA	2.3 ± 1.6 cB	4.1 ± 1.2 aB
	WA	95.3 ± 2.3 aA	80.4 ± 3.4 bcA	16.5 ± 4.1 abA	3.1 ± 1.7 aB
	WA + V5w2	92.2 ± 1.6 aA	89.1 ± 2.6 abA	7.8 ± 1.6 bcB	3.1 ± 1.7 aB
Ratoon crop (harvest)	Control	88.9 ± 1.7 aA	66.5 ± 5.1 aA	25.7 ± 3.7 aA	7.7 ± 1.9 aA
	V5w2	93.1 ± 2.3 aA	75.5 ± 4.8 aB	16.5 ± 3.3 aA	8.0 ± 2.3 aA
	WA	94.0 ± 2.8 aA	75.8 ± 6.2 aA	18.2 ± 4.5 aA	6.0 ± 3.2 aA
	WA + V5w2	87.9 ± 6.9 aA	67.1 ± 8.2 aB	24.9 ± 4.8 aA	8.0 ± 4.3 aA

Values represent means ± standard error. For each parameter and crop cycle, means followed by the same lower-case letter(s) indicate no treatment difference (per column). While for each treatment, means with the same upper-case letter indicate no difference between cropping cycles (per row) at $p = 0.05$.

There was a significant effect of the cropping cycle on the proportion of snapped plants ($\chi^2 = 8.38$, $df = 1$, $p = 0.003$), where more plants snapped in the ratoon crop (7.4%) compared to the mother crop (3.4%). However, the proportion of snapped plants did not differ between banana cultivar ($\chi^2 = 3.10$, $df = 1$, $p = 0.07$) or endophyte treatment ($\chi^2 = 0.35$, $df = 3$, $p = 0.94$). Furthermore, no interaction effect between cropping cycle, banana cultivar and endophyte treatment was observed ($\chi^2 = 1.34$, $df = 3$, $p = 0.71$) (Table 2).

The length of the cropping cycle (days from planting to harvest) was shorter for banana cultivar Mbwazirume ($\leq$433) compared to cultivar Grande Naine ($\geq$476) in both the mother crop ($F = 97.37$, $df = 1$, $p < 0.001$) and the ratoon crop ($F = 34.65$, $df = 1$, $p < 0.001$). However, there was no effect of endophyte treatment on the cropping cycle length in mother crop ($F = 2.32$, $df = 3$, $p = 0.07$) and the ratoon crop ($F = 0.60$, $df = 3$, $p = 0.61$). Similarly, no interaction effect of endophyte treatment and banana cultivar was observed in both cropping cycles ($F \leq 2.00$, $df = 3$, $p \geq 0.11$).

The proportion of toppled banana plants between the two cropping cycles differed ($\chi^2 = 13.67$, $df = 1$, $p < 0.001$), with more toppled plants observed in the ratoon crop (21.3%) compared to the mother crop (12.5%). However, no difference was recorded between cultivars ($\chi^2 = 0.39$, $df = 1$, $p = 0.53$). However, a significant interaction effect between crop cycle and endophyte treatment was observed ($\chi^2 = 14.13$, $df = 3$, $p = 0.002$). Consequently, data on the proportion of toppled plants were analysed independently for each cropping cycle but it was pooled across banana cultivars within each cropping cycle. In the mother crop, endophyte inoculation with V5w2 or a combination of V5w2 and WA reduced plant toppling ($\leq$7.8%) compared to non-inoculated plants (23.3%) ($\chi^2 = 32.17$, $df = 3$, $p < 0.001$). However, plant toppling in the ratoon crop did not differ between endophyte inoculated and non-inoculated plants ($\chi^2 = 5.35$, $df = 3$, $p = 0.14$) (Table 2).

3.3. Nematode Densities

At 3-, 6- and 9-MPT, nematode densities were similar between banana cultivars ($F \leq 2.59$, $df = 1$, $p \geq 0.11$), with no interaction observed between banana cultivar and the endophyte treatment ($F \leq 1.26$, $df = 1$, $p \geq 0.28$). Consequently, data on nematode density were pooled across banana cultivars for each sampling occasion. The densities of *R. similis* differed between endophyte treatments at 3-($F = 2.68$, $df = 1$, $p = 0.04$), 6-($F = 5.41$, $df = 1$, $p = 0.001$) and 9-($F = 3.44$, $df = 1$, $p = 0.01$) MPT. Similarly, the density of *H. multicinctus* varied between endophyte treatments at 6-MPT ($F = 2.70$, $df = 1$, $p = 0.04$), but not at 3-($F = 0.19$, $df = 1$, $p = 0.90$) or 9-MPT ($F = 1.37$, $df = 1$, $p = 0.25$) (Table 3). While inoculation of the banana plants with V5w2 significantly suppressed the total nematode densities at all the three sampling occasions ($F \geq 2.95$, $df = 3$, $p \leq 0.03$), the combined inoculation of plants with both V5w2 and WA significantly reduced the total nematode density at 6-MPT ($F = 5.65$, $df = 3$, $p = 0.001$) and 9-MPT ($F = 11.44$, $df = 3$, $p = 0.009$) (Table 3). The proportion of samples from which *Meloidogyne* spp. were recovered were few (<7%), so data on *Meloidogyne* spp. infection were not separately analysed but incorporated in the computation and statistical analysis of the total nematode density.

Table 3. Nematode densities (nematodes/100 g root weight) and percentage root damage (root necrosis) of banana plants cultivar Mbwazirume and cultivar Grande Naine (data pooled) inoculated with endophytes *Fusarium oxysporum* isolate V5w2 and *Beauveria bassiana* isolate WA individually and in combination at five growth stages after establishment of banana plants in the field.

Growth Stage	Treatment	*R. similis*	*H. multicinctus*	Total Nematode *	Root Necrosis (%)
3-MPT	Control	3319 ± 645 a	122 ± 50 a	3528 ± 657 a	24.2 ± 1.1 a
	V5w2	1528 ± 308 b	81 ± 30 a	1622 ± 324 b	12.8 ± 1.0 c
	WA	2800 ± 619 ab	116 ± 38 a	2934 ± 626 ab	17.7 ± 0.9 b
	WA + V5w2	2175 ± 373 ab	109 ± 44 a	2341 ± 395 ab	14.5 ± 0.7 bc
6-MPT	Control	5567 ± 780 a	205 ± 78 a	5780 ± 796 a	33.0 ± 1.4 a
	V5w2	2892 ± 410 b	43 ± 16 b	2954 ± 415 b	26.4 ± 1.9 b
	WA	3350 ± 502 b	76 ± 24 ab	3485 ± 512 ab	29.5 ± 1.9 ab
	WA + V5w2	3129 ± 281 b	112 ± 28 ab	3281 ± 291 b	28.0 ± 1.5 ab
9-MPT	Control	8203 ± 1064 a	275 ± 103 a	8559 ± 1072 a	34.3 ± 1.9 a
	V5w2	5409 ± 610 ab	125 ± 78 a	5550 ± 613 b	22.1 ± 1.4 b
	WA	7238 ± 811 ab	94 ± 34 a	7366 ± 806 ab	34.2 ± 2.9 a
	WA + V5w2	5113 ± 588 b	131 ± 54 a	5269 ± 601 b	24.0 ± 1.7 b
Mother crop (harvest)	Control	8024 ± 509 a	2867 ± 373 a	11069 ± 561 a	52.6 ± 1.6 a
	V5w2	5301 ± 614 b	1761 ± 265 a	7202 ± 666 b	41.8 ± 1.5 b
	WA	6200 ± 646 ab	3187 ± 534 a	9531 ± 867 ab	50.8 ± 1.6 a
	WA + V5w2	4489 ± 368 b	2559 ± 317 a	7153 ± 440 b	42.8 ± 1.3 b
Ratoon crop (harvest)	Control	9158 ± 1063 a	1050 ± 167 a	10375 ± 1194 a	49.6 ± 1.9 a
	V5w2	8685 ± 813 a	940 ± 119 a	9679 ± 892 a	48.3 ± 2.2 a
	WA	9320 ± 1151 a	1072 ± 169 a	10517 ± 1288 a	52.1 ± 1.5 a
	WA + V5w2	8148 ± 700 a	1160 ± 152 a	9450 ± 796 a	49.7 ± 1.9 a

Values represent means ± standard error. At each growth stage, means with the same letter(s) for each variable are not significantly different by Tukey's honestly significant difference (HSD) test at $p < 0.05$. V5w2: plants inoculated with *F. oxysporum* isolate V5w2, WA: plants inoculated with *B. bassiana* isolate WA, WA + V5w2: plants inoculated with a combination of WA and V5w2, Control: Non-endophyte inoculated plants. * Total nematode is the sum of *R. similis*, *H. multicinctus* and *Meloidogyne* spp. MPT: months post transplanting.

For the mother plant, inoculation with V5w2 and a mixture of V5w2 and WA suppressed *R. similis* and the overall total nematode densities at harvest ($F \geq 2.57$, $df = 3$, $p \leq 0.05$). Nevertheless, neither the individual nematode species nor the total nematode densities differed between the endophyte inoculated and non-inoculated plants at harvest for the ratoon crop ($F \leq 3.94$, $df = 3$, $p \geq 0.26$).

3.4. Nematode Root Necrosis Damage

At 3-, 6- and 9-MPT, nematode root necrosis damage between the two banana cultivars was similar ($\chi^2 \leq 0.77$, $df = 1$, $p \geq 0.37$), with no significant interactions observed between cultivar and endophyte treatment at each of the sampling occasions ($\chi^2 \leq 1.55$, $df = 1$, $p \geq 0.67$) and so the data were pooled across banana cultivars for each sample occasion. The inoculation of endophytes resulted in lower root necrosis damage at 3-($\chi^2 = 81.17$, $df = 3$, $p < 0.001$), 6-($\chi^2 = 8.21$, $df = 3$, $p = 0.04$) and 9-($\chi^2 = 29.45$, $df = 3$, $p < 0.001$) MPT (Table 3).

At plant harvest, necrosis damage of the ratoon crop was higher than for the mother crop ($\chi^2 = 5.99$, $df = 1$, $p = 0.01$). There was also a significant interaction effect between cropping cycle and endophyte treatment ($\chi^2 = 11.65$, $df = 3$, $p = 0.008$), but no effect of banana cultivar ($\chi^2 = 0.92$, $df = 1$, $p = 0.33$). Thus, nematode necrosis damage data at crop harvest was split between the two cropping cycles but pooled across cultivars within each cycle. At harvest, the nematode necrosis damage differed between endophyte treatments for the mother crop ($\chi^2 = 40.68$, $df = 3$, $p < 0.001$), but not for the ratoon crop ($\chi^2 = 1.98$, $df = 3$, $p = 0.57$) (Table 3).

3.5. Banana Weevil Damage

Tunneling damage caused by the banana weevil larvae to the outer corm (OC), inner corm (IC), outer pseudostem (OP) and inner pseudostem (IP) differed between the two banana cultivars ($\chi^2 \geq 4.35$, $df = 1$, $p < 0.03$). Overall, more damage was recorded on banana cultivar Mbwazirume (10.4%) compared to cultivar Grande Naine (6.7%). Similarly, there was a significant effect of crop cycle on OC ($\chi^2 = 303$, $df = 1$, $p < 0.001$), IC ($\chi^2 = 63.55$, $df = 1$, $p < 0.001$), OP ($\chi^2 = 63.1$, $df = 1$, $p < 0.001$) and IP ($\chi^2 = 12.83$, $df = 1$, $p < 0.001$). Weevil larvae tunneling damage was significantly greater in the ratoon crop (10.6%) than in the mother crop (7.4%). However, there was no significant difference in damage between the endophyte inoculated and non-inoculated plants in the mother crop ($\chi^2 \leq 3.28$, $df = 3$, $p \geq 0.34$) or ratoon crop ($\chi^2 \leq 7.02$, $df = 3$, $p \geq 0.07$) (Table 4).

Table 4. Percentage weevil damage to banana plants cultivar Mbwazirume and cultivar Grande Naine (data merged) inoculated with endophytes *Fusarium oxysporum* isolate V5w2 and *Beauveria bassiana* isolate WA individually and in combination at the harvest stage of two cropping cycles.

Growth Stage	Treatment	Outer Corm (OC)	Inner Corm (IC)	Outer Pseudostem (OP)	Inner Pseudostem (IP)
Mother crop (harvest)	Control	5.80 ± 0.28 aB	2.35 ± 0.25 aB	2.98 ± 0.23 aB	1.35 ± 0.19 aB
	V5w2	6.56 ± 0.57 aB	2.27 ± 0.46 aB	3.43 ± 0.47 aB	1.43 ± 0.40 aB
	WA	5.28 ± 0.55 aB	2.14 ± 0.48 aB	2.73 ± 0.39 aB	1.01 ± 0.25 aB
	WA + V5w2	5.68 ± 0.48 aB	1.81 ± 0.28 aB	2.32 ± 0.37 aB	1.04 ± 0.27 aB
Ratoon crop (harvest)	Control	15.27 ± 1.29 aA	5.40 ± 0.89 aA	8.36 ± 1.85 aA	2.96 ± 1.66 aA
	V5w2	12.06 ± 2.26 aA	3.52 ± 1.27 aA	7.02 ± 1.83 aA	3.54 ± 0.93 aA
	WA	16.26 ± 1.77 aA	4.77 ± 1.80 aA	8.35 ± 2.57 aA	2.55 ± 1.39 aA
	WA + V5w2	15.90 ± 2.56 aA	6.57 ± 2.30 aA	9.93 ± 2.57 aA	3.67 ± 1.18 aA

Values represent means ± standard error. For each plant part and within each crop cycle, means followed by the same lower-case letter indicate no significant difference between endophyte treatments (per column). While for each treatment, means with the same upper-case letter indicate no difference between the cropping cycle. Means separated by Tukey HSD test at $p = 0.05$ (per row).

3.6. Plant Yield

Bunch weight and crop yield differed between cropping cycles ($F \geq 16.77$, $df = 1$, $p < 0.001$); in addition, there was a significant interaction between cropping cycle and endophyte treatment for bunch weight ($F = 3.44$, $df = 3$, $p = 0.01$). Consequently, data on bunch weight and crop yield were analysed independently for each cropping cycle. In the mother crop, banana plants inoculated with either V5w2 or a combination of V5w2 and WA produced heavier bunches and a higher yield compared to the non-inoculated plants ($F \geq 5.32$, $df = 3$, $p \leq 0.006$). However, neither bunch weight nor crop yield were significantly different between the two banana cultivars ($F \leq 2.89$, $df = 1$, $p \geq 0.08$). Conversely, neither bunch weight nor yield of the ratoon crop differed between endophyte inoculated

and non-inoculated plants ($F \leq 0.93$, $df = 3$, $p \geq 0.42$) (Figure 2). Bunch weights for the banana cultivar Grande Naine were heavier than cultivar Mbwazirume in the ratoon crop ($F = 7.33$, $df = 1$, $p = 0.007$).

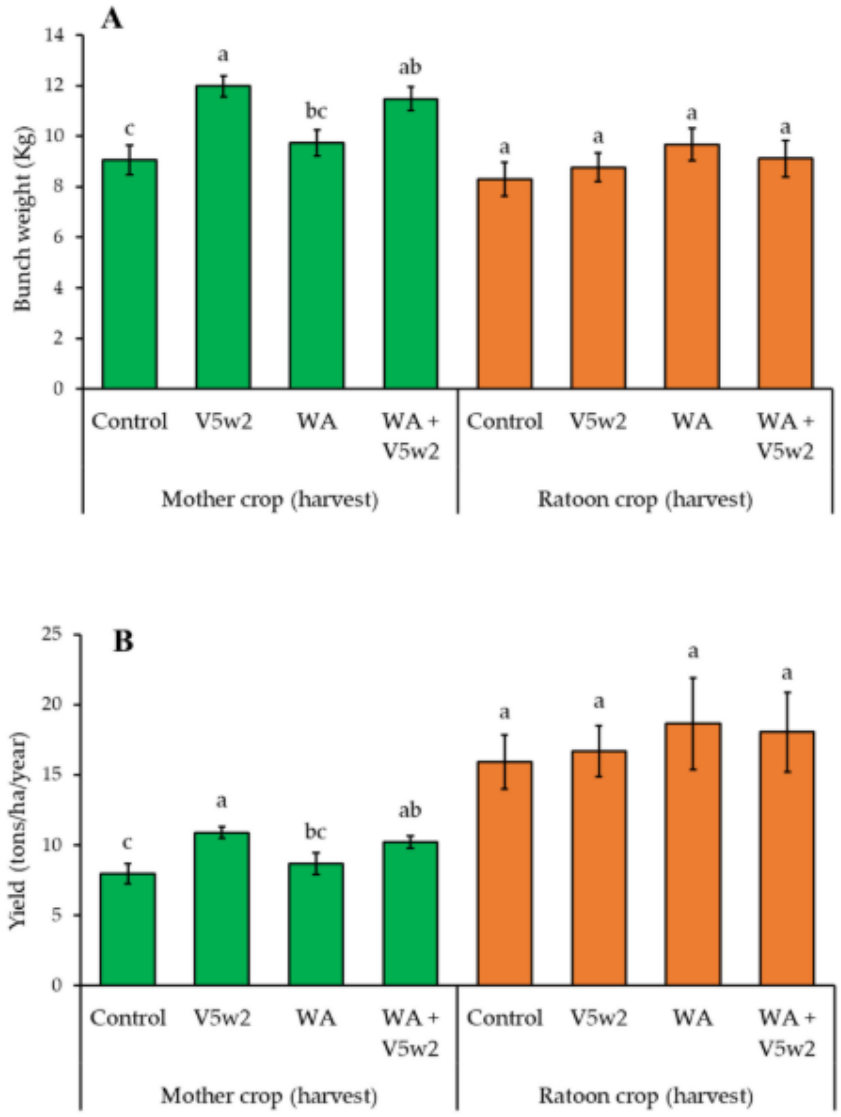

Figure 2. Effect of endophyte inoculation on bunch weight (**A**) and yield (**B**) of banana plants cultivars Mbwazirume and Grande Naine (data pooled) across two growth cycles (mother crop and ratoon crop). Within each crop cycle, means followed by the same letter(s) are not significantly different by Tukey's honestly significant difference (HSD) test at $p < 0.05$.

4. Discussion

The inoculation of banana tissue cultured plants with *F. oxysporum* isolate V5w2 individually or in combination with the *B. bassiana* isolate WA protected plants against nematode infection and damage, and increased the bunch weight and overall yield of the mother crop, under field conditions in Uganda. However, the dual inoculation of plants with *F. oxysporum* and *B. bassiana* did not significantly reduce nematode infection nor

improve yield beyond the inoculation with *F. oxysporum* alone, an indication that the single inoculation of plants with *F. oxysporum* is optimal. Furthermore, there was no observed carry-over effect of the endophytes on nematode suppression and yield on the ratoon crop. Nematode infection of banana can cause up to 30% reduction in bunch weight, more than 60% plant toppling [47], extended crop cycle duration to harvest and reduced annual yields by over 50% [48]. Furthermore, Ssango et al. [49] showed that root necrosis is negatively correlated with bunch weight and yield. In our study, inoculation of the banana plants with the fungal isolate V5w2 resulted in more than 34% reduction in nematode infection levels and decreased nematode root necrosis by >19%, while the total number of harvested plants increased by up to 27%, demonstrating the protective effect of endophytes against PPN infection and related damage in banana.

Keeping other factors constant, a well-developed and healthy banana root system supports optimum water and nutrient uptake, leading to healthy plant growth and vigor and consequently large bunches. Enhancement of plants with endophytes by inoculating with V5w2 reduced nematode infection levels and root necrosis damage. This protection translated into bigger bunches than in the control plants. Furthermore, the healthier root system meant better plant anchorage, which explains the reduced toppling observed in plants inoculated with endophytes and the higher number of harvested plants compared with the controls. The reduction in nematode densities on V5w2-inoculated plants, however, did not significantly influence the proportion of flowered plants between treatments. In banana, nematode damage is most obvious after flowering, at the fruit filling stage when the banana plants rely on an efficient root system to supply nutrients and water to the developing bunch and support the weight of the filling fruit, hence the plant topples at the weight of its own fruit.

In Kenya, Waweru et al. [25] reported a reduction in *Pratylenchus goodeyi* infection, reduced root necrosis damage and increased bunch weight and yield of Giant Cavendish and Grand Naine dessert banana cultivars following enhancement with endophytic *F. oxysporum* isolate V5w2. In addition to testing the fungal endophyte in a different location from where Waweru et al. [25] conducted their study, our study demonstrates the potential use of endophytes to manage multiple nematode species (*R. similis*, *H. multicinctus* and *Meloidogyne* spp.) in dessert banana cultivars as well as in cooking bananas (EAHB), indicating the broader spectrum of applicability for fungal endophyte(s). Consequently, these studies complement each other and can be used to fast track the registration and incorporation of the *F. oxysporum* isolate V5w2 into IPM management strategies against PPNs in bananas in the East African region.

Yield is a factor of bunch weight, crop cycle length, and plant density (the number of harvested plants per hectare per year) [50,51], where bunch weight and plant density are positively correlated with yield, while crop cycle length is negatively correlated. Our study's highest reported yield value (18.6 t ha^{-1} $year^{-1}$), following endophyte enhancement, is lower than the reported potential yield value of 60 t ha^{-1} $year^{-1}$. This low yield could be attributed to the deliberate and uniform infection of all plantlets with PPNs before their establishment in the field, which generally would not happen under farmer's practice. While our findings revealed no effect of endophyte treatment on the crop cycle length, inoculation of the plants with V5w2 improved bunch weight and increased plant survival (number of harvested plants) in the mother crop. This observation ultimately explains the higher yield of the endophyte treated plants compared to control plants. The lack of yield differences between endophyte-treated and control plants in the ratoon crop indicates that the influence of endophyte inoculation wanes and reduces over time and that repeated applications may be necessary to boost endophyte enhancement. Inoculation of banana plants with *B. bassiana* isolate WA alone, however, had no effect on the number of harvested plants, or bunch weight. Moreover, while we reported no effect of fungal treatment on plant growth parameters, the observed difference in growth parameters between the two banana cultivars could largely be attributed to agronomic and genomic differences between the two banana cultivars.

Previous studies have indicated a high colonization of banana plants by the *B. bassiana* isolate WA with no penalty to plant growth [19], coupled with high virulence against the banana weevil and a high sporulation potential on culture media [28,29]. In our assessment of endophyte colonization at the transplanting stage, we confirmed the establishment of both fungal isolates (*B. bassiana* and *F. oxysporum*) in the banana root system. Furthermore, both isolates persisted in the banana roots for 6-months after transplanting into the field. Other studies have also reported successful colonization of banana roots following inoculation with single [20,21] and multiple [22] isolates of non-pathogenic *F. oxysporum*, and single isolates of *B. bassiana* [19,52] under screenhouse conditions. Studies assessing the colonization potential of non-pathogenic *F. oxysporum* isolates in tomato (*S. lycopersicon*) [53], and maize (*Zea mays*) [54], among other crops, have also been demonstrated. Similarly, studies have demonstrated the endophytic potential of *B. bassiana* isolates in a range of crops, such as pine (*Pinus* spp.) [55], cocoa (*Theobroma cacao*) [56] corn [57], tomato [58]. Such reports indicate the potential application of endophytic *F. oxysporum* and *B. bassiana* across plant species.

Upon inoculation, fungal spores of non-pathogenic *F. oxysporum* endophytic isolates are induced to germinate, and fungal hyphae are triggered to produce branches upon contact with the plant root system. The fungal hyphae invade the root system through cracks and wounds in the epidermis or a direct penetration at the root tip. Hyphae of non-pathogenic *F. oxysporum* colonize and establish in the cortical region of the plant root without extending into the stele [59]. Studies have demonstrated a downregulation of plant defence pathways and mechanisms during the initial colonization of the plant by the mutualistic microbes [60]. However, later induction of the defence signaling pathways prevents the microbe from transcending the 'mutualistic' limits [61], which may lead to a gradual reduction in the level of mutualistic endophytes in the plant system. The observed gradual reduction, with time, in colonization levels of both V5w2 and WA fungal isolate could be explained by a possible induction of signaling pathway(s) with a suppressive action against the mutualistic endophytes. Paparu et al. [62] demonstrated an upregulation of the phenylpropanoid enzymes phenylalanine ammonia lyase (PAL) and peroxidase (POX) in roots of banana plants inoculated with the non-pathogenic *F. oxysporum* isolate V5w2. However, the same enzymes were upregulated in leaves of banana plants challenged with the pathogenic *F. oxysporum* f. sp. *cubense* [63]. While PAL and POX have been shown to play a positive role in the plant defence against the pathogenic *F. oxysporum* f. sp. *cubense* tropical race four [64], it can be assumed that the exact defence mechanism can be applied against the non-pathogenic endophytic fungus, leading to a gradual reduction in colonization level. Furthermore, with the increased growth and development of the plant root system, it is possible that the fungal hyphae do not easily establish in the newly formed roots, and thus the chance of recovering the inoculated fungal isolate(s) diminishes with the increased mass of the root system.

While our study confirmed recovery of the fungal endophytes from the banana roots until 6-months post field establishment, several studies have demonstrated the persistence of defence mechanisms and production of metabolites initiated by fungal endophytes even beyond the time when the fungal endophyte can be recovered from the plant tissue [65]. These post-exposure mechanisms could explain the continued suppression of nematode infection levels until the harvest stage of the mother crop cycle in plants singly inoculated with V5w2 or in combination with WA. In addition, the continued suppression of nematode numbers could explain the observed reduction in the number of toppled plants, which consequently increased the proportion of harvested plants in the mother crop.

While, for fear of instant death of the experimental banana plants due to banana weevil infestation, there was no direct infection of the young plantlets with the banana weevils. Assessment through pseudostem trapping [30] at 6-months after transplanting into the field confirmed natural infestation of the experimental plants by the banana weevil. We suspect that this natural infestation came from the mature weevil-infested banana fields that neighboured the trial field.

Previous screenhouse studies have demonstrated the potential use of endophytic *B. bassiana* for the management of the banana weevil [23,24]. Akello et al. reported a reduction in weevil damage to tissue-cultured banana plants inoculated with endophytic *B. bassiana* and challenged with banana weevil larvae [24] and adults [23] in two separate studies. However, in these studies, they also observed a significant reduction in the survivorship of all growth stages of the weevil with respective mycosis, an indication that *B. bassiana* mainly acted against the banana weevil as an endophytic entomopathogen. In fact, Akello et al. [23] concluded that: "Our study therefore demonstrates for the first time that through direct parasitism, endophytic *B. bassiana* kills the eggs, larvae, pupae, and adults of *C. sordidus*". The failure to observe differences in banana weevil damage between the different endophyte treatments in our study could be attributed to the fact that using fungal endophytes against the coleopteran banana weevil would only be effective if the fungus persists in the plant tissue to entomopathogenically infect the weevil. Consequently, a repeated in-field application of the endophytic entomopathogen *B. bassiana* could help boost its inoculum levels, which would help to entomopathogenically infect the weevil, reducing their field population and damage to banana. Thus, the best way to test the efficacy of fungal isolates against the banana weevil might be when used as entomopathogens or endophytic entomopathogens. Studies about the endophytic use of *B. bassiana* for the management other plant pests for example: aphid (*Myzus persicae*) infection in tobacco (*Nicotiana tabacum*) [66], and the leaf miners (*Liriomyza sativae* and *L. trifolii*) in French beans (*Phaseolus vulgaris*) [54,67] resulted in improved plant vigour and development. Similarly, the use of endophytic *B. bassiana* resulted in the management of the Asian corn borer (*Ostrinia furnacalis*) in maize [68] and diamond back moth (*Plutella xylostella*) infection of cabbage (*Brassica oleracea*) [69]. However, studies on the endophytic potential of *B. bassiana* against coleopteran insects are very limited.

5. Conclusions

This study demonstrated that inoculation of the East African highland banana cultivar Mbwazirume and the dessert banana cultivar Grande Naine with the non-pathogenic endophytic fungus *F. oxysporum* isolate V5w2 suppresses nematode infection and increases banana productivity. We found that application of V5w2 alone was sufficient in nematode suppression and boosting banana yield, indicating high potential for its development into an environmentally friendly biopesticide. However, future research will be necessary to elucidate the mechanisms underpinning nematode suppression and increased banana yield observed after treatment with V5w2. The influence of single or combined application of the assessed endophytes on soil microbiome and soil health should also be assessed to better understand other benefits that could be derived from this potential biopesticide. Furthermore, repeat in-field application of the fungal isolates should be assessed for boosting the fungal inoculum following field establishment over successive crop cycles. Lastly, due the low yield value reported in our study in comparison to the reported potential yield value, it would be appropriate that future similar trials include a control treatment with no pest challenge; this will help depict farmer management practice.

Author Contributions: Conceptualization and trial design, J.K. (James Kisaakye), J.K. (Joseph Kisitu), S.N., H.F., D.C., L.C., S.S. and S.H.; data collection, curation and analysis, J.K. (James Kisaakye), J.K. (Joseph Kisitu) and S.N.; resources, J.K. (James Kisaakye), J.K. (Joseph Kisitu), S.N., H.F., S.S., L.C., D.C. and S.H.; writing—original draft preparation, J.K. (James Kisaakye); writing—review and editing, J.K. (James Kisaakye), J.K. (Joseph Kisitu), S.N., H.F., D.C., L.C., S.S. and S.H.; supervision, H.F., D.C., L.C., S.S. and S.H.; project administration, S.H., S.S. and D.C.; funding acquisition, D.C., L.C., S.S., H.F., and S.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by H2020 European union under the project "Microbial uptakes for sustainable management of major banana pests and diseases" (MUSA): grant number, 727624. James Kisaakye. was supported by the German Academic Exchange Service (DAAD) through the African Regional Postgraduate Programme in Insect Science (ARPPIS).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data supporting the findings and conclusions are contained within the article.

Acknowledgments: We gratefully acknowledge the financial support for this research by the following organizations and agencies: UK's Foreign, Commonwealth & Development Office (FCDO); the Swedish International Development Cooperation Agency (Sida); the Swiss Agency for Development and Cooperation (SDC); the Federal Democratic Republic of Ethiopia; the Government of the Republic of Kenya; and the CGIAR Research Program for Roots, Tubers and Bananas (CRP-RTB).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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CHAPTER 5: Article 3

Endophytic fungi improve management of the burrowing nematode in banana (*Musa* spp.) through enhanced expression of defence-related genes

Prepared for submission to **Nematology**

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Summary - The root burrowing nematode, *Radopholus similis,* is reputedly the most damaging nematode pest of banana and responsible for major production losses. In this study, the endophytic potential of 13 fungal isolates was assessed for the management of *R. similis* in East African highland bananas (*cv*. Ng'ombe). All isolates successfully colonised tissue-cultured banana roots, with isolates from *Trichoderma, Fusarium* and *Hypocrea* producing the highest (≥49.1%) and *Beauveria* isolates the lowest (≤14.4%) colonisation. The fungal endophytes *T. asperellum* (ICIPE 700) and *H. lixii* (ICIPE 697) were the most effective in reducing *R. similis* densities (>81%) relative to the non-inoculated control. However, the combined inoculation of ICIPE 700 and ICIPE 697, led to greater suppression of *R. similis* (>21%) relative to individual inoculation. Suppression of *R. similis* following inoculation of banana roots with ICIPE 700 and/or ICIPE 697 was associated with the significant upregulation of the defence-related gene *PR-1*, the cell signalling gene calmodulin-Ca^{2+} and the cell wall strengthening gene *β-1,3-glucan synthase*. This study demonstrates the potential for nematode management in bananas with fungal endophytes, especially using the isolates ICIPE 700 and ICIPE 697 when combined.

Keywords: *Radopholus similis,* biological control, endophyte, microbial antagonists, tissue culture, gene expression

Plant-parasitic nematodes (PPN) are a major threat to banana (*Musa* spp.) production in the tropics and subtropics. The East African highland banana (EAHB), commonly grown in the Great Lakes region, are a key staple food and cash crop. However, they are highly susceptible to PPN. Nematode infection of banana leads to reduced bunch weight (Talwana *et al.*, 2003), extends the crop cycle duration and can substantially affect yield (Fogain, 2000). Globally, *Radopholus similis*, is viewed as the most devastating nematode parasite of banana (Sikora *et al.*, 2018), but it occurs in a complex with other species, often consisting of *Helicotylenchus multicinctus*, *Meloidogyne* spp. and *Pratylenchus* spp. among others (Coyne & Kidane, 2018; Nyang'au *et al.*, 2021; Speijer *et al.*, 2001; Wang & Hooks, 2009).

Chemical nematicides, such as carbofuran and methyl bromide have been used for the management of banana nematodes (Bujulu *et al.*, 1983). However, these have been linked to multiple health and environmental hazards (Gallegos-Avila *et al.*, 2010; Satar *et al.*, 2005), hence their use is discouraged or banned (WHO, 2010). Clean, healthy planting materials are key to the management of PPN in banana. Three techniques exist for the generation of healthy planting materials: 1) paring banana suckers and dipping in boiling (100°C) water for 30 seconds, 2) macropropagation and 3) tissue culture (TC) technique. Although the use of clean planting materials helps reduce the initial nematode infection and retards population density build-up over crop cycles, the banana plants remain prone to in-field infection.

Beneficial fungal endophytes colonise host plants without inducing any penalty to the host or pathogenic symptoms, existing in a harmonious association with their plant hosts. Various studies have demonstrated the beneficial effects of the endophyte-banana plant associations in improving host resistance to both biotic and abiotic constraints, including PPN (Athman *et al.*, 2007; Paparu *et al.*, 2009; Waweru *et al.*, 2014, 2013), fungal and bacterial diseases (Cheng *et al.*, 2020; Thangavelu & Gopi, 2015) among others.

The use of TC banana plants has numerous benefits, such as providing clean, healthy planting material which is free of pests and diseases. The shoot tip culture technique (Vuylsteke, 1998), however, also eliminates any beneficial microbes that would otherwise naturally occur and confer protection against pests and diseases (Pereira *et al.*, 1999). Inoculation of beneficial microbes into TC banana plants at the weaning stage can facilitate their re-establishment into the plant (Kisaakye *et al.*, 2022; Paparu *et al.*, 2006b). Identifying the most useful endophytes, however, requires a thorough screening of potential, naturally occurring microbial isolates to assess their performance against key target pest(s) and to confirm the non-phytotoxicity of the potential beneficial microbial isolate(s) to the banana plant. The current study was therefore conducted to assess the endophytic potential of selected, local isolates of fungal strains for the management of *R. similis* infection of banana in East Africa. The most appropriate spore concentration for effective management of *R. similis* infection was determined, with a focus on single and combined inoculation of the selected candidate isolates, and the mode of action of the candidate isolates assessed, with a bias on the expression profiles of selected defence-related genes in banana following inoculation with fungal isolate(s) and infection with- (or without) *R. similis*.

Materials and Methods

FUNGAL ISOLATES

Thirteen fungal isolates: *Beauveria bassiana* (8), *Trichoderma asperellum* (2), *Trichoderma atroviride* (1), *Fusarium proliferatum* (1) and *Hypocrea lixii* (1) were screened for their endophytic potential against *R. similis* infection in banana (*Musa* sp.). Fungal cultures were sourced from culture repositories of the International Centre of Insect Physiology and Ecology (*icipe*) and Real IPM Company Ltd (Table 1). The identities of the selected fungal isolates

were previously determined based on morphological features, as described by Goettel *et al.* (2000) and Humber (2012), and molecular tools based on the conserved internal transcribed spacer (ITS) regions of the respective fungal isolate DNA.

The fungal isolates had been preserved on potato dextrose agar (PDA) media blocks in a sterile solution of 10% (v/v) glycerol in 2 ml Eppendorf tubes at -80°C. Fungal spores were revived by culturing on sterile PDA medium (39 g/l distilled water) in sterile 90 mm plastic Petri dishes and maintained in the incubator at 25 ± 2°C for 3 - 4 weeks until sporulation. For each isolate, a fungal spore suspension was prepared by washing each Petri dish with 10 ml of sterile distilled water and the spores scraped off using a sterile metal spatula. Suspensions of individual isolates were separately collected into sterile 30 ml universal bottles containing 3 mm diameter glass beads to form the stock suspensions. Spore viability for each respective fungal isolate was determined as described by Inglis *et al.* (2012).

TISSUE CULTURE BANANA PLANTS

EAHB *cv*. Ng'ombe (genomic group, EA-AAA) was used in this study. TC plants at the deflasking stage were sourced from a commercial tissue culture laboratory: Stokman Rozen Kenya Ltd. (SRK), Naivasha, Kenya. The plants were generated using the shoot-tip culture method by Vuylsteke (1998). Upon deflasking, the plant roots were washed free of rooting media with distilled water and planted in 66 multi-cell plug plastic propagation trays (50 × 28 × 4.3 cm: L × W × H) containing a steam sterilized potting mixture of forest soil and composted manure (2:1, v/v).

NEMATODE CULTURE

A *R. similis* nematode population used in this study was isolated from infected roots of dessert banana (*cv*. Cavendish) in Homa Bay County, Kenya. Nematodes were extracted from roots using a modified Baermann method (Coyne *et al.*, 2018; Hallmann & Subbotin, 2018); adult females were then used to establish monoxenic nematode cultures on carrot discs (Coyne *et al.*, 2014). The *R. similis* population identity was confirmed based on morphological features as described by Luc, (1987) and Loof, (1991). The nematodes were sub-cultured onto fresh carrot discs once every 6 - 8 weeks and maintained in a temperature-controlled incubator (27 ± 0.5°C) until required for use.

SCREENING OF FUNGAL ISOLATES AGAINST *RADOPHOLUS SIMILIS* INFECTION IN BANANA

To screen fungal isolates for their endophytic potential against *R. similis*, approximately 50 ml of spore concentration 1.0×10^7 spores/ml was prepared for each individual fungal isolate; this formed the 'working spore suspension', which was used for drenching the banana plants in the 66 multi-cell plug plastic propagation trays. Each plantlet was individually inoculated with the fungal isolates by drenching each seedling plug with the spore suspension at approximately 10% (v/v) of the volume of the potting mixture; this was equivalent to approximately 4 ml of spore suspension per plant. Estimation of the inoculation volume was based on the maximum volume the potting mixture in the seedling plug could absorb without leaking. For each plant, the spore suspension was carefully applied to the potting mixture from above, using a pipette, to gradually soak into and completely drench the potting mixture.

Drenching with the fungal suspension was performed on two occasions: 1) at the deflasking stage: on the day of establishment in the seedling trays, and 2) at 4 weeks post-deflasking. Control plants were drenched with an equivalent volume of sterile distilled water. Plants were

maintained in a humidity chamber (relative humidity > 80%, Temp = 25 ± 3℃) for 5 weeks post-deflasking, then transferred singly into 2 litre plastic pots filled with the same steam sterilized potting mixture used for maintaining the tissue culture banana plantlets. The plants were maintained in the greenhouse and watered regularly. Endophyte inoculated and control plants were each infected with 1000 *R. similis* (juveniles and adult females) at 4 weeks after transfer into the 2 litre pots. Infection of banana plants with *R. similis* was performed using the method described by Speijer and De Waele (1997). Plants were maintained in the greenhouse at 25 ± 3°C and a natural photo period of 12:12 h (light:dark). The treatments were arranged in a completely randomised block design and the experiment was terminated 8 weeks after infection with *R. similis*. Each treatment was replicated six times and the experiment repeated once, except for fungal isolate ICIPE 712, which was not assessed in experiment 2 due to poor sporulation of the isolate.

At experiment termination, each plant was gently removed from the pots and the roots rinsed free of adhering soil using tap water. The roots were cut from the banana corm using a kitchen knife, and plant pseudostem height, number of functional leaves, shoot fresh weight and root fresh weight growth parameters recorded.

To assess fungal root colonisation, three roots from each plant were randomly removed, and colonisation determined as described by Kisaakye *et al.* (2022). The remaining roots were chopped into ca. 0.5 cm pieces, thoroughly mixed and nematodes extracted from a 5 g root sub-sample using the modified Baermann method for 48 h (Coyne *et al.*, 2018; Hallmann & Subbotin, 2018). Nematode suspensions were reduced to 10 ml and nematode densities estimated from 3 × 2 ml aliquots using a compound microscope (×20 magnification) (Leica Microsystems, Wetzlar, Germany) and a 2 ml De Grisse counting dish (De Grisse, 1963). The final (Pf) and initial (Pi) nematode densities were used to estimate the nematode reproduction factor (RF) for each banana plant:

$$\text{RF} = \frac{\text{Final nematode population (Pf)}}{\text{Initial nematode population (Pi)}}$$

DETERMINATION OF FUNGAL ISOLATE TREATMENT AND SPORE CONCENTRATION FOR OPTIMAL EFFICACY AGAINST *RADOPHOLUS SIMILIS*

Two fungal isolates were selected and used to establish the optimal level of spore concentration for management of *R. similis* infection in banana when applied individually and in combination. The isolates were selected based on two criteria: 1) high level of suppression to *R. similis* infection of banana, and 2) high level of root colonisation without hampering plant growth. Based on these criteria, two isolates: ICIPE 697 and ICIPE 700 were selected for spore concentration and isolate combination studies.

Efficacy of the selected fungal isolates were assessed at different spore concentrations individually and in combination. Each fungal isolate and the combination were applied at four spore concentrations: 1.0×10^5, 1.0×10^6, 1.0×10^7 and 1.0×10^8 spores/ml, prepared from the stock suspensions. Inocula of the individual isolate treatments contained spores of a single isolate. While inocula of the combined isolate treatments contained half the spores of each isolate but with a total spore concentration equal to that of the individual isolate treatments at the respective level of spore concentrations.

Inoculation of the banana plantlets with the fungal inocula, and subsequent infection with *R. similis* were performed as described previously. Each treatment was replicated 12 times, arranged in a completely randomized block design, and the experiment was conducted twice. The plants were maintained in the greenhouse and watered regularly for 8 weeks after infection with *R. similis*. At experiment termination, nematodes were extracted and quantified as described earlier.

TREATMENT OF BANANA PLANTS FOR GENE EXPRESSION ANALYSIS

To establish the expression profile of selected defence-related genes in endophyte inoculated and/or *R. similis* infected banana plants, TC banana plantlets *cv*. Ng'ombe were planted in seedling trays and separately inoculated with a spore suspension (1.0×10^7 spores/ml) of the fungal isolates ICIPE 697, ICIPE 700 individually and in combination at deflasking and 4 weeks post-deflasking stages, as described earlier. Control plants were inoculated with distilled water. The plants were transferred into 2 litre plastic pots at 5 weeks post deflasking. Each of the four treatments: ICIPE 697, ICIPE 700, ICIPE 697+ICIPE 700 and untreated control comprised a total of 72 plants. For each treatment, 18 plants were individually inoculated with 1000 *R. similis* (juveniles and adult females) 1 week post transfer into the 2 litre pots (Fig. 1), and the plants were maintained in the greenhouse and watered regularly for a further 2 weeks.

COLLECTION OF ROOT SAMPLES FOR GENE EXPRESSION ANALYSIS

Treatments were sub-sampled and banana roots collected for assessment of the expression levels of the selected defence-related genes. Sample collection was performed at 6 time points (Fig. 1): 1) Day 2: at two days after the first endophyte inoculation, 2) Day 28: at 4 weeks after the first endophyte inoculation but before second inoculation, 3) Day 30: at two days after the second endophyte inoculation, 4) Day 42: at two weeks after second inoculation with fungal endophytes (1 week after transfer of plants into 2 litre pots) and just before infection with *R. similis*, 5) Day 44: at two days after infection with or without *R. similis*, and 6) Day 56: at 2 weeks after infection of plants with or without *R. similis*. At each sample collection time, the plant roots were gently washed under running tap water to ensure that they were free of any adhering soil, then rinsed with sterile distilled water. The roots were blotted dry with sterile

tissue paper, immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Each treatment consisted of three biological replicates, each consisting of roots from three plants pooled together at harvest.

RNA EXTRACTION AND cDNA SYNTHESIS

Extraction of total RNA from banana roots was conducted using the Isolate II RNA Mini Kit (Bioline, London, UK) according to the manufacturer's instructions. The purity and concentration of extracted RNA were determined by measuring the absorbance using a Nanodrop 2000 Spectrophotometer (Thermo Fischer Scientific, Wilmington, USA). Only the RNA extract with a $A_{260\ nm}$/ A_{280nm} ratio ~2 was considered pure and was used for cDNA synthesis. The cDNA was synthesised using the SensiFAST™ cDNA Synthesis Kit (Bioline) according to manufacturer's instructions. The resultant cDNA products were assayed for genomic DNA contamination using PCR and the actin-specific primer set actinF (5'-ACCGAAGCCCCTCTTAAC-CC-3') and actinR (5'-GTATGGCTGACACCATCACC-3'), and the PCR products separated by electrophoresis through a 1.5% (w/v) agarose gel (Bioline) stained with 10 mg/ml ethidium bromide (Sigma-Aldrich, Gmbh, Germany). The pure cDNA products were stored at -80°C for use in downstream processes.

REAL TIME-PCR PRIMERS

The expression level of three defence-related genes: pathogenesis-related gene (*PR-1*), *calmodulin-*Ca^{2+} and *β-1,3-glucan synthase* (Table 2) in roots of the EAHB *cv*. Ng'ombe (EA-AAA) were determined. *PR-1* was previously reported to be upregulated in the nematode susceptible Cavendish banana *cv*. Williams (genomic group AAA) (Van den Berg *et al.*, 2007).

Similarly, all three target genes were previously found to be upregulated in the nematode tolerant banana *cv*. Kayinja (genomic group ABB) (Paparu *et al.*, 2007). The *PR-1* primer sequences were previously identified by Van den Berg *et al.* (2007), while primer sequences for *calmodulin-*Ca^{2+} and *β-1,3-glucan synthase* were previously identified by Paparu *et al.* (2013). The endogenous control gene, *Musa* 25S rRNA, previously identified by Van den Berg *et al.* (2007) was used as a reference gene since its expression remains relatively constant. All primers were synthesized as balanced pairs by Macrogen Inc. Europe Laboratory, The Netherlands. For each primer sequence, the optimal annealing temperature was determined using gradient PCR, run in the Mastercycler Nexus gradient thermal cycler (Eppendorf, Germany), using the following conditions: initial denaturation for 2 minutes at 95°C, followed by 40 cycles each consisting of 30 seconds denaturation at 95°C, 45 seconds annealing at a gradient temperature range of 54.4 - 64.9°C, and a final extension step of 10 minutes at 72°C. PCR products were separated in a 1.5% (w/v) agarose gel (Bioline) containing 10 mg/ml ethidium bromide (Sigma-Aldrich, Gmbh, Germany). Gel bands were visualised and documented using KETA GL imaging system transilluminator (Wealtec Corp, Meadowvale Way Sparks, Nevada, USA).

GENE EXPRESSION ANALYSIS USING qRT-PCR

Quantitative real-time reverse transcriptase (qRT-PCR) was used to quantify the expression levels of the target genes. This was performed using the SensiFAST™ SYBR® Hi-ROX Kit (Bioline) using the first strand cDNA samples as template. A 10 µl reaction mixture for PCR amplification contained 5 µl SensiFAST SYBR® Hi-ROX mix, 2 µl cDNA template, 0.5 µl of forward and reverse primer (400 nM) and 2 µl PCR grade water. The non-template control treatment contained PCR-grade water instead of cDNA template. The qRT-PCR assays were

performed in a Stratagene Mx3005P qPCR instrument (Agilent Technologies, California, USA). All reactions were performed in three technical replicates and three independent biological replicates. The PCR conditions were performed as follows: pre-incubation at 95°C for 10 minutes, followed by 40 cycles, each consisting of 30 seconds denaturation at 95°C, 20 seconds annealing at the respective optimised annealing temperature for each gene (Table 2) and a final extension of 20 seconds at 72°C.

DATA ANALYSIS

All data analyses were performed using R (Version 4.0.5) statistical software (R Core Team, 2021). In the experiment to screen for the endophytic potential of fungal isolates against *R. similis* infection of banana, nematode density, nematode reproduction (RF) and plant height data were subjected to a two-way analysis of variance (ANOVA) to investigate the effects and interactions between experiment repeat and endophyte treatment. Prior to ANOVA, nematode density data were $\log_{10}$ transformed, while nematode reproduction (RF) data were square root transformed to conform to the requirements of normality (Shapiro & Wilk, 1965) and homogeneity of variances (O'Neill & Mathews, 2002). No transformation was performed on plant height data. Other plant growth parameters: number of functional leaves, root fresh weight and shoot fresh weight were fitted with generalised linear model (GLM) with gaussian distribution to check the main effects of experiment repeat and endophyte treatment, and the interaction effect. Due to the binary nature of endophyte colonisation data: colonised *vs*. non-colonised, the data were fitted using GLM with binomial distribution (Warton & Hui, 2011).

In the experiment to establish the ideal endophytic isolate combination and fungal spore concentration for optimal efficacy against *R. similis* infection in banana, nematode density data were subjected to a three-way ANOVA to establish the main effects of experiment repeat,

fungal endophyte treatment and fungal spore concentration, and the interaction effects. Nematode density data were square root transformed prior to ANOVA to conform to the requirements for normality of data and homogeneity of variances. Nematode reproduction (RF) data were fitted to GLM with gaussian distribution to establish the main effects of experiment repeat, fungal endophyte treatment and fungal spore concentration, and the interaction effects. Apart from shoot fresh weight data, which were square root transformed and fitted to a three-way ANOVA, data on other plant growth parameters: number of functional leaves, plant height and root fresh weight were fitted to GLM with gaussian distribution to check the main effects of experiment repeat, endophyte treatment and fungal spore concentration, and the interaction effects. Significance of the GLM models was established using an analysis of deviance (Wald chi-square test) followed by computation of least-square means using the 'emmeans' package (Lenth *et al.*, 2021), and group mean separation performed by Tukey's (Honest Significance Difference: HSD) multiple comparisons using the 'cld' function of the 'multcomp' package (Hothorn *et al.*, 2021).

The RT-PCR expression data were normalised using the endogenous control gene, *Musa* 25s rRNA using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008) and calibrated using the non-inoculated control at each time point to generate fold change (relative gene expression) at each time point. Gene fold change data were fitted to GLM with gaussian distribution to check the main effects of fungal endophyte treatment and *R. similis* infection, and the interaction effects on the relative expression of the target genes in endophyte inoculated and/or *R. similis* infected plants at each respective time point. Significance of the GLM models was established using an analysis of deviance (Wald chi-square test) followed by comparison of group means using the 'glht' function of the 'multcomp' package (Hothorn *et al.*, 2021), separation of group means was performed by Tukey's (HSD) multiple comparisons.

Results

SCREENING FUNGAL ISOLATES AGAINST *RADOPHOLUS SIMILIS* INFECTION OF BANANA

Fungal colonisation of plant roots

There was no significant effect of experiment repeat on root colonisation by the fungal endophytes ($\chi^2 = 0.05$, $p = 0.82$). On the contrary, root colonisation was significantly influenced by the fungal isolates ($\chi^2 = 1100.5$, $p < 0.001$). However, there was no interaction effect of experiment repeat and endophyte treatment ($\chi^2 = 3.37$, $p = 0.99$). Consequently, data were pooled across experiment repeats prior to further analysis. Colonisation of banana roots by each of the *Trichoderma* spp. isolates (ICIPE 700, SD 228 TRC 900 & ICIPE 710), the *H. lixii* isolate (ICIPE 697) and the *F. proliferatum* isolate (ICIPE 712) was significantly higher than root colonisation by the *B. bassiana* isolates (Fig. 2). No fungal colonisation was observed in the untreated control plants.

Nematode density and reproduction

There was a significant effect of experiment repeat on nematode densities ($F = 70.65$, $p < 0.001$) (Table 3). The nematode density (pooled across treatments) in experiment 1 (4,013 *R. similis*/100 g root weight) was significantly higher than experiment 2 (2,164 *R. similis* per 100 g root weight). Consequently, nematode density data for each experiment was independently subjected to statistical analysis and not pooled for the two experiments. There was a significant effect of endophyte treatment on nematode densities in experiment 1 ($F = 24.53$, $p < 0.001$) and 2 ($F = 9.18$, $p < 0.001$). In experiment 1, the fungal isolates ICIPE 700, ICIPE 697, SD 228 TRC 900 and ICIPE 710 significantly suppressed nematode densities by 86, 84, 80 and 64% relative to untreated control. While the relative suppression in nematode densities in experiment 2 was 87, 81, 69 and 57% respectively.

Generally, there was an overall suppression of nematode reproduction (RF), however, this further differed significantly between the two experiment repeats ($F = 18.98$, $p < 0.001$) (Table 3). Reproduction of nematodes in experiment 1 (RF = 0.52) was significantly lower than experiment 2 (RF = 0.76). There was a significant effect of fungal endophyte treatment in experiment 1 ($F = 3.94$, $p < 0.001$) and 2 ($F = 8.28$, $p < 0.001$), respectively. The fungal isolates ICIPE 700, ICIPE 697 and SD 228 TRC 900 significantly and consistently suppressed nematode reproduction in experiments 1 (RF $\leq$ 0.2) and 2 (RF $\leq$ 0.4) compared to untreated control.

Plant growth

There was a significant effect of experiment repeat on plant height ($F = 191.8$, $p < 0.001$), root fresh weight ($\chi^2 = 249.2$, $p < 0.001$) and shoot fresh weight ($\chi^2 = 246.5$, $p < 0.001$) (Table 4). These three growth parameters were significantly higher in experiment 2 than experiment 1. In experiment 1, there was no effect of endophyte treatment on plant height ($F = 12.77$, $p = 0.06$), root fresh weight ($\chi^2 = 16.73$, $p = 0.21$) and shoot fresh weight ($\chi^2 = 18.79$, $p = 0.13$), while plant height ($F = 3.56$, $p < 0.001$), root fresh weight ($\chi^2 = 22.89$, $p = 0.03$) and shoot fresh weight ($\chi^2 = 34.85$, $p < 0.001$) were significantly influenced by fungal endophyte treatment in experiment 2. To the contrary, there was no effect of experiment repeat on the number of functional leaves of the banana plants ($\chi^2 = 0.88$, $p = 0.35$). However, there was an overall significant effect of endophyte treatment ($\chi^2 = 28.25$, $p = 0.008$), but no interaction effect ($\chi^2 = 1.17$, $p = 0.13$).

DOSE RESPONSE STUDY

Nematode infection

In the experiment to assess the effect of single and combined inoculation of TC banana plants with the fungal endophytes *H. lixii* (ICIPE 697) and *T. asperellum* (ICIPE 700) and the ideal spore concentration for optimal management of *R. similis* infection in the banana, there was a significant effect of experiment repeat (F = 100.92, df = 1, $p < 0.001$) on *R. similis* densities. Overall, the mean nematode density (pooled across treatments) in experiment 2 (3,565 *R. similis* /100 g root weight) was significantly higher than in experiment 1 (2,215 *R. similis* /100 g root weight). Thus, nematode counts data were split between experiment repeat prior to further analysis.

There was a significant effect of endophyte treatment on nematode density in experiment 1 (F = 3.74, df = 2, p = 0.03) and 2 (F = 6.68, df = 2, p = 0.002). Similarly, fungal spore concentration significantly influenced nematode density in experiment 1 (F = 38.99, df = 4, $p < 0.001$) and 2 (F = 21.10, df = 4, $p < 0.001$). However, there was no significant interaction between endophyte treatment and fungal spore concentration for experiment 1 (F = 0.41, df = 8, p = 0.91) or 2 (F = 1.44, df = 8, p = 0.20) (Fig. 3).

In experiment 1, the combined inoculation of ICIPE 697 and ICIPE 700 led to a significantly higher suppression (55.2%) in *R. similis* densities compared to ICIPE 697 (40.1%) and ICIPE 700 (42.6%) relative to the control. Similarly, in experiment 2, a 47.1% suppression in *R. similis* densities was recorded in plants inoculated with the combination of ICIPE 697 and ICIPE 700, which was significantly higher than ICIPE 697 (31.1%) and ICIPE 700 (30.8%), respectively.

In both experiments, and for all endophyte treatments, *R. similis* densities were inversely proportional to fungal spore concentration, attaining the lowest nematode density at a spore concentration of 1.0×10^7 spores/ml. However, a further increment in spore concentration

beyond 1.0×10^7 spores/ml lessened the protective role of the fungal endophytes against *R. similis* infection of banana (Fig. 3).

There was a significant effect of experiment repeat on nematode RF ($\chi^2 = 192.15$, $df = 1$, $p < 0.001$) (Fig. 4). While there was a general suppression of nematode reproduction in both experiments, but RF values were lower in experiment 1 (RF = 0.28) compared to experiment 2 (RF = 0.88). Consequently, nematode RF data for each of the two experiments were analysed separately.

There was no effect of fungal endophyte treatment on RF in experiment 1 ($\chi^2 = 4.87$, $df = 2$, $p = 0.09$) and 2 ($\chi^2 = 3.99$, $df = 2$, $p = 0.14$) (Fig. 4). However, fungal spore concentration significantly influenced RF in experiment 1 ($\chi^2 = 72.52$, $df = 4$, $p < 0.001$) and 2 ($\chi^2 = 38.5$, $df = 4$, $p < 0.001$), respectively. Furthermore, there was no significant interaction for experiment 1 ($\chi^2 = 3.26$, $df = 8$, $p = 0.92$) and 2 ($\chi^2 = 9.85$, $df = 8$, $p = 0.28$). In both experiments, a substantial reduction in RF was evident as the fungal spore concentration increased, reaching the lowest RF at 1.0×10^7 spores/ml. A further increment in spore concentration from 1.0×10^7 to 1.0×10^8 spores/ml did not yield additional suppression in nematode reproduction; indeed, a slight increase in nematode reproduction was observed with increase in spore concentration for both experiments.

Plant growth

All plant growth parameters (plant height, shoot fresh weight, root fresh weight and number of functional leaves) were significantly higher in experiment 2 than experiment 1 ($F = 6.08$ or $\chi^2 \geq 4.17$, $p \leq 0.04$) (Table 5). However, there was no significant effect of fungal spore concentration on plant growth in experiment 1 ($F = 0.06$ or $\chi^2 \leq 8.07$, $p \geq 0.05$) or 2 ($F = 0.07$ or $\chi^2 \leq 3.16$, $p \geq 0.37$). Consequently, data for all plant growth parameters was analysed independently for each experiment but it was pooled across fungal spore concentration within

each experiment. Except for plant height in experiment 1 (χ^2 = 8.56, p = 0.04), there was no significant effect of fungal endophyte treatment on plant growth parameters for experiment 1 (F = 0.74 or $\chi^2 \leq 4.08$, $p \geq 0.13$) and 2 (F = 0.31 or $\chi^2 \leq 4.37$, $p \geq 0.11$), respectively. Furthermore, there was no significant interaction for any growth parameter for experiment 1 (F = 1.20 or $\chi^2 \leq 4.59$, $p \geq 0.31$) or 2 (F = 0.45 or $\chi^2 \leq 6.05$, $p \geq 0.42$).

GENE EXPRESSION

The relative expression of the cell signalling gene, *calmodulin-Ca^{2+}*, was significantly upregulated in the roots of banana plant at 2 days (≥ 7.36 fold) and 28 days (≥ 1.34 fold) post initial inoculation of the plants with the fungal endophytes ICIPE 697 and ICIPE 700, applied individually or in combination ($\chi^2 \leq 38137$, $p < 0.001$), except for the individual application of ICIPE 697, which did not differ significantly from the non-inoculated plants at 28 days (p = 0.23) (Fig. 5 A). Boosting of banana plants with fungal endophytes at 28 days led to a further significant upregulation of the *calmodulin-Ca^{2+}* gene in all fungal-treated plants at 30- (≥9.3-fold; χ^2 = 634, $p < 0.001$) and 42- (≥ 2.04-fold; χ^2 = 234, $p < 0.001$) days. The expression profile of the *calmodulin-Ca^{2+}* gene was maintained at significantly higher levels in the endophyte inoculated plants than the non-inoculated plants at day 44 (χ^2 = 231, $p < 0.001$) and day 56 (χ^2 = 120, $p < 0.001$), although this was at a relatively lower-fold difference compared to earlier time points (1.29 - 1.52-fold). However, the expression of the *calmodulin-Ca^{2+}* gene in plants inoculated with a mixed inoculum of both endophytes did not differ from the non-inoculated plants at day 44 (p = 0.79) and 56 (p = 0.16). On the contrary, when plants dually inoculated with the fungal isolates were infected with *R. similis* at day 42, the expression of this gene was maintained relatively higher in these plants (≥ 1.45-fold; $p < 0.001$) relative to endophyte-free control. Furthermore, infection of the non-inoculated plants with *R. similis* at day 42, led to a

significant suppression of the *calmodulin-Ca^{2+}* gene at day 44 (0.68-fold; $p < 0.001$) and day 56 (0.6-fold; $p < 0.01$), relative to the control plants.

Inoculation of banana plants with the fungal endophytes ICIPE 697 and ICIPE 700 singly or in combination led to an up regulation of the *β-1,3-glucan synthase* gene at day 2 (≥ 3.5-fold; $\chi^2 = 289.3$, $p < 0.001$) and day 28 (≥ 3.9-fold; $\chi^2 = 205.9$, $p < 0.001$) relative to the non-inoculated control (Fig. 5 B). Re-inoculation of banana plants with the fungal endophytes at day 28 further enhanced the expression levels of the *β-1,3-glucan synthase* gene at day 30 (≥ 5.1-fold; $\chi^2 = 457.2$, $p < 0.001$) and day 42 (≥ 5.6-fold; $\chi^2 = 215.6$, $p < 0.001$), relative to the endophyte-free control. Furthermore, the expression level of the *β-1,3-glucan synthase* gene was maintained significantly higher in endophyte inoculated plants at day 44 (≥ 5.6-fold; $\chi^2 = 1350.7$, $p < 0.001$) and day 56 (≥ 5.9-fold; $\chi^2 = 1769.5$, $p < 0.001$). Similarly, infection of endophyte inoculated plants with *R. similis* at day 42 yielded a significantly higher relative expression of the *β-1,3-glucan synthase* gene at day 44 (≥ 6.0-fold; $p < 0.001$) and day 56 (≥ 6.2-fold; $p < 0.001$), relative to the endophyte-free control. On the contrary, infection of endophyte-free plants with *R. similis* at day 42 yielded no difference in the expression levels of the *β-1,3-glucan synthase* gene at day 44 (1.0-fold; $p = 1$) and day 56 (1.0-fold; $p = 1$), relative to endophyte-free control.

The expression level of the pathogenesis-related gene (*PR-1*) was increased by >2-fold at day 2 ($\chi^2 = 122.2$, $p < 0.001$) and >1.8-fold at day 28 ($\chi^2 = 51.7$, $p < 0.01$) in endophyte inoculated plants, relative to the non-inoculated control. Re-inoculation of banana plants with the fungal endophytes at day 28 maintained the expression levels of the *PR-1* gene significantly higher at day 30 (≥ 2.6-fold; $\chi^2 = 132.4$, $p < 0.001$), day 42 (≥ 2.4-fold; $\chi^2 = 68.0$, $p < 0.001$), day 44 (≥ 3.5-fold; $\chi^2 = 233.5$, $p < 0.001$) and day 56 (≥ 3.2-fold; $\chi^2 = 193$, $p < 0.001$), relative to the endophyte-free control. Similarly, infection of endophyte inoculated plants with *R. similis* at day 42 further maintained the expression level of the *PR-1* gene at a significantly high level at day 44 (≥ 4.8-fold; $\chi^2 = 233.5$, $p < 0.001$) and day 56 (≥ 6.9-fold; $\chi^2 = 193.1$, $p < 0.001$).

Furthermore, infection of endophyte free plants with *R. similis* at day 42 significantly increased the expression of the *PR-1* gene at day 44 (≥ 4.8-fold; $p < 0.001$) and day 56 (≥ 6.9-fold; $p < 0.001$), relative to untreated control (Fig. 5 C).

Discussion

The current study demonstrates that the combined inoculation of *T. asperellum* isolate ICIPE 700 and *H. lixii* isolate ICIPE 697 at 1.0×10^7 spores/ml is ideal for the suppression of *R. similis* infection in the EAHB *cv*. Ng'ombe. We also established that increasing the spore concentration beyond 1.0×10^7 spores/ml led to the reduced effectiveness of the fungal endophytes. We further revealed that the potential of these two fungal isolates to suppress *R. similis* infection is achieved through enhancement of the plant's defence mechanisms by upregulation of the defence-related gene *PR-1*, the cell signalling gene *calmodulin-*$Ca2^+$ and the cell wall strengthening gene *β-1,3-glucan synthase*.

To identify microbial isolates with likely biological activity against a target pest, it is essential to conduct a preliminary screening of the candidate microbial isolates against the target pest. In our study, the potential of 13 fungal isolates to endophytically suppress *R. similis* infection and reproduction in banana TC plants *cv*. Ng'ombe was assessed. In both experiments, *B. bassiana* isolates displayed the lowest efficacy against *R. similis* infection, in comparison to the *Trichoderma* spp. (ICIPE 700, ICIPE 710 & SD 228 TRC 900), *H. lixii* (ICIPE 697) and *F. proliferatum* (ICIPE 712) isolates. The potential for a fungal isolate to endophytically express its antagonistic efficacy against a target pathogen largely depends on its ability to establish a mutualistic endophytic association with its host plant, as this mutualistic association helps modify the host plant's physiological and biochemical processes and ultimately induce the host's defence mechanism against target pests and pathogens (Contreras-Cornejo *et al.*,

2016; Khare *et al.*, 2018; Kiarie *et al.*, 2020; Li *et al.*, 2021; Paparu *et al.*, 2007; Waweru *et al.*, 2014). Thus, failure of the *Beauveria* isolates to supress *R. similis* infection in our study could be attributed to the low levels of root colonisation attained by the *Beauveria* isolates. On the other hand, all the *Trichoderma* spp. and the *H. lixii* (ICIPE 697) isolate(s) tested in our study supressed *R. similis* densities and reproduction. This suppression of nematode density and reproduction could be attributed to the relatively high level of root colonisation exhibited by these isolates.

The potential for *Trichoderma* spp. to endophytically manage *R. similis* infection in banana has been studied previously (e.g. zum Felde *et al.*, 2005, 2006; Vargas *et al.*, 2015). However, only limited information is available on the endophytic potential of *Hypocrea* spp. for nematode management in banana. In the experiment to screen the endophytic potential of the fungal isolates against *R. similis* infection of banana, we observed between 56 - 86% reduction in *R. similis* infection of banana upon drenching the banana roots with 10 ml of 1.0×10^7 spores/ml of either *Trichoderma* spp. or *H. lixii* isolate(s), with the *T. asperellum* isolate ICIPE 700 and the *H. lixii* isolate ICIPE 697 recording the highest level of nematode suppression at ≥85.8 and ≥81.2%, respectively. Similar to our findings, zum Felde *et al.* (2005) observed up to 80% reduction in *R. similis* infection of banana (*cv*. Grande Naine) in a pot experiment following inoculation of the banana plantlets with *Trichoderma* spp. isolates.

Fungal endophytes form mutualistic associations with their host plants. They exert their antagonistic activity through various means, which are expressed as modifications to host plant physiological and biochemical processes (Khare *et al.*, 2018; Li *et al.*, 2021; Paparu *et al.*, 2013; Plett & Martin, 2018). Inoculation of the banana plants with ICIPE 700 and ICIPE 697 individually or in combination led to activation of the cell wall strengthening gene (*β-1,3-glucan synthase*) and the general defence pathogenesis-related gene (*PR-1*), irrespective of whether the plants were infected with (or without) *R. similis*. However, there was no

upregulation of the *β-1,3-glucan synthase* gene when the endophyte free banana plants were infected with *R. similis*, an indication that upregulation of this defence gene was dependent on inoculation of the plants with the fungal endophytes.

Callose, a polysaccharide of β-1,3-glucan, is a key component of plant cell walls and plays a vital role in cell wall strengthening (Chang *et al.*, 2021; Hayashi, 1989). The burrowing nematode, *R. similis* is a migratory endoparasite. The nematode moves inter- and intra-cellularly, feeding on the cell cytoplasm, which it gains access to by piercing the plant cell wall with its stylet. Upon inoculation of banana plants with the fungal endophytes ICIPE 700 or ICIPE 697, we observed a reduction in the levels of *R. similis* infection. The observed suppression of *R. similis* infection could be attributed to deposition of extra callose in the plant cell walls following the activation and increased expression of the *β-1,3-glucan synthase* gene in the endophyte inoculated plants. The role of β-1,3-glucans and callose deposition in the suppression of *R. similis* infection of banana has been reported (Valette *et al.*, 1997).

A persistent expression of the *PR-1* gene was observed in endophyte inoculated banana plants until 42 days post inoculation (DPI), with a slight increment in the expression level at 44 and 56 DPI when the plants were infected with *R. similis*. Similar to our findings Paparu *et al.* (2007), reported an increase in the expression level of *PR-1* when the nematode-tolerant banana *cv*. Kayinja (genomic group ABB) was inoculated with the endophytic non-pathogenic *Fusarium oxysporum* isolate V5w2 and infected with (or without) *R. similis*, an indication that the *PR-1* gene can be upregulated in different banana cultivars, and by different genera (or isolates) of fungal endophytes.

If a given fungal isolate is to be considered a candidate for the endophytic control of crop pests and pathogens, it is essential that such an isolate establishes a mutualistic endophytic association with its host plant. In the current study, all the tested fungal isolates colonised the

banana roots, although at varying levels. Generally, we recorded relatively low colonisation levels for all the *B. bassiana* isolates (Colonisation ≤ 14%) compared to the *Trichoderma* spp. (ICIPE 700 ICIPE 710 & SD 228 TRC 900), *H. lixii* (ICIPE 697) and *F. proliferatum* (ICIPE 712) isolates, which all recorded colonisation levels of ≥ 49%. The low colonisation levels by the *B. bassiana* isolates could be attributed to genetic incompatibility between the tested *B. bassiana* isolates and banana or the banana cultivar (Ng'ombe: EA-AAA) used in this study, or the method of endophyte application. In an experiment to understand the role of genotypic variations in host plant-fungal endophyte compatibility, Saikkonen *et al.* (2010) established that fungal endophyte colonisation of red fescue grass (*Festuca rubra*) was partly matched to genotypic compatibility between the host grass and the colonising endophyte.

In an experiment to compare root dip, rhizome injection and soil-solid substrate as methods for introducing the *B. bassiana* isolate G41 as an endophyte in banana, Akello *et al.* (2007) reported that the root dip method was the most appropriate method for maximum colonisation of banana roots by *B. bassiana*, achieving root colonisation levels ≥ 49%. Similarly, Akello *et al.* (2008, 2009) attained root colonisation levels of ≥ 40%, when *B. bassiana* isolate G41 was introduced into banana plants using the root dip method. Thus, the low level of banana root colonisation by *B. bassiana* isolates reported in our study (≤ 14%) could be further attributed to the method of application (soil drench) as opposed to the root dip method demonstrated in previous studies. Indeed, when comparing soil drench and root dip methods for introducing *B. bassiana* isolate G41 as an endophyte in roots of TC banana plants, Kato (2013) established that the level of root colonisation in dipped plants was double that of drenched plants.

Similar to our findings, fungal isolates belonging to the *Trichoderma* spp. group have been shown to successively colonise roots and corms of banana (*Musa* sp.) (Thangavelu & Gopi, 2015), and roots, stems and leaves of pineapple (*Ananas comosus*) (Kiriga *et al.*, 2018), onion (*Allium cepa*) (Muvea *et al.*, 2014), french bean (*Phaseolus vulgaris*) (Paradza *et al.*, 2021),

tomato (*Solanum lycopersicum*) (Paradza *et al.*, 2021), maize (*Zea mays*) (Batool *et al.*, 2020; Kiarie *et al.*, 2020), among other crops, with resultant antagonistic activity against specific plant pests and pathogens.

In our study, we demonstrated that inoculation of the EAHB *cv*. Ng'ombe with a combination of *T. asperellum* isolate ICIPE 700 and *H. lixii* isolate ICIPE 697 at 1.0×10^7 spores/ml is optimal for the suppression of *R. similis* infection, which is achieved through enhancement of the defence mechanisms of the banana plant. However, studies to elucidate other possible modes of action will help better understand the functioning of these isolates. Additionally, further studies to explore the potential of these two candidate isolates, *T. asperellum* isolate ICIPE 700 and *H. lixii* isolate ICIPE 697, in the management of other banana pests and diseases would help understand the full benefits of these candidate fungal isolates in the management of other biotic constraints in banana.

Acknowledgement

This research was funded by H2020 European union under the project "Microbial uptakes for sustainable management of major banana pests and diseases" (MUSA): grant number, 727624. The authors gratefully and acknowledge the financial support for this research from the UK's Foreign, Commonwealth and Development Office (FCDO); the Swedish International Development Cooperation Agency (Sida); the Swiss Agency for Development and Cooperation (SDC); the Federal Democratic Republic of Ethiopia; and the Government of the Republic of Kenya. The Real IPM company Ltd. is acknowledged for supply of two of the fungal isolates used in this study. Appreciation is extended to colleagues in the Arthropod Pathology Unit (APU) and the *Nem*Africa team for their technical support. The funders had no role in the design, data collection, interpretation, or decision to publish the results.

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Table 1. Identity of selected fungal endophytic isolates used in the study.

Fungal species	Isolate	Year of isolation	Isolation host/source	Locality (Country) of isolation	Repository
Beauveria bassiana	ICIPE 273	2006	Soil	Mbita (Kenya)	*icipe*, Kenya
	ICIPE 284	2005	Soil	Mauritius	*icipe*, Kenya
	ICIPE 609	2008	Soil	Meru (Kenya)	*icipe*, Kenya
	ICIPE 621	2008	Soil	Kericho (Kenya)	*icipe*, Kenya
	ICIPE 622	2008	Soil	Kericho (Kenya)	*icipe*, Kenya
	ICIPE 644	2007	Soil	Mauritius	*icipe*, Kenya
	ICIPE 660	2008	Soil	Kemokock (Kenya)	*icipe*, Kenya
	SD 229-Bb01	2008	Soil	Thika (Kenya)	Real IPM, Kenya
Trichoderma asperellum	ICIPE 700	2009	Maize (roots & stalk)	Kenya	*icipe*, Kenya
	SD 228-TRC 900	2005	Tomato (roots)	Thika (Kenya)	Real IPM, Kenya
Trichoderma atroviride	ICIPE 710	2014	Onion	Kenya	*icipe*, Kenya
Fusarium proliferatum	ICIPE 712	2014	Onion	Kenya	*icipe*, Kenya
Hypocrea lixii	ICIPE 697	2014	Maize (roots & stalk)	Kenya	*icipe*, Kenya

Table 2. Oligonucleotide sequences used in the expression analysis of defence-related genes in roots of banana *cv*. Ng'ombe (EA-AAA) inoculated with fungal endophyte(s) and infected with or without 1000 *Radopholus similis*.

Target gene	**Function**	**Primer Sequence (5′-3′)**	**Primer melting temperature (°C)**	**Annealing temp (°C) ***	**Amplicon size (bp)**
Calmodulin-Ca^{2+}	Cell signalling (Defence)	GTAGACTGCGTACCGACAAG GTGGAGGAAACAAGAGGAAG	60.5 58.4	62	150
β-1,3-glucan synthase	Cell wall strengthening (Callose synthesis)	TGTAGACTGCGTACCGACA CCATGGGAAGGATAAGGA	57.3 53.9	63	163
Pathogenesis-related (*PR-1*)	General defence	TCCGGCCTTATTTCACATTC GCCATCTTCATCATCTGCAA	56.4 56.4	59	126
Musa 25S rRNA	Reference gene	ACATTGTCAGGTGGGGAGTT CCTTTTGTTCCACACGAGATT	58.4 57.4	59	106

* Determined by gradient PCR.

Table 3. *Radopholus similis* densities (per 100 g root) and reproduction factor (RF) in banana plants *cv*. Ng'ombe at 13 weeks post inoculation with fungal endophytes and 8 weeks post infection with 1000 *R. similis*.

Fungal Isolate	*R. similis* /100 g root weight		Reproduction factor (RF)	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Control	6015 ± 601 abcA	3383 ± 197 abA	0.9 ± 0.2 aA	1.2 ± 0.2 abB
ICIPE 273	3519 ± 458 b-eA	2767 ± 315 abcA	0.7 ± 0.3 abA	0.7 ± 0.1 bcdB
ICIPE 284	7038 ± 1003 abA	4808 ± 442 aA	0.6 ± 0.1 abB	1.6 ± 0.2 aA
ICIPE 609	4361 ± 991 a-eA	1467 ± 350 bcdB	1.0 ± 0.3 aA	0.6 ± 0.1 bcdB
ICIPE 621	4442 ± 589 a-dA	2758 ± 569 abcB	0.5 ± 0.1 abB	1.1 ± 0.2 abcA
ICIPE 622	7346 ± 594 aA	2260 ± 484 abcB	0.8 ± 0.3 abA	0.7 ± 0.2 bcdB
ICIPE 644	3239 ± 721 cdeA	1825 ± 422 a-dB	0.4 ± 0.1 abA	0.7 ± 0.2 bcdB
ICIPE 660	5628 ± 603 abcA	3125 ± 600 abcB	0.9 ± 0.2 aA	1.1 ± 0.2 abcB
SD 229-Bb01	6959 ± 910 abA	2158 ± 229 abcB	0.5 ± 0.1 abA	0.8 ± 0.1 abcdB
ICIPE 700	854 ± 100 gA	444 ± 188 eB	0.1 ± 0 bA	0.4 ± 0.1 cdeB
SD 228 TRC 900	1192 ± 43 fgA	1040 ± 329 cdeA	0.2 ± 0 bA	0.1 ± 0.1 eB
ICIPE 710	2149 ± 451 efA	1456 ± 226 bcdA	0.2 ± 0.1 abA	0.5 ± 0.1 bcdeB
ICIPE 712	2476 ± 252 def	*	0.3 ± 0.1 ab	*
ICIPE 697	960 ± 178 gA	635 ± 112 deA	0.2 ± 0.1 bA	0.3 ± 0.1 B

Values represent means ± standard error. For each parameter and within each experiment, means followed by the same lower-case letter(s) indicate no significant difference between fungal isolate(s). For each fungal isolate, means followed by same upper-case letter indicate no significant difference between experiment 1 and 2. Means were separated by Tukey's (HSD) at $p < 0.05$. *Not enough fungal spores were available to conduct experiment 2 due to poor sporulation of the fungal isolate.

Table 4. Growth of banana plants *cv*. Ng'ombe (EA-AAA) at 13 weeks post inoculation with fungal endophytes and 8 weeks post infection with 1000 *Radopholus similis*.

Fungal Isolate	Plant height (cm)		Fresh root weight (g)		Fresh shoot weight (g)		Number of functional leaves†
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	
Control	14.5 ± 1.0 aB	17.7 ± 0.8 bA	16.2 ± 3.8 aB	36.7 ± 4.4 abA	38.7 ± 7.7 aB	76.1 ± 9.3 abA	7.9 ± 0.3 ab
ICIPE 273	15.1 ± 1.6 aA	17.3 ± 0.5 bA	17.7 ± 4.7 aA	27.7 ± 4.6 bA	41.1 ± 9.0 aB	63.6 ± 5.6 bA	7.2 ± 0.4 b
ICIPE 284	13.3 ± 1.2 aB	18.2 ± 0.5 abA	9.4 ± 2.9 aB	33.3 ± 2.2 abA	22.6 ± 5.0 aB	87.9 ± 6.9 abA	7.3 ± 0.3 ab
ICIPE 609	15.3 ± 0.6 aB	20.2 ± 0.8 abA	21.3 ± 3.4 aB	45.9 ± 2.9 aA	42.2 ± 6.4 aB	104.6 ± 9.3 aA	8.2 ± 0.3 ab
ICIPE 621	15.2 ± 1.0 aA	17.5 ± 0.9 bA	10.8 ± 1.9 aB	42.4 ± 5.8 abA	36.5 ± 4.9 aB	62.7 ± 9.2 bA	7.4 ± 0.3 ab
ICIPE 622	12.8 ± 0.8 aB	18.1 ± 0.6 abA	11.0 ± 4.3 aB	29.0 ± 5.0 bA	26.5 ± 5.4 aB	66.8 ± 9.5 abA	7.5 ± 0.3 ab
ICIPE 644	12.7 ± 0.7 aB	21.6 ± 1.0 aA	10.7 ± 1.3 aB	43.4 ± 5.7 abA	26.2 ± 1.0 aB	100.7 ± 14.8 abA	7.3 ± 0.4 ab
ICIPE 660	14.3 ± 0.5 aB	20.4 ± 0.5 abA	16.4 ± 2.8 aB	37.2 ± 3.7 abA	37.4 ± 4.2 aB	90.9 ± 2.5 abA	8.0 ± 0.4 ab
SD 229-Bb01	11.2 ± 0.6 aB	19.3 ± 0.8 abA	8.3 ± 2.1 aB	36.5 ± 1.8 abA	23.1 ± 3.7 aB	88.2 ± 8.6 abA	7.5 ± 0.2 ab
ICIPE 700	12.5 ± 0.9 aB	17.5 ± 0.4 bA	16.5 ± 1.9 aB	34.5 ± 1.2 abA	37.4 ± 6.0 aB	68.4 ± 3.6 abA	8.6 ± 0.3 a
SD 228 TRC 900	11.9 ± 1.2 aB	17.6 ± 0.7 bA	13.3 ± 3.9 aB	40.7 ± 1.5 abA	35.0 ± 8.2 aB	85.7 ± 2.0 abA	8.3 ± 0.3 ab
ICIPE 710	13.5 ± 1.4 aB	18.4 ± 0.9 abA	11.1 ± 4.5 aB	34.1 ± 5.5 abA	26.5 ± 8.0 aB	69.1 ± 9.2 abA	7.3 ± 0.2 ab
ICIPE 712	11.9 ± 1.4 a	*	12.1 ± 3.6 a	*	26.4 ± 6.9 a	*	7.2 ± 0.5 b
ICIPE 697	15.6 ± 1.6 aA	17.5 ± 0.8 bA	19.0 ± 5.2 aB	41.0 ± 2.8 abA	49.5 ± 12.3 aB	80.2 ± 6.4 abA	8.0 ± 0.3 ab

Values represent means ± standard error. For each growth parameter and within each experiment, means followed by the same lower-case letter(s) indicate no significant difference between fungal isolate(s). For each fungal isolate, means followed by same upper-case letter indicate no significant difference between experiment 1 and 2. Means separated by Tukey's (HSD) at $p < 0.05$. † Data pooled across experiments; *Not enough fungal spores were available to conduct experiment 2 due to poor sporulation of the fungal isolate.

Table 5. Growth of banana plants *cv*. Ng'ombe (EA-AAA) inoculated with endophytes: *Hypocrea lixii* (ICIPE 697) and *Trichoderma asperellum* (ICIPE 700) individually and in combination, and infected with 1000 *Radopholus similis*.

Experiment repeat	Treatment	Plant height (cm)	Fresh shoot weight (g)	Fresh root weight (g)	Number of functional leaves
1	Untreated control	12.0 ± 0.5 ab	32.7 ± 2.6 a	14.2 ± 1.4 a	6.39 ± 0.14 a
	ICIPE 697	11.1 ± 0.4 b	28.5 ± 1.9 a	10.5 ± 1.0 a	6.15 ± 0.14 a
	ICIPE 700	12.8 ± 0.5 a	33.8 ± 2.8 a	13.6 ± 1.3 a	6.40 ± 0.11 a
	ICIPE 697 + ICIPE 700	12.0 ± 0.3 ab	30.4 ± 1.8 a	11.9 ± 0.8 a	6.38 ± 0.10 a
	Grand mean*	12.0 ± 0.2 B	31.3 ± 1.1 B	12.4 ± 0.6 B	6.32 ± 0.06 B
2	Untreated control	12.7 ± 0.3 a	35.1 ± 2.1 a	25.4 ± 2.0 a	6.92 ± 0.12 a
	ICIPE 697	12.6 ± 0.3 a	35.1 ± 1.6 a	24.1 ± 1.4 a	6.90 ± 0.15 a
	ICIPE 700	12.4 ± 0.3 a	33.4 ± 2.0 a	25.3 ± 1.7 a	6.56 ± 0.13 a
	ICIPE 697 + ICIPE 700	12.5 ± 0.3 a	33.7 ± 1.8 a	25.5 ± 1.8 a	6.52 ± 0.15 a
	Grand mean*	12.5 ± 0.1 A	34.3 ± 0.9 A	25.1 ± 0.8 A	6.71 ± 0.07 A

Values represent mean ± standard error. For each growth parameter, means followed by the same lower-case letter(s) indicate no significant difference between treatments. Means followed by the same upper-case letter indicate no significant difference between experiment 1 and 2. Means separated by Tukey (HSD) test at $p < 0.05$. *Mean pooled across treatments for each growth parameter within each experiment.

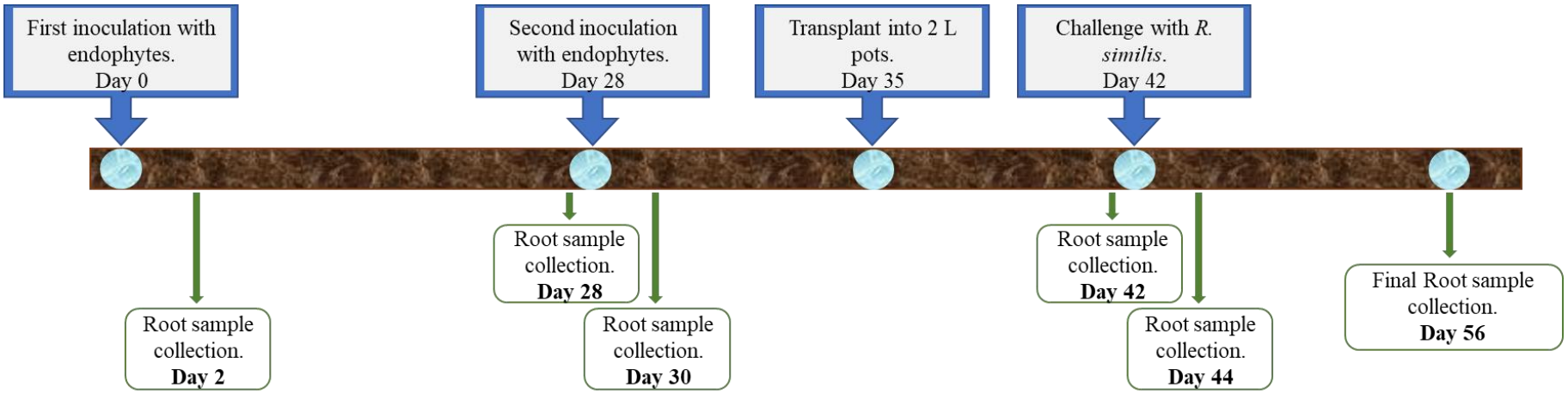

Fig. 1. Timeline for inoculation of banana roots [Ng'ombe (EA-AAA)] with fungal endophytes and infection with *Radopholus similis*, and root sample collection for qRT-PCR analysis.

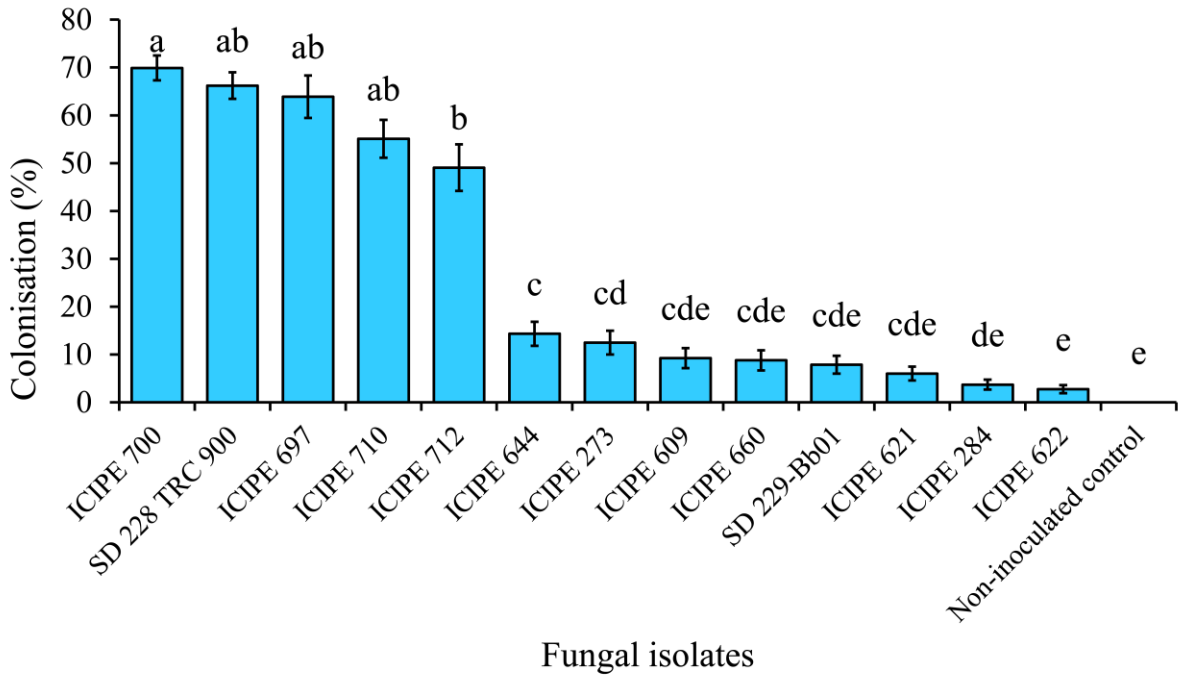

Fig. 2. Colonisation of banana root tissue of *cv*. Ng'ombe (EA-AAA) by endophytic fungal isolates at 8 weeks after inoculation. Bars followed by the same letter(s) indicate no significant difference. Means separated by Tukey's (HSD) at $p < 0.05$. Data pooled across 2 experiments

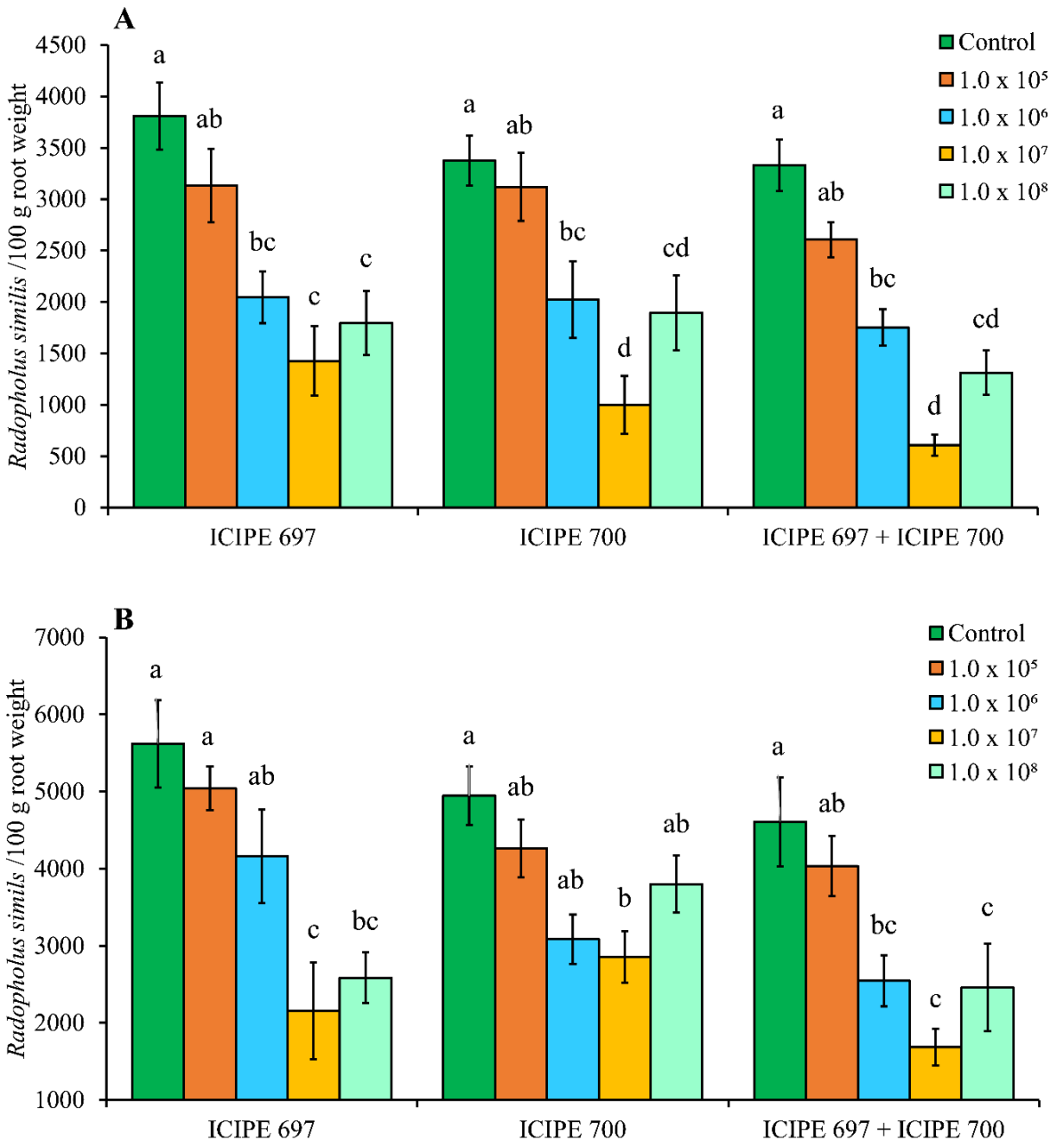

Fig. 3. *Radopholus similis* densities (per 100 g root) in banana *cv*. Ng'ombe (EA-AAA) for experiment 1 (A) and 2 (B) inoculated with endophytes *Hypocrea lixii* (ICIPE 697) and *Trichoderma asperellum* (ICIPE 700) individually and in combination at spore concentrations 1.0×10^5, 1.0×10^6, 1.0×10^7 and 1.0×10^8 spores/ml. Control: untreated control. For each experiment and within each endophyte treatment, bars followed by same letter(s) are not significantly different by Tukey's (HSD) test at $p < 0.05$.

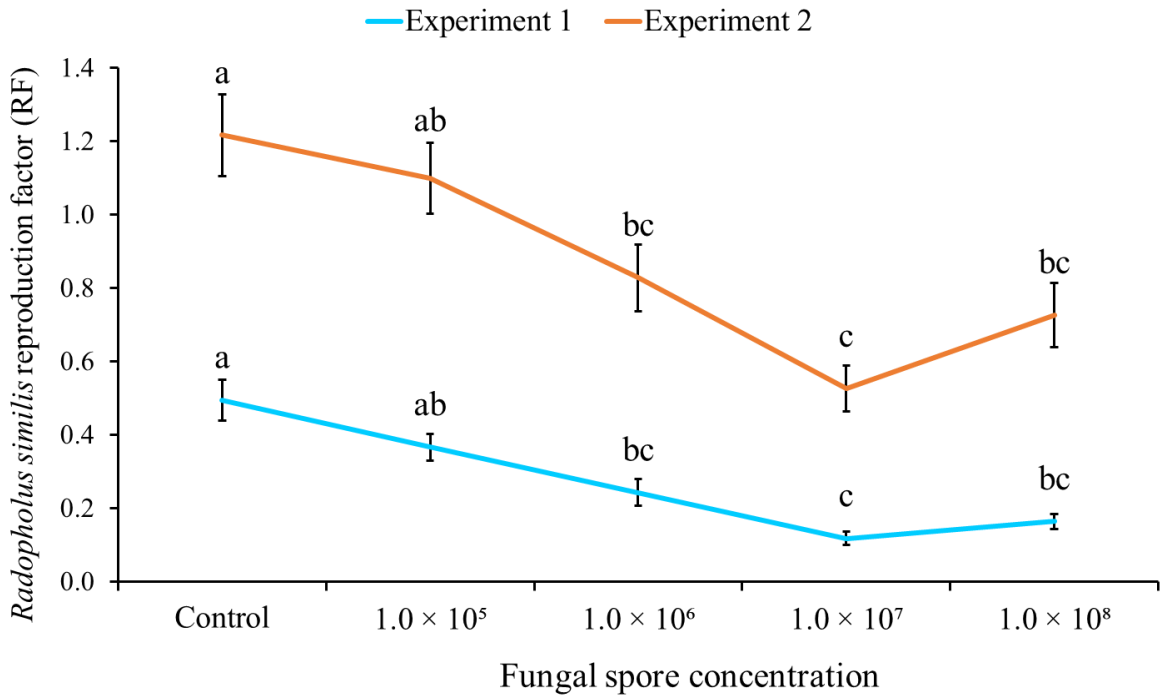

Fig. 4. *Radopholus similis* reproduction in banana plants *cv*. Ng'ombe (EA-AAA) inoculated with fungal endophytes *Hypocrea lixii* (ICIPE 697) and *Trichoderma asperellum* (ICIPE 700) (data pooled) at five spore concentrations: 1.0×10^5, 1.0×10^6, 1.0×10^7 and 1.0×10^8 spores/ml. Control: untreated control. For each experiment repeat, fungal spore concentrations with the same letter(s) are not significantly different by Tukey's (HSD) test at $p < 0.05$.

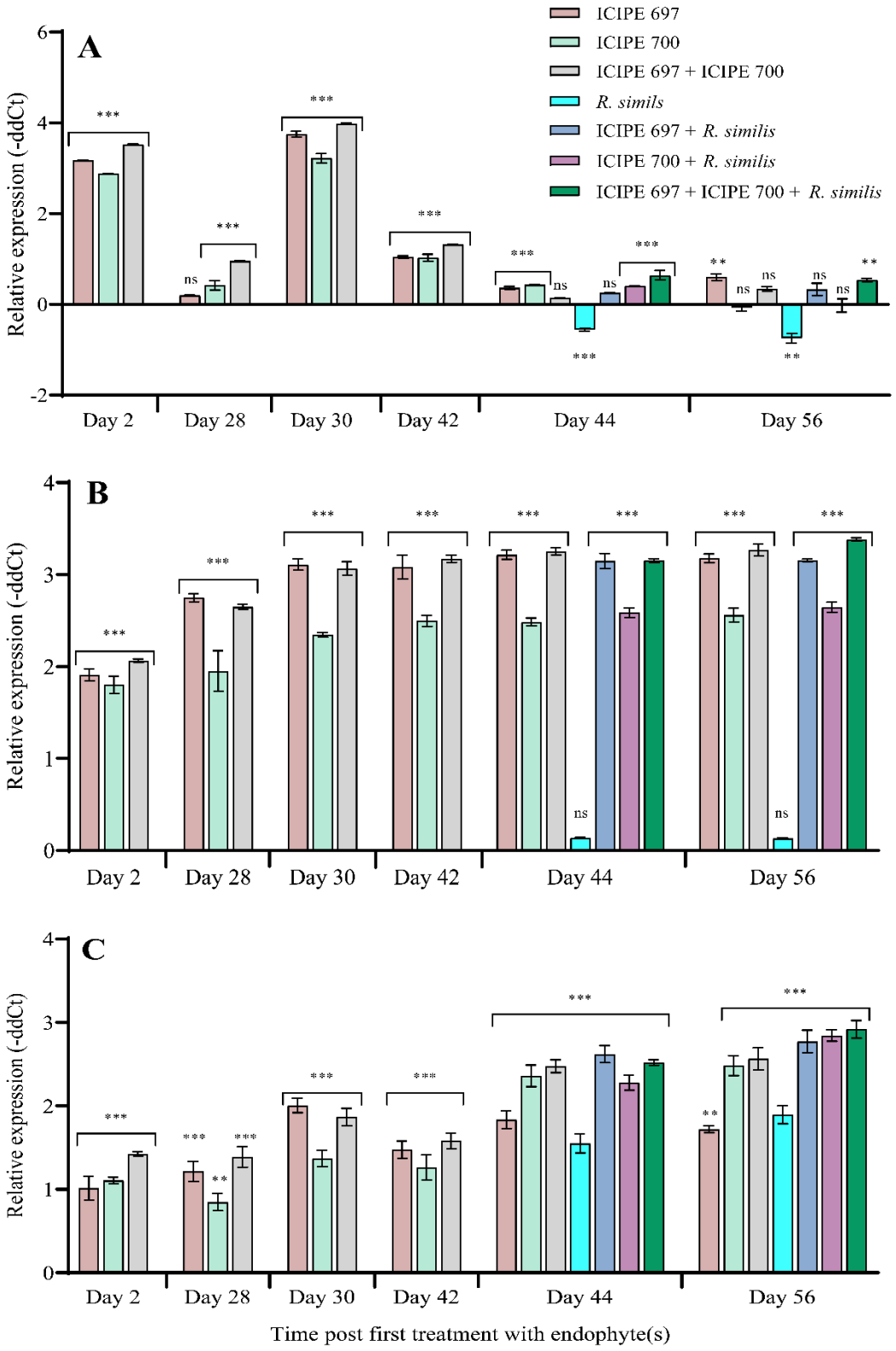

Fig. 5. Expression of defence-related genes *calmodulin-Ca*$^{2+}$ (A), *β-1,3-glucan synthase* (B) and *PR-1* (C) in roots of fungal endophyte inoculated banana plants *cv.* Ng'ombe (EA-AAA) infected with or without 1000 *Radopholus similis* relative to untreated control plants. At each day, asterisks indicate statistical significance relative to untreated control as shown by Tukey's (HSD) test (***: $p < 0.0001$, **: $p < 0.01$, ns: no significant difference).

CHAPTER 6: Conclusions and Recommendations

6.1 Testing the hypotheses

The overall objective of this study was to evaluate the efficacy of local candidate fungal antagonists against the banana nematode, *R. similis*, and the banana weevil (BW), *Cosmopolites sordidus* in East African highland banana (EAHB). The study was successful in achieving the overall objective and the associated specific objectives. The outcomes of each specific objective are summarised per associated hypothesis:

1) **Indigenous EPF isolates previously isolated from the plant's rhizosphere or endorhiza possess varying levels of antagonistic activity against BW**

The literature review section of this thesis highlighted studies where indigenous EPF isolates were tested and found to exhibit varying levels of pathogenicity to the adult BW. Similarly, among the twenty EPF isolates tested (Chapter 3: Article 1), eight isolates were pathogenic to the adult BW, causing >50% mortality, ten isolates caused 6.7 – 30% mortality, while two isolates were non-pathogenic to the adult BW (Kisaakye *et al.*, 2021). Furthermore, following BW mortality due to EPF infection, the weevil cadavers killed by the different fungal isolates produced varying levels of fungal spores upon incubation. Thus, the stated hypothesis is therefore accepted.

2) **Endophytic fungi previously isolated from the plant's rhizosphere or endorhiza possess antagonistic activity against *R. similis* infection of banana.**

The antagonistic activity of fungal endophytic isolates was tested in chapter 4 (Article 2) and chapter 5 (article 3). In chapter 4, the tested fungal endophytes supressed PPN (primarily composed of *R. similis*) infection of banana under field conditions. Upon scoring for root necrosis, it was observed that endophyte-inoculated plants had less necrotic damage compared to uninoculated control plants (Kisaakye *et al.*, 2022). Similarly, in chapter 5, all the tested 13 fungal isolates supressed *R. similis* infection of banana without compromising plant growth. Therefore, the stated hypothesis is accepted.

3) The antagonistic potential of endophytic fungi against *R. similis* infection of banana is enhanced when multiple fungal isolates are used as opposed to single isolate.

Dual inoculation of banana plants with fungal endophytic isolates was assessed under field conditions (Chapter 4) and in pots (Chapter 5). The inoculation of banana TC plants with the non-pathogenic *F. oxysporum* isolate V5w2 or a combination of V5w2 and the *B. bassiana* isolate WA equally suppressed *R. similis* infection of banana, an indication that inoculation of banana plants with V5w2 alone was sufficient in nematode suppression and boosting banana yield. On the contrary, in chapter 5, when the *T. asperellum* isolate ICIPE 700 and the *H. lixii* isolate ICIPE 697 were inoculated into banana plants individually and in combination, the combined inoculation of ICIPE 700 and ICIPE 697 led to greater suppression of *R. similis* (>21%) relative to individual inoculation. An indication that effectiveness of multiple fungal isolates against *R. similis* infection of banana varies with the type of fungal isolates used.

4) Mode of action of endophytic fungus against *R. similis* infection is mediated by the up- or down regulation of defence-related genes in the banana roots.

The expression profile of three defence-related genes was assessed in endophyte inoculated and uninoculated plants (Chapter 5). Inoculation of the EAHB *cv.* Ng'ombe with the *T. asperellum* isolate ICIPE 700 or *H. lixii* isolate ICIPE 697 individually and in combination significantly enhanced upregulation of the defence-related gene *PR-1*, the cell signalling gene *calmodulin-*$Ca^{2+}$and the cell wall strengthening gene *β-1,3-glucan synthase* and suppressed *R. similis* infection of banana, an indication that upregulation of the defence genes is linked to the suppression of *R. similis* infection of banana. Hence the stated hypothesis is accepted.

5) Endophyte enhanced bananas are more resilient against *R. similis* infection in the field, yield more and last longer.

The EAHB *cv*. Mbwazirume was inoculated with the fungal isolates V5w2 and WA individually and in combination and growth in the field for two cropping cycles (Chapter 4). Enhancement of the banana plants with the fungal endophytes significantly suppressed *R. similis* density by >34%, with a 7% reduction in plant toppling and 22% increase in yield. These observations positively impact banana resilience against *R. similis* infection, yield and plant longevity, respectively. Therefore, the stated hypothesis is accepted.

6.2 Conclusions

Amongst the 20 EPF isolates screened in the laboratory for management of BW, *B. bassiana* isolates caused >50% mortality of the adult BW. The *B. bassiana* isolates: ICIPE 273, ICIPE 648 and ICIPE 660 were the most pathogenic, killing >80% of BW within the shortest time (LT_{50} ≤11.1 days) compared to other isolates tested. Furthermore, these three *B. bassiana* isolates produced the highest number of fungal spores (≥3.6×10^7 spores) per BW cadaver and were able to cause BW mortality in the field. Based on these results, this study concluded that ICIPE 273, ICIPE 648 and ICIPE 660 are pathogenic to BW and hence potential candidates for management of BW. However, the low pathogenicity observed in the field, linked to the rapid decline in spore viability, is an indication that effectiveness of the EPF isolates was compromised by elements of the environment (Bouamama *et al.*, 2010; Guilherme *et al.*, 2015). Thus, further studies need to be conducted to improve and maintain fungal spore viability in the field.

When the non-pathogenic *F. oxysporum* (isolate V5w2) and the *B. bassiana* (isolate WA) were inoculated (individually and in combination) into EAHB *cv.* Mbwazirume and the dessert banana *cv.* Grande Naine, it was observed that V5w2 alone was the most effective in suppressing *R. similis* and *H. multicinctus* infection of banana, reduced plant toppling and root necrosis, and ultimately improved yield of the first crop cycle. Similarly, upon inoculation of V5w2 in banana plants in Kenya, Waweru *et al.* (2014) reported a reduction in *P. goodeyi* infection, reduced root necrosis damage and increased bunch weight and yield of Grande Naine and Giant Cavendish bananas. This is an indication that the *F. oxysporum* isolate V5w2 has potential for development into an eco-friendly endophytic biopesticide against parasitic nematode in banana fields where BW is not a problem. However, integration of endophytic V5w2 with another isolate(s) or other environmentally friendly BW control strategies would be appropriate in fields where PPN and BW co-exist. On the other hand, the potential of V5w2 for management of banana diseases needs to be explored to understand its full benefits.

Colonisation of banana plants by V5w2 declined drastically between field planting (45% root colonisation) and 6 months post planting (3% root colonisation), and no root colonisation was detected after 6 months. Similar results were obtained when the EAHB *cvs*. Kibuzi and Nabusa were inoculated with *F. oxysporum* isolate V2w2 (Paparu *et al.*, 2008). This is an indication that one-time application of the fungal endophyte is not appropriate to sustain fungal

colonisation in the banana roots. Consequently, repeat application of the fungal endophyte in the field could help boost root colonisation.

In the study to understand the mode of action of fungal endophytes, suppression of *R. similis* in ICIPE 700 and/or ICIPE 697 inoculated banana plants was linked to the enhanced upregulation of the defence-related gene *PR-1*, the cell signalling gene *calmodulin-*Ca^{2+} and the cell wall strengthening gene *β-1,3-glucan synthase*. Paparu *et al.* (2007) had earlier reported an upregulation of the three defence genes upon inoculation of the nematode tolerant banana *cv*. Kayinja (genomic group ABB) with V5w2. This clearly indicates that activation of defence-related genes is key in the modulation of nematode infection of banana by antagonistic fungal endophytes.

6.3 Recommendations

1) Suitable mechanisms of delivery through an effective formulation of the three potential entomopathogens, ICIPE 273, ICIPE 648 and ICIPE 660 is needed. This will help maintain high spore viability in the field and thus increase the length of the effective period and the overall efficacy in the field. Similarly, methods of mass-producing and subsequent formulation of the endophytic *F. oxysporum* isolate V5w2 need to be explored.
2) Repeat in-field application of endophytic fungi *F. oxysporum* isolate V5w2 should be performed. This will provide knowledge on whether in-field application helps boost the inoculum levels and how this impacts nematode infection and yield of successive crop cycles. In field application can be achieved through drenching of the soil around the banana plant with a spore suspension of the fungal isolate.
3) Prior to developing ICIPE 273, ICIPE 648 and ICIPE 660 as entomopathogenic biopesticides for management of the BW, and V5w2, ICIPE 697 and ICIPE 700 as endophytes against parasitic nematodes in banana, it is highly recommended that their effect on non-target beneficial insects, including natural enemies be tested. Additionally, it is critical that these candidate isolates be tested in different agro-ecological zones in the region to fully understand their full performance and response in the different agro-ecologies in the region.

4) The impact of these candidate fungal isolates (both entomopathogens and endophyte) on the soil microbiome and overall soil health should be studied to understand the full benefits that can be derived from these promising biopesticides.
5) There is need to test compatibility of the candidate isolates for both BW and PPN with other control measures practiced by the farmers for development of a sustainable pest management strategy.
6) As most banana farmers in East Africa practice little or no pest management there is need to raise awareness and knowledge on the importance of BW and PPN. Training banana farmers and extension workers on the benefits of using environmentally friendly microbial products will help raise awareness and increase usage.

6.4 References

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APPENDICES

Appendix A

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