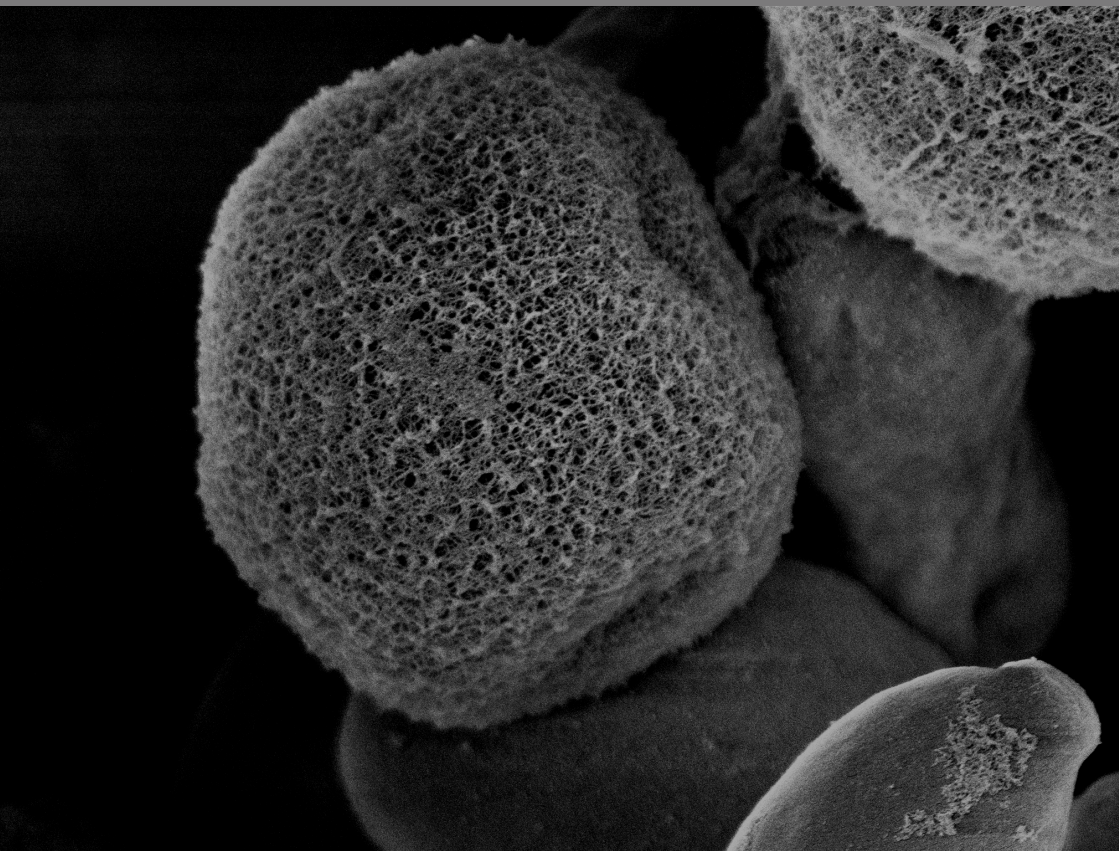
Mechanistic aspects of the eco-physiology of *Fusarium oxysporum* f. sp. *cubense* TR4

Evans Were



Faculty of Agricultural Sciences

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Mechanistic aspects of the eco-physiology of *Fusarium oxysporum* f. sp. *cubense* TR4

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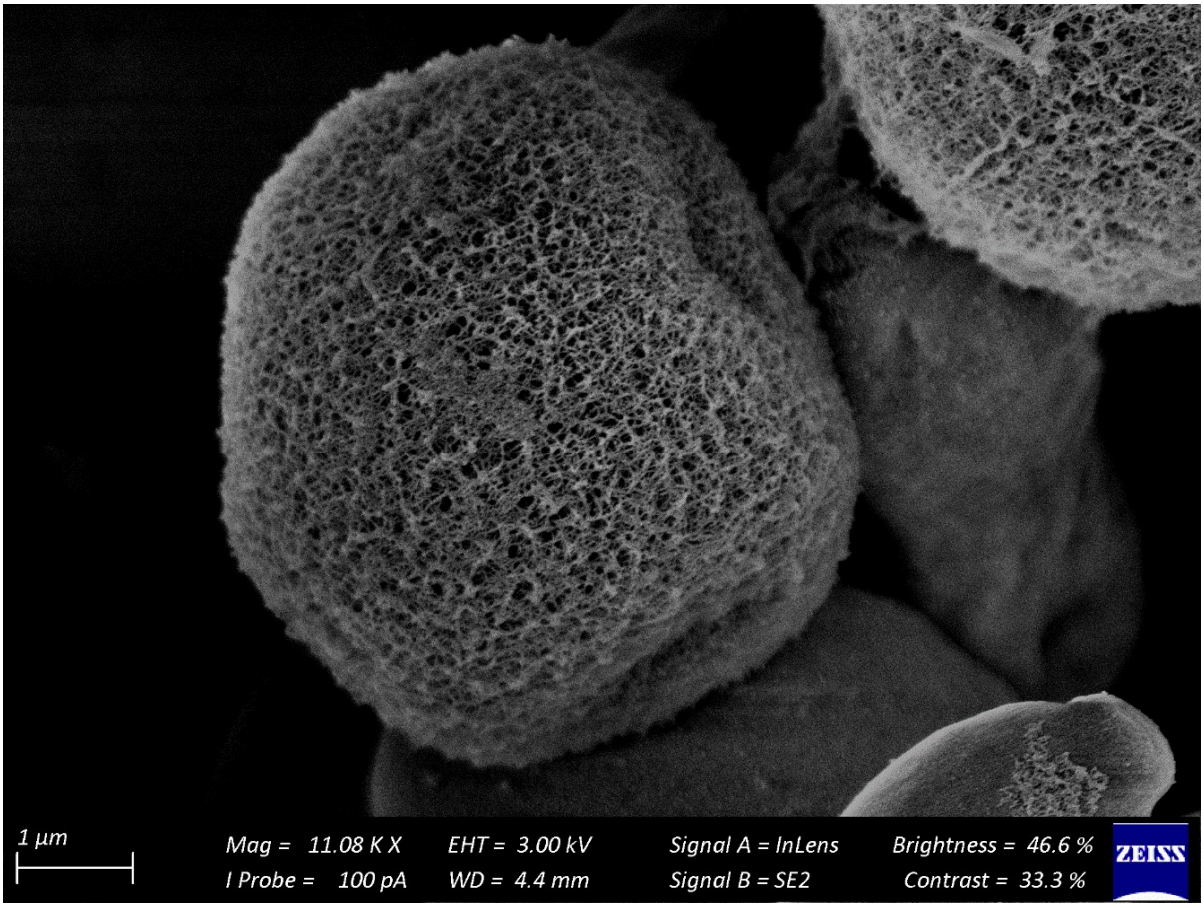

Cover image: electron micrograph of a dormant chlamydospore of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4). The image was taken using the Zeiss Merlin Scanning Electron Microscope, on 26th September, 2019 (*Source*: Evans Were).

“No fruit dies so vile and offensive a death as the banana...”

— Kiran Desai

The Inheritance of Loss, 2006

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This is the LORD’s doing; it is marvelous in our eyes. Psalm 118:23 (ESV)

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Acronyms

ATP	Adenosine 5'–triphosphate
BCA	Biological control agent
cAMP	Cyclic 3',5'–adenosine monophosphate
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
FAO	Food and agriculture organization of the United Nations
HPLC–DAD	High–performance liquid chromatography–diode array detection
IFPRI	International Food Policy Research Institute
IYPH	International year of plant health
LC–MS	Liquid chromatography–mass spectrometry
LC–ESI/MS	Liquid chromatography–electrospray ionization mass spectrometry
mRNA	Messenger RNA
m*/*z	Mass–to–charge ratio
PCA	Principal component analysis
PGPB	Plant growth promoting bacteria
qPCR	Real time quantitative polymerase chain reaction
RT–qPCR	Reverse transcription real–time quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase
SEM	Scanning electron microscope
SPK	Spitzenkörper
spp.	Species pluralis
UN	United Nations
v*/*v	Volume of solute/volume of solution
w*/*v	Mass of solute/volume of solution

Summary

Banana and plantain (*Musa* spp.), here termed as bananas, are a source of food security and income for more than 400 million people globally. Banana production is threatened by Fusarium wilt disease, caused by the soilborne root-infecting fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc). Foc Tropical Race 4 (Foc TR4) is considered the most virulent race of Foc and has gained notoriety due to its inexorable spread and devastating impact on banana cultivation. Host infection occurs when pathogen propagules, called chlamydospores, germinate and produce hyphae that penetrate host roots and subsequently invade host tissues. Infection occurs in a narrow zone of soil immediately adjacent to the roots, called rhizosphere. The rhizosphere is notable for the extensive interactions between roots, the microbiome, and soil physico-chemical factors. Banana rhizosphere interactions are poorly understood, yet profoundly influence infection and development of Fusarium wilt. It is speculated that a better understanding of banana rhizosphere interactions will improve management of Fusarium wilt through the reduction of the abundance and/or efficacy of inoculum or enhance the disease suppressiveness of soils. Hence, the overarching objective of this doctoral study was to contribute to the fundamental ecological understanding of banana rhizosphere interactions related to Foc.

The first study of this thesis analysed literature from four electronic databases (AGRIS, CAB Direct, SciVerse Scopus, ProQuest) to bring together the relatively scant data available on banana rhizosphere interactions and to highlight the key knowledge gaps. Analysis of 2,281 publications revealed the complexity of banana rhizosphere interactions and the driving factors of Fusarium wilt, for which the mechanisms remain poorly understood. Data from the literature shows that management of Fusarium wilt through rhizosphere manipulation is a dominant element albeit with limited success in the field. Notably, the data from literature shows that biological control agents (bacterial and fungal strains) are highly effective *in vitro*

and in the greenhouse with a mean efficacy of 77.1% and 73.5%, respectively, but efficacy remains below 25.0% under field conditions.

The second study of this thesis provides empirical evidence for suppression of Foc TR4 by root-secreted phenolic acids of non-host plants. Hydroponic culture and targeted metabolite analysis of root exudates of two legumes, *Desmodium uncinatum* and *Mucuna pruriens*, identified phenolic compounds such as benzoic-, *t*-cinnamic-, and *p*-hydroxybenzoic acid with inhibitory potential. These phenolic compounds suppressed Foc TR4 by inhibition of chlamydospore germination, production of new spores, and hyphal growth, and specifically also the biosynthesis of fusaric acid and beauvericin toxins, which are essential in the biology of the fungus.

The third study of this thesis provides empirical evidence that the process of chlamydospore germination in Foc TR4 is developmentally orchestrated and iron-dependent. Scanning electron microscopy showed that iron-starved chlamydospores are unable to form a germ tube and exhibit reduced metabolic activity. Moreover, germination exhibits plasticity regarding extracellular pH, where over 50% germination occurs between pH 3 and pH 11. This suggests that disease suppression by manipulation of soil pH may not necessarily act via alteration of iron bioavailability. The requirement for iron was further investigated by assessing the expression of two genes (*rnr1* and *rnr2*) that encode ribonucleotide reductase (RNR), the enzyme that controls cell growth through DNA synthesis. Expression of *rnr2* was significantly induced in iron-starved chlamydospores compared to the control.

The fourth study assessed the production of microbial iron-sequestering metabolites (siderophores) as a potential mechanism to counteract iron starvation. Specifically, ferrichrome, a hydroxamate siderophore, was synthesized exclusively in the mycelia of iron-starved cultures, which suggests *de novo* biosynthesis. Moreover, amino acid precursors for siderophore biosynthesis (ornithine, arginine) were altered by iron starvation.

Collectively, this doctoral thesis extends the fundamental understanding of the biology and ecology of Foc TR4 and provides a base for realizing the potential of rhizosphere manipulation for management of Fusarium wilt.

Zusammenfassung

Bananen und Kochbananen (*Musa*-Arten), die der Ernährungssicherung und dem Einkommen von weltweit mehr als 400 Millionen Menschen dienen, sind von der Fusarium-Welke bedroht, die auf dem bodenbürtigen Pilz *Fusarium oxysporum* f. sp. *cubense* (Foc) beruht. Die Rasse Foc Tropical Race 4 (Foc TR4) gilt als diejenige mit der höchsten Virulenz und ist bekannt für ihre rasche Ausbreitung und die verheerenden Wirkungen in Bananenplantagen. Zur Fortpflanzung bildet das Pathogen sogenannte Chlamydosporen, die nach ihrer Keimung Hyphen produzieren. Die Infektion erfolgt in der Rhizosphäre, wo die Hyphen über die Wurzeln in das Wirtsgewebe eindringen. Die Rhizoshpäre ist bedeutend für die intensiven Interaktionen zwischen Wurzel und dem Mikrobiom sowie den physikalisch-chemischen Faktoren im Boden. Über die Rhizosphäre-Interaktionen bei Bananen ist noch wenig bekannt. Sie haben jedoch erheblichen Einfluss auf den Befall und die Entwicklung der Fusarium-Welke. Es ist davon auszugehen, dass genauere Kenntnisse der Bananen-Rhizospäre-Interaktionen das Management der Fusarium-Welke verbessern werden, und zwar durch die Reduktion der Abundanz und/oder der Wirksamkeit des Inokulums, oder durch die Erhöhung der krankheitsunterdrückenden Wirkung des Bodens. Das übergeordnete Ziel dieser Doktorarbeit war es entsprechend, zum fundamentalen Verständnis der Bananen-Rhizospäre-Interaktionen im Zusammenhang mit Foc TR4 beizutragen.

Die erste Studie dieser Arbeit umfasste die Literaturanalyse aus elektronischen Datenbanken (AGRIS, CAB Direct, SciVerse Scopus, ProQuest), um die relativ spärlich verfügbaren Daten zu den Interaktionen in der Bananen-Rhizosphäre im Zusammenhang mit der Fusarium-Welke zusammenzustellen und die Wissenslücken aufzuzeigen. Die Analyse von 2,281 Publikationen zu Bananen-Rhizosphäre Interaktionen und den bestimmenden Faktoren für die Fusarium-Welke zeigte die Komplexität der wenig verstandenen Mechanismen. Literaturdaten ergaben, dass Manipulationen der Rhizosphäre die vorherrschenden Ansätze darstellen, jedoch mit begrenzten Erfolgen unter Feldbedingungen.

Biologische Kontrollagenten (Bakterien- und Pilz-Stämme) sind sehr effektiv *in vitro* und unter Gewächshausbedingungen mit durchschnittlichen Wirksamkeiten von 77.1% bzw. 73.5%. Unter Feldbedingungen lag die Wirksamkeit jedoch unter 25%.

Die zweite Studie dieser Arbeit liefert empirische Beweise für die Unterdrückung von Foc TR4 durch Phenolsäuren, die von den Wurzeln von nicht-Wirtspflanzen abgegeben wurden. In Hydrokulturen und in gezielten metabolischen Analysen der Wurzelexudate zweier Leguminose-Arten (*Desmodium uncinatum* und *Mucuna pruriens*) zeigten Phenolverbindungen wie Benzoe-, *t*-Zimt- und *p*-Hydroxybenzoe-Säure ein inhibitorisches Potenzial. Diese Verbindungen unterdrückten Foc TR4 durch Hemmung der Chlamydosporenkeimung, der Neuproduktion von Sporen, des Hyphenwachstums und insbesondere der Biosynthese von Fusarinsäure und toxischen Beauvericinen, die in der Biologie des Pilzes essenziell sind.

Die dritte Studie dieser Arbeit lieferte den empirischen Beweis, dass der entwicklungsgesteuerte Prozess der Chlamydosporen-Keimung bei Foc TR4 eisenabhängig ist. Im Rasterelektronenmikroskop zeigte sich, dass Chlamydosporen unter Eisenmangel keinen Keimschlauch bilden und eine reduzierte metabolische Aktivität aufweisen. Außerdem weist die Keimung eine Plastizität hinsichtlich des extrazellulären pH-Wertes auf, wobei mehr als 50% der Keimungen zwischen pH 3 und pH 11 erfolgten. Dies deutet darauf hin, dass die Krankheitsunterdrückung durch die Manipulation des Boden-pH-Wertes nicht notwendigerweise durch Veränderung der Bioverfügbarkeit von Eisen erfolgt. Der Bedarf an Eisen wurde anhand der Expression zweier Gene (*rnr1* und *rnr2*) weiter untersucht. Diese Gene kodieren die Ribonukleotid-Reduktase (RNR), d.h. das Enzym, welches das Zellwachstum durch DNA-Synthese kontrolliert. Die Expression von *rnr2* wurde bei Chlamydosporen unter Eisenmangel signifikant stärker induziert als in der Kontrolle.

Die vierte Studie dieser Arbeit untersuchte die Produktion mikrobieller, eisen-absondernden Metaboliten (Siderophoren) als möglichen Mechanismus, der dem Eisenmangel entgegenwirkt. Es wurde im Speziellen gezeigt, dass Ferrichrom, eine Hydroxamat-Siderophore, ausschließlich im Mycel von Kulturen mit Eisenmangel

synthestisiert wurde und somit eine *de novo* Biosysnthese nahelegt. Darüber hinaus wurden auch Aminosäure-Vorstufen für die Siderophoren-Biosynthese (Ornithin, Arginin) durch Eisenmangel verändert. Insgesamt erweitert diese Doktorarbeit das grundlegende Verständnis der Biologie und Ökologie von Foc TR4 und liefert somit eine Grundlage für die Nutzung des Potenzials zur Manipulation der Rhizosphäre für das Management der Fusarium-Welke.

1. General introduction

1.1 The burden of phytopathogens on global food security

Global food security remains a major concern in a changing world (Godfray et al., 2010; Viana et al., 2022). The global human population is predicted to grow to 9.7 billion people by 2050 and surpass 10 billion people by 2100 (UN, 2017). Population growth will exert unprecedented demand on global resources, especially food (Viana et al., 2022). Currently, 811 million people (or 10.3% of the global population) are hungry, while another 1.3 billion people (17%) suffer from micronutrient deficiencies (FAO et al., 2021). Moreover, the COVID-19 pandemic is anticipated to propel over 150 million people into poverty (Laborde et al., 2021). This could make healthy diets less affordable particularly in the developing countries, and make the Sustainable Development Goal (SDG) of zero hunger by 2030 (SDG2), seemingly out of reach (Laborde et al., 2021; Zhu et al., 2022). Therefore, innovative strategies are needed to keep abreast of population growth and to bring gain on food security (Hickey et al., 2019; Dobermann et al., 2022).

Food security exists if and only if “all people at all times have physical, social, and economic access to sufficient, safe, and nutritious food that meets their dietary needs and food preferences for an active and healthy life” (FAO, 2009). The four pillars of food security are food availability, access, stability, and utilization (Ingram, 2011). Global food security is threatened by phytopathogens, particularly fungi, which persistently undermine crop yield, quality, and trade (Avery et al., 2019; Viljoen et al., 2020; Ristaino et al., 2021; Trivellone et al., 2022; van Westerhoven et al., 2022).

The global fungal diversity constitutes a vast kingdom of an estimated 1.5 to 12 million fungal species (Baldrian et al., 2021; Phukhamsakda et al., 2022). However, only 150,600 fungal species are formally known (Baldrian et al., 2021; Phukhamsakda et al., 2022). About 10% of all known fungal species are phytopathogens that can cause disease in over 10,000

different plant species (Savary et al., 2019; Liu et al., 2022). Fungal phytopathogens destroy up to 30% of crop products, sufficient to feed 600 million people annually (Avery et al., 2019; Trivellone et al., 2022). Losses associated with fungal phytopathogens can disrupt food availability, thereby leading to fluctuations in food production which, in turn, can compromise food stability (Rizzo et al., 2021). Comparably, food access and utilisation are directly affected by mycotoxin-producing fungi, such as *Aspergillus flavus* and *Fusarium verticillioides*, which compromise food quality, safety, and trade (Munkvold et al., 2021). Indeed, the consumption of mycotoxin-contaminated food can cause liver cancer, kwashiorkor, and stunted growth (Munkvold et al., 2021; Giaquinto et al., 2022). Collectively, fungal phytopathogens threaten global food security by limiting crop yield, quality, and trade (Avery et al., 2019; Fones et al., 2020; Ristaino et al., 2021; Trivellone et al., 2022). In recognition of this, the UN declared the year 2020 as the International Year of Plant Health (IYPH) (FAO, 2020). The overarching purpose of the IYPH was to raise awareness of plant health and its impact on society (FAO, 2020).

1.2 *Fusarium oxysporum* f. sp. *cubense*, a devastating fungal pathogen of banana

Musa species (grouped into banana and plantain), hereafter called banana, is an important crop globally (Heslop-Harrison and Schwarzacher, 2007). Bananas are also the most important traded fruit globally in terms of export value (Voora, et al., 2020). For example, in 2017, the banana export trade (excluding plantains) was 22.7 million tonnes representing almost 20% of global production that year (Voora, et al., 2020). The value of this trade was worth USD 11 billion, which is higher than the export value of any other exported fruit (Voora, et al., 2020).

Bananas produce fruit all year-round and play an important role in the food security and income of more than 400 million people in the tropical and subtropical regions of the world. For example, East African Highland bananas constitute an important cash crop and staple food in the Great Lakes Region of Africa (Burundi, DR Congo, Rwanda, Tanzania, and

Uganda) (Akankwasa et al., 2021). More than 1000 banana varieties are cultivated in 135 countries on over 11 million hectares, with production quantity generally exceeding 150 million tonnes (Fig. 1.1; FAOSTAT, 2022).

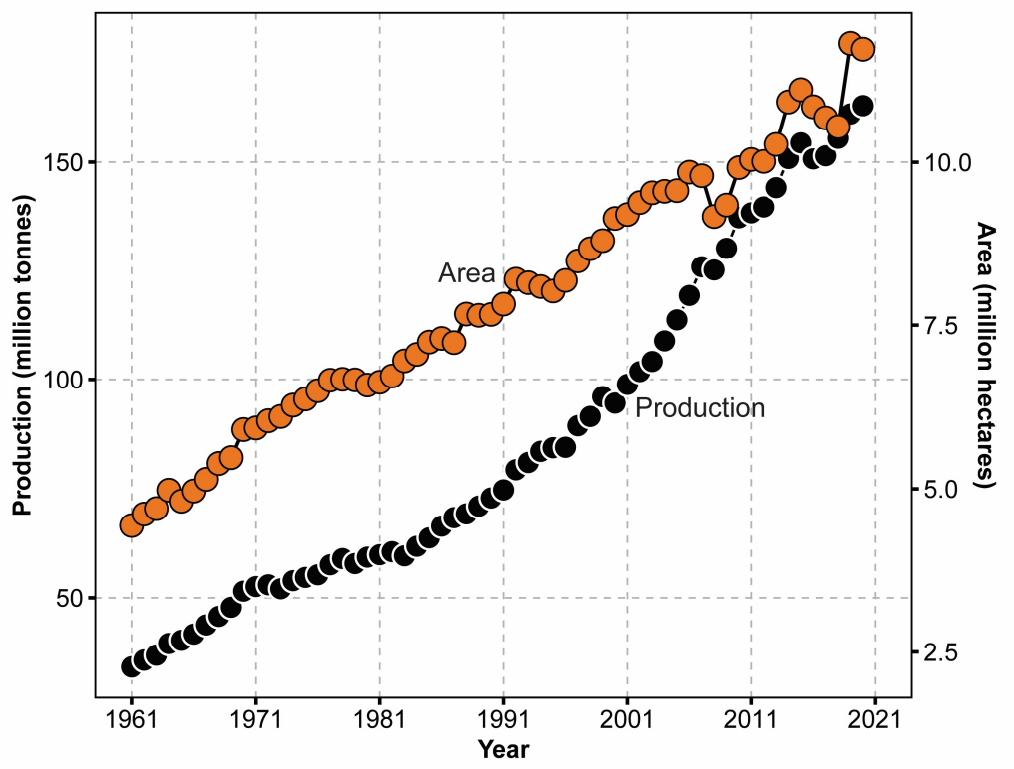

Figure 1.1. Temporal trends in global production of banana and area under banana cultivation from 1961 to 2020 (*Source*: Data elaborated from FAOSTAT, accessed 20th April, 2022).

Globally, sustainable production of banana is constrained by pests and diseases, particularly Fusarium wilt disease. Fusarium wilt of banana is caused by the notorious, soilborne root-infecting fungal pathogen, *Fusarium oxysporum* f. sp. *cubense* (Foc) (Viljoen et al., 2020). Foc is one of the most important *formae speciales* of the *F. oxysporum* species complex (Dean et al., 2012). *Fusarium oxysporum* is a genetically heterogeneous polytypic morphospecies, which includes pathogenic and non-pathogenic fungal strains (O'Donnell et al. 1998; Gordon, 2017; Edel-Hermann and Lecomte, 2019). Pathogenic isolates of *F. oxysporum* are highly host-specific and are, therefore, classified as *formae speciales* (Dean et al., 2012; Edel-Hermann and Lecomte, 2019).

Formae speciales of *F. oxysporum* can cause disease on single hosts in over 120 crop species (Dean et al., 2012). The diversity in non-pathogenic *F. oxysporum* strains unable to cause disease to agricultural crops is even greater (O'Donnell et al. 1998; Nel et al. 2006). The genomes of *F. oxysporum* species are compartmentalized into core chromosomes that are highly similar among *F. oxysporum* strains, and accessory chromosomes that are not similar (Ma et al. 2013; Constantin et al., 2021). Moreover, non-pathogenic strains of *F. oxysporum* have the same core genome as the pathogenic strains, but typically possess fewer effector candidate genes and associated non-autonomous transposons (*mimps*) than the pathogenic strains (Constantin et al., 2021).

Formae speciales of *F. oxysporum* frequently comprise several pathogenic races, which are defined by virulence on differentially-resistant host cultivars (Edel-Hermann and Lecomte, 2019). There are three such races of Foc (Foc race 1, race 2, and race 4) that are recognized globally based on pathogenicity to specific banana cultivars. Foc Tropical Race 4 (Foc TR4) is considered the most virulent, due its pathogenicity on diverse banana cultivars (Ordonez et al., 2015; Pegg et al., 2019). Foc TR4 has gained notoriety due to its extremely devastating impact and inexorable spread, which make Foc TR4 a threat to the livelihoods of over 400 million people that depend on banana for food security and income (Ordonez et al., 2015; van Westerhoven et al., 2022).

Fusarium wilt of banana was first observed in Australia in 1876 (Drenth and Kema, 2021). The disease, however, gained significance in the 20th century, when Foc race 1 exterminated thousands of hectares of Gros Michel bananas in Central America, which, at that time, dominated the international banana export trade (Drenth and Kema, 2021). Losses of Gros Michel bananas due to Fusarium wilt in the 1900s were estimated at 2.3 billion USD (Altendorf, 2019). Gros Michel bananas were eventually substituted by Cavendish cultivars, which quenched the epidemic. Cavendish bananas were considered resistant to Fusarium wilt until the disease re-emerged, initially in subtropical banana production areas and later in the tropics (Viljoen et al., 2020; Drenth and Kema, 2021). Symptoms on Cavendish bananas were

first observed in South Africa, Australia, and the Canary Islands (Viljoen et al., 2020; Drenth and Kema, 2021).

After 1967, Cavendish bananas became severely affected in Taiwan (Su et al., 1986). Fusarium wilt became an imminent threat to the production of Cavendish bananas, when symptomatic plants were discovered in the early 1990s in tropical Malaysia and Indonesia on commercial plantations without a previous history of the disease (Lee et al., 2001; Hwang and Ko, 2004). Disease symptoms on Cavendish in the tropics initially appeared to be restricted to Southeast Asia and the Northern Territory of Australia until 2012, where after the disease was detected in the Middle East, Mozambique, Colombia, and recently in Peru (García-Bastidas et al., 2019; Viljoen et al., 2020; Acuña et al., 2021; Drenth and Kema, 2021). The inexorable spread and devastating impact of Foc TR4 has created a pressing need to understand the ecology and biology of this fungus so as to fine-tune or develop interventions for disease management.

1.3 Ecology of *Fusarium oxysporum* f. sp. *cubense*

The source of primary Foc inoculum are hardy, thick-walled asexual spores called chlamydospores, which can remain quiescent in Foc-infested soil for decades (Rishbeth, 1955; Ma et al., 2013). When suitable conditions are encountered, chlamydospores undergo a revival cellular process called germination, producing hyphae that penetrate host roots and subsequently invade aerial plant parts, which leads to wilting and death of the infected plant (Rishbeth, 1955; Viljoen et al., 2020). Stover (1962) in Central America and Rishbeth (1955) in Jamaica reported that Fusarium wilt quickly recurred in replanted plantations, which had been previously abandoned for a period of more than 20 years. Similarly, Trujillo and Snyder (1963) observed that the propagules of Foc were distributed sparsely and unevenly through the soils of infested banana plantations, existing as chlamydospores inside decaying host-root tissues. Besides, non-host plants, such as native grass species and weeds, have been reported to harbour Foc without apparent disease symptoms (Waite and Dunlap, 1953;

Pittaway et al., 1999; Hennessy et al., 2005; Catambacan and Cumagun, 2022). The commensal colonization of non-host plants by Foc may further contribute to the persistence of the pathogen in soil (Hennessy et al., 2005; Catambacan and Cumagun, 2022).

The process of Foc infection and severity of Fusarium wilt are influenced by complex below-ground interactions between roots, root-associated microbial communities (such as bacteria, fungi, protists, as well as free-living and plant-parasitic nematodes), and soil physico-chemical factors (Stover, 1962; Almeida et al., 2018; Dita et al., 2018). These interactions occur in a narrow zone of soil immediately adjacent to roots, called the rhizosphere (Hartmann et al., 2008). Interactions in the banana rhizosphere profoundly influence the rhizosphere ecology of Foc and ultimately disease (Stover, 1962; Dita et al., 2018).

For soil-borne fungal phytopathogens, the rhizosphere environment can have a critical influence on the development of an epidemic. For example, the environment may influence disease by imposing a direct effect on pathogen fitness, or indirectly through a host-mediated effects. Thus, an underlying tenet of fungal ecology is that an increased understanding of how a pathogen responds to perturbations, such as alterations in nutrient bioavailablity, moisture, or pH in its environment, is fundamentally important and can lead to new interventions for disease management (Minerdi et al., 2011; Alabouvette et al., 2009; Hartmann, 2022). The complexity of banana below-ground interactions can be described using the disease triangle model. The disease triangle remains as a core principal of phytopathology, as it describes the complex interactions between the environment, the pathogen, and the host plant (McNew, 1960; Scholthof, 2007).

The disease triangle is a visual holistic tool that enables the integrative understanding of the infection and disease development from three interconnected perspectives: host, pathogen, and the environment (McNew, 1960; Scholthof, 2007). The concept of the disease triangle was formalized in the 1960s by George McNew, who broadly defined the parameters of the disease triangle as “the inherent susceptibility of the host, the inoculum potential of the parasite, and the impact of the environment on parasitism and pathogenesis” (Scholthof, 2007).

Previous reports show that the incidence and severity of Fusarium wilt can be influenced by interactions in the banana rhizosphere. For instance, high disease incidence and severity have been associated with soil microbial and physico-chemical properties, such as nutrient bioavailability, moisture, and pH (Peng et al., 1999; Aguilar et al., 2000; Turner et al., 2007). In addition, parasitic nematodes have been reported to make the ensuing damage worse than it would have been with either Foc or nematodes alone (Almeida et al., 2018). Understanding the impact of banana rhizosphere interactions on Fusarium wilt could help to further understand the molecular mechanisms of infection and disease progression, and could guide effective management of Fusarium wilt disease in the field. By review of available literature (Chapter 2), this doctoral thesis sought to bring together the relatively scant data available on banana below-ground interactions in relation to Fusarium wilt, the known mechanisms, and to highlight key knowledge gaps for progressive research.

Generally, the destruction of infected plants, isolation of infested areas, and disinfection of farm machinery represent the mainstay of management of Foc TR4 (Dita et al., 2018). However, these measures are ineffective and unsustainable, as evidenced by the inexorable spread and devastating impact of Foc TR4 (Viljoen et al., 2020; Acuña et al., 2021; Drenth and Kema, 2021). Fusarium wilt can be effectively managed by use of genetically resistant, but commercially acceptable banana cultivars are currently not available for Foc Tropical Race 4 (Foc TR4) (Dita et al., 2018; Viljoen et al., 2020). Therefore, management of Fusarium wilt TR4 largely relies on cultural practices aimed at reducing the inoculum load in soil, enhance the disease suppressiveness of soils, and limit pathogen spread (Dita et al., 2018; Bubici et al., 2019; Viljoen et al., 2020; Segura et al., 2021). For instance, Fusarium wilt has been suppressed through rotation of banana with other crops, such as chilli pepper *(Capsicum frutescens* L.), rice (*Oryza sativa* L.), maize (*Zea mays* L.), or pineapple (*Ananas comosus* [L.] Merr.) (Hwang, 1985; Wang et al., 2015; Hong et al., 2020). Intercropping banana with Chinese leek (*Allium tuberosum* Rottler) or the pinto peanut (*Arachis pintoi* Krap. et Greg. nom. nud.) has also been reported to suppress Fusarium wilt (Pattison et al., 2014; Zuo, et al., 2015).

Intercrop or rotation crops in banana production systems may suppress Fusarium wilt through the reduction of pathogen population and the inhibition of the early developmental stages of Foc, but the evidence and mechanisms for this is meagre (Buddenhagen, 2009; Zhang et al., 2013; Li et al., 2020). Intercrops, particularly legumes such as *Desmodium uncinatum* (Jacq.) DC and *Mucuna pruriens* (L.), have potential for developing sustainable banana production systems (McIntyre et al., 2001; Ocimati et al., 2019; Blomme et al., 2022). *D. uncinatum* and *M. pruriens* produce diverse root-secreted metabolites, such as phenolics with several functions, such as nutrient acquisition and suppression of soil-borne pests and pathogens (McIntyre et al., 2001). However, phenolic acids present in root exudates of *D. uncinatum* and *M. pruriens* and their effect on the early developmental stages of Foc TR4 have not been explored. Therefore, as a first step to field studies, this doctoral thesis sought to determine the profile of root-exuded phenolics of *D. uncinatum* and *M. pruriens* and to assess the impact of the phenolics on the early developmental stages of Foc TR4 (Chapter 3).

The suppressiveness of soils to Fusarium can be enhanced, for instance, by manipulation of soil pH to 7.0 or close thereto (Dita et al., 2018). Among the factors that may contribute to this effect is the reduced access to micronutrients, such as iron and the enhanced competition from other rhizosphere microbial communities (Peng et al., 1999; Segura et al., 2021). Iron bioavailability and soil pH could influence Fusarium wilt, in part through their effect on chlamydospore germination (Peng et al., 1999; Domínguez et al., 2001). Germination of fungal spores is a key developmental stage in the life cycle of fungal pathogens (Hayer et al., 2014; Turgeman et al., 2016). However, the mechanism of spore germination in Foc TR4 is largely unknown. Moreover, most studies on spore germination in Foc (Li et al., 2011; Meldrum et al., 213; Deng et al., 2015) have been conducted using conidia, which may not be appropriate substitutes for chlamydospores.

Germination of fungal spores is marked by heightened metabolic activity, which involves the synthesis of biomolecules, such as proteins and DNA, to rebuild hyphae from the disintegrating spore (Deng et al., 2015; Sephton-Clark and Voelz, 2018). The process of DNA

synthesis is regulated by the enzyme ribonucleotide reductase (RNR), which converts ribonucleotides to deoxyribonucleotides (dNTPs), thereby providing the precursors required for DNA synthesis and repair (Greene et al., 2020; Steenwyk, 2021). In *F. oxysporum*, RNR has only two non-identical homodimeric subunits, a big subunit Rnr1 and a small one-Rnr2, which are encoded by the genes *rnr1* and *rnr2*, respectively (Cohen et al., 2019). The catalytic activity of RNR and the assembly of both Rnr1 and Rnr2 subunits require iron (Furukawa et al., 1992).

The effects of iron bioavailability and pH on the process of chlamydospore germination are poorly understood. A better understanding of the effect of pH could allow for an enhancement of the benefit of pH manipulation. Chapter 4 of this doctoral thesis investigated the possible role of iron in the process of chlamydospore germination. Iron is essential for almost all organisms (Andrews et al., 2003). Iron functions as a catalytic component of enzymes involved in key cellular processes, such as DNA synthesis and repair, regulation of gene expression, as well as protein and amino acid biosynthesis (Andrews et al., 2003). Consequently, alteration of the iron nutrition of Foc through the application of synthetic iron chelators such, as HBED and EDDHA, which sequester iron, has potential for suppression of Fusarium wilt. For example, in a pot experiment, Fusarium wilt was suppressed when the synthetic iron chelator EDDHA was applied to Foc-infested soils (Peng et al., 1999). However, in a field experiment, application of HBED and EDDHA did not alter disease, suggesting that the iron nutrition of Foc was not affected by the synthetic iron chelators (Orr et al., 2021).

Plant pathogenic microorganisms have evolved efficient mechanisms for iron acquisition and uptake, such as the synthesis of low-molecular-weight secondary metabolites called siderophores, which can sequester iron with high affinity (Hilder and Kong, 2010). However, the siderophore-mediated mechanism of iron acquisition in Foc TR4 is largely unknown. Chapter 5 of this doctoral thesis investigated the production of siderophores as a possible mechanism by which Foc TR4 may adapt to conditions of limited, iron such as iron starvation, which can be induced by synthetic iron chelators.

1.4 Objectives and guiding hypotheses

The aim of this doctoral thesis was to contribute to a fundamental understanding of the ecology of *F. oxysporum* f. sp. *cubense* (Foc). The first study of this doctoral thesis is a systematic literature review. In this study, the rhizosphere ecology of Foc was investigated by bringing together the relatively scant data available on banana below-ground interactions in relation to Fusarium wilt, and to highlight key knowledge gaps for progressive research. On the basis of this, *in vitro* studies were performed with Foc TR4 to address the following specific objectives:

i. Determine the profile and cytotoxicity of phenolic compounds in root exudates of *D. uncinatum* and *M. pruriens* on Foc TR4.
ii. Assess the effects of iron bioavailability and pH on the process of chlamydospore germination in Foc TR4.
iii. Assess changes in amino acid profiles and siderophore biosynthesis in Foc TR4 in response to iron starvation.

Accordingly, this research was guided by the following hypotheses:

i. The root metabolome of *D. uncinatum* and *M. pruriens* contains phenolic compounds with antimicrobial activity that suppress Foc TR4 by inhibition of pathogen development.
ii. Iron is essential for DNA synthesis in germinating Foc TR4 chlamydospores and scarcity leads to selective optimization of ribonucleotide reductase function by enhanced expression of *rnr1* and *rnr2* genes.
iii. Iron starvation triggers a compensatory increase in siderophore biosynthesis in Foc TR4 to scavenge iron. This counteractive response requires arginine that is synthesized and subsequently converted to ornithine, the biogenic precursor of siderophores.

1.5 Thesis outline and methods

The main body of this doctoral thesis is written in chapter format, where each chapter addresses a specific objective, as depicted in Fig. 1.2. The format of the individual chapters is consistent with the style of the journal, in which the chapter was originally published or submitted for peer-review.

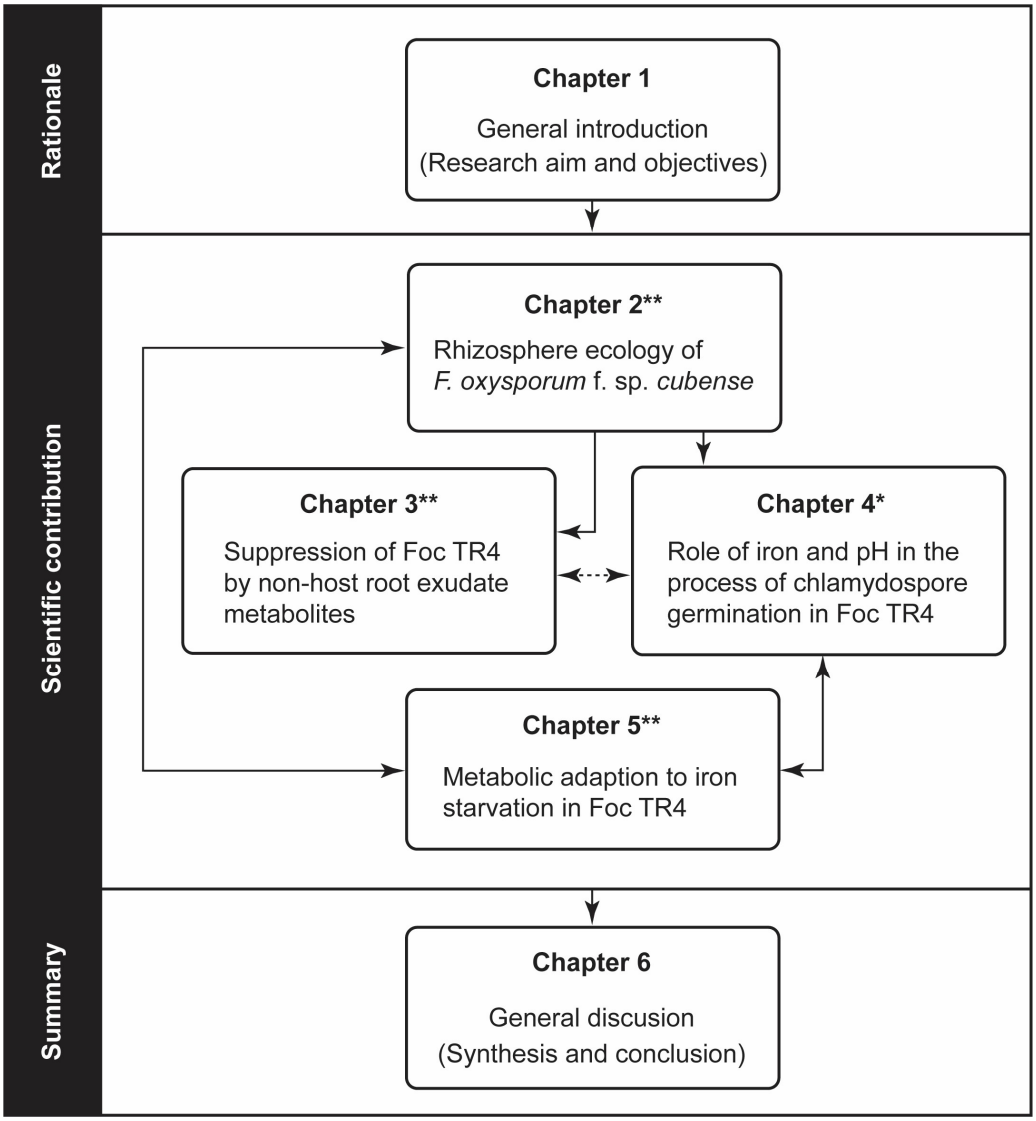

Figure 1.2. Schematic outline and structure of this doctoral thesis. Solid lines indicate a direct link between chapters, while dashed lines indicate a conceptual or thematic link between the chapters. Double asterisk (**) denote published chapters, while a single asterisk (*) denotes chapters submitted to a journal for peer-review.

The first study of this doctoral thesis is a desk review of available literature on banana below-ground interactions in relation to Fusarium wilt which, are summarized in Chapter 2. This chapter uses a qualitative and a quantitative approach to collect, summarize and visualize the data. Chapter 2 is supplemented by Chapters 3, 4, and 5, which respectively address objectives i, ii, and iii through *in vitro* laboratory experiments.

Objective i of this doctoral thesis is attained through Chapter 3. In this chapter, root-exuded phenolics of two legume species: *D. uncinatum* and *M. pruriens* are characterized to determine their effects on Foc TR4. A hydroponics-based method using climate chambers was employed to collect root exudates and to overcome technical challenges that are frequently encountered from the heterogeneity of soils and soil substitutes (Oburger and Jones, 2018). Hydroponic plant culture involves growing plants in an axenic environment with roots immersed into a nutrient solution into which metabolites are exuded (Oburger and Jones, 2018). To identify root-exudate phenolics of *D. uncinatum* and *M. pruriens*, the nutrient solution was collected and targeted metabolite analysis was performed using HPLC with diode array UV detection (HPLC-DAD). *In vitro* bioassays (using pure synthetic compounds) together with scanning electron microscopy and bright-field microscopy were used to assess the effect of the phenolics on the early developmental stages of Foc TR4 such as chlamydospore germination, mycelial growth, and conidia formation. Moreover, LC-MS and HPLC-DAD were used to assess the effect of phenolics on the secretion of two key toxins, fusaric acid and beauvericin in Foc TR4.

Objective ii of this doctoral thesis is addressed through Chapter 4. This chapter utilizes a time-course *in vitro* experiment to determine the temporal developmental sequence of germinating chlamydospores as a first step to understand this process in Foc TR4. The temporal developmental sequence was assembled using scanning electron microscopy and bright-field microscopy. To assess the requirement of iron in chlamydospore germination, a synthetic lipophilic iron chelator, 2,2′-dipyridyl, is used to simulate iron starvation. Chapter 4 also employs reverse transcription quantitative polymerase chain reaction (RT-qPCR) to

explore the role of iron in DNA synthesis during chlamydospore germination by quantifying the expression of RNR genes.

Objective iii is attained through Chapter 5. This chapter builds on Chapter 4 to explore siderophore production as a potential counteractive mechanism, by which Foc TR4 could subvert synthetic iron chelators and thereby evade iron starvation. *In vitro* experiments were performed to determine the production of the hydroxamate siderophore, ferrichrome, and the metabolic changes associated with siderophore biosynthesis. Furthermore, this study employs targeted metabolomics using LC-MS, followed by multivariate analysis to determine the profile of amino acids and to assess the iron-sparing response in Foc TR4 when challenged with iron starvation.

Finally, Chapter 6 discusses the salient issues arising from this doctoral thesis. The chapter also provides concluding remarks, including a summary of our current understanding of banana below-ground interactions in relation to Fusarium wilt and suggestions for progressive research arising from the findings of this doctoral study.

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2. Back to the roots: understanding banana below-ground interactions is crucial for effective management of Fusarium wilt*

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2.1 Abstract

Global banana production is affected by Fusarium wilt, a devastating disease caused by the soilborne root-infecting fungus, *Fusarium oxysporum* f. sp. *cubense* (Foc). Fusarium wilt is notoriously difficult to manage because infection arises through complex below-ground interactions between Foc, the plant, and the soil microbiome in the root–soil interface, defined as the rhizosphere. Interactions in the rhizosphere play a pivotal role in processes associated with pathogen development and plant health. Modulation of these processes through manipulation and management of the banana rhizosphere provides an auspicious prospect for management of Fusarium wilt. Yet, a fundamental understanding of interactions in the banana rhizosphere is still lacking. The objective of this review is to discuss the state-of-the-art of the relatively scant data available on banana below-ground interactions in relation to Fusarium wilt and, as a result, to highlight key research gaps. Specifically, we seek to understand (a) the biology of Foc and its interaction with banana; (b) the ecology of Foc, including the role of root-exuded metabolites in rhizosphere interactions; and (c) soil management practices and how they modulate Fusarium wilt. A better understanding of molecular and ecological factors influencing banana below-ground interactions has implications for the development of targeted interventions in the management of Fusarium wilt through manipulation of the banana rhizosphere.

Keywords: banana, chlamydospore, exudates, Foc, rhizosphere, root.

2.2 Introduction

Banana (*Musa* spp.) is a giant perennial herb with a pantropical distribution (Heslop-Harrison & Schwarzacher, 2007). Bananas originated in South-east Asia following interspecific and intraspecific hybridization between two wild diploid species, Musa acuminata (AA) and M. balbisiana (BB). These two species contributed the A and B genomes, respectively, resulting in more than 1000 banana varieties comprising diploids (AA, AB, BB), triploids (AAA, AAB, ABB) and tetraploids (ABBB) (Heslop-Harrison & Schwarzacher, 2007). Most of the cultivated bananas are triploids, which include sweet dessert bananas such as Cavendish (AAA), Gros Michel (AAA), Apple (Silk AAB), Pome (AAB) and Pisang Awak (ABB), and cooking bananas such as Matooke bananas (*Musa* spp. genomic group AAA), plantains (AAB) and Bluggoe (ABB) (Heslop-Harrison & Schwarzacher, 2007).

Bananas are cultivated in more than 135 countries and on approximately 11 million ha. In 2020, the global banana production quantity reached 163 million tonnes, an increase from 154.4 million tonnes in 2015 (FAOSTAT, 2022). Commercial production is done on large-scale monoculture plantations, with considerable agricultural inputs such as inorganic fertilizers, pesticides and irrigation. In contrast, subsistence production is done on small plots (0.2–4 ha) in mixed-cultivar systems, or intercropped with other perennial and annual crops, and with minimal or even no inputs (Bellamy, 2013; Kimunye et al., 2020). Bananas play an important role in food security and provide income for over 400 million people globally. About 87% of bananas produced globally are consumed locally or sold in informal markets. Cavendish (AAA) cultivars such as Grande Naine, Williams and Valery constitute almost half of the bananas cultivated in the world and dominate banana exports (Heslop-Harrison & Schwarzacher, 2007).

Globally, bananas are threatened by Fusarium wilt disease caused by the notorious soilborne, root-infecting fungus, *Fusarium oxysporum* f. sp. *cubense* (Foc; Fig. 2.1 A) (Viljoen et al., 2020).

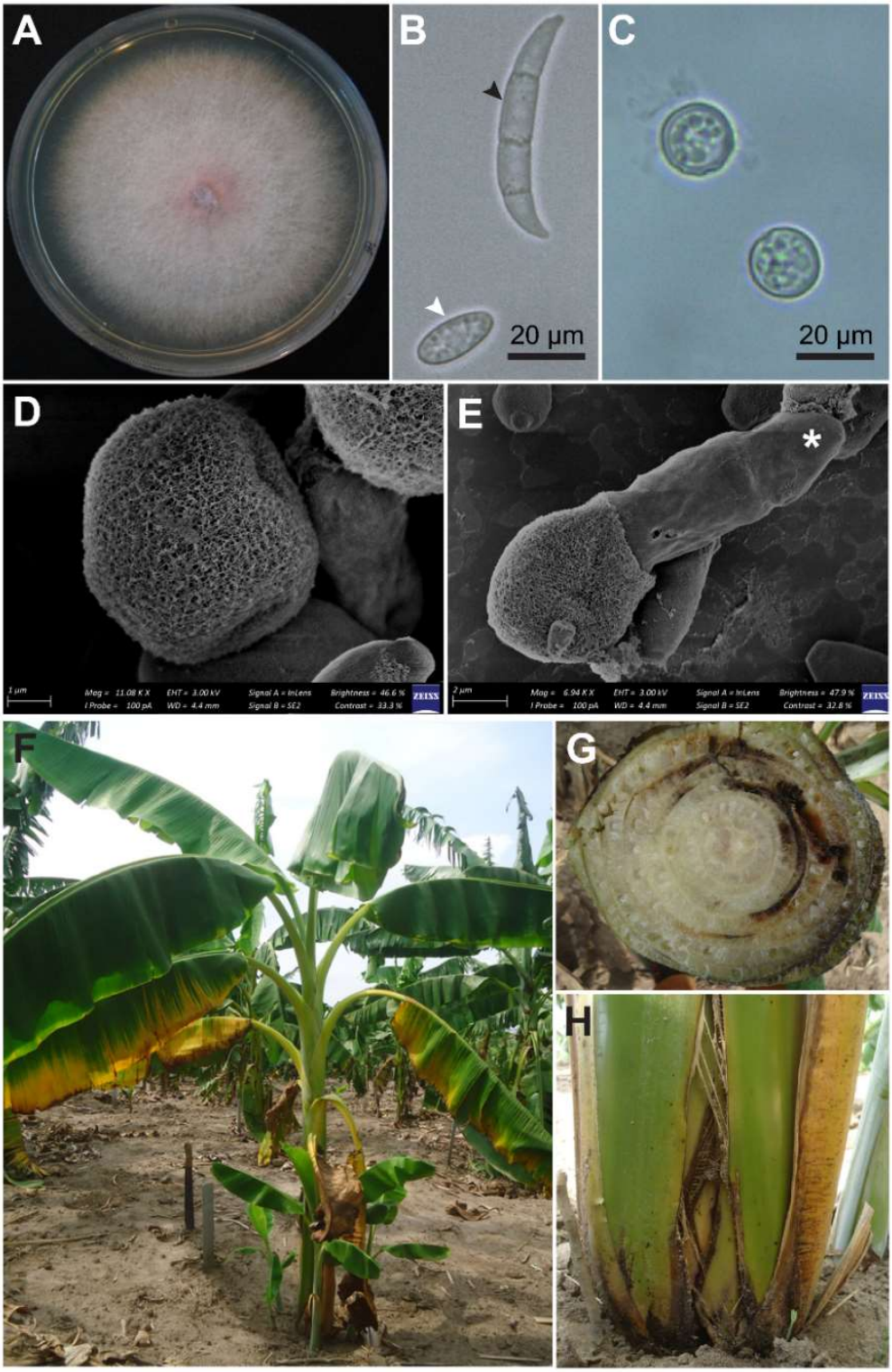

Figure 2.1. *Fusarium oxysporum* f. sp. *cubense* (Foc) and banana Fusarium wilt disease. A colony of Foc growing on potato dextrose agar medium (**A**); Bight-field microscopy micrographs of spore types produced by Foc: macroconidia (black arrowhead) and microconidia (white arrowhead), (**B**) and chlamydospores (**C**). Scanning Electron Microscopy micrographs of a dormant (**D**) and germinating chlamydospore (**E**) with a protruding germ tube (white asterisk). Typical symptoms of banana Fusarium wilt disease: wilting of leaves (**F**) and a rotting pseudostem (**G** and **H**) (*Source*: Evans Were).

Three physiological races of Foc are pathogenic to banana (Ploetz, 2015). The strain that caused significant losses of Gros Michel bananas in Latin America in the 20th century is Foc race 1, which is also pathogenic to other dessert bananas such as Apple (Silk AAB), Pome (AAB) and Pisang Awak (ABB) bananas. Foc race 2 affects Bluggoe (ABB) and other cooking bananas. Foc races 1 and 2 are not virulent to Cavendish bananas. Cavendish bananas are affected by Foc race 4, which can also cause disease to banana cultivars susceptible to Foc races 1 and 2 (Ploetz, 2015). Foc race 4 is subdivided into Foc "subtropical" race 4 (STR4) and "tropical" race 4 (TR4). Foc STR4 causes disease in Cavendish bananas in the subtropics due to predisposition to abiotic stresses such as low temperatures (Moore et al., 1993; Viljoen, 2002). However, unlike Foc STR4, Foc TR4 is virulent to Cavendish bananas under all environmental conditions (Ploetz, 2015). Like other *F. oxysporum* species, Foc produces three kinds of asexual spores: microconidia, macroconidia and chlamydospores (Fig. 1B-E) (Leslie & Summerell, 2006). Chlamydospores serve as the source of primary Foc inoculum in soil, whereas micro- and macroconidia are responsible for colonization and multiplication of Foc in vascular tissues of the plant (Ploetz, 2015).

The process by which Foc infects banana roots occurs in the rhizosphere, a narrow zone of soil approximately 2 mm immediately adjacent to the roots, and is influenced by roots primarily through root exudates (Beckman, 1987; Li et al., 2011). Root exudates stimulate the germination of Foc chlamydospores, producing hyphae that infect host roots and subsequently colonize the plant vascular tissues, which results in water stress, severe leaf chlorosis and wilting of the plant (Beckman, 1987; Ploetz, 2015; Stover, 1962). Chlorosis and wilting progress from the older to younger leaves, after which the leaves droop at the petiole (Fig. 1F) (Viljoen et al., 2020). Pseudostems of infected plants are sometimes characterized by longitudinal splits in the lower portion of the outer leaf sheaths (Fig. 2.1 G, H). When split open, the pseudostems reveal numerous yellow to dark red strands caused by infected xylem vessels (Viljoen et al., 2020). In the field, infected plants show symptoms as early as 3 months after planting but are most severely affected at the flowering stage (Viljoen et al., 2020). Depending on the phase and magnitude of infection, infected plants may die before producing

fruit (Rishbeth & Naylor, 1957; Viljoen et al., 2020). As the plant dies, it decomposes releasing masses of chlamydospores into the soil. Chlamydospores remain quiescent in soils, thereby limiting the cultivation of susceptible banana varieties in Foc-infested soils for decades (Rishbeth & Naylor, 1957).

Losses associated with Fusarium wilt during the era of Gros Michel were estimated at $2 billion (Ploetz, 2015) while losses caused by Foc TR4 are very considerable. Annual economic losses due to Foc TR4 were estimated at $253 million in Taiwan (Aquino et al., 2013) and $121 million in Indonesia (Hermanto et al., 2009). In 2013, Foc TR4 was detected in Africa on a commercial banana plantation in Mozambique (Viljoen et al., 2020). The pathogen destroyed the commercial banana plantation, killing nearly a million plants at a rate of about 15,000 plants per week, which prompted measures to be instituted to curb the spread of Foc TR4 (Viljoen et al., 2020). In China, Li et al. (2013) reported a fourfold increase (40,000 hectares) in banana production area infested by Foc within a period of years from 2002. Scheerer et al. (2018) projected that by 2040, 17% of the current banana-growing area could be infested by Foc TR4. This could result in losses worth about $10 billion. Foc TR4 has continued to spread inexorably throughout the world's banana-growing regions (Drenth & Kema, 2021) causing very considerable damage and impact on banana production.

Banana varieties resistant to Foc are the most effective and efficient strategy to reduce the impact of Fusarium wilt (Viljoen et al., 2020; Zorrilla-Fontanesi et al., 2020). However, disease resistant hybrids are undermined by inferior fruit characteristics such as taste and postharvest properties (Viljoen et al., 2020). For instance, FHIA-01 (Goldfinger), FHIA-02 and FHIA-18 hybrids are resistant to Foc TR4, but not popular among consumers because they have a lower pulp-to-peel ratio and are not as sweet as Grand Naine or Williams (Dadzie, 1998; Smith et al., 2014). Similarly, variants of Giant Cavendish (GCTCVs) developed in Taiwan were shown to be partially resistant to Foc TR4 (Hwang & Ko, 2004), but have a longer cycle time than Cavendish cultivars. The majority of disease-resistant hybrids are produced through conventional breeding, which is a tedious and slow process that can take more than 15 years (Tenkouano et al., 2011).

Genetic engineering may provide multiple and durable resistance to plant pathogens (Collinge & Sarrocco, 2022). Genetic engineering has been shown to provide resistance to Foc in commercial banana cultivars without altering the desired fruit and agronomic traits (Dale et al., 2017; Paul et al., 2011). However, a key challenge facing genetically modified bananas is the concern of regulators and consumers regarding the possible adverse effects of transgenes on human health and/or the environment (Ishii & Araki, 2016). More recently, technologies such as gene-editing using the CRISPR/Cas9 system are profoundly revolutionizing the ability to precisely engineer the banana genome by introducing genetic modifications to favour a desired trait in a relatively short time (Tripathi et al., 2020). However, it remains uncertain if banana modified with gene-editing will be acceptable to consumers. Moreover, the current regulatory uncertainties may further impact research and development of genome-edited crops, which could interfere with international trade in commodity crops including banana (Conko et al., 2016).

Infection of host roots by Foc arises through complex interactions in the banana rhizosphere, including interactions with the microbiome, parasitic nematodes and soil physicochemical factors (Dita et al., 2018; Stover, 1962). Moreover, management of Fusarium wilt is particularly difficult because (a) Foc is a soilborne pathogen that can persist in infested soils for decades even in the absence of the host; (b) Foc can spread easily, for instance through contaminated soil, farm machinery, irrigation water or via infected planting material; and (c) Foc is a vascular pathogen that, once established within the plant vascular tissues, becomes inaccessible to control measures such as nonsystemic fungicides and non-endophytic biological control agents (BCAs) (Bubici et al., 2019; Dita et al., 2018; Rishbeth, 1955; Stover, 1962; Viljoen et al., 2020). Because of this, containment (exclusion and early quarantine) measures are the only effective strategy for the control of Fusarium wilt when disease-resistant varieties are not available (Dita et al., 2018; Viljoen et al., 2020). However, once the disease is established in a field, containment becomes impractical and management must focus on integrating the available disease management strategies (Dita et al., 2018; Pattison et al., 2018).

Previous studies showed that manipulation of the banana rhizosphere can suppress Fusarium wilt (Fernández-Falcón et al., 2004; Nowembabazi et al., 2021; Peng et al., 1999). This includes alteration or supplementation of soil microbiota into the rhizosphere along with adjustment of soil physicochemical properties. For the former case, Fusarium wilt was suppressed by application of microbial inoculants such as strains of Bacillus, Pseudomonas and Trichoderma (Catambacan & Cumagun, 2021; Fu et al., 2016; Yadav et al., 2021). For the latter case, increasing the bioavailability of nutrients such as calcium, phosphorus and zinc, or decreasing iron bioavailability was shown to alleviate the impact of Fusarium wilt and to promote plant growth and vigour (Fernández-Falcón et al., 2004; Nowembabazi et al., 2021; Peng et al., 1999).

Integrating rhizosphere manipulation with existing disease management strategies could thus be suggested as an option to reduce the build-up of Foc inoculum in soil, prevent pathogen spread and alleviate the impact of Fusarium wilt on susceptible banana varieties. However, the prospect of rhizosphere manipulation has been compromised largely by inconsistent efficacy in the field (Orr et al., 2021; Thangavelu & Mustaffa 2010; Wibowo et al., 2013). This is attributed to several factors arising from interactions in the banana rhizosphere including interactions with the microbiome, parasitic nematodes and soil physicochemical factors (Belgrove et al., 2011; Orr & Nelson, 2018; Peng et al., 1999). The rhizosphere is intriguingly complex and dynamic (Sasse et al., 2018). As a result, ecological interactions in the banana rhizosphere in relation to Fusarium wilt remain poorly understood. Therefore, realizing the potential of rhizosphere manipulation for suppression of Fusarium wilt requires a comprehensive understanding of the versatile interactions and mechanisms in the banana rhizosphere.

The objective of this review is to bring together the relatively scant data available to date on the subject of banana below-ground interactions in relation to Fusarium wilt and to highlight the key research gaps. Specifically, we sought to understand (a) the biology of Foc and its interaction with banana; (b) the ecology of Foc, including the role of root-exuded metabolites in rhizosphere interactions; and (c) soil management practices and how they modulate

Fusarium wilt. First, we summarize the scope of research on the banana rhizosphere by showing the key research domains. Then, we describe abiotic and biotic interactions and how they potentially influence Fusarium wilt. In so doing, we illustrate how the known mechanisms involved in these interactions can yield distinct effects on Fusarium wilt. Details related to the epidemiology of Fusarium wilt and pathogen genomics are covered to a lesser extent in this review because these have been thoroughly reviewed elsewhere (Dita et al., 2018; Pegg et al., 2019; Viljoen et al., 2020).

2.3 Literature search and article selection

A literature search was conducted in four electronic databases: AGRIS, CAB Direct, SciVerse Scopus and ProQuest, which represent the main comprehensive databases for research in agricultural and life sciences. Databases were searched from their first entries up to 15 November 2021. The search strategy consisted of compiling four search strings, one for each category (banana, Foc, Fusarium, rhizosphere), while combining these with the Boolean operator “AND” to obtain only the intersection. Due to limited resources for translation, publications in languages other than English were excluded. Duplicates were identified using queries targeting identical digital object identifiers, titles, authors or first 50 characters of the abstract. Articles without an abstract or articles clearly indexed either as review, editorial or errata were excluded. Frequencies and percentages were used to summarize the nominal data.

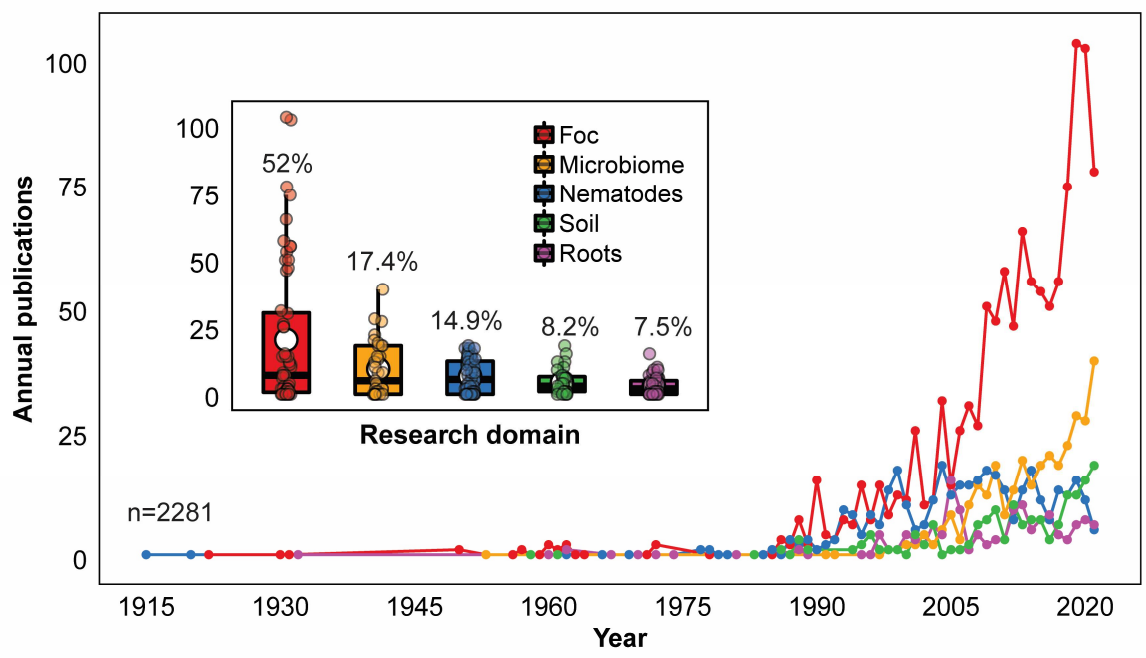

Figure 2.2. Temporal trend of publications on major research domains of banana rhizosphere and *Fusarium oxysporum* f. sp. *cubense*. Research articles retrieved from the databases AGRIS, CAB Direct, SciVerse Scopus and ProQuest published from 1915 to 2021. Boxplots (inset) show the upper and lower quartile, median (bold horizontal bar), mean (white circle) and whiskers (vertical lines). The points represent the cumulative number of articles for each research domain, and percentages, per year.

A total of 3409 titles and abstracts were retrieved from the databases, of which 2281 studies on the banana rhizosphere were eligible for data extraction. Through synthesis of the data, four general research domains were identified: (a) studies on Foc (n = 1186); (b) studies on nematodes (n = 396); (c) studies on the microbiome (n = 341); (d) studies on soil physicochemical properties (n = 187); and (e) studies on banana roots (n = 171). Publications on Foc mainly focused on pathogen biology, host–pathogen interactions and disease management and showed a continuous increase over time (Fig. 2.2). This could be attributed to the increased importance of Foc and the increased digitalization of information. Management of Fusarium wilt is a dominant element of banana rhizosphere research (Fig. 3A).

Biological control dominates the strategies for management of Fusarium wilt with 222 (40.4%) publications. This was followed by studies on breeding or screening bananas for resistance to Foc with 144 publications (26.2%). Studies on soil amendments, agronomic practices and genetic modification accounted for 83 (15.1%), 68 (12.4%) and 32 (5.8%) of the publications, respectively (Fig. 2.3A). Nearly half (48.0%) of the studies on biological control have been conducted under in vitro laboratory conditions, while 39.5% were pot (greenhouse) trials, and less 15% were field studies (Fig. 2.3B). Generally, BCA strains were highly effective in vitro and in the greenhouse, with a mean efficacy of 77.1% and 73.5%, respectively, whereas efficacy was less than 25.0% in the field (Fig. 2.3B). In the field, the majority of studies reported only a delay in the development of disease symptoms and plants still succumbed to Fusarium wilt.

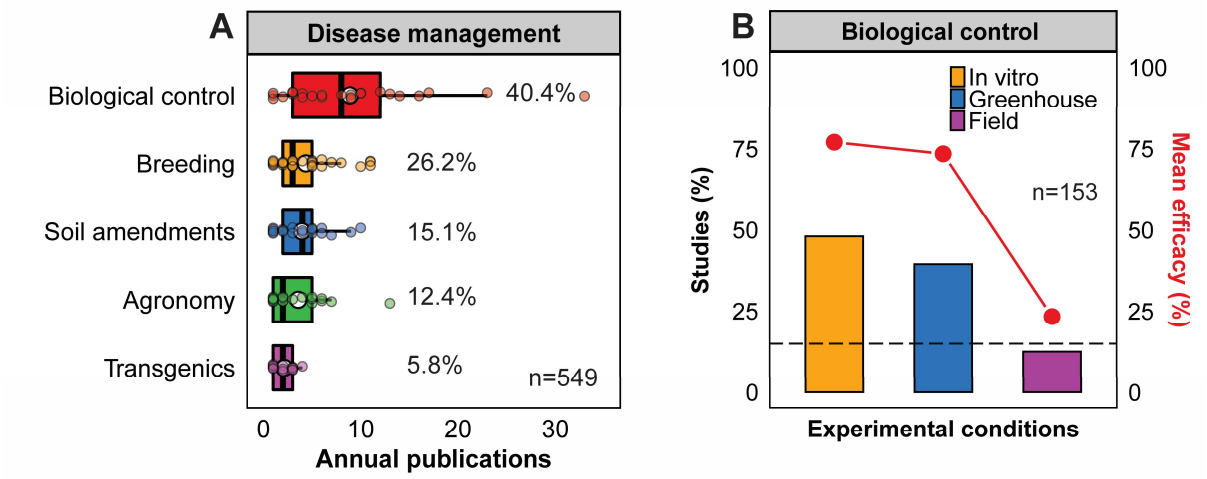

Figure 2.3. Publication on strategies for management of banana Fusarium wilt. (**A**) Boxplots show the upper and lower quartile, median (bold horizontal bar), mean (white circle) and whiskers (vertical lines). The points represent the cumulative number of articles for each category and percentages. (**B**) Studies on biological control of Fusarium wilt are conducted under in vitro laboratory conditions, greenhouse (or pot) trials and in the field, and exhibit varying efficacies.

2.4 Interactions in the banana rhizosphere

2.4.1 The rhizosphere

The characteristics of the rhizosphere, a narrow volume of soil surrounding plant roots, may be drastically different from those of the bulk soil (i.e., soil without roots). Generally, the rhizosphere consists of three distinct regions: the rhizosphere per se (the soil closely adhered to the root), the rhizoplane (root surface) and the root (Sasse et al., 2018). The banana plant consists of a subterranean corm from which new suckers and more than 500 adventitious main roots (cord roots) emerge and branch to form numerous smaller secondary and tertiary lateral roots (Blomme, 2000). Cord roots originate from the corm in groups of about four and can grow up to a length of about 5 m. Cord roots are 4–10 mm in diameter, relatively straight and cylindrical, and possess a prominent root cap of up to 10 mm thick at the root tip (Blomme, 2000; Lecompte et al., 2002). Cord roots possess root hairs that occur 4–6 cm behind the root tip and can grow over 2 mm long. Secondary and tertiary lateral roots are the most active portion of the banana root system (Blomme, 2000; Lecompte et al., 2002). Functionally, the banana root system is important for anchorage, acquisition of nutrients and water, and for the synthesis and storage of some plant hormones (Blomme, 2000; Lecompte et al., 2002).

Generally, the cytoarchitecture of banana cord roots and lateral roots is similar and consists of concentric cell layers (Fig. 2.4). The outermost epidermis/rhizodermis is followed by the cortex, endodermis, pericycle and the central cylinder (stele), which contains phloem and xylem vascular tissues (Lecompte et al., 2002). Because of this internal structure, it can be anticipated that root cell layers respond to infection by Foc or nonpathogenic soil microorganisms and symbionts in a transcriptionally distinctive manner. Yet, the functional significance of the banana root cytoarchitecture remains elusive and little is known about the biochemical and structural contribution of particular cell layers in the establishment of pathogenic, nonpathogenic and beneficial interactions with microorganisms in the banana rhizosphere.

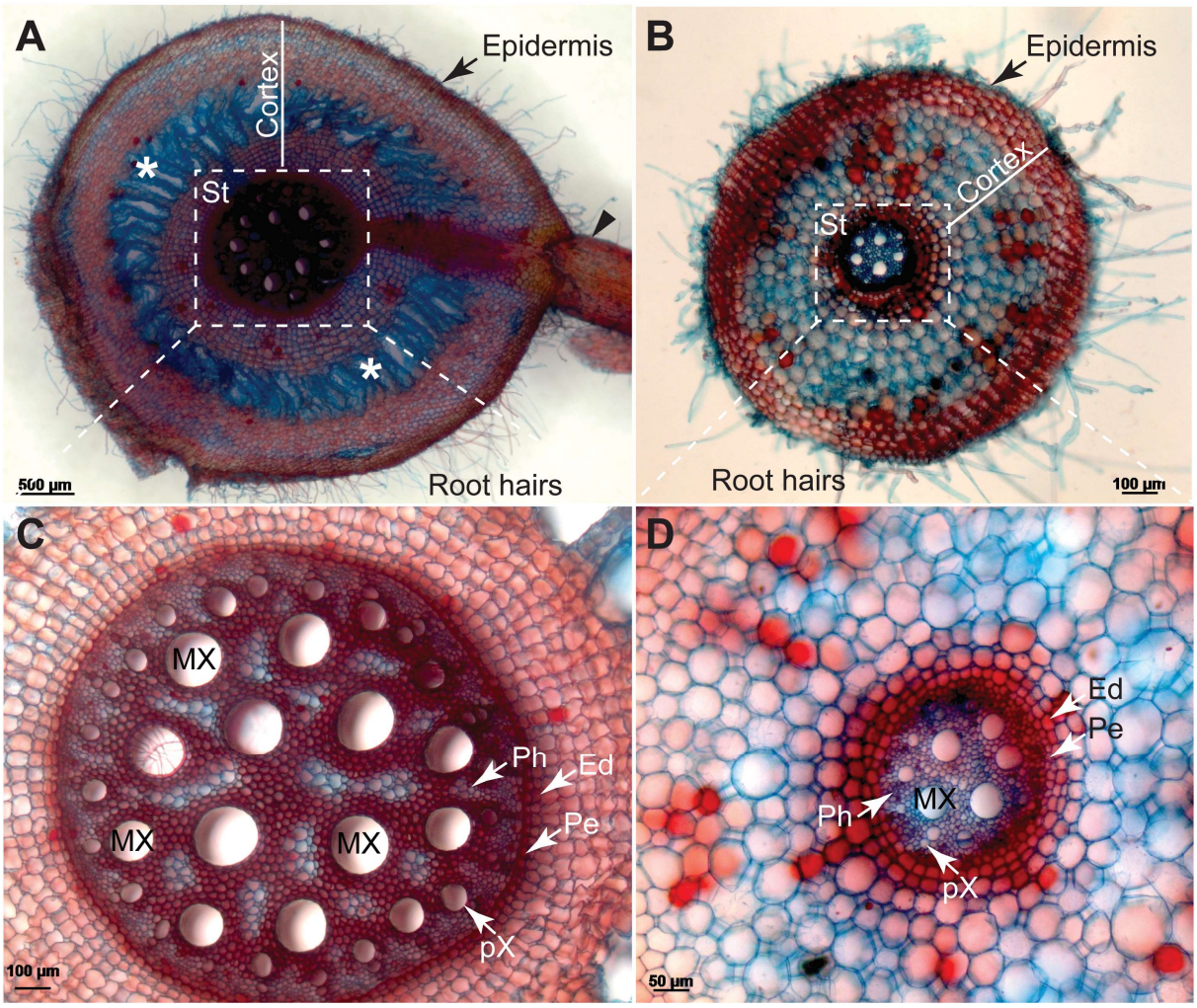

Figure 2.4. Bright-field microscopy micrographs showing the structural organization of a primary root (about 7 cm from the root tip) and a secondary root of Cavendish banana cultivar Grande Naine stained with Safranin O and Alcian blue 8GX. Lignified tissues are stained in red while celluloses are stained in blue. Transverse section of a primary root (**A**) with an emerging secondary root (black arrowhead; **B**). Transverse sections of a primary (**C**) and secondary (**D**) root focusing only on the root stele. The root layers are marked: epidermis; cortex; Ed, endodermis; Pe, pericycle; St, stele. Aerenchyma is marked by asterisks. MX, metaxylem; pX, peripheric xylem vessel; Ph, phloem cells (*Source*: Evans Were).

Plant roots influence the rhizosphere primarily through root exudates and rhizodeposits, which can attract or deter soil microorganisms (Sasse et al., 2018). Rhizodeposits include volatile compounds, sloughed root cap cells and debris (Sasse et al., 2018). On the other hand, root exudates include an assortment of primary and secondary metabolites of both low (<1 kDa) and high (>1 kDa) molecular weight (Badri & Vivanco, 2009). Root exudation and rhizodeposition in banana with regard to Fusarium wilt remain largely unexplored. Moreover, assessment of the mechanisms and effective concentration of root-secreted metabolites in the banana rhizosphere under field conditions presents a substantial challenge.

2.4.2 Interaction of Foc with banana roots

Infection of banana roots by Foc occurs in two phases: a primary determinative phase and a secondary determinative phase (Beckman, 1987). The primary determinative phase occurs in the rhizosphere and entails germination of Foc chlamydospores, hyphal growth, penetration of host roots and colonization of the root cortex and endodermis. These events are followed by the secondary determinative phase in which the fungus colonizes and establishes in the vascular bundles leading to disease (Beckman, 1987). While the secondary determinative phase has been extensively studied, little is known about the primary determinative phase. The source of primary Foc inoculum is hardy thick-walled asexual spores called chlamydospores, which can remain quiescent in soil for decades (Rishbeth, 1955). Generally, the thick wall surrounding a chlamydospore is tacitly agreed upon to confer resistance to adverse conditions that are often encountered in the soil. Foc can also survive in soil by saprophytic colonization of plant debris, or by commensal colonization of nonhost plants without apparent disease symptoms (Hennessy et al., 2005; Pittaway et al., 1999). Generally, the factors that contribute to the formation, quiescence and persistence of chlamydospores in soil remain elusive.

When suitable conditions are encountered, chlamydospores in soil undergo a revival cellular process called germination (Fig. 2.1D, E) and produce hyphae that infect host roots (Ploetz, 2015; Rishbeth, 1955). Chlamydospore germination is a crucial step in the life cycle of Foc, yet this process remains largely uncharacterized. Moreover, most of the studies on spore germination in Foc have been conducted using conidia (Deng et al., 2015; Li et al., 2011), which may not be appropriate substitutes for chlamydospores.

Previous studies suggest that chlamydospore germination is stimulated by metabolites related to amino acids or sugars in root exudates of banana (Buxton, 1962; Li et al., 2011) or when in contact with pieces of fresh uncolonized plant residue (Stover, 1962). However, a direct underpinning of root exudate metabolites that can stimulate chlamydospore germination is still lacking. In the study of Buxton (1962), root exudates of the susceptible banana cultivar Gros Michel did not inhibit spore germination, whereas root exudates of the resistant cultivar Lacatan had an inhibitory effect, which diminished with the age of the plant. Further analysis revealed a higher composition of sugars in root exudates of Gros Michel than Lacatan. Moreover, only 13 of the 18 amino acids identified were common in root exudates of both cultivars. Buxton (1962) therefore suggested that the differences in root exudate metabolites may contribute to host resistance against Foc race 1 in the rhizosphere through their effects on chlamydospore germination.

After germination, hyphae grow and colonize the rhizoplane prior to infection of roots (Li et al., 2017). This is followed by the secondary determinative phase in which hyphae penetrate host roots and subsequently establish in the vascular bundles (Beckman, 1987; Stover, 1962). Penetration of roots occurs mostly directly through the tips (cap region) of lateral roots at distances of about 5–15 cm from the corm (Li et al., 2017; Pegg et al., 2019; Rishbeth, 1955; Wardlaw, 1961). Infection rarely occurs through cord roots, and the radial spread of the pathogen by direct root-to-root contact is unlikely (Rishbeth, 1955). Upon infection, roots undergo extensive transcriptome reprogramming (Bai et al., 2013). In particular, transcripts of genes associated with defence such as cell wall lignification are highly and more rapidly expressed in roots of tolerant/resistant plants compared to susceptible plants (Bai et al., 2013).

Invasion of host tissues is facilitated by a repertoire of carbohydrate-active enzymes, which degrade the host cell wall (Qin et al., 2017).

Inside host roots, hyphae colonize the cortex and endodermis, and invade the xylem vessels of lateral roots through pits. The root xylem acts as a barrier that mounts defence responses to intercept pathogen ingress (Rishbeth & Naylor, 1957). Other host defence responses include the production of antifungal compounds, suberization and lignification of the plant cell wall, production of gels and tyloses, and the accumulation of gums (Beckman, 1987; Mace & Wilson, 1964; Rishbeth & Naylor, 1957). Paratracheal cells (parenchyma cells surrounding the xylem vessels) as well as the surrounding tissue exude gums containing mostly polysaccharides and phenolic metabolites (Mace, 1963; Mace & Wilson, 1964). Phenolic metabolites may possess limited toxicity, but their oxidation products are both toxic and denaturants of fungal enzymes (Beckman, 2000).

Successful colonization of roots is followed by colonization of the corm, which marks a key stage in pathogen establishment (Beckman, 1987; Stover, 1962). Foc penetrates the stele and invades the xylem vessels. Mycelia remain within xylem vessels and produce conspicuous masses of microconidia, which germinate and produce more hyphae that further colonize the plant, spreading within the cell apoplast and causing profound cytological distortions (Beckman, 1987). Consequently, the pathogen's mycelium and conidia, as well as the gels and gums produced by the plant in response to infection, clog the host's xylem vessels. The activated host resistance responses lead to crushing of xylem vessels by the proliferating adjacent parenchyma cells via callose deposition (Beckman, 1987). Ultimately, this results in the blockage of water and nutrient transport, leading to plant wilting and death (Li et al., 2017). Wilting is further exacerbated by fusaric acid, a toxin produced by Foc (Liu et al., 2020).

2.4.3 Interaction with rhizosphere microbiome

Communities of plant-associated microorganisms such as bacteria, fungi, archaea and protists constitute the plant microbiome (Compant et al., 2019). The plant and its microbiome function together physically and have intertwined metabolism such that they are together termed the holobiont (Compant et al., 2019). Plants rely on the rhizosphere microbiome to facilitate nutrient acquisition, in exchange for carbon-rich root exudates for nutrition (Compant et al., 2019; Sasse et al., 2018). Furthermore, interactions between beneficial microorganisms and plant roots can result in systemic host resistance to pathogens, due to activation of induced systemic resistance (Bakker et al., 2013). As a result, there is growing interest to elucidate how banana-associated microbial communities impact Fusarium wilt. However, a large proportion of bacteria and fungi associated with banana remain unidentified and their functional potential unknown (Kaushal, Swennen, et al., 2020a; Köberl et al., 2015). Traditional culture-dependent approaches and advanced culture-independent approaches, such as next-generation sequencing and DNA metabarcoding (Table 2.1), have been used to profile microbial assemblages associated with banana under different management practices (Kaushal, Swennen, et al., 2020a; Köberl et al., 2015; Yuan, Hong, et al., 2021a; Yuan, Wang, et al., 2021b).

Soil management practices, such as the application of organic amendments, intercropping or crop rotation, have been shown to modulate the structure and composition of banana-associated microbial communities (Yuan, Hong, et al., 2021a; Yuan, Wang, et al., 2021b) that can be harnessed for effective disease suppression (Tables 2.2 and 2.3). Generally, bulk soil harbours diverse bacterial and fungal communities, which progressively decline in the rhizosphere and roots of the banana plant, suggesting that bulk soil serves as the pool of microbial communities (Kaushal, Mahuku, et al., 2020b). Nevertheless, the majority of the bacterial and fungal communities present in bulk soil have also been frequently found in the rhizosphere, and as endophytes inside the roots and corm of the banana plant (Kaushal, Swennen, et al., 2020a; Köberl et al., 2015).

Table 2.1. Summary and study methods for articles assessing banana-associated microbiota.

Reference	Banana cultivar	Study description and target microbial communities	Microbiome characterization approach		Most relevant remarks/key findings
			Culture-dependent[a]	Culture-independent[b]	
Rossmann et al. (2012)	Diverse cultivars, including East African Highland bananas (AAA)	Field study on small-holder farms with varying management; community structure and function of bacteria and fungi in bulk soil, rhizosphere and pseudostem microhabitats	SNA, PDA, MacConkey, TSM, King's B media	PCR-SSCP fingerprinting of bacterial 16S rRNA and fungal ITS genes; pyrosequencing of bacterial 16S rRNA gene; qPCR quantitation of *Enterobacteriaceae* targeting 16S rRNA gene; ARDRA; FISH-CLSM	Microhabitat-specific microbial communities were noted across sites and management practices; antagonistic bacteria enriched in plant tissues whereas fungi were predominant in soil; the pseudostem contained abundant and diverse bacteria genera of *Enterobacteriaceae*
Kaushal et al. (2020a)	Sukari Ndizi (AAB)	Field study on small-holder farms with varying management; community structure, diversity and assemblage of bacteria and fungi in the rhizosphere, roots and corms of plants with and without symptoms	–	Illumina MiSeq sequencing of V3-V4 region of bacterial 16S rRNA gene and fungal ITS2	High bacterial and fungal diversity and richness noted in the rhizosphere, decreasing in root and corm; bacteria and fungi in roots and corm primarily originate from the rhizosphere; disease status was a key driver of fungal community composition
Kaushal et al. (2020b)	Mchare (AA), Sukari Ndizi (AAB)	Root endophytic bacteria of plants with and without symptoms	–	Illumina MiSeq sequencing of V3-V4 region of bacterial 16S rRNA gene	*Proteobacteria*, *Bacteroidetes*, and to a lesser extent *Actinobacteria*, dominated root endophytic bacteria; root endophytic bacteria were greatly impacted by disease
Deltour et al. (2017)	Maçã (AAB)	Field sites under agroforestry management and mixed cultivars; culturable and nonculturable bacterial communities of bulk soil	LB agar, Komada media	DGGE analysis of V3 region of bacterial 16S rRNA gene amplicons; sequencing of V3 region of bacterial 16S rRNA gene	Soil suppressiveness could not be linked to culturable bacterial communities; soil suppressiveness was linked to clay content, pH, cultivar diversity, and negatively correlated with silt, sand, the density of cv. Maçã and soil cover by graminoids
Shen et al. (2014)	Cavendish cv. Brazil (AAA)	Field sites with organic amendments including bio-organic fertilizer (BIO); bacterial communities of bulk soil	–	Pyrosequencing of V1-V3 region of 16S bacterial rRNA gene	Disease suppression after BIO application linked to a shift within soil bacterial community (e.g., specific enrichment of genera *Gemmatimonas* and *Sphingomonas*)

Table 2.1. (*Continued*)

Reference	Banana cultivar	Study description and target microbial communities	Microbiome characterization approach		Most relevant remarks/key findings
			Culture-dependent[a]	Culture-independent[b]	
Zhou et al. (2019)	Cavendish cv. Brazil (AAA)	Field study; bacterial and fungal communities of diseased and disease-free plants	Komada medium	Illumina MiSeq sequencing of V3-V4 region of bacterial 16S rRNA gene and fungal ITS1	Abundance of bacteria genera *Bacillus*, *Lactococcus* and *Pseudomonas* in disease-free soils was twice that of diseased soils; fungal genus *Mortierella* was the most abundant (34.6%) in disease-free soils, compared to 15.4% in diseased soils
Fu et al. (2016)	Cavendish cv. Brazil (AAA)	Field study with continuous application of bio-organic fertilizer (BIO); rhizosphere culturable bacterial communities	R2A agar; Biolog EcoPlate (for community-level physiological profiling based on the carbon source utilization)	DGGE analysis of V3 region of bacterial 16S rRNA gene amplicon	BIO application reduced disease incidence by stabilizing metabolic potential and community structure of culturable bacteria genera (e.g., *Bacillus* and *Pseudomonas*)
Sudarma and Suprapta (2011)	–	Culturable actinomycetes, bacteria, fungi and Foc antagonists in bulk soil of diseased and disease-free fields	PDA, NA, Kenknight media	–	Actinomycete genera (e.g., *Actinomyces* and *Streptomyces*), bacteria genera (e.g., *Bacillus* and *Pseudomonas*) and fungal genera (e.g., *Trichoderma, Aspergillus*, *Penicillium*) were abundant in soils of disease-free compared to diseased fields
Huang et al. (2015)	–	In vitro study; changes in *F. oxysporum,* bacterial and fungal communities of bulk soil after reductive soil disinfestation (RSD)	–	Illumina MiSeq sequencing of the V4 hypervariable region of bacterial 16S rRNA gene and fungal ITS1; qPCR quantitation of total bacteria, fungi, and Foc; DGGE analysis of bacterial 16S rRNA gene and fungal ITS amplicons	Bacterial genera (e.g., *Ruminococcus*, *Coprococcus*), and to a less extent *Clostridium* were dominant and increased while *F. oxysporum* decreased during RSD; after RSD, fungal genera (e.g., *Podospora* and *Zopfiella*) increased but *Fusarium* decreased further

Table 2.1. (*Continued*)

Reference	Banana cultivar	Study description and target microbial communities	Microbiome characterization approach		Most relevant remarks/key findings
			Culture-dependent[a]	Culture-independent[b]	
Fu et al. (2017)	Cavendish cv. Brazil (AAA)	Field study; temporal effects of bio-organic fertilizer (BIO) and compost application on composition and abundance of rhizosphere bacterial and fungal communities	–	Pyrosequencing of V4-V5 region of bacterial 16S rRNA gene and fungal ITS; qPCR quantitation of total bacteria, fungi, Foc, and biocontrol agent NJN-6 (*Bacillus amyloliquefaciens*) in BIO	BIO amendment increased abundance of bacteria, but decreased abundance of fungi and *F. oxysporum*; BIO amendment enriched bacterial genera (e.g., *Sphingobium*, *Gp6*, *Gp4*, *Lysobacter*, *Sphingopyxis* and *Dyadobacter*), but depleted *Gp1*, *Ralstonia*, *Burkholderia* and *Mucilaginibacter*
Köberl et al. (2015, 2017)	Gros Michel (AAA)	Field study of impact of biogeography and agroforestry on gammaproteobacterial microbiome in the rhizosphere, roots, pseudostem and leaves of healthy plants and plants with symptoms	–	Illumina MiSeq sequencing of hypervariable V4 region of bacterial 16S rRNA gene	Diversity and community members of gammaproteobacteria identified as potential indicators of healthy banana plants; bacterial genera (e.g., *Pseudomonas* and *Stenotrophomonas*) increased in healthy plants; *Enterobacteriaceae* preferentially increased in diseased plants.
Shen et al. (2018)	Cavendish cv. Brazil (AAA)	Field study on sites under varying lengths of banana monoculture; bacterial and fungal communities of bulk soil	–	Illumina MiSeq sequencing of the V4 region of bacterial 16S rRNA gene and fungal ITS1	Monocropping increased Foc accumulation; disease incidence and high fungal richness were significantly correlated with successive cropping; fungal genus *Mortierella* dominated soil fungal communities.
Effendi et al. (2019)	–	Field study; bacterial communities from rhizosphere of Foc-infected and non-infected banana plants	–	Illumina MiSeq sequencing of the V4 region of bacterial 16S rRNA gene	Bacteria phyla *Acidobacteria* and *Verrucomicrobia* were associated with healthy rhizosphere soil
Fu et al. (2019)	Cavendish (AAA)	Field study; bacterial and fungal communities of rhizosphere of healthy and diseased plants	–	Illumina MiSeq sequencing of the V4 region of bacterial 16S rRNA gene and fungal ITS1 region; qPCR quantitation of bacteria and fungi	Bacterial and fungal diversity was not different between the rhizosphere of healthy and diseased plants; diversity of microbial communities markedly more stable in rhizosphere of healthy plants than diseased plants

Table 2.1. (*Continued*)

Reference	Banana cultivar	Study description and target microbial communities	Microbiome characterization approach		Most relevant remarks/key findings
			Culture-dependent[a]	Culture-independent[b]	
Sun et al. (2013)	Foc-susceptible cultivar Baxi and tolerant cultivar Fj01	Field study; rhizosphere bacterial communities and activities of soil phosphatase and urease	Biochemical analysis of soil enzymes (phosphatase and urease)	T-RFLP and qPCR analysis of bacterial 16S rRNA gene	Higher bacterial diversity, abundance and urease activity noted in the rhizosphere of Fj01 compared to Baxi
Cabanás et al. (2021)	Dwarf Cavendish (AAA)	Field and pot studies; endophytic bacterial and fungal communities from roots of mother plants and suckers; Foc antagonists	NA, PDA media	illumina MiSeq sequencing of V3-V4 region of bacterial 16S rRNA gene and fungal ITS2 region	Relative abundance of bacterial genus *Pseudomonas* was higher (but not significant) in suckers than in mother plants; *Rhizobium*, *Streptomyces* and *Actinophytocola* were higher (but not significant) in mother plants than suckers
Xia et al. (2011)	–	Epiphytic and endophytic *Trichoderma* associated with banana roots	TSM and its modified form	AFLP analysis of ITS and translation elongation factor (*tef1*)	*T. asperellum*, *T. virens*, *T. koningiopsis*, *H. lixii*, *T. atroviride* were identified as key epiphytes; *T. asperellum*, *T. virens*, *T. brevicompactum*, *H. lixii* were identified as key endophytes; *T. asperellum, H. lixii*, *T. virens* were found in both niches
Shen et al. (2015a)	Cavendish cv. Brazil (AAA)	Field study; bacterial communities as an indicator of Fusarium wilt-suppressive soils in orchards with differing disease incidences	–	Pyrosequencing sequencing of V4-V5 region of bacterial 16S rRNA gene	Bacteria genera exhibited significant difference among orchards; bacteria genera (e.g., *Chthonomonas*, *Pseudomonas* and *Tumebacillus*) were significantly enriched in suppressive soils, whereas *Gp2* was significantly reduced; enrichment of *Gp5* and *Pseudomonas* together with soil available phosphorus were significantly correlated with disease suppression
Rames et al. (2018)	Ducasse banana (syn. Pisang Awak, ABB)	Field study; changes in the soil microbiome in banana under ground cover management compared to bare soil	Soil biochemical analysis (β-glucosidase activity, fluorescein diacetate hydrolysis, labile C)	T-RFLP and qPCR analysis of bacterial 16S rRNA gene	Disease suppression tended to increase over time under ground covers compared to bare soil; fungal richness was correlated with disease suppression and activity of β-glucosidase in soils under ground covers

Table 2.1. (*Continued*)

Reference	Banana cultivar	Study description and target microbial communities	Microbiome characterization approach		Most relevant remarks/key findings
			Culture-dependent[a]	Culture-independent[b]	
Shen et al. (2015b)	Cavendish cv. Brazil (AAA)	Field study; response of rhizosphere microbial community soil application of bio-organic fertilizer (BIO), compost and chemical fertilizer over time	–	Pyrosequencing of V4-V5 region of bacterial 16S rRNA gene and fungal ITS region	BIO application induced disease suppression via manipulation of microbial community composition; *Acidobacteria* (*Gp1* and *Gp3*) were significantly enriched whereas abundance of *Rhizobium* and *Fusarium* decreased
Thangavelu and Jayanthi (2009)	Variety of commercial cultivars and wild bananas	Field sampling and pot study; nonpathogenic *Fusarium oxysporum* (np*Fo*) isolates from banana rhizosphere	Komada, PDA media	RFLP analysis of the fungal ITS region	In a pot trial, np*Fo* isolates promoted plant growth and reduced severity of Fusarium wilt by up to 89.4% (in tissue culture plants) and 77.8% (in sucker-derived plants) compared to controls; in the field, np*Fo* isolates (Ra-1 and Ro-3) reduced disease by up to 84% compared to controls
Chou et al. (2017)	Cavendish (AAA)	Field sampling; functional diversity and dominant populations of bacteria in bulk soils of long-term organic and conventional farms	TSA, NFA, TPA, SMA media; community-level physiological profiling using Biolog EcoPlate	16S rRNA sequencing	Diversity and dominant soil bacterial population (β-*proteobacteria*, *acidobacteria* and α-*proteobacteria*) was significantly higher in organic than conventional farms
Sánchez-Zúñiga et al. (2021)	Gros Michel (AAA)	Field study; farms under different management (organic, organic intermediate conventional, abandoned)	Community-level physiological profiling using Biolog EcoPlate	Sanger sequencing of species-specific amplicons	Farm under conventional management had the greatest presence of beneficial indicator microorganisms

[a]Culture media: PDA, potato dextrose agar; SNA, synthetic nutrient agar; TSM, Trichoderma-selective medium; LB, Luria-Bertani medium; TSA, tryptic soy agar; NFA, nitrogen-free agar; TPA, tricalcium phosphate agar; SMA, skimmed milk agar; R2A, Reasoner's 2A medium; NA, nutrient agar.
[b]Molecular techniques: AFLP, amplified fragment length polymorphism; ARDRA, amplified rDNA restriction analysis; DGGE, denaturing gradient gel electrophoresis; FISH-CLSM, fluorescence in situ hybridization coupled with confocal laser scanning microscopy; qPCR, quantitative polymerase chain reaction; SSCP, single-stranded conformational polymorphism; T-RFLP: terminal restriction fragment length polymorphism. Marker genes: conserved genes (commonly 16S ribosomal RNA [rRNA], internal transcribed spacer [ITS] and 18S rRNA) that typically contain a highly variable region that can be used for detailed identification. The variable region is flanked by highly conserved regions that can serve as binding sites for the PCR primers.

Table 2.2. Soil nutrient management practices, their effect on Fusarium wilt disease of banana, and proposed mechanisms.

Soil parameter	Effect on disease	Proposed mechanisms	References
Iron (Fe)	High Fe bioavailability increases disease severity	Fe promotes chlamydospore germination leading to root infection	Domínguez et al. (2001), Peng et al. (1999)
Soil pH	Low (acidic) pH increases disease severity. High (neutral or slightly alkaline) pH decreases disease severity	pH influences nutrient bioavailability (e.g., Fe) in the rhizosphere by altering the cation exchange capacity of the soil. pH profoundly influences metabolic activity of rhizosphere microorganisms Low pH (pH 3.8–4.0) is ideal for in vitro culture of Foc from soils and may stimulate chlamydospore germination. Low pH increases bioavailability of micronutrients (e.g., Fe) and Mg, which are known to influence host susceptibility to Fusarium wilt	Segura-Mena et al. (2021), Stover (1962) Komada (1975), Peng et al. (1999)
Potassium (K)	High K decreases disease severity	K is essential for strengthening plant cells, thereby limiting Foc infection and ingress; K is essential for expression of host defence-related proteins	Navajothy et al. (2011), Nowembabazi et al. (2021)
Nitrogen (N)	Nitrate (NO_3^-) fertilizers decrease disease severity Ammonium (NH_4^+) fertilizers increase disease severity	NO_3^- enhances host resistance by increasing lignification of plant tissues; improves absorption of other nutrients required for host resistance thereby maintaining a higher photosynthetic rate Selective absorption of NH_4^+ over NO_3^- coupled with a high demand and uptake of K results in acidification of the rhizosphere due to extrusion of protons from banana roots	Delvaux et al. (2005), Epp (1987), Nasir et al. (2003)
Phosphorus (P)	High P is associated with disease suppression	P is important for root development	Shen et al. (2015b)

Table 2.2. (*Continued*)

Soil parameter	Effect on disease	Proposed mechanisms	References
Magnesium (Mg)	High Mg reduces disease severity	Mg is a cofactor for enzymes in many cellular process in the plant including photosynthesis	Hu et al. (2021), Wu et al. (2020)
Zinc (Zn)	Zn deficiency is associated with high disease severity	Zn supposedly improves formation of tyloses in the xylem which occlude Foc and limit pathogen ingress	Borges-Pérez et al. (1991), Fernández-Falcón et al. 2004), Hecht-Buchholz et al. (1998)
Silicon (Si)	Si application reduces disease severity	Si stimulates defence responses of the phenylpropanoid pathway (e.g., lignin, phenylalanine ammonialyase, polyphenoloxidase, peroxidase, β-1,3-glucanase and chitinase)	Fortunato et al. (2012, 2014)
Calcium (Ca)	Ca application reduces disease severity; high Ca increases susceptibility	Ca is involved in cellular signalling processes that are essential for defence plant response; Ca is an important component of plant cell walls	Peng et al. (1999)

Table 2.3. Common plant species reported to suppress Fusarium wilt disease of banana and the proposed mechanisms of suppression

Plant species	Banana cropping system	Effect on Fusarium wilt	Proposed mechanism	Reference
Cassava (*Manihot esculenta*)	Rotation/intercrop	Reduced disease incidence	Lowering of Foc inoculum in soil	Buddenhagen (2009)
Chinese leek (*Allium tuberosum*)	Intercrop/rotation	Suppressed Fusarium wilt	Antifungal organic volatiles (e.g., 2-methyl-2-pentenal) and organosulfur compounds (e.g., dimethyl trisulfide) in root exudates inhibit chlamydospore germination and mycelia growth	Li et al. (2020), Zhang et al. (2013)
			Antifungal metabolites in root exudates suppress Foc through induction of reactive oxygen species; decreasing mitochondrial transmembrane potential; decreasing ergosterol biosynthesis; induction of autophagy	Zuo et al. (2015, 2017)
Pinto peanut (*Arachis pintoi*), carpet grass (*Axonopus affiinis*), greenleaf desmodium (*Desmodium intortum*)	Intercrop/groundcover	Reduced disease severity and incidence by 20%; delayed onset of symptoms	Augmentation of the diversity and activity of soil microbial communities; reduction of Foc inoculum in soil	Pattison et al. (2014), Rames et al. (2018)

Table 2.3. (*Continued*)

Plant species	Banana cropping system	Effect on Fusarium wilt	Proposed mechanism	Reference
Pineapple (*Ananas comosus*), maize (*Zea mays*)	Rotation	Pineapple–banana rotation was more effective than maize–banana rotation	Reduction of Foc inoculum in soil; augmentation of beneficial soil microorganisms (e.g., *Acidobacteria*, *Planctomycetes*, *Burkholderia*, *Talaromyces* and *Clonostachys*)	Wang et al. (2015), Yuan et al. (2021b)
Rice (*Oryza sativa*)	Rotation	Reduced disease incidence from initial 30%–50% to 8.1%–17.6% in the first year, and 0.8%–6.3% in the subsequent 2.5–3 years	–	Hwang (1985)
Coffea spp.; Fabaceae trees (*Inga* spp. and *Erythrina poeppigiana*)	Intercrop/agroforestry	–	–	Köberl et al. (2015, 2017)
Chilli pepper (*Capsicum frutescens*)	Rotation	Reduced disease incidence and Foc inoculum in soil	Reduction of Foc inoculum through stimulation of the abundances of antagonistic bacterial genera (e.g., *Gemmatimonas*, *Pseudomonas*, *Sphingobium* and *Sphingomonas*)	Hong et al. (2020)

Kaushal, Swennen, et al. (2020a) reported that the rhizosphere, roots and corm of Sukari Ndizi (AAB) with symptoms of Fusarium wilt exhibited lower bacterial and fungal diversity compared to symptomless plants. It was suggested that the impact of disease stress on banana plants may have altered the quality and/or quantity of available root exudates in the rhizosphere (Kaushal, Swennen, et al., 2020a). In support of this, Yuan et al. (2018) reported that Cavendish banana challenged with Foc exhibited enhanced exudation of phenolics such as phthalic acid, salicylic acid (SA) and cinnamic acid. The phenolics stimulated the expression of genes associated with antibiotic production and biofilm formation in the biocontrol bacterium, *Bacillus amyloliquefaciens* NJN-6 (Yuan et al., 2018).

Organic acids and plant hormones are key components of root exudates known to stimulate chemotaxis, a phenomenon in which cell movement is directed in response to an extracellular chemical gradient (Li et al., 2012). Fumaric acid in root exudates of banana stimulated biofilm formation and colonization of banana roots by the plant growth-promoting bacterium *Bacillus subtilis* N11 (Zhang et al., 2014). Similarly, in chemotaxis *in vitro* assays, bacterial species from the genera *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Providencia* and *Pseudomonas*, isolated from the banana rhizosphere, were attracted by SA (Li et al., 2012). Chemotaxis is an essential component of bacterial aggregation and biofilm formation, which are crucial for establishing and maintaining intimate spatial association with the plant, thereby improving plant growth and health (Li et al., 2012). Moreover, biofilm formation in bacterial BCAs may provide protection from predation and fluctuating environmental conditions (Li et al., 2012).

In Cavendish banana, inoculation with an incompatible Foc race 1 showed enhanced resistance to a compatible Foc TR4, when subsequently inoculated in the roots (Wu et al., 2013). The enhanced resistance to Foc TR4 was associated with elevated levels of SA and expression of PR-1 and several other defence-related genes in roots compared to the roots of plants not pre-inoculated with Foc race 1 (Wu et al., 2013). However, how these host defences may affect the banana rhizosphere microbiome is largely unknown. Studies in the model plant *Arabidopsis thaliana* have demonstrated that the fungal endophyte *Trichoderma* T-78

colonized an SA-impaired mutant faster than the wild type (Martínez-Medina et al., 2017). Similarly, involvement of jasmonic acid signalling in the assembly of the rhizosphere microbiome was reported in jasmonic acid-defective *A. thaliana* mutants (Carvalhais et al., 2015).

Microbiome interactions typically occur via complex networks consisting of a plethora of interdependent individuals that interact in a mutualistic, synergistic or parasitic mode. Ou et al. (2019) highlighted the importance of constructing microbial networks in the soil to manage banana Fusarium wilt. Microbial network analysis can be highly instrumental to zoom into specific microbial consortia such as communities of beneficial microorganisms. However, in-depth insight in plant metabolites and traits that shape and sustain the rhizosphere microbiome, which in turn contribute to defence, could emerge through carefully designed experiments in which metagenomics, metatranscriptomics and root metabolomics approaches are integrated (Hultman et al., 2015). Given the decreasing cost of metagenome sequencing, adoption of high-throughput sequencing and network analysis may reveal the role of microbiome variability across field sites and banana genotypes. Metagenome-wide association studies, coupled with machine-learning predications of both bulk soil and the rhizosphere, could enable the prediction of plant productivity at different field sites based on site-specific microbiome data (Yuan et al., 2020). This information could inform plant pathologists and banana breeders to make well-informed choices regarding field sites for variety testing, selection and performance in soils infested with Foc. Thus, in future, analysis of interactions involving genotype × environment × microbiome will probably emerge.

2.4.4 Interaction with nematodes

Banana is affected by parasitic nematodes that differ in their lifestyle and feeding habits, and often occur in mixed populations (Coyne et al., 2018). Parasitic nematodes of banana include the root-knot nematode (*Meloidogyne* spp.), root-lesion nematodes (*Pratylenchus* spp.), spiral nematode (*Helicotylenchus multicinctus*) and the burrowing nematode *Radopholus similis*, which is considered the nematode of major concern (Coyne et al., 2018). Interactions between parasitic nematodes and Foc seem to vary considerably across banana genotypes. For example, in the field, Epp (1987) found no association between the incidence and severity of Fusarium wilt and the infestation of Cavendish with nematodes (*R. similis*, *Meloidogyne* spp.). Conversely, Loos (1959) reported that *R. similis* aggravated the severity of Fusarium wilt and early expression of disease symptoms in Gros Michel. Similarly, Almeida et al. (2018) found that banana fields severely affected by Fusarium wilt were heavily infested with *Pratylenchus* spp.

In pot trials, synergistic albeit inconsistent interactions between Foc and *R. similis* have been reported (Chaves et al., 2014; Dinesh et al., 2014; Rocha et al., 2020). Unlike *R. similis*, *Meloidogyne* spp. are sedentary endoparasites that infect host roots resulting in abnormal vessel elements and disruption of the arrangement and continuity of the vascular tissues (Coyne et al., 2018). Moreover, giant cells associated with the feeding of *Meloidogyne* spp. serve as a nutrient sink, which may predispose the roots to infection by Foc. Collectively, as parasitic nematodes and Foc occupy a similar ecological niche in the banana rhizosphere, they may develop complex interactions depending on the species of nematode. While there are far fewer reports so far, concomitant infection with Foc and parasitic nematodes may aggravate Fusarium wilt, for instance, through nematode-induced wounds, which can provide a portal for root entry by Foc (Almeida et al., 2018). Future studies are needed to untangle the mechanisms underlying the interactions between parasitic nematodes and races of Foc, for example, by analysis of metabolites secreted by nematode-infected roots in relation to the

lifestyle of the nematode. Such information would foster appropriate use of the Fusarium wilt-resistant or tolerant banana cultivars in soils infested with both Foc and nematodes.

2.4.5 Interaction with physico-chemical soil properties

Optimal supply of nutrients is essential for optimal growth and health of banana plants (Nowembabazi et al., 2021; Shen, Ruan, Chao, et al., 2015a). The nutritional status can affect plant metabolic activity including the expression of host defences against invading pathogens (Marschner et al., 2011). Several studies have demonstrated that manipulation of the bioavailability of nutrients in soil (e.g., nitrogen, phosphorus, potassium, magnesium, manganese, iron, zinc) and soil pH can reduce the incidence and severity of Fusarium wilt (Table 2.2). However, the mechanisms by which nutrient bioavailability and soil chemical properties may impact Fusarium wilt are complex and poorly understood. Moreover, assessing the impact of the infinitely varied forms of nutrients on Fusarium wilt remains a challenge due to the influence of other abiotic and biotic factors in the soil environment. Further studies are needed to assess the independent effects of physicochemical soil factors on Foc, the banana plant and the banana microbiome.

Bioavailability of iron in soil is one of the critical factors that has been linked to the severity of Fusarium wilt (Domínguez et al., 1996; Orr & Nelson, 2018). Although iron is abundant in soil, its bioavailability is generally limited (c.10^{-18} M), and well below the optimal requirement for plants (10^{-9}–10^{-4} M) and microorganisms (10^{-7}–10^{-5} M) (Robin et al., 2008). In a pot trial, Peng et al. (1999) reported that application of the synthetic iron chelator EDDHA reduced disease severity and germination of Foc chlamydospores by one-third to one-half. Iron chelators with a high stability constant (K), such as HBED (K = $10^{39.7}$) and EDDHA (K = $10^{33.9}$), sequester iron with high affinity (Robin et al., 2008). This limits iron bioavailability and can thereby cause iron starvation to Foc (Peng et al., 1999). However, in a field study, Orr et al. (2021) suggested that the iron nutrition of Foc is not effectively altered by chelators, as no decrease in disease severity was observed when HBED and EDDHA were applied to Foc-

infested soils. Studies are needed to establish the requirement of iron in the germination of Foc chlamydospores and to determine if Foc can subvert iron chelators and thereby evade iron starvation.

Soil pH is another factor that has been reported to modulate Fusarium wilt (Table 2.2). The optimum soil pH for growing bananas is approximately 5.0–7.5, while soil pH values of 5.6–6.0 have been recommended for management of Fusarium wilt (Dita et al., 2018; Domínguez et al., 2001). A high incidence of Fusarium wilt has been associated with lower soil pH, whereas soils suppressive to Fusarium wilt have a neutral or slightly alkaline pH (Deltour et al., 2017; Stover, 1962). In a pot trial, Peng et al. (1999) found that disease progression was slower in acidic soils than alkaline soils (pH 8). This was attributed to the influence of pH on chlamydospore germination, as high pH favoured chlamydospore germination. The effects of pH on Fusarium wilt are multifaceted and complex as pH also controls the bioavailability of nutrients and trace elements and affects the activities of extracellular enzymes and the reactivity of soil organic matter (Paul et al., 2006). Future studies are needed to further understand the impact of soil pH on banana root exudation, chlamydospore germination and growth of Foc, and the rhizosphere microbiome.

Other abiotic soil factors, such as soil type, temperature and water content have been shown to affect the severity of Fusarium wilt, but the precise mechanisms are not well known (Pattison et al., 2014). Regarding soil water content, Aguilar et al. (2000) reported that waterlogged soils result in hypoxia or anoxia, which greatly restricts oxygen to banana roots. Under oxygen-deficient conditions, the activities of enzymes involved in phenol metabolism are enhanced, resulting in the formation of lysigenous aerenchyma in the mid cortex (Fig. 4A) from about 50 mm behind the root tip. Lysigenous aerenchyma is a gas space created by death of cells in the root cortex that enables efficient diffusion of oxygen within plants and thereby reduces the energy costs associated with root cells. Aguilar et al. (2003) suggested that aerenchyma may also provide a by-pass for Foc hyphae around barriers in the stele. Collectively, manipulation of physicochemical soil properties is a promising strategy for management of Fusarium wilt. Understanding the interplay between the soil and banana-

associated microbiota will expand our knowledge on the impact of soil abiotic factors on biological control of Fusarium wilt.

Soil management practices, such as crop rotation and intercropping, have been reported to suppress Fusarium wilt (Table 2.3). Disease suppression has been linked to root-secreted metabolites, which in part target the early stages of Foc development (Table 2.3), yet the underlying mode of inhibition remains unclear. Zuo et al. (2015) suggested that the inhibitory effect of *Allium tuberosum* volatiles could be intensified by root-secreted phenolic compounds, but the effect of phenolic compounds on the early development of Foc is not completely understood. Phenolic compounds are abundantly secreted by legumes (Fabaceae), such as *Desmodium uncinatum* and *Mucuna pruriens* (Hooper et al., 2015). Accordingly, legumes could be useful intercrops when developing sustainable banana production systems (McIntyre et al., 2001; Ocimati et al., 2019). In this line, it will be relevant to identify legumes with root-secreted metabolites that can suppress Foc development and disease incidence in the field.

2.5 Conclusion and outlook

Research on banana Fusarium wilt has a long history. However, it is remarkable how little progress has been made towards understanding the rhizosphere ecology of Foc. Research is needed to integrate previous advancements with consideration of disease management, host genetics and the biology of Foc to synthesize a clarified view of how banana below-ground interactions influence Fusarium wilt. The rhizosphere is a complex adaptive environment of closely interacting biological, chemical and physical components. Management and manipulation of the banana rhizosphere has great potential for improving plant growth and suppression of Fusarium wilt. To realize the potential of rhizosphere manipulation for suppression of Fusarium wilt, a comprehensive understanding of banana below-ground interactions and the mechanisms involved is required.

Author contributions

FR and AV conceived the project, sourced the funding and provided the resources. EW collected, curated and analysed the data, and drafted the original manuscript. FR and AV, critically revised and gave further inputs to the final manuscript.

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Conflict of interest

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

Data availability

All data for this study are included in the article.

2.6 References

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3. Phenolics mediate suppression of *Fusarium oxysporum* f. sp. *cubense* TR4 by legume root exudates*

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3.1 Abstract

Fusarium oxysporum Schlecht f. sp. *cubense* (E.F. Smith) Snyder & H.N. Hansen (Foc) is a notorious soil-borne fungal pathogen that causes Fusarium wilt disease of bananas (*Musa* spp. L.). Foc, especially Tropical Race 4 (TR4), is particularly devastating due its ability to infect diverse banana cultivars including Cavendish bananas. Fusarium wilt is difficult to manage, but intercropping has been reported to efficiently suppress Fusarium wilt through exudation of root metabolites with antagonistic effects on Foc. Yet, the metabolites that inhibit Foc and the underlying mode of action remain unclear. Hydroponic culture and targeted metabolite profiling were used to investigate the potential of root-exuded phenolic acids and flavonoids of two legume species, *Desmodium uncinatum* (Jacq.) DC. and *Mucuna pruriens* (L.) DC., to inhibit growth and the biosynthesis of the phytotoxins beauvericin and fusaric acid (FA) in Foc TR4. Of the twelve metabolites targeted, four phenolic acids (benzoic, *t*-cinnamic, *p*-coumaric, *p*-hydroxybenzoic) were common in root exudates of both legumes, whereas vanillin and the flavonoid quercetin were only detected in root exudates of *M. pruriens*. Bioassays using synthetic benzoic-, *t*-cinnamic-, or *p*-hydroxybenzoic acid, or a mixture thereof, showed a concentration-dependent suppressive effect on Foc TR4. Low concentrations of phenolic acids (0.01 and 0.1 mM) inhibited chlamydospore germination and the production of macro- and micro-conidia, as well as the biosynthesis of FA. Mycelial growth of Foc TR4 was only inhibited at high concentrations (1 mM) of benzoic acid, t-cinnamic acid, and a mixture of the three phenolics. This study demonstrates that phenolics in root exudates of *D. uncinatum* and *M. pruriens* suppress Foc TR4 by directly inhibiting the early stages of pathogen development. Intercropping banana with *D. uncinatum* and *M. pruriens* may thus be considered as a valuable option for suppression of Foc TR4.

Keywords: *Desmodium uncinatum*, *Mucuna pruriens*, Foc, Benzoic, *t*-cinnamic, *p*-hydroxybenzoic

3.2 Introduction

Fusarium oxysporum Schlecht f. sp. *cubense* (E.F. Smith) Snyder & H.N. Hansen (Foc) is a devastating root-infecting fungal pathogen that causes Fusarium wilt disease of banana (*Musa* spp. L.) (Viljoen et al., 2020). Three races of Foc exist, of which Foc Tropical Race 4 (TR4) is considered the most destructive. Foc TR4 is particularly damaging to Cavendish bananas, which comprise about 50% of all bananas produced worldwide, but also affects several banana varieties that are susceptible to Foc races 1 and 2. Foc TR4 thus poses a serious threat to the livelihoods of nearly 400 million people that depend on banana for food security and income (Pegg et al., 2019). Fusarium wilt can be managed successfully by replacing susceptible with resistant banana varieties (Dita et al., 2018), if the fruit of such varieties are acceptable to growers and consumers. Unfortunately, most banana hybrids produced through conventional (classical) breeding are often undermined by inferior fruit characteristics such as taste and cycle time (Tenkouano et al., 2011; Viljoen et al., 2020). Although genetic engineering could overcome such limitations (Paul et al., 2011; Dale et al., 2017), a major concern of consumers and regulators is the possible adverse effects of transgenes on human health and/or the environment (Ishii and Araki, 2016).

Manipulation of the banana rhizosphere has shown promising results and may provide a prospect for management of Fusarium wilt. Greenhouse and *in vitro* studies have reported microorganisms that are antagonistic against Foc (Bubici et al., 2019). For instance, Yadav et al. (2021) reported that *Bacillus licheniformis* strain CSR-D4 strongly inhibited the growth of Foc TR4 *in vitro*. The inhibitory effect was attributed to the production of antifungal metabolites such as bacillomycin, fengycin, surfactin, and iturin (Yadav et al., 2021). Non-pathogenic strains of *F. oxysporum* were also reported to colonise banana roots, establish asymptomatically as endophytes, and suppress Foc (Belgrove et al., 2011; Nel et al., 2006). Besides, the disease can also be suppressed by intercropping banana with cassava (*Manihot esculenta* L. Crantz), Chinese leek (*Allium tuberosum* Rottler) and pinto peanut (*Arachis pintoi* Krap. et Greg. *nom. nud.*), or by rotation with pineapple (*Ananas comosus* [L.] Merr.), or rice

(*Oryza sativa* L.) (Buddenhagen, 2009; Huang et al., 2012; Pattison et al., 2014; Wang et al., 2015; Li et al., 2020). Disease suppression was linked to root-secreted compounds (primary and secondary metabolites), but the underlying mode of inhibition remains unclear.

Foc inoculum survives in infested soil primarily as hardy thick-walled spores called chlamydospores. Chlamydospores remain dormant and survive in Foc-infested soils for decades, but germinate when susceptible banana varieties are planted in such soils (Rishbeth, 1955; Ploetz, 2015). Germination represents the early developmental stage of Foc, and results in the production of actively growing hyphae that produce two other types of asexual spores, called macroconidia and microconidia. During its development, Foc produces virulence factors such as plant cell wall-degrading enzymes and the phytotoxins beauvericin and fusaric acid (FA) (Liu et al., 2020). Beauvericin and FA are essential for infection and colonisation of banana roots (Li et al., 2013; Portal González et al., 2021), and possess antimicrobial activity that may enhance the ecological fitness of *F. oxysporum* spp. through the inhibition of competing soil microorganisms (Notz et al., 2002; Spraker et al., 2018). Thus, the early stages of Foc development are essential for host infection and are the target of interventions for the suppression of Fusarium wilt through manipulation of the rhizosphere (Peng et al., 1999; Dita et al., 2018; Bubici et al., 2019).

Plants secrete up to 50% of their photosynthesized carbon and 15% of their nitrogen into the rhizosphere in the form of rhizodeposits and root exudates (root metabolome) (Haichar et al., 2016). Root exudates contain a myriad of primary and secondary metabolites of low (<1000 Da) and high (>1000 Da) molecular weight (Oburger and Jones, 2018; Vives-Peris et al., 2019). Root-secreted metabolites of intercrops are important in the suppression of Fusarium wilt, partly by inhibiting the early stages of Foc development (Buddenhagen, 2009; Huang et al., 2012). Zhang et al. (2013) identified five organic volatiles and organosulfur compounds, including 2-methyl-2-pentenal, and four organosulfur compounds (dimethyl trisulfide, dimethyl disulfide, dipropyl disulfide, dipropyl trisulfide) from Chinese leek (*Allium tuberosum*) that inhibited Foc. The strongest inhibition was obtained with 2-methyl-2-pentenal and dimethyl trisulfide. Zuo et al. (2015) suggested that the inhibitory effect of *A.*

tuberosum volatiles could be intensified by root-secreted phenolic compounds, but the effect of phenolic compounds on the early development of Foc is not completely understood.

Phenolic compounds are ubiquitous plant secondary metabolites and a class of natural chemicals containing one or more hydroxyl groups attached to an aromatic ring (Cheynier et al., 2013). They are divided into flavonoids (including flavones, flavanones, and flavanols) and phenolic acids (Cheynier et al., 2013). Phenolic compounds possess antioxidant properties and antimicrobial activity, and can suppress fungal pathogens *in vitro*, suggesting a role in the suppression of soil-borne pathogens such as *F. oxysporum* spp. (Michielse et al., 2012;Yuan et al., 2018). Phenolic compounds can suppress fungal pathogens by inhibiting spore germination, hyphal growth, and the synthesis of virulence factors such as toxins (Michielse et al., 2012; Ling et al., 2013; Schöneberg et al., 2018; Gautier et al., 2020; Oufensou et al., 2020). Phenolic compounds may be synthesized during normal growth and development, or may accumulate in plant tissues in response to various abiotic (e.g., heat, drought) and biotic (e.g. beneficial and antagonistic microorganisms) stress (Haichar et al., 2016). Legumes (*Fabaceae*) can secrete large quantities of phenolic compounds due to their root biomass, root density, and synergistic interaction with beneficial microorganisms (Duchene et al., 2017; Sasse et al., 2018). *Desmodium uncinatum* (Jacq.) DC and *Mucuna pruriens* (L.) DC secrete flavonoids and phenolic acids that have been demonstrated to play an important role in the suppression of weeds and nematodes (Tsanuo et al., 2003; Arim et al., 2006; Hooper et al., 2015). Consequently, legumes could be useful intercrops when developing sustainable crop production systems, especially for banana (McIntyre et al., 2001; Wink, 2013; Ocimati et al., 2019; Muoni et al., 2019; Blomme et al., 2020).

Despite the importance of phenolic compounds in plant defense against fungal pathogens, there is limited knowledge about the effect of root-secreted phenolics on the early stages of Foc development. We hypothesized that the root metabolome of legumes contains phenolics with antimicrobial activity that may inhibit development of Foc TR4. On the basis of this hypothesis, the specific objectives of this research were to (i) assess the cytotoxicity of root exudates from *D. uncinatum* and *M. pruriens* against Foc TR4, (ii) fingerprint the profiles

of phenolic acids and flavonoids in root exudates of *D. uncinatum* and *M. pruriens*, and (iii) assess the effect of identified metabolites on chlamydospore germination, mycelial growth, production of macro- and microconidia, and the synthesis of beauvericin and FA by Foc TR4.

3.3 Materials and methods

3.3.1 Fungal strain and plant material

An isolate of *Fusarium oxysporum* Schlecht f. sp. *cubense* (E.F. Smith) Snyder & H.N. Hansen Tropical Race 4 (Foc TR4) (VCG 01213/16) was obtained from the Department of Plant Pathology, Stellenbosch University, South Africa. The fungus was preserved at −80 °C on 30% (*v*/*v*) glycerol, and revived by culturing on potato dextrose agar (PDA) at 28 °C for 5 days. Seeds of *Desmodium uncinatum* (Jacq.) DC. (CIAT 728) were provided by the International Centre for Tropical Agriculture (CIAT) in Cali, Colombia. Seeds of *Mucuna pruriens* (L.) DC. were purchased from Gardengoods (Johannesburg, South Africa).

3.3.2 Reference standards and chemicals

Twelve reference standards (10 phenolic acids and two flavonoids) were used for the fingerprinting of root-exuded phenolics of *D. uncinatum* and *M. pruriens* (Table 1). All solvents for metabolite analysis were of HPLC-MS grade. Other chemicals, unless otherwise stated, were purchased from Carl Roth (Karlsruhe, Germany). For metabolite fingerprinting, mix-standards with concentrations of 1, 5, and 10 mg L^{-1} were prepared. For analysis of beauvericin the concentrations of standards were 0.1 and 1 mg L^{-1}, and for FA it was 1, 5 and 10 mg L^{-1}.

3.3.3 Plant hydroponic culture, root exudate sampling, and extraction of root exudate metabolites

Seeds of *D. uncinatum* and *M. pruriens* were surface sterilized (Paradiso et al., 2017) and germinated at 28 °C for 5 days. After germination, 25 seedlings of similar size were selected and cultured in half-strength Hoagland's nutrient solution in a growth chamber (Percival Scientific, Boone, IA, USA) under a 16-h light cycle (irradiance at 100 µmol $m^{-2}s^{-1}$, 25 °C, and 50% relative humidity) for 14 days. The nutrient solution was then changed to full-strength, and the plants cultured for an additional 28 days. The nutrient solution was replenished every 3 days and aerated every 15 min using a membrane pump (KNF Neuberger, Freiburg, Germany). The composition of macronutrients in the nutrient solution was 5 mM KNO_3, 2 mM KH_2PO_4, 1 mM NH_4NO_3, 7 mM $Ca(NO_3)_2 \times 4H_2O$, and 2 mM $MgSO_4 \times 7H_2O$. The composition of micronutrients was 90.4 mM H_3BO_3, 0.5 mM $MnSO_4$, 1.5 mM $ZnSO_4$, 0.8 mM Cu SO_4, 0.34 mM $(NH_4)_6Mo_7O_{24}$, and 63 mM Fe-EDTA.

To collect root exudates, plants were removed from the hydroponic system and their roots gently immersed in sterile distilled water (at 25 °C) for 1 min, followed by dipping in 0.2 mM $CaCl_2$ (pH 6.2) to remove the nutrient solution. A sponge wrapped around the stem of each plant was used to stabilise the plants on an acrylic lid mounted on a 1-L glass tank (Assistent, Sondheim, Germany). The glass tank was filled with 0.2 mM $CaCl_2$ (pH 6.2) into which the plant roots were entirely immersed for 6 h to collect root exudates. Collection of root exudates started 3 h after the onset of light (Pramanik et al., 2000). The $CaCl_2$ solution was filtered using filter paper (Grade 595 ½; 185 mm, Schleicher & Schuell, Dassel, Germany) and the root exudate solution was then transferred to 1-L polypropylene bottles (Kautex Textron, Bonn, Germany), and stored at −20 °C. Roots were removed and weighed to determine fresh root mass.

Root-exuded phenolic metabolites were extracted from the $CaCl_2$ solution using liquid-liquid phase extraction with ethyl acetate. To do this, samples were mixed with 300 mL of ethyl acetate in 2-L separatory funnels and shaken vigorously for 3 min. After 1 min of repose, the

ethyl acetate-phase was filtered over anhydrous Na_2SO_4 to remove any residual water. This process was repeated with 200 mL of ethyl acetate. Extracts were pooled and concentrated at 40 °C using a rotary evaporator (Büchi, Flawil, Switzerland). Extracts were then transferred to pre-weighed 2-mL tubes and reduced to a dried pellet under a nitrogen gas stream on a thermoblock at 40 °C (Barkley, Leopoldshoehe, Germany). For metabolite fingerprinting, the pellet was suspended in 1 mL of acetonitrile:water (1:1). For assessing the cytotoxic potential of root exudates, the pellet was weighed and re-suspended in dimethyl sulfoxide (DMSO) to a final concentration of 1 mg mL^{-1}. The resulting solution was filtered into 50-mL Falcon tubes using a syringe filter (0.2 µm; Sartorius, Göttingen, Germany), and stored at 4 °C in the dark.

3.3.4 Cytotoxicity bioassay of crude root exudates

The cytotoxic potential of *D. uncinatum* and *M. pruriens* root exudates was assessed using Foc TR4 chlamydospores. The chlamydospores were produced according to the methodology described by Goyal et al. (1973) and purified using density gradient centrifugation. For density gradient centrifugation 20 mL of Percoll gradients (25, 50, and 80% *w/v*), prepared in 0.1% (*v/v*) Triton X-100, were gently layered in 50-mL Falcon tubes. The tubes were centrifuged at 3000 revolutions per minute (r.p.m.) for 10 min at 4 °C (Allegra X-15R centrifuge, Beckman Coulter, Fullerton, CA, USA). The chlamydospore suspension was then gently overlaid onto the upper 80% gradient, and the tubes further centrifuged for 40 min. Gradients were collected separately and washed thrice with 30 mL of cell wash buffer [phosphate-buffered saline (pH 7.6); MP Biomedicals, Irvine, CA, USA] containing 0.01% (*v/v*) Triton X-100. Following collection, chlamydospores were pooled and resuspended in 10 mL of Tris buffer (50 mM Tris-HCl, pH 7.4).

Cytotoxicity assays were conducted using the Alamar Blue kit (Bio-Rad, Hercules, CA, USA) that measures activity of the mitochondrial respiratory chain as a readout of cell viability. The assay was performed following the manufacturer's protocol. Briefly, root exudates were serially diluted in Barz broth (Hutner et al., 1950; Barz, 1971) to final concentrations of 0, 0.01,

0.1 and 1 µg mL^{-1}, in 24-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany). About 10^4 chlamydospores were added to each well. Plates were incubated on an orbital shaker (Edmund Bühler, Hechingen, Germany) rotating at 120 r.p.m. in the dark at 28 °C for 24 h. Absorbance was measured using a microplate reader (Tecan, Maennedorf, Switzerland), and readings were normalized to the DMSO control where cell viability was set to 100%.

3.3.5 Effect of crude root exudates on radial mycelial growth

A high concentration of root exudates (100 µg mL^{-1}) was used to assess the effect on mycelial growth of Foc TR4. Bioassays were conducted in 90-mm Petri plates, where PDA was mixed with root exudates (100 µg mL^{-1}). For the controls, PDA was mixed with DMSO [10% (*v*/*v*)]. Plates were inoculated with a 5-mm-diameter mycelial disc taken from the periphery of a 5-day-old Foc culture, and incubated at 28 °C. After 10 days, the colony diameter was measured using a digital Vernier calliper (NeikoTools, Chesterton, IN, USA) (Hendricks et al., 2017). In addition, the morphology of the colonies and hyphae was examined with bright-field microscopy using a Leica DM750 microscope equipped with a Leica ICC50 HD camera (Leica Microsystems, Heerbrugg, Switzerland).

3.3.6 Metabolite fingerprinting

Fingerprinting of root-exuded phenolics of *D. uncinatum* and *M. pruriens* was conducted using the Accela HPLC system coupled with the Accela Photodiode Array (DAD)-detector (Thermo Fisher Scientific, San Jose, CA, USA). Separation of metabolites was performed on a YMC-Triart C18 column (3.0 × 100 mm, 3 µm particle size) (YMC, Kyoto, Japan). Eluents were 10 mM formate buffer at pH 3.7 (eluent A) and acetonitrile (eluent B). The applied eluent gradient is provided in the supporting information (Table S3.1). The flow rate was 0.5 mL min^{-1}, and the injection volume was 6 µL. The selected DAD wavelength range was 215–600 nm.

Reference standards were run as a standard mixture with concentrations of 1, 5, and 10 mg L^{-1}. Peaks were identified by the specific absorbance maximum of each metabolite (Table 3.1). Identified metabolites were quantified by linear regression analysis of the corresponding peak areas using the Quanbrowser software (Thermo Fisher Scientific, San Jose, CA, USA). Metabolite concentration was expressed as µg g^{-1} fresh root weight.

3.3.7 Selection of metabolites for bioassays

Three metabolites (benzoic acid, *t*-cinnamic acid, and *p*-hydroxybenzoic acid), which were common in root exudates of *D. uncinatum* and *M. pruriens* (Table 1), were selected and assessed for their effect on Foc TR4. Stock solutions (100 mM) of each compound, and a mixture of the three compounds, were prepared in DMSO. The solutions were filter-sterilized as described earlier, and stored at 4 °C in the dark. For bioassays, concentrations of 0.01, 0.1, and 1.0 mM were selected based on a previous study (Siqueira et al., 1996) and measurements in this study.

3.3.8 Chlamydospore germination, radial mycelial growth, and conidiation bioassays

Chlamydospore germination assays were conducted in 24-well cell culture plates containing 900 µL of Barz broth and 10^4 chlamydospores per well, as described earlier. The broth was supplemented with phenolic acids, and DMSO was used as control. After incubation for 7 h, samples were retrieved in 2 mL Eppendorf tubes and chilled on ice. Germinated chlamydospores were counted using a haemocytometer and a Leica DM750 microscope equipped with a Leica ICC50 HD camera (Leica Microsystems). Micrographs were acquired using Leica Application Suite Software, converted to 8-bit grayscale or 24-bit RGB, and annotated using CorelDraw 12.0 (Corel, Ottawa, Canada). Fine details of germinating chlamydospores were captured using scanning electron microscopy (SEM).

For SEM analysis, chlamydospores were suspended in 1 mL of ice-cold fixative [2.5% (*v/v*) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2)] and kept at 4 °C for at least 1 h. After post-fixation in 1% (*v/v*) osmium tetroxide for 1 h, samples were bloc-stained overnight in 1% (*v/v*) aqueous uranyl acetate at 4 °C. Samples were then gradually dehydrated in a series of ethanol solutions of 50, 70, 90, and 100% (*v/v*), and finally dried using hexamethyldisilazane. Dried pellicles were mounted onto 12-mm aluminium SEM stubs using conductive carbon tape. To enhance conductivity, samples were sputter-coated with 8 nm of gold using a low vacuum coater (EM ACE200; Leica, Vienna, Austria). SEM was performed using the Zeiss Merlin scanning electron microscope (Carl Zeiss, Jena, Germany) with a Gemini-type field emission gun electron column (FEG-SEM) equipped with two Oxford Instruments X-MaxN 150 SDDs. Typical imaging conditions were magnification of 1–3 × 10^4, a working distance of 5–10 mm, 2–3 kV, a beam current 100–200 pA and using an In Lens secondary electron detector. Micrographs were captured in TIF format using a pixel averaging noise reduction algorithm and SmartSEM software (Carl Zeiss).

The experiment for assessing radial mycelial growth was conducted in 90-mm-diameter Petri dishes containing PDA mixed with phenolic acids, as described earlier. Colony diameter was measured using a digital Vernier calliper (NeikoTools). Production of macroconidia and microconidia was assessed as described by Ohara et al. (2004). Macroconidia and microconidia were enumerated using a haemocytometer and a bright field microscope (Axioskop; Zeiss, Oberkochen, Germany) (Fey et al., 2007).

3.3.9 Analysis of beauvericin and fusaric acid biosynthesis

Cultures were prepared in 250-mL Erlenmeyer flasks containing 50 mL of Czapek-Dox broth (Sigma-Aldrich, Sternheim, Germany). The broth was inoculated with Foc TR4 and incubated as described earlier. Culture filtrates were harvested by filtering using Grade 595 ½; 185 mm diameter filters (Schleicher & Schuell, Dassel, Germany). The pH of the filtrate was determined, and the samples stored at 4 °C until analysis. To extract toxins from the filtrate, the samples were homogenized for 1 min in acetonitrile-water-glacial acetic acid (79:20:1, *v/v/v*) using a homogenizer (Workcenter T10 basic, IKA®, Wilmington, NC, USA). The mixture was re-homogenized after 2 min of repose and centrifuged for 10 min at 12000 r.p.m before reducing to dryness under a nitrogen gas stream. Dried crude extracts were reconstituted in acetonitrile/water (50% *v/v*) prior to analysis.

Analysis of beauvericin was performed by HPLC/ESI-MS using the Accela HPLC-LTQ Velos mass spectrometer (Thermo Scientific, San Jose, CA, USA). The LC separation was performed with a YMC Triart C18 column (3.0 × 100 mm, 3 µm particle size) (YMC, Kyoto, Japan). The eluents were: water with 0.2% (*v*/*v*) formic acid in 10% (*v*/*v*) acetonitrile (eluent B) and 0.2% (*v*/*v*) formic acid (eluent C). The flow rate was 0.5 mL min^{-1}, and the injection volume was 1 µL. The applied eluent gradient is provided in the supporting information (Table S3.2). The mass spectrometer was operated in the full scan mode with the range of *m/z* 100–900. Beauvericin was identified as protonated molecular ion with *m/z* 784 $(M + H)^+$. Quantification of beauvericin was performed by linear regression of the corresponding peak areas using the Quanbrowser software (Thermo Fisher Scientific, San Jose, CA, USA).

Analysis of FA was conducted using the Accela HPLC system coupled with an Accela DAD-detector (Thermo Fisher Scientific, San Jose, CA, USA). The eluents were: water with 0.2% (*v*/*v*) formic acid in 10% (*v*/*v*) acetonitrile (eluent B) and 0.2% (*v*/*v*) formic acid (eluent C). The flow rate was 0.5 mL min^{-1}, and the injection volume was 3 µL. The applied gradient is shown in the supporting information (Table S3.3). The selected DAD wavelength range was 220–350 nm. FA was identified by its specific absorbance maximum of 271 nm. Quantification

of FA in the samples was performed by linear regression of the corresponding peak areas using the Quanbrowser software (Thermo Fisher Scientific, San Jose, CA, USA).

3.3.10 Statistical analysis

Data analysis and visualisation were performed with R (v.4.0.2, R Development Core Team, 2020). Data were visually inspected for normality and homoscedasticity using diagnostic residual plots and Q-Q plots to assess potential violation of linear modelling assumptions (Kozak and Piepho, 2018). In addition, the assumptions of normality and homoscedasticity were verified using Shapiro-Wilk test and Levene's test, respectively. When the assumptions for parametric tests were met, analysis of variance (ANOVA) and the post hoc Tukey's range test were used to test significant differences in the means. In case data were nonparametric, Mann-Whitney U and Kruskal-Wallis tests were applied. A subsequent post hoc Dunn's test was conducted for all pairs of comparisons between groups using the Benjamini-Hochberg adjustment for multiple testing (adjusted p value) (Benjamini and Hochberg, 1995). Where applicable, comparisons were made between treatments and the control. Unless otherwise stated, all values represent mean ± standard error of the mean (s.e.m) values.

3.4 Results

3.4.1 Root exudates of *D. uncinatum* and *M. pruriens* exhibit cytotoxicity against Foc TR4

Root exudates of *D. uncinatum* and *M. pruriens* stimulated cellular metabolic activity of Foc TR4 chlamydospores at a concentration of 5 µg mL^{-1}, but cellular metabolic activity was reduced as the concentration of the exudates increased ($p < 0.0001$) (Fig. 3.1A).

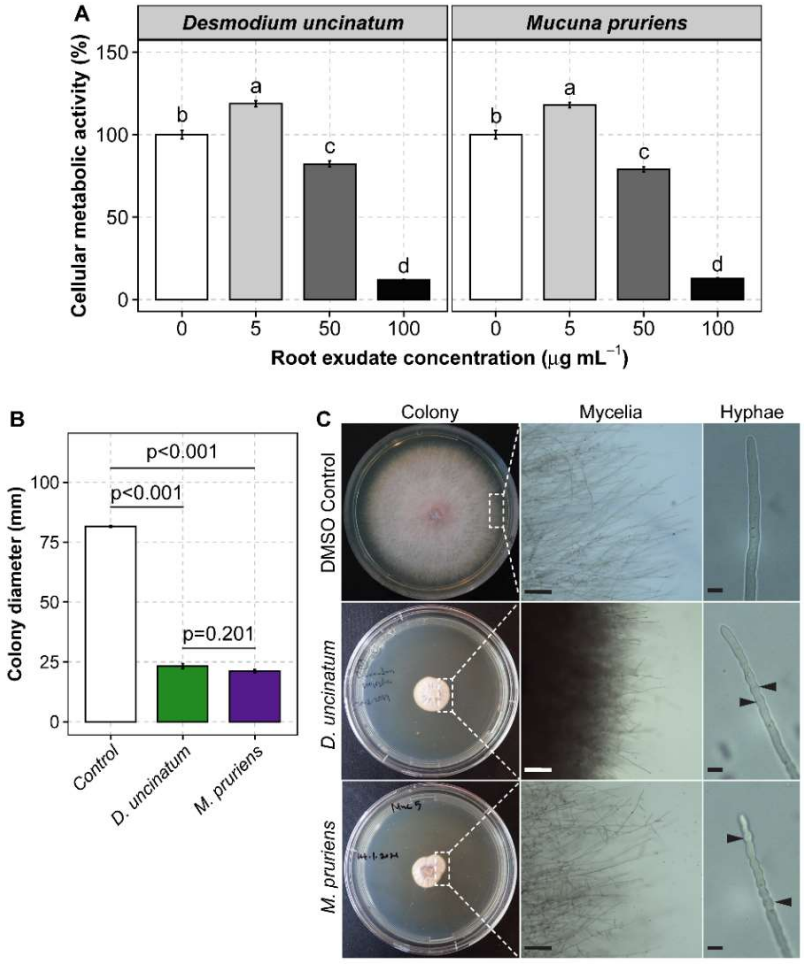

Figure 3. 1. The cytotoxic effect of *Desmodium uncinatum* and *Mucuna pruriens* root exudates on *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4). Cytotoxic effect of root exudates on chlamydospores assessed by measuring cellular metabolic activity (**A**). Mycelia growth inhibition (**B**) revealing compact masses of aberrant hyphae marked by hyperbranching (**C**) and a pattern of repetitive bulges along the hyphal walls (black arrowheads).

The radial mycelial growth of Foc TR4 cultures was also reduced ($p < 0.0001$) in the presence of root exudates at a concentration of 100 µg mL^{-1} (Fig. 1B). Aerial hyphae were notably reduced in the presence of root exudates (Fig. 3.1C), with compact masses of aberrant hyphae marked by hyperbranching and a pattern of repetitive bulges along the hyphal walls (Fig. 3.1C). Similar hyphal morphologies were not observed in the control cultures.

3.4.2 Identification of *D. uncinatum* and *M. pruriens* root-exuded phenolic metabolites

A total of twelve compounds (Table 3.1), including the phenolic acids benzoic, *t*-cinnamic, *p*-coumaric, and *p*-hydroxybenzoic, were targeted in root exudates of *D. uncinatum*. Of these, *t*-cinnamic acid and benzoic acid were predominant at 0.674 ± 0.285 and 0.324 ± 0.068 µg g^{-1} fresh root weight, respectively. In contrast, the phenolic acids, benzoic, *t*-cinnamic, *p*-coumaric, *p*-hydroxybenzoic, and vanillin, and the flavonoid quercetin, were detected in root exudates of *M. pruriens*. The predominant phenolic acids in *M. pruriens* were *t*-cinnamic acid and benzoic acid at 0.228 ± 0.091 and 0.057 ± 0.025 µg g^{-1} fresh root weight, respectively. Unlike benzoic, *t*-cinnamic, *p*-hydroxybenzoic acid, *p*-coumaric was not detected in all root exudate samples of *D. uncinatum*.

Table 3. 1. Metabolite concentration of phenolic compounds and flavonoids in root exudates of *Desmodium uncinatum* and *Mucuna pruriens* cultured in a hydroponic system.

Metabolite	**R_T (min)**	**$UV\lambda_{max}$ (nm)**	**Metabolite concentration in root exudates (µg g^{-1} fresh root)**	
			Desmodium uncinatum	*Mucuna pruriens*
Benzoic acid	6.13	229	0.324 ± 0.068^{ab}	0.057 ± 0.025^{b}
Caffeic acid	4.29	322	nd	nd
t-cinnamic acid	7.75	275	0.674 ± 0.285^{a}	0.228 ± 0.091^{a}
p-coumaric acid	5.34	309	0.056 ± 0.027^{b}	0.013 ± 0.006^{b}
t-ferulic acid	5.64	322	nd	nd
Gallic acid	1.27	270	nd	nd
p-hydroxybenzoic acid	3.94	254	0.081 ± 0.021^{b}	0.002 ± 0.002^{b}
Protocatechuic acid	2.56	258	nd	nd
Naringenin	8.4	288	nd	nd
Quercetin	7.64	370	nd	0.051 ± 0.008^{b}
Umbelliferon	5.8	324	nd	nd
Vanillin	5.48	280	nd	0.032 ± 0.007^{b}

R_T= Retention time; nd = not detected. Means in the same column followed by the same letter are not significantly different according to Tukey's range test.

3.4.3 Effect of phenolic acids on chlamydospore germination, mycelial growth, and production of conidia

Chlamydospore germination was inhibited by benzoic acid ($p<0.05$), *t*-cinnamic acid ($p<0.05$) and a mixture of benzoic acid, *t*-cinnamic acid ($p<0.05$) and *p*-hydroxybenzoic, but not *p*-hydroxybenzoic acid alone ($p=0.0625$) (Fig. 3.2A). Inhibition of chlamydospore germination was dependent on metabolite dose, with only benzoic acid reducing germination significantly at the lowest dose (0.01 mM) compared to the control ($p<0.05$). Scanning electron microscopy (SEM) revealed inhibition-associated abnormalities in the morphology of chlamydospores and germ tubes. Treated chlamydospores were remarkably shrivelled, deformed, and exhibited profoundly distorted or raptured germ tubes (Fig. 3.2B), whereas no such effects were observed in the controls (Fig. 3.2C).

Radial mycelial growth was significantly stimulated by *t*- cinnamic acid at 0.01 mM ($p=0.047$) and 0.1 mM ($p=0.011$), whereas 1 mM had no effect on mycelial growth of Foc TR4 (Fig. 3.2A, D). The metabolite *p*-hydroxybenzoic acid did not have any effect on mycelial growth. In contrast, benzoic acid and a combination of the three metabolites had no effect on mycelial growth at low doses, but exhibited a notable inhibition for benzoic acid ($p<0.0001$) and a combination of the three compounds ($p<0.0001$) at 1 mM. The number of macroconidia produced by Foc TR4 PDA mixed with benzoic acid, *t*- cinnamic acid and *p*-hydroxybenzoic acid, and a combination thereof, was lower than for the control ($p<0.0001$) (Fig. 4.2E).

An average of 49.938 ± 1.258 × 10^3 macroconidia mL^{-1} were found in the control treatment compared to less than 20 × 10^3 mL^{-1} in the metabolite-mixed treatments. A reduction of microconidia was found when PDA was mixed with benzoic acid at concentrations higher than 0.01 mM, and with *t*- cinnamic acid at 1 mM ($p<0.0001$) (Fig. 3.2E). *p*-Hydroxybenzoic acid had no effect on the production of Foc TR4 microconidia. The phenolic acid mixture was at least two orders of magnitude more effective than individual compounds in suppressing the production of macro- and microconidia. The lowest dose of the phenolic acid mixture (0.01 mM) reduced the production of macroconidia to an average of 13.0 ± 0.632 × 10^3 and of

microconidia to 626.6 ± 24.459 × 10^3 spores, compared to 53.0 ± 1.048 × 10^3 macroconidia and 999.4 ± 18.859 × 10^3 microconidia for the DMSO control.

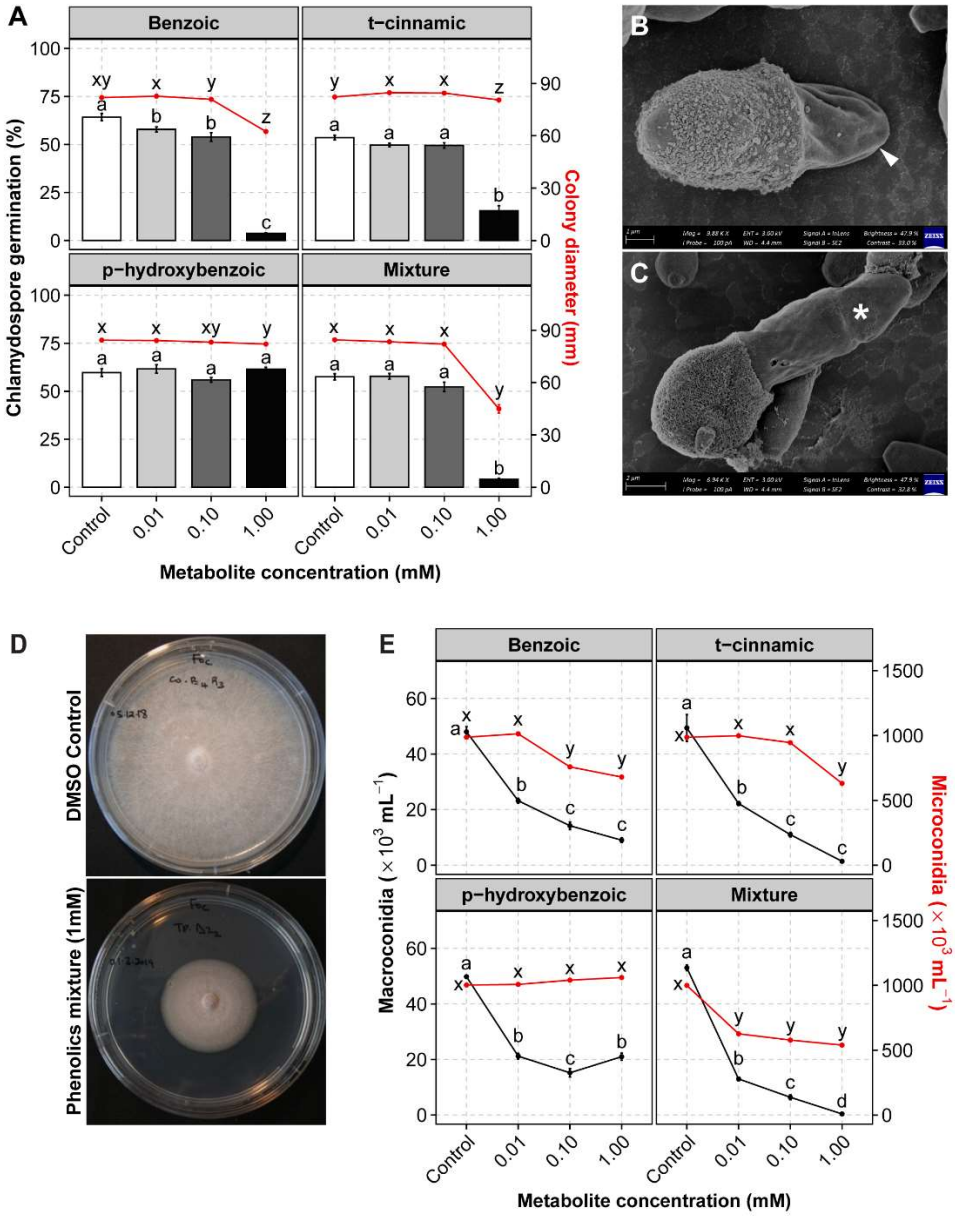

Figure 3. 2. Effect of phenolic acid metabolites detected in root exudates of *Desmodium uncinatum* and *Mucuna pruriens* on chlamydospore germination and radial mycelial growth of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) (**A**-**D**). (**B**) Electron micrographs of a germinating Foc TR4 chlamydospore with a protruding germ tube (white asterisk) and (**C**) a chlamydospore germinating in the presence of a mixture of phenolic acids at 1 mM revealing a distorted germ tube (white arrowhead). (**D**) A colony of Foc TR4 growing on potato dextrose agar (PDA), and (**E**) PDA supplemented with different phenolic acids at 1 mM (**D**). The phenolics mixture includes benzoic acid, *t*-cinnamic acid, and *p*-hydroxybenzoic acid.

3.4.4 Effect of phenolic acids on biosynthesis of fusaric acid and beauvaricin

Biosynthesis of beauvericin was stimulated by benzoic acid, while *t*-cinnamic acid, *p*-hydroxybenzoic acid, and a combination of the three metabolites, stimulated beauvericin at low concentrations and inhibited the toxin at 1 mM (Fig. 3.3A). Conversely, the biosynthesis of FA was progressively reduced by increasing concentrations of the phenolic acids individually and combined (Fig. 3.3B). Biosynthesis of FA in Foc TR4 was completely abolished when treated with 1 mM of the metabolite combination ($p<0.0001$).

Interestingly, the pH (6.7 ± 0.2) of the broth medium was elevated during fungal growth. Hence, it was hypothesised that the repression of FA biosynthesis was pH-dependent, and not due to effects brought on by impaired fungal growth after treatment. To proof this hypothesis, Foc TR4 was cultured in the same broth buffered at low (5.0) or high (7.0) pH using MES (2-(*N*-morpholino) ethanesulfonic acid). When FA concentration and fungal biomass were determined, an 8-fold increase in FA concentration was found at pH 7 compared to pH 5 ($p=0.0008$) for all phenolic acid treatments. Fungal biomass was, however, not affected by pH ($p>0.05$) (Fig. 3.3C, D). The repression of FA biosynthesis by phenolic acids thus appears not to be linked to a reduction in fungal biomass. Rather, dissipation of cytosolic pH was assumed as a mechanism by which phenolic acids repress FA biosynthesis in Foc TR4.

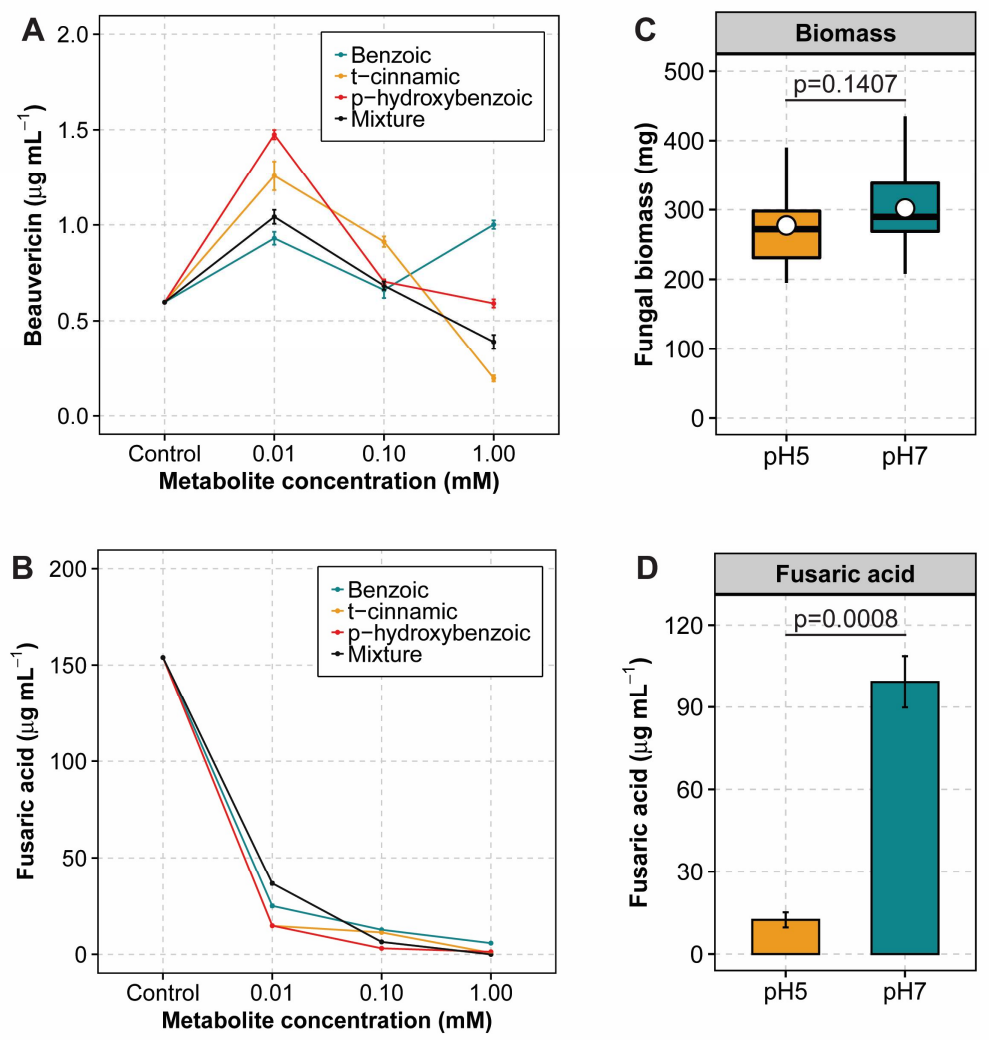

Figure 3. 3. Effect of phenolic acid metabolites on the biosynthesis of beauvericin (**A**) and fusaric acid (FA) (**B**) mycotoxins in *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4). Influence of extracellular pH (pH 5 and pH 7) on FA biosynthesis and fungal biomass (**C**) and FA biosynthesis (**D**) in Foc TR4. Boxplots show the upper and lower quartile, median (bold horizontal bar), mean (white circle), and whiskers (vertical lines).

3.5 Discussion

Plants release a myriad of primary and secondary metabolites into the rhizosphere through the process of root exudation (Haichar et al., 2016; Oburger and Jones, 2018). While root exudation was suggested to play an important role in the suppression of Fusarium wilt disease in banana intercropping systems (Buddenhagen, 2009; Pattison et al., 2014), the suppressive effect of root exudates and the metabolites involved remain poorly understood. Here, we show that root exudates of two legumes, *D. uncinatum* and *M. pruriens*, exhibit cytotoxicity against Foc TR4. The cytotoxicity can be attributed metabolites present in root exudates of the two legumes. In support of this, targeted metabolite analysis revealed differences in phenolics (phenolic acids and flavonoids) present in root exudates of *D. uncinatum* and *M. pruriens*. Phenolics represent one of the major classes of plant secondary metabolites with antioxidant properties, antimicrobial activity, and have been reported to suppress soil-borne pathogens including *F. oxysporum* spp. (Michielse et al., 2012; Yuan et al., 2018). The difference in the composition of phenolics in root exudates of the two legumes may be attributed to plant genotype. Plant genotype is a well recognized factor that strongly influences the metabolite composition and quantity of root exudates along with the microbiome present in the rhizosphere (Micallef et al., 2009; Zhalnina et al., 2018; Iannucci et al., 2021).

Benzoic acid exhibited the highest inhibitory effect on chlamydospore germination, production of conidia, and mycelial growth in Foc TR4. The difference in antimicrobial effect of phenolic acids has been previously reported for other *Fusarium* spp. (Schöneberg et al., 2018; Gautier et al., 2020), and may be attributed to the position of hydroxyl moiety on the aromatic ring or to the antioxidant capacities of the phenolic acids (Synowiec et al., 2021). Antioxidant compounds may act via nonspecific mechanisms such as disruption of plasma membrane function which can lead to a modification of membrane permeability (Pagnussatt et al., 2014; Morales et al., 2017).

The compact masses of aberrant hyphae with distinct structural aberrations could be due to disruption of the Spitzenkörper, a pleomorphic complex that regulates hyphal growth

and morphogenesis (Riquelme et al., 2018). The Spitzenkörper is formed by aggregation of cytoskeletal components together with transport vesicles containing precursors for cell surface expansion and growth at hyphal tips (Riquelme et al., 2018). Phenolic acids such as *p*-coumaric acid and caffeic acid, where the former was detected in root exudates of *D. uncinatum* and *M. pruriens*, have been reported to disrupt the Spitzenkörper and ultimately inhibit hyphal growth (Neves et al., 2005).

The reduced number of conidia suggests that phenolic acids inhibit conidia production in Foc TR4 which is consistent with previous reports on the suppression of conidia production in *Fusarium oxysporum* spp. by phenolics (Wu et al., 2008; Michielse et al., 2012). Generally, the prelude to conidia production in *F. oxysporum* spp. is the formation aerial hyphae. Macroconidia are mostly produced in sporodochia that occur on aerial hyphae (Ohara et al., 2004). The reduced number of macroconidia could be attributed to the reduction in aerial hyphae which was also observed in Foc TR4 cultures treated with phenolic acids. A reduction in the formation of aerial hyphae was reported to reduce the production of macroconidia in *Fusarium oxysporum* (Ohara and Tsuge, 2004). The biological significance of this finding would be that a decrease in macroconidia production could limit chlamydospore formation, because chlamydospores often develop from the cytological modification in segments of macroconidia (Ohara and Tsuge, 2004; Leslie and Summerell, 2006).

The dramatic decrease in FA in the filtrate of Foc TR4 cultures supplemented with phenolic acids suggests repression of FA biosynthesis. This finding apparently contrasts a report in *F. oxysporum* f. sp. *niveum*, in which *t*-cinnamic acid promoted mycotoxin biosynthesis (Wu et al., 2008) and could be attributed to the difference in fungal strains used. Suppression of FA biosynthesis in Foc TR4 may negatively affect the establishment of Foc TR4 in the rhizosphere, as FA plays an important role in the inhibition of competing soil microorganisms during colonisation of the rhizosphere (Notz et al., 2002; Bacon et al., 2005; Spraker et al., 2018). Moreover, our findings suggest that biosynthesis of FA in Foc TR4 may be tightly linked to pH, where an alkaline pH is ideal for biosynthesis of FA, as previously reported in other *Fusarium* spp. (Masachis et al., 2016; Sánchez-Rangel et al., 2018; López-

Díaz et al., 2018). Thus, the suppression of FA biosynthesis by phenolic acids could, in part, be attributed to the acidic, apolar, and planar characteristics allowing phenolic acids to readily traverse the plasma membrane and become protonated, thereby acidifying the cytosol (Pagnussatt et al., 2014; Morales et al., 2017). In support of this, buffering the growth medium at pH 5 dramatically reduced the amount of FA produced by Foc TR4 cultures.

The increased concentration of beauvericin in the supernatant of Foc TR4 cultures supplemented with low concentration of phenolic acids suggests a stimulation of beauvericin biosynthesis. A mechanistic explanation for this stimulation remains unclear, but could reflect a hormesis effect which is the protective response induced in cells and organisms exposed to low doses of toxic compounds (Calabrese et al., 2010; Belz and Sinkkonen, 2019). Stimulation of mycotoxin biosynthesis or fungal growth by low doses of phenolic acids was reported previously (Wu et al., 2008) and could in part be attributed to catabolism of phenolic acids by the fungus. However, the possibility that Foc TR4 is able to catabolise phenolic acids, releasing metabolic intermediates that stimulate fungal metabolism and growth, could not be corroborated in our experimental settings. Our findings highlight the role of legume root-exudate metabolites in pathogen suppression. With this, intercropping banana with *D. uncinatum* and *M. pruriens* may be considered for prospective development of sustainable strategies for management of Foc TR4.

3.6 Conclusion

This study examined the role of root-secreted metabolites in the suppression of Foc TR4, emphasizing the suppression of pathogen development and metabolism. Three phenolic acids; benzoic acid, *t*-cinnamic acid, and *p*-hydroxybenzoic acid; were common in root exudates of *D. uncinatum* and *M. pruriens*. Depending on the compound and its concentration, phenolic acids inhibited chlamydospore germination, hyphal growth, production of macroconidia and microconidia and the synthesis of beauvericin and FA toxins in Foc TR4. A key message of this study was the ability of Foc TR4 to alkalinize the growth medium (external

environment), suggesting a metabolic adaptation for the production of virulence factors such as FA.

Author contributions

FR and AV conceived the project, sourced the funding and, provided the resources. EW designed performed the experiments, curated and analysed the data, and drafted the original manuscript. EW and JS performed metabolite analysis. FR, JS, and AV, critically revised and gave further inputs to the final manuscript.

Data availability statement

All data generated or analysed during this study are included in this article.

Declaration of competing interest

The authors declare no potential conflict of interest.

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4. Role of iron and pH in the process of chlamydospore germination in *Fusarium oxysporum* f. sp. *cubense* TR4*

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4.1 Abstract

Fusarium wilt disease of banana, caused by the notorious soil-borne pathogen *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4), is extremely difficult to manage. Manipulation of soil pH or application of synthetic iron chelators can suppress the disease through iron starvation, which inhibits the germination of pathogen propagules called chlamydospores. However, the effect of iron starvation on chlamydospore germination is largely unknown. In this study, scanning electron microscopy was used to assemble the developmental sequence of chlamydospore germination and to assess the effect of iron starvation and pH *in vitro*. Germination occurs in three distinct phenotypic transitions (swelling, polarized growth, outgrowth). Outgrowth, characterized by formation of a single protrusion (germ tube), occurred at 2 to 3 h, and a maximum value of 69.3% to 76.7% outgrowth was observed at 8 to 10 h after germination induction. Germination exhibited plasticity with pH as over 60% of the chlamydospores formed a germ tube between pH 3 and pH 11. Iron-starved chlamydospores exhibited polarized-growth arrest, characterized by the inability to form a germ tube. Gene expression analysis of *rnr1* and *rnr2*, which encode the iron-dependent enzyme ribonucleotide reductase, showed that *rnr2* was upregulated ($p < 0.0001$) in iron-starved chlamydospores compared to the control. Collectively, these findings suggest that extracellular pH is crucial for chlamydospore germination in Foc TR4. Moreover, inhibition of germination by iron starvation may be linked to a different mechanism, rather than repression of the function of ribonucleotide reductase, the enzyme that controls growth by regulation of DNA synthesis.

Keywords: germ tube, outgrowth, pH, polarized, ribonucleotide reductase, quiescence

4.2 Introduction

Chlamydospores are hardy thick-walled asexual spores produced by diverse fungi, including *Fusarium oxysporum* f. sp. *cubense* (Foc), the notorious root-infecting pathogen causing Fusarium wilt disease of banana (Viljoen et al. 2020). *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4), considered the most virulent and devastating race of Foc. Foc TR4 is spreading inexorably and poses a threat to the food security and income of nearly 400 million people that depend on banana (Zheng et al., 2018; Viljoen et al. 2020; van Westerhoven et al. 2022). Chlamydospores are the primary source of Foc inoculum and can remain quiescent in infested soils for decades. When suitable conditions are encountered, chlamydospores in soil undergo a revival cellular process called germination (Pegg et al. 2019; Were et al. 2022a). Chlamydospore germination is a crucial step in the infection of host roots and development of Fusarium wilt (Pegg et al. 2019). Fusarium wilt can be suppressed by targeting chlamydospore germination through manipulation of soil pH to 7.0 or close thereto, and by reduction of the bioavailability of iron through application of iron chelators (Peng et al. 1999; Dita et al. 2018; Segura-Mena et al. 2021). However, the mechanism of inhibition and the distinct role of iron and pH on chlamydospore germination are largely unknown.

Quiescence in fungal spores is a non-proliferative cellular state of reversible cell cycle arrest (Rittershaus et al. 2003; Blatzer and Latgé 2021). Quiescence is characterized by reduced metabolic activity and accumulation of storage molecules, such as trehalose (Wyatt et al. 2013; Hayer et al. 2014). The transition from quiescence to germination generally occurs in three distinct phenotypic transitions: dormancy to swelling (isotropic increase in size), swelling to polarized growth, and the emergence of a germ tube (outgrowth) (Sephton-Clark and Voelz 2018). Outgrowth marks the end of the germination process. It is considered the first development step in the formation of a fungal colony and a key developmental stage in the life cycle of fungi (Sephton-Clark and Voelz 2018). Spore germination has been widely investigated in fungal pathogens (Hayer et al. 2014; Turgeman et al. 2016). However, spore germination in Foc TR4 is largely unknown. Moreover, most studies on spore germination in

Foc have been conducted using conidia (Li et al. 2011; Meldrum et al. 213; Deng et al. 2015), which may not be appropriate substitutes for chlamydospores.

Spore germination is marked by heightened metabolic activity during which biomolecules are synthesised to rebuild hyphae from the disintegrating spore (Deng et al. 2015; Sephton-Clark and Voelz 2018; Balotf et al. 2021). For example, outgrowth in filamentous fungi occurs after nuclear division which, in turn, is dependent on DNA synthesis (Cohen et al. 2019; Greene et al., 2020; Steenwyk, 2021). The synthesis of DNA requires ribonucleotide reductase (RNR), the enzyme which converts ribonucleotides to deoxyribonucleotides (dNTPs) which are the precursors for DNA synthesis and repair (Greene et al. 2020; Steenwyk 2021).

In *F. oxysporum*, RNR has two non-identical homodimeric subunits, a large subunit Rnr1 and a small one-Rnr2, which are encoded by the genes *rnr1* and *rnr2*, respectively (Cohen et al. 2019). The Rnr1 subunit contains the catalytic site and allosteric sites that control enzyme activity and specificity (Greene et al. 2020). On the other hand, the Rnr2 subunit contains a non-heme iron centre, which generates a tyrosyl free radical that is essential for catalysis. Iron is an indispensable micronutrient for all eukaryotic organisms and an essential cofactor of RNR (Furukawa et al. 1992; Colombo et al., 2014; Fukada et al. 2019). Given the significance of iron, we hypothesized that iron is fundamental for outgrowth in Foc TR4 and that iron scarcity leads to selective optimization of RNR function by enhanced expression of *rnr1* and *rnr2* genes. Accordingly, the specific objectives were to assess (i) the effect of iron starvation on outgrowth and the expression of *rnr1* and *rnr2* genes, and (ii) the effect of extracellular pH on outgrowth.

4.3 Materials and methods

4.3.1 Fungal strain, culture conditions, and production of chlamydospores

Fusarium oxysporum f. sp. *cubense* Tropical Race 4 (TR4) VCG 01213/16 was obtained as a frozen stock on 30% (*v*/*v*) glycerol from the Department of Plant Pathology Stellenbosch University, South Africa. The isolate was preserved at -80°C and revived by culturing on potato dextrose agar (PDA) at 28°C for 5 days. All chemicals, unless otherwise stated, were purchased from Carl Roth (Karlsruhe, Germany).

4.3.2 Chlamydospore germination time-course assay

Chlamydospores were produced from the culture and purified as previously described (Goyal et al. 1973; Were et al. 2022b). A time-course experiment was conducted to determine the developmental sequence of germinating chlamydospores of Foc TR4. The experiment was conducted in 24-well culture plates (Costar, Cambridge, MA, USA) using Barz broth (Were et al. 2022b). Samples for phenotypic analysis of germination were retrieved after 30 min and thereafter every hour for duration of 10 h post induction of germination (h.p.i). Samples were retrieved in 2-mL tubes, chilled on ice, and centrifuged at 13000 r.p.m. for 10 min at 4°C (Eppendorf 5810R Centrifuge, Eppendorf, Hamburg, Germany). Chlamydospores were washed with ice-cold cell wash buffer (PBS-T: 137 mM, NaCl; 2.7 mM, KCl; 2 mM, KH_2PO_4; 0.005% *v/v*, Triton X-100; pH 7.4) by vortexing for 1 min, followed by centrifugation at 13000 r.p.m. for 10 min at 4°C. This procedure was repeated using sterile deionized water.

A developmental sequence of germinating chlamydospores was assembled by bright-field microscopy using a LeicaDM750 microscope equipped with a Leica ICC50 HD camera (Leica, Heerbrugg, Switzerland). Germinated, polarized, and round chlamydospores were counted using a haemocytometer and a Zeiss Axioskop microscope (Carl Zeiss, Jena, Germany). Micrographs were captured using a LeicaDM750 microscope, converted to 8-bit grayscale or

24-bit RGB, and annotated using CorelDraw 12.0 (Corel, Ottawa, Canada). This procedure allowed for the monitoring of the progression of chlamydospore germination.

4.3.3 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to obtain ultra-structural details of germinating chlamydospores. Chlamydospore specimens for SEM analysis were prepared as described previously (Were et al., 2022b). Specimens were visualized with a Zeiss Merlin scanning electron microscope (Carl Zeiss) with a Gemini-type field emission gun electron column (FEG-SEM) equipped with two Oxford Instruments X-MaxN 150 SDDs. Typical imaging conditions were magnification of 1-3 × 10^4, a working distance of 5-10 mm, 2-3 kV, a beam current 100-200 pA and using an In Lens secondary electron detector. Micrographs of specimens were captured in TIF format using a pixel averaging noise reduction algorithm and SmartSEM software (Carl Zeiss).

4.3.4 Cellular metabolic activity and germination in iron-starved chlamydospores

The effect of iron-depletion on chlamydospore germination was assessed by determining the percent germination and cellular metabolic activity of iron-starved chlamydospores. Iron starvation was induced using 2,2'-dipyridyl, a synthetic lipophilic iron chelator that sequesters both extracellular and intracellular Fe^{2+} and Fe^{3+} pools (Romeo et al., 2001). A stock solution of 2,2'-dipyridyl (20 mM) was prepared in dimethyl sulfoxide (DMSO), filter-sterilized using a 0.22 µm syringe filter (Sartorius, Göttingen, Germany), and stored in the dark at 4°C.

Cellular metabolic activity was determined using the Alamar Blue kit (Bio-Rad, Hercules, CA, USA), that measures activity of the mitochondrial respiratory chain as a readout of cell viability. The assay was conducted in Barz medium supplemented with different concentrations of 2,2'-dipyridyl [1, 10, 100, 200 µM and control (10% *v*/*v* DMSO)] by following the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). Absorbance was measured using

a microplate reader (Tecan, Maennedorf, Switzerland). Absorbances were normalized to the DMSO control, where cell viability was set to 100%. The effect of iron-depletion on chlamydospore germination was conducted in 24-well plates as described earlier using Barz supplemented with 2,2'-dipyridyl [1, 10, 100, 200 µM and control (10% *v*/*v* DMSO)]. Plates were incubated for ten hours and germinated chlamydospores were determined as described earlier. To further establish the effect of iron starvation on the chlamydospore germination process, a two-step experiment (iron deplete and iron replete) was conducted. First, in the iron deplete experiment, chlamydospores (10^3 per well) were incubated in 24-well culture plate containing Barz broth supplemented with 2,2'-dipyridyl (100 µM). After 5 h, rounded, polarized, and outgrown chlamydospores was determined as described earlier. In the second, iron-replete experiment, chlamydospores from the iron- deplete were washed as described earlier to remove 2,2'-dipyridyl and further incubated for 5 h in fresh Barz medium. Afterward, rounded, polarized, and outgrown chlamydospores was determined as described earlier.

4.3.5 RNA extraction and cDNA synthesis

Chlamydospore suspensions from two wells were pooled into 2-mL tubes and washed as described earlier. Then, samples were stabilized by immediately suspending in 500 µL of RNAlater RNA (Qiagen, Hilden, Germany) and stored at -80°C. Samples were retrieved and thawed on ice prior to extraction of total RNA. Total RNA was isolated from chlamydospores using the RNeasy kit following the manufacture's protocol (Qiagen). RNA samples were subsequently reverse transcribed to cDNA using a QuantiTect Reverse Transcription Kit following the manufacture's protocol (Qiagen).

4.3.6 Quantitative RT-PCR

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed on a StepOne Real-Time PCR system (Applied BioSystems, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primer pairs (Table 4.1) (Cohen et al. 2019). All samples were amplified in triplicate using the following thermal cycling conditions: 95°C for 20 s, 40 cycles of priming at 54°C for 20 s, and elongation at 72°C for 20 s. Cycle thresholds were obtained using the StepOne software (v2.0, Applied BioSystems, CA, USA). Relative gene expression levels were normalized to that of the *Fusarium oxysporum* actin-lateral binding (*ALb*) gene using the ΔΔCt method (Yuan et al. 2006).

Table 4.1. Primers used for analysis of the expression of ribonucleotide reductase genes (*rnr1* and *rnr2*) and actin-lateral binding (*ALb*) gene in chlamydospores of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Cohen et al. 2019).

Target gene	**Primer name**	**Sequence (**5'→3')
rnr1 (ribonucleotide reductase large subunit, Rnr1)	rnr1-F	CATCAAGGCTGATGTTGAGG
	rnr1-R	CTTGACACCCAACTCTTCTTCC
rnr2 (ribonucleotide reductase small subunit, Rnr2)	rnr2-F	TACTTTGGATCCGGAAGCTG
	rnr2-R	TTTTCATCCACCCTGAGTCC
ALb (actin-lateral binding protein)	ALb-F	GGTTTCCCTTCAGCCTTTTC
	ALb-R	CGGAGCTGGTTCATTTTCTC

4.3.7 Statistical analyses

Data analysis and visualisation were performed with R, v.4.0.2 (R Development Core Team, 2020). Prior to statistical analysis, data was checked for normality and homoscedasticity (Kozak and Piepho 2018). Homoscedasticity of the data was verified using Levene's test, whereas the normality the data was verified using the Shapiro-Wilk test and diagnostic plots (histograms and Q-Q plots). Comparisons were made between treatments and the control. Statistical significance was declared if $p < 0.05$. Significant differences between treatments and the control were tested using analysis of variance (ANOVA) and subsequent post hoc analysis using Tukey's Honest Significant Difference (Tukey HSD) test while the unpaired Welch's t-test was used to compare amino acids profiles. All data are expressed as mean ± standard error of the mean (Kozak and Piepho 2020).

4.4 Results

4.4.1 Germination Foc TR4 chlamydospore is asynchronous

Chlamydospores appeared round and enveloped in a double layered wall: a thin inner wall and a thick outer wall when examined with brightfield microscopy (Fig. 4.1A). The outer wall was composed of distinctly fibrillar material when examined by SEM (Fig. 4.1D-F). Germination was characterized by three distinct phenotypic transitions: swelling, to polarized growth, to outgrowth (Fig. 4.1A-H). Swelling occurred within 0.5 to 1 hours post induction of germination (h.p.i) during which the rounded appeared larger than the dormant chlamydospores and possessed a highly granulated cytoplasm (Fig. 4.1A, G).

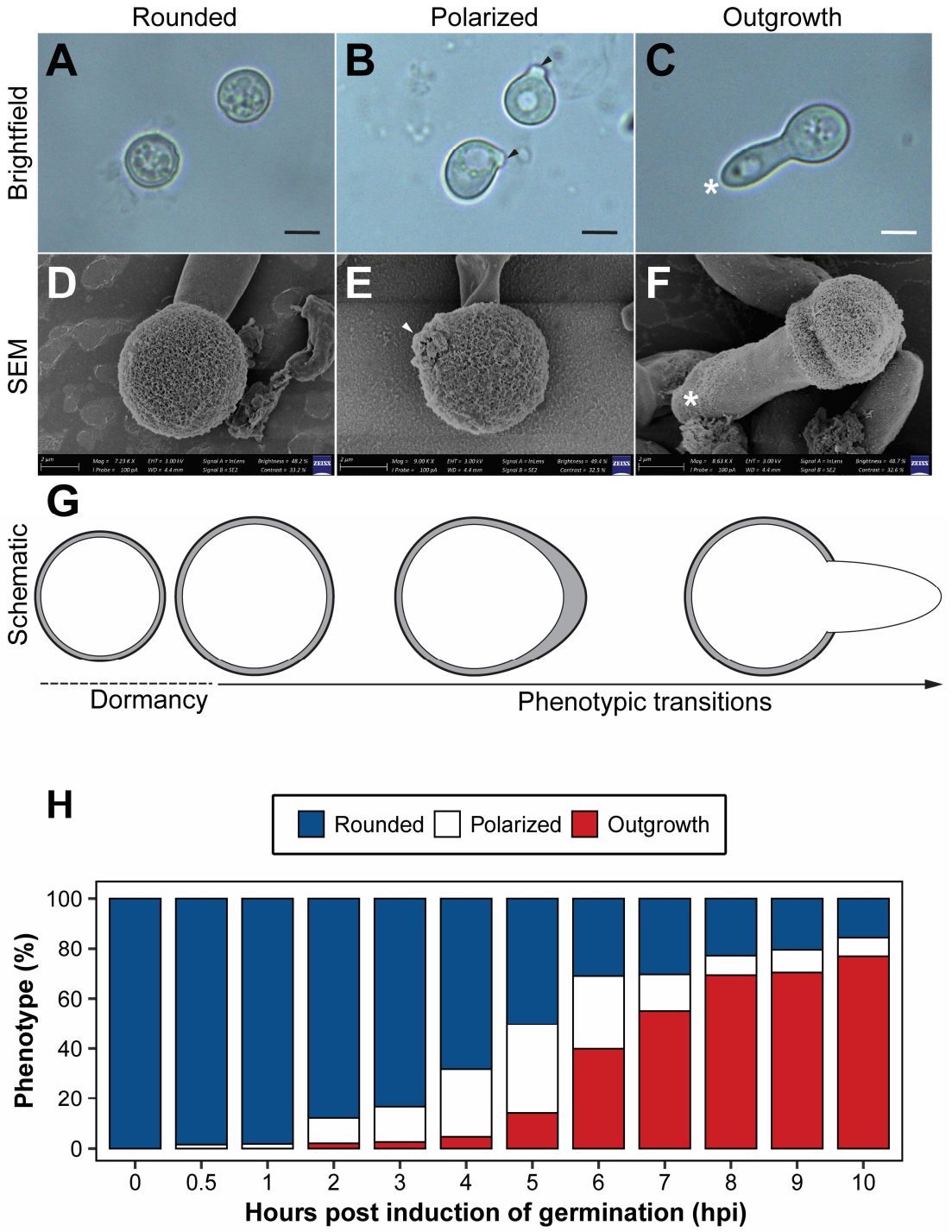

Figure 4.1. Bright-field and scanning electron microscopy micrographs showing the transitions during germination of chlamydospores of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4): rounded (**A**, **D**), polarized growth (**B**, **E**), and outgrowing (**C**, **F**). Schematic illustration of the chlamydospore germination process in Foc TR4 (**G**). The site of polarity establishment (indicated by black arrow heads) on polarized chlamydospores is the point where the germ tube (indicated by white arrow heads) emerges during outgrowth. Scale bar = 20 µm. Phenotypic changes during the process of germination of chlamydospores in Foc TR4 (**H**).

At 2 to 3 h.p.i, deposition and concentration of cellular material was observed at a single specific point on the chlamydospore inner wall (Fig. 4.1B). This cellular material whose molecular identity was not determined, appeared as a bright spot towards which growth was directed thereby defining the spore front and domain. This domain was maintained, resulting in a polarized chlamydospore with a pear-shaped form (Fig. 4.1B, E). Outgrowth, the most conspicuous transition, was detected at 2 to 3 h.p.i and a maximum value of 69.3% to 76.7% chlamydospores produced a single protrusion (germ tube) between 8 to 10 h.p.i (Fig. 4.1C, F and H). At this point, chlamydospores were considered to be fully germinated.

Notably, the germ tube emerged from the chlamydospore by the extension of the inner spore wall after local rapture of the outer wall at the site of polarity establishment (Fig. 4.1B). The germ tube progressively grew into hyphae that extended along a polar axis (Fig. 4.1C). Importantly, although swelling, polarized growth, and outgrowth occurred subsequently, but not simultaneously as evidenced by the rapid remarkable transition that was observed in some chlamydospores. This suggested that chlamydospore germination in Foc TR4 is an asynchronous process (Fig. 4.1H).

4.4.2 Iron is essential for metabolic activity and outgrowth in chlamydospores

At low concentration (1 and 10 µM) of 2,2'-dipyridyl, cellular metabolic activity was stimulated in chlamydospores, but this decreased ($p < 0.0001$) as the concentration of 2,2'-dipyridyl increased (Fig. 4.2A). Similarly, chlamydospore germination decreased ($p < 0.0001$) as the concentration of 2,2'-dipyridyl increased (Fig. 4.2A, B). Iron-starved chlamydospores underwent through swelling but halted their developmental progression, thereby accumulating at polarized growth stage (Fig. 2A, B). This polarized growth-arrest was characterized by the inability to form a germ tube. Only 12.61% of chlamydospores switched to outgrowth in the presence of 2,2'-dipyridyl, compared to 25.47% in the control ($p < 0.001$).

Chlamydospores that exhibited polarized growth-arrest contained perceptibly smaller and fewer cytoplasmic inclusions indicating defects in replication or differentiation of cellular

organelles. In marked contrast, control chlamydospores exhibited no discernible change in cytoplasmic inclusions and progressed normally through germination resulting in the formation of a germ tube (outgrowth). Chlamydospores exhibiting polarized growth-arrest were rescued by iron repletion and germination was fully restored (p = 0.0509) (Fig. 4.2B).

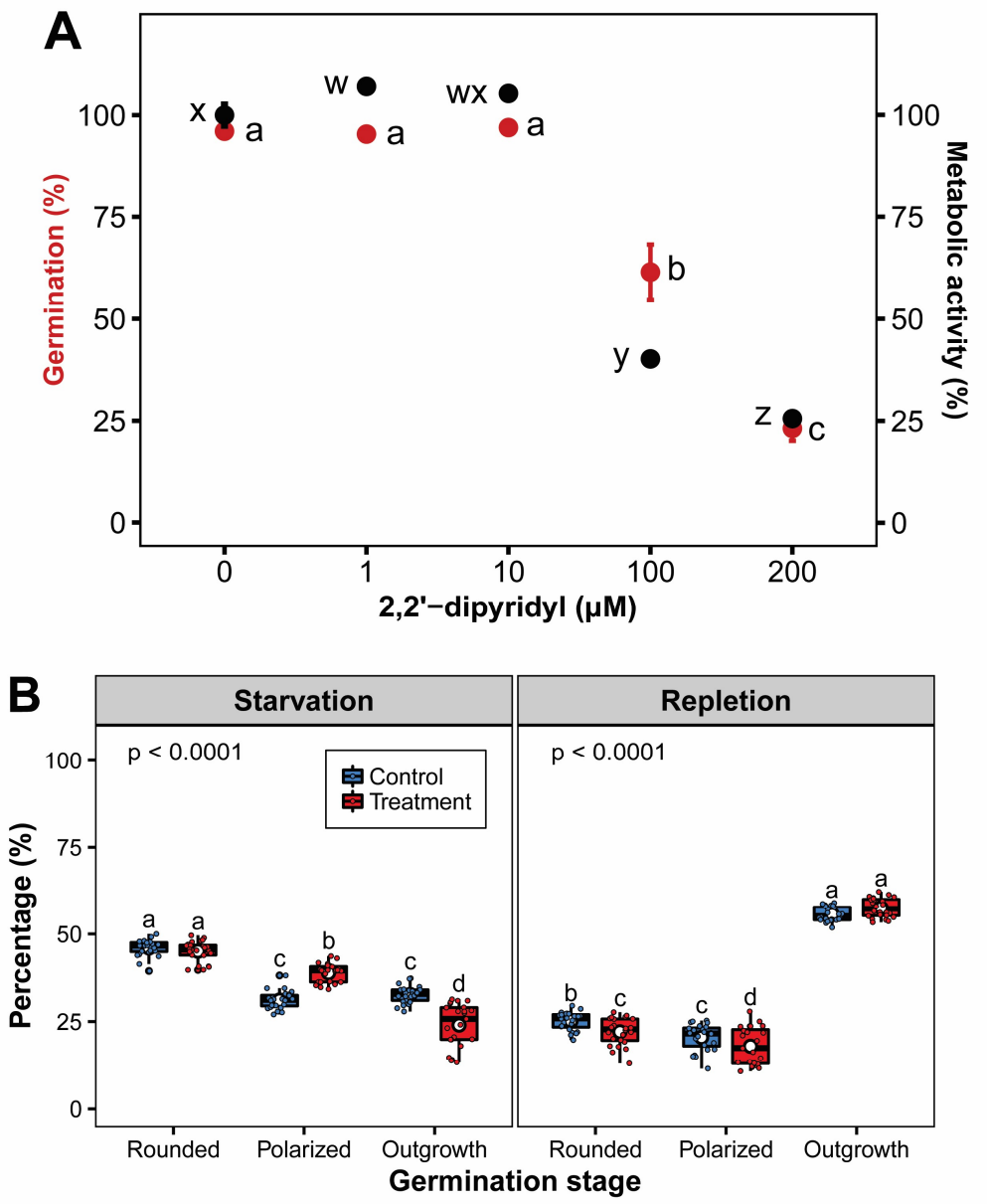

Figure 4.2. Effect of iron starvation on cellular metabolic activity (**A**) and germination (**B**) on chlamydospores of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4. Boxplots show the upper and lower quartile, median (bold horizontal bar), mean (white circle), and whiskers (vertical lines).

4.4.3 Expression of the *rnr2* gene is up-regulated in iron-starved chlamydospores

Expression of the *rnr2* gene was up-regulated in iron-starved chlamydospores, as compared with the control ($p < 0.0001$) (Fig. 4.3A). In contrast, expression of the *rnr1* gene did not differ between in iron-starved chlamydospores and the control ($p > 0.05$) (Fig. 4.3A).

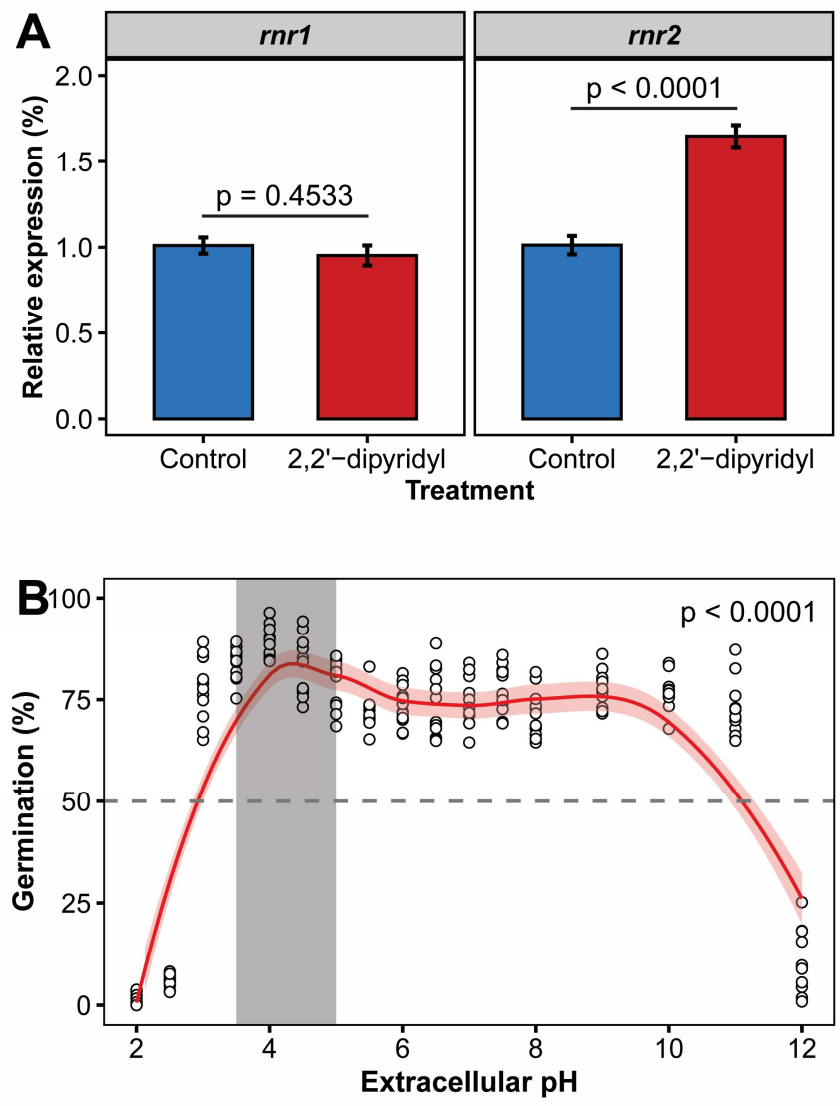

Figure 4.3. Effect of iron starvation on the expression of genes *rnr1* and *rnr2* that encode the Rnr1 and Rnr2 subunits, respectively of the ribonucleotide reductase enzyme in chlamydospores of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (**A**). Effect of pH on chlamydospore germination (**B**).

4.4.4 Chlamydospore germination exhibits plasticity in extracellular pH

Chlamydospore germination was influenced by extracellular pH ($p < 0.0001$) (Fig. 4.3B). Germination was apparent over a wide pH range and exhibited double maxima; a primary peak (optimal) on the acid side from pH 3.5 to 4.5, where 75.3% to 94.3% of chlamydospores formed a germ tube. A secondary peak was noted close to neutrality between pH 8 and 9.5, where 67.8% to 84% of chlamydospores formed a germ tube (Fig. 4.3B). An increase of 0.5 pH units from pH 2.5 to 3.0 induced a 14-fold increase in germination ($p < 0.0001$). Notably, chlamydospores appeared small in broth below pH 3.0 compared to chlamydospores above pH 10.0. Moreover, disintegration of incipient germ tubes was frequently observed below pH 3.0. Above pH 10.0, however, produced germ tubes were often large with a small terminal globular structure, resembling a newly formed chlamydospore.

4.5 Discussion

Chlamydospore germination is crucial in the infection of banana roots by the notorious fungal pathogen Foc TR4 and severity of Fusarium wilt disease (Dita et al. 2018; Pegg et al. 2019). Fusarium wilt can be suppressed by targeting chlamydospore germination through iron starvation caused by application of iron chelators or manipulation of soil pH, but the underlying mechanism of inhibition remains elusive (Peng et al. 1999; Segura-Mena et al. 2021). The findings of this study verify that iron-deficient chlamydospores are unable to form a germ tube.

Consistent with previous work (Plante et al. 2017), we found that depletion of iron results in polarized growth arrest and a concomitant decrease in cellular metabolic activity. Our data suggest that chlamydospore germination is a developmental process that requires iron. Spore germination in Foc TR4 entails resumption of metabolism and *de novo* synthesis of macromolecules to rebuild hyphae from the disintegrating spore (Deng et al. 2015). Macromolecular biosynthesis and energy generation require a unique enzymatic repertoire and bioavailability of cofactors, such as iron (Philpott et al. 2012). Polarized-growth arrest observed in Foc TR4 chlamydospores may reflect attenuation of cell cycle progression arising from a paucity of dNTPs available for DNA synthesis (Renton and Jeitner 1996; Cohen et al. 2019; Greene et al. 2020). DNA synthesis immediately precedes mitosis and subsequently nuclear division, which is indispensable for outgrowth in many fungi (Fukada et al. 2019). Nuclear division and migration of the daughter nucleus into the germ tube are contingent on mitosis control (Renton and Jeitner 1996; Cohen et al. 2019; Greene et al. 2020). Iron is required for assembly and activity of ribonucleotide reductase, an enzyme that catalyses the rate-limiting step in the production of dNTPs, the precursors for DNA synthesis (Cohen et al. 2019; Greene et al. 2020). Thus, the enhanced expression of *rnr2* suggests that iron scarcity leads to selective optimization of RNR function at the expense of other non-essential iron-dependent processes, to allow for DNA synthesis and repair. Similar findings have also been reported in *Saccharomyces cerevisiae* (Sanvisens et al. 2011).

Germination of Foc TR4 chlamydospores occurred over a wide pH range, which is consistent with studies in *F. oxysporum* (Webb 1950), *Harpophora maydis* (Degani and Goldblat 2014), and *Rhizopus delemar* (Turgeman et al. 2016). Maximum germination observed in the acidic and alkaline pH may denote different mechanism by which pH modulates germination. Specifically, the isoelectric point of protoplasmic proteins at this pH is at a low point, but increases under acidic and alkaline conditions (Caracuel et al. 2003; Turgeman et al. 2016). Moreover, interference with cellular processes, including protein synthesis, protein folding and therefore enzyme activity, cell wall remodelling and reduced availability of nutrients, are likely involved (Gaitanaki et al. 1990; Caracuel et al. 2003; Turgeman et al. 2016). Under field conditions, however, extreme acidic and alkaline conditions rarely occur. Nevertheless, from a biological perspective, our findings suggest a robust adaptation of Foc TR4 to ambient pH. Thus, from a virulence perspective, this might reflect the potential of Foc TR4 to infect plants in diverse soils.

Chlamydospore germination in Foc TR4 was characterized by the developmental sequence of three discrete morphological changes transitioning to swelling, polarized growth, and outgrowth. These transitions did not occur uniformly in a chlamydospore population, suggesting that the process of chlamydospore germination in Foc TR4 is asynchronous. Germination asynchrony may be a strategy for biological bet-hedging an unpredictable environment (Stelkens et al. 2016), or may denote heterogeneity within a spore population (Wyatt et al. 2013). Asynchronous germination has also been reported in *Penicillium marneffei* (Zuber et al. 2003), *Saccharomyces paradoxus* (Stelkens et al. 2016), and *Cryptococcus neoformans* (Ortiz et al. 2021).

Phenotypic transitions observed in germinating chlamydospores of Foc TR4 are reminiscent of filamentous fungi, but differ in the timing between transitions (Sephton-Clark and Voelz 2018). This difference could reflect strain variability of the pathogen or distinct experimental systems. Chlamydospore swelling at the onset of germination suggests an increase in spore volume, which is typical of germinating spores (Sharma et al. 2016; Hayer et al. 2014). Increase in spore volume results from hydration of the spore and is a fundamental

step in the germination process (Hayer et al. 2014; Sephton-Clark and Voelz, 2018). Hydration of the spore can be passive, following hydrolysis of trehalose to glucose (Sharma et al. 2016) or ATP-driven via aquaporins (Turgeman et al. 2016).

Polarized growth in filamentous fungi is well established (Ghose et al. 2021). A polar site is defined inside the cell (polarity establishment), from where growth is directed along a polar axis (polarity maintenance) (Ghose et al. 2021). Polarized growth is regulated by spatio-temporal activation of Rho-family guanosine triphosphatases (GTPases) and requires specialized GTP-binding proteins, called septins. Septins localize in a cortical region and serve as positional landmarks for activation and recruitment of the polarity machinery (Bonazzi et al. 2014; Ghose et al. 2021; Hall and Wallace 2022). This defines the cell's front, thereby creating a polarity domain and protein complex, termed the polarisome (Ghose et al. 2021; Mishra et al. 2022). The polarisome is sustained by a constant supply of exocytic vesicles containing precursors for cell surface expansion and growth of the cell tip (Mishra et al. 2022). Vesicles aggregate at the cell tip and together with cytoskeleton components form the Spitzenkörper, a highly dynamic and pleomorphic complex that regulates hyphal growth and morphogenesis (Bonazzi et al. 2014; Ghose et al. 2021; Mishra et al. 2022). Hence, for now, it may be only speculated that the bright spot observed in polarized chlamydospores represents the polarisome, but further confirmative analysis will be necessary.

4.6 Conclusion

Our results suggest that the process of chlamydospore germination in Foc TR4 is developmentally orchestrated and iron-dependent. Our findings highlight the role of iron and pH in the process of chlamydospore germination and suggest that in soil, disease suppression by manipulation of soil pH may act via other mechanisms besides the alteration of iron bioavailability.

Author contributions

FR and AV conceived the project, sourced the funding, provided the resources, and supervised the research. EW designed performed the experiments, curated and analysed the data, and drafted the original manuscript. FR, JS, and AV, critically revised and gave further inputs to the final manuscript.

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5. *De novo* synthesis of ferrichrome by *Fusarium oxysporum* f. sp. *cubense* TR4 in response to iron starvation*

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5.1 Abstract

Manipulation of iron bioavailability in the rhizosphere of banana may suppress Fusarium wilt, caused by the notorious soil-borne fungal pathogen, *Fusarium oxysporum* f. sp. *cubense* (Foc). However, iron starvation induced by the application of synthetic iron chelators does not effectively suppress Fusarium wilt. It is not clearly known whether Foc can subvert iron chelators and thereby evade iron starvation through production of low molecular weight secondary metabolites, called siderophores. *In vitro* studies were conducted using iron-deficient growth medium and medium supplemented with a synthetic iron chelator, 2,2'-dipyridyl, to mimic iron starvation in Foc Tropical Race 4 (Foc TR4). The concentration of extracellular siderophores increased three-fold ($p<0.05$) in the absence of iron. Liquid chromatography-mass spectrometry analysis detected the hydroxamate siderophore, ferrichrome, only in the mycelia of iron-starved cultures. Moroever, iron-starved cultures exhibited a reduction in total cellular protein concentration. In contrast, out of the 20 proteinogenic amino acids, only arginine increased ($p<0.05$) under iron starvation. Our findings suggest that iron starvation does not cause a remodelling of amino acid metabolism in Foc, except for arginine, which is required for biosynthesis of ornithine, the precursor for siderophore biosynthesis. Collectively, our findings suggest that the biosynthesis of siderophores, particularly ferrichrome, could be a counteractive mechanims for Foc TR4 to evade iron starvation.

Keywords: arginine, Foc TR4, siderophores, ornithine

5.2 Introduction

Fusarium oxysporum f. sp. *cubense* (Foc) is a notorious soil-borne fungal pathogen that causes Fusarium wilt disease of banana (Viljoen et al., 2020). There are three races of Foc that are pathogenic to banana, but Foc Tropical Race 4 (TR4) is considered the most destructive as it is pathogenic to diverse banana cultivars and is extremely difficult to manage (Dita et al., 2018; Zuo et al., 2018). Infection of banana by Foc and severity of Fusarium wilt are linked to iron bioavailability in soils (Domínguez et al., 1996; Orr and Nelson, 2018). Accordingly, Fusarium wilt was suppressed when the synthetic iron chelator, EDDHA, was applied to Foc-infested soils (Peng et al., 1999). Iron chelators with a high stability constant (K) such as HBED ($K = 10^{39.7}$) and EDDHA ($K = 10^{33.9}$) sequester iron with high affinity (Robin et al., 2008), which limits iron bioavailability, and can thereby cause iron starvation. Orr et al. (2021), however, suggested that the iron nutrition of Foc is not effectively altered by chelators, as no decrease in the severity of Fusarium wilt was observed after the application of HBED and EDDHA to Foc-infested soils. It is unclear whether Foc can subvert iron chelators and thereby evade iron starvation.

Bioavailable iron in soil is generally limited (~10^{-18} M), and well below the optimal requirement for plants (10^{-9} to 10^{-4} M) and microorganisms (10^{-7} to 10^{-5} M) (Robin et al., 2008; Colombo et al., 2014). This foments competition for iron between microorganisms in the rhizosphere (Gu et al., 2020; Jarmusch et al., 2021). Generally, microorganisms utilize three strategies to scavenge iron. The first strategy involves acidification of the extracellular milieu to enhance iron solubility, while the second strategy involves the reduction of ferric iron (Fe^{3+}) to the more soluble ferrous iron (Fe^{2+}). The third strategy entails biosynthesis and secretion of low molecular weight (<1000 Da) secondary metabolites, called siderophores, which bind Fe^{3+} with high affinity and specificity (Miethke and Marahiel, 2007; Hilder and Kong, 2010). Siderophores occur in three categories based on the differences between their iron-binding moieties: catechols, carboxylates, and hydroxamates (Hilder and Kong, 2010). Hydroxamate siderophores can be assigned to four structural families: rhodoturalic acid, coprogen,

fusarinine, and ferrichrome (Hilder and Kong, 2010). There are more than 260 siderophores listed in the online database of microbial siderophores (http://bertrandsamuel.free.fr/siderophore_base/index.php). The only siderophores reported in Foc are three hydroxamate siderophores: (N,N',N''-triacetylfusarinin C, fusigen, and fusarinine C), which all share the basic N^5-acyl-N^5-hydroxyornithine structural unit and are synthesized from ornithine (Anke et al., 1973; Beckmann et al., 2013). Ornithine is a non-proteinogenic amino acid and the obligatory precursor of fungal siderophore biosynthesis (Hider and Kong, 2010). Ornithine is produced either in the mitochondria from glutamate, or in the cytosol from the hydrolysis of arginine (Dietl et al., 2020; Misslinger et al., 2021).

Iron serves as a cofactor for enzymes involved in key metabolic processes such as DNA synthesis and repair, and the biosynthesis of amino acids and proteins (Herlihy et al., 2020). Thus, for optimal growth, microorganisms require ample supply of iron and must be able to adapt when faced with limited iron bioavailability (Philpott et al., 2012; Herlihy et al., 2020; Liu et al., 2021). The mechanisms for adaptation to limited iron bioavailability in Foc are poorly understood. However, studies in model fungi such as *Aspergillus* spp. show that fungal pathogens respond to limited iron bioavailability by altering cellular metabolism to conserve iron (Philpott et al., 2012) or by up-regulating systems devoted to iron acquisition, such as siderophore biosynthesis (Philpott et al., 2012; Brault and Labbé, 2020). For instance, during iron starvation, the corn smut pathogen *Ustilago maydis* may down-regulate protein synthesis and divert the cellular pool of amino acids to the biosynthesis of siderophores such as the hydroxamate-type siderophore, ferrichrome (Emery 1971; Philpott et al., 2012). Ferrichrome is a cyclic hexapeptide consisting of three N^{δ}-acyl-N^{δ}-hydroxyornithines and three amino acids. One amino acid is always glycine, while the remaining two can be a combination of alanine, serine, or glycine.

Ferrichrome is a key fungal siderophore that is important for iron scavenging and storage (Emery, 1971; Matzanke et al., 1987). For instance, *Schizosaccharomyces pombe* secretes ferrichrome to the extracellular environment to scavenge and storage iron (Schrettl et al., 2004). Ferrichrome was detected both in the mycelia of *U. sphaerogena* and culture filtrate,

suggesting a role in iron scavenging and storage (Budde and Leong, 1989). In *U. maydis*, ferrichrome plays and important role of iron storage and accounts for 50% of the cellular iron pool (Matzanke et al., 1987). Despite the importance of siderophores in iron scavenging and storage, siderophore biosynthesis by iron-starved Foc is not well understood. It was hypothesised that iron starvation would trigger a compensatory increase in siderophore biosynthesis in Foc to scavenge iron. Such a response would require that arginine is synthesized and subsequently converted to ornithine, the biogenic precursor of siderophores. The objectives of this research were, therefore, to (1) examine the production of extracellular siderophores during iron repletion, (2) assess the biosynthesis of ferrichrome, and (3) determine whether the amino acid precursors for siderophore biosynthesis are altered during iron starvation.

5.3 Materials and methods

5.3.1 Fungal strain and culture conditions

A strain of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) VCG 01213/16 was used in this study. The strain was obtained from the Department of Plant Pathology, Stellenbosch University, South Africa and preserved on 30% (*v*/*v*) glycerol at - 80°C. Fungal cultures for routine experiments were prepared from the frozen stock by culturing at 28°C for 5 days on potato dextrose agar (PDA) (Carl Roth, Karlsruhe, Germany).

5.3.2 Chemicals and reference standards

Reagents for high performance liquid chromatography (HPLC) and mass spectrometry (LC-MS) were purchased from Sigma-Aldrich (Steinheim, Germany). The analytical standard for ferrichrome was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The

analytical standard for amino acid analysis (amino acid standard H) was purchased from Thermo Scientific (Rockford, IL, USA). Other chemicals, unless otherwise stated, were purchased from Carl Roth.

5.3.3 Analysis of siderophores

Fungal cultures were prepared in 250-mL Erlenmeyer flasks containing 50 mL of minimal medium (MM) (Correll et al., 1987), supplemented with $FeSO_4$ (0, 10, 100, and 200 µM). The culture medium was inoculated with a 5-mm-diameter block of PDA with mycelia taken from the edge of a five-day-old culture. After inoculation, cultures were incubated at 28°C and rotated at 120 revolutions min^{-1} (r.p.m) for ten days (Edmund Bühler, Hechingen, Germany). Fungal mycelia were harvested by filtration using filter paper (113P, Ø 320 mm). The filter papers with mycelia were dried at 60°C for four days to determine fungal biomass, while culture filtrates were used to quantify extracellular siderophores produced by Foc using the SideroTec kit and following the manufacturer's protocol (Emergen Bio, Maynooth, Ireland).

5.3.4 Iron starvation assay

The experiment was conducted in 250-mL Erlenmeyer flasks containing 50 mL of MM with 10 µM $FeSO_4$. To mimic Fe starvation, the culture medium was supplemented with the synthetic iron chelator, 2,2'-dipyridyl (Breuer et al., 1995; Noguchi et al., 2020), while control cultures were supplemented with dimethyl sulfoxide (DMSO) (0.5% *v*/*v*). Unlike HBED and EDDHA, 2,2'-dipyridyl has lipophilic properties and therefore can sequester extracellular and intracellular Fe pools (Romeo et al., 2001). The stock solution of 2,2'-dipyridyl (1 mM) was prepared in DMSO and filter-sterilized (0.2 µm; Sartorius, Göttingen, Germany) before adding to the cultures. Cultures were incubated as described earlier, after which mycelia and culture filtrates were collected. Culture filtrates were immediately stored at -20°C, while mycelia samples were transferred to 50-mL Falcon tubes and washed with 30 mL of pre-chilled PBS

buffer, pH 7.4 (MP Biomedicals, Irvine, CA, USA) containing 0.01% (*v*/*v*) Triton X-100. Afterwards, mycelial pellets were snap frozen in liquid nitrogen and immediately stored at -80°C till lyophilization using the Lyovac GT2 lyophilizer (SRK-Systemtechnik, Riedstadt, Germany). Lyophilized mycelial samples (100 mg) were processed as described previously (Mohammadi and Kazemi, 2002) and used for analysis of ferrichrome, proteins, and amino acids profiling.

5.3.5 Protein quantification and amino acid profiling

Quantification of fungal soluble proteins was determined using the Bradford kit following the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). Absorbance was measured using a microplate reader (Tecan, Maennedorf, Switzerland). Profiling of the 21 proteinogenic amino acids and the non-proteinogenic amino acid, ornithine, was performed by liquid chromatography-mass spectrometry (LC-MS) using the Accela HPLC-LTQ Velos mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was performed with the AccQ-Tag column, 150 mm × 3.9 mm, 4 µm (Waters, Milford, MA, USA) using mobile phases of 10 mM ammonium formate with 2% (*v*/*v*) methanol, pH 6.3 (eluent A) and acetonitrile (solvent B) (Supplementary Table S5.1). The injection volume was 3 µL, the column flow rate was 500 µL min^{-1}, and the column temperature was set at 33 °C. The derivatised amino acid standards were measured with individual concentrations. The mass spectrometer was operated in the full scan mode with the range of *m*/*z* 110-630. Derivatised amino acids were identified as protonated molecular ions with individual *m*/*z* values (Supplementary Table S5.2). Quantification of amino acids was performed by linear regression of the corresponding peak areas using the Quanbrowser software (Thermo Fisher Scientific).

5.3.6 Analysis of ferrichrome

Ferrichrome was analysed by LC/ESI-MS using the Accela HPLC-LTQ Velos mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was performed using a Kinetex XB-C18 column, 150 × 4.6 mm, 2.6 µm (Phenomenex, Torrance, CA, USA). The eluents were 10 mM Ammonium formate buffer, pH 4.9 with 2% (*v*/*v*) methanol (eluent A) and acetonitrile with 0.2% (*v*/*v*) formic acid (eluent B) (Supplementary Table S5.3). The injection volume was 6 µL, the column flow rate was 500 µL min^{-1}, and the column oven temperature was set at 40 °C. Ferrichrome standards were measured with the concentration of 1, 10, and 100 μgL^{-1}. The mass spectrometer was operated in the full scan mode with the range of *m/z* 500-1000. Ferrichrome was identified as protonated sodium adduct ion with *m/z* 712 $(M+Na)^+$. Quantification of ferrichrome was performed by linear regression of the corresponding peak areas using the Quanbrowser software (Thermo Fisher Scientific).

5.3.7 Data analysis

Data analysis and visualisation were performed with R (v.4.0.2, R Development Core Team, 2020). Prior to statistical analysis, data was checked for normality and homoscedasticity. Homoscedasticity of the data was verified using Levene's test, whereas the normality the data was verified using the Shapiro-Wilk test and diagnostic plots. Comparisons were made between treatments and the control. Statistical significance was declared if $p<0.05$. Significance differences between treatments and the control were tested using analysis of variance (ANOVA) and subsequent post hoc analysis using Tukey's Honest Significant Difference (Tukey HSD) test or unpaired Welch's t-test. All data are expressed as mean ± standard error of the mean. The amino acid dataset was log_{10}-transformed and scaled using the Pareto scaling method (van den Berg et al., 2006) prior to rincipal component analysis (PCA).

5.4 Results

5.4.1 Iron bioavailability regulates biosynthesis of extracellular siderophores

In the iron-deficient medium, the fungal biomass of Foc was low but increased with iron supplementation ($p<0.05$) (Fig. 5.1A), demonstrating that iron is a limiting factor for the growth of Foc. Conversely, iron supplementation of the growth medium reduced siderophore production ($p<0.05$) (Fig. 5.1B).

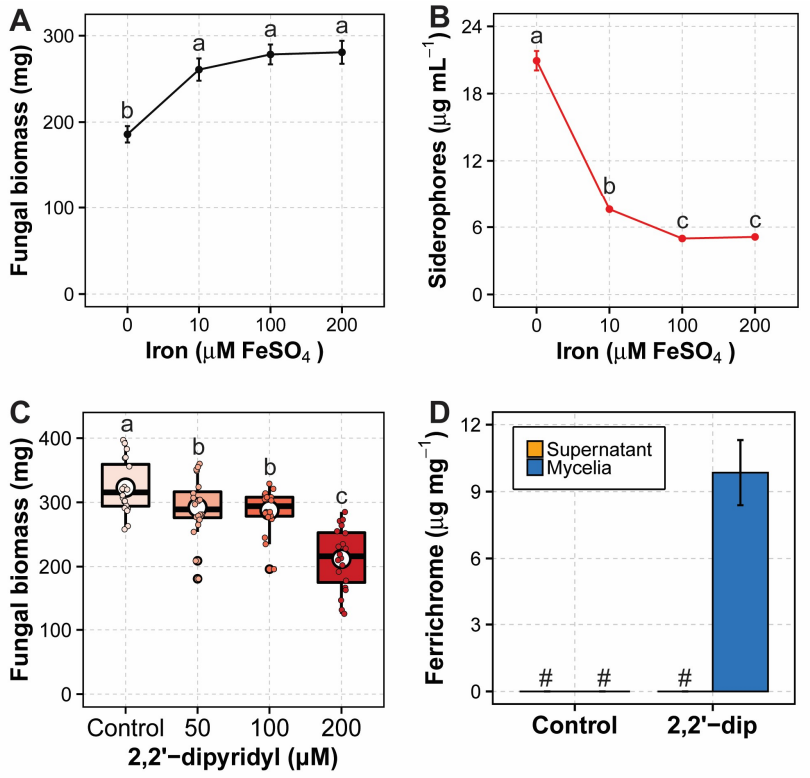

Figure 5.1. Fungal biomass (**A**) and extracellular siderophores (**B**) of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 in a growth medium containing increasing concentration of $FeSO_4$. Fungal biomass of Foc in cultures grown in culture medium containing 10 µM $FeSO_4$ and supplemented with increasing concentrations of the iron chelator 2,2'-dipyridyl to mimic iron starvation (**C**). The hydroxamate siderophore, ferrichrome, was detected only in mycelia of iron-starved Foc. # denotes ferrichrome not detected (**D**).

Siderophore concentration in the filtrate of iron-deficient cultures was 20.94 ± 0.87 µg mL^{-1}, but decreased three-fold, reaching 7.61 ± 0.11 µg mL^{-1} and 5.13 ± 0.09 µg mL^{-1} in the presence of 10 µM and 200 µM $FeSO_4$, respectively (Fig. 5.1B). Regression analysis revealed a negative correlation between the production of extracellular siderophores with the concentration of iron in the growth medium (R^2 = - 0.560, p=0.0004).

5.4.2 Iron starvation induces de novo biosynthesis of ferrichrome

To mimic iron starvation, the growth medium was supplemented with the iron chelator 2,2'-dipyridyl. Fungal biomass was reduced in Foc cultures supplemented with 200 µM of 2,2'-dipyridyl ($p<0.05$), but not in cultures supplemented with 50 µM and 100 µM of 2,2'-dipyridyl (Fig. 5.1C). Based on these findings, 2,2'-dipyridyl (100 µM) was selected to further investigate the effect of iron starvation on the biosynthesis of proteins, amino acids, and ferrichrome in Foc. LC-MS analysis detected ferrichrome in the mycelia of iron-starved cultures, but not in the filtrate (Fig. 5.1D). The concentration of ferrichrome in the mycelia of iron-starved cultures was 9.85 ± 1.46 µg mg^{-1}, whereas no ferrichrome was detected in either the mycelia or the filtrate of control cultures (Fig. 5.1D).

5.4.3 Iron starvation increases cellular arginine with a concomitant decrease in ornithine

The metabolomic signature of proteinogenic amino acids in iron-starved and control cultures was similar, except for arginine which increased ($p<0.05$) in iron-starved cultures compared to the control cultures (Fig. 5.2). The mycelia of iron-starved cultures contained 3.62 ± 0.45 µg mg^{-1} of arginine compared with 2.05 ± 0.36 µg mg^{-1} in the control (Fig. 5.2). The concentration of proteinogenic amino acids such as alanine, glutamine, and glutamate tended to increase in the mycelia of iron-starved cultures compared to the control cultures ($p>0.05$) (Fig. 5.2).

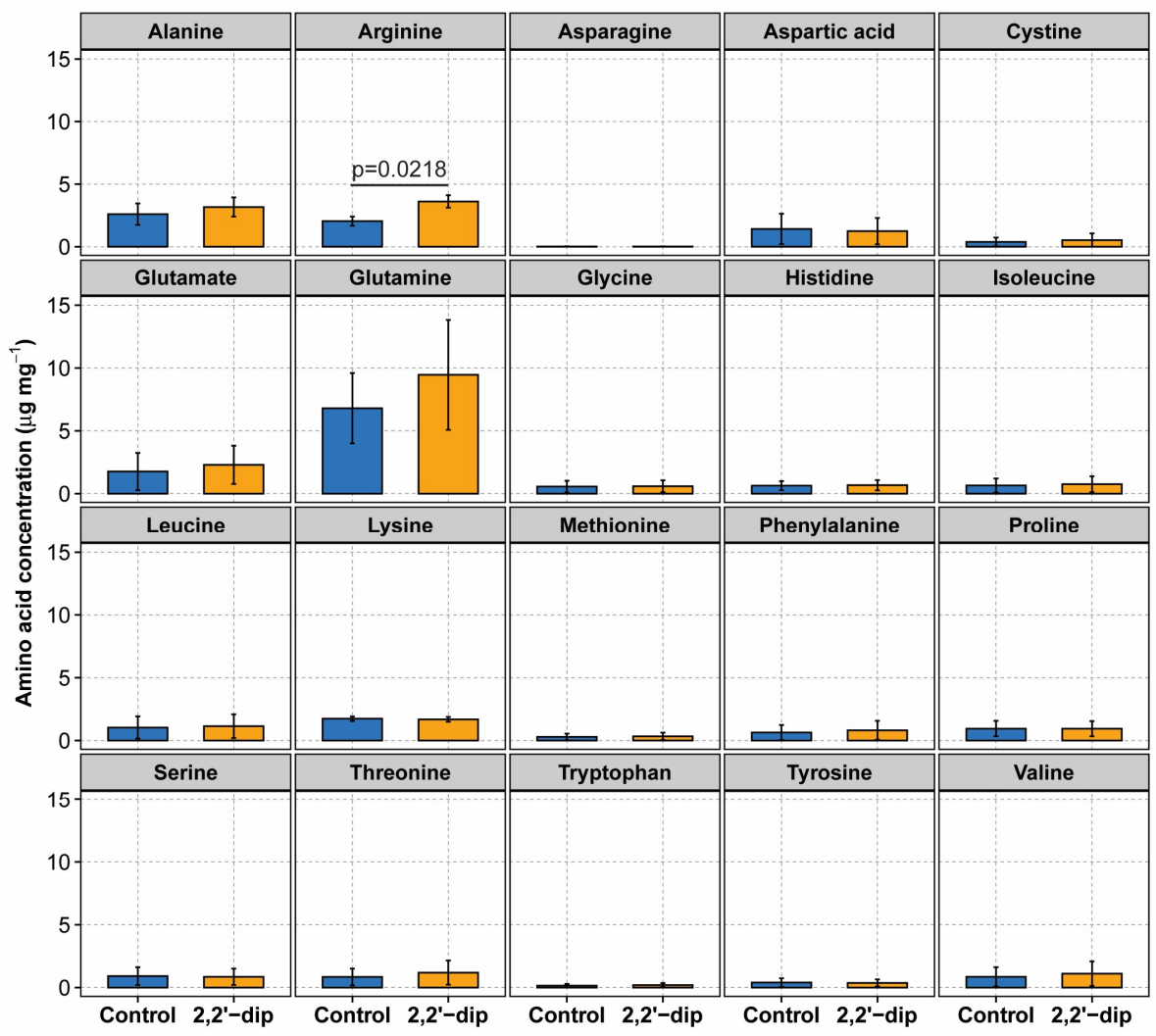

Figure 5.2. Metabolic fingerprint of proteinogenic amino acids in the mycelia of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 cultured in a growth medium containing 10 µM $FeSO_4$. The growth medium was supplemented with 100 µM 2,2'-dipyridyl (yellow bars) to mimic iron-starvation or controls with iron only (blue bars).

The PCA scores showed the first and second principal components, respectively, to account for 87.9% and 5.1% of variance in the data set, with no clear separation between the control and iron-starved groups (Fig. 5.3A). Arginine had a strong influence on PC1, while methionine, phenylalanine and tyrosine had a stronger influence on PC2 (Fig. 5.3B).

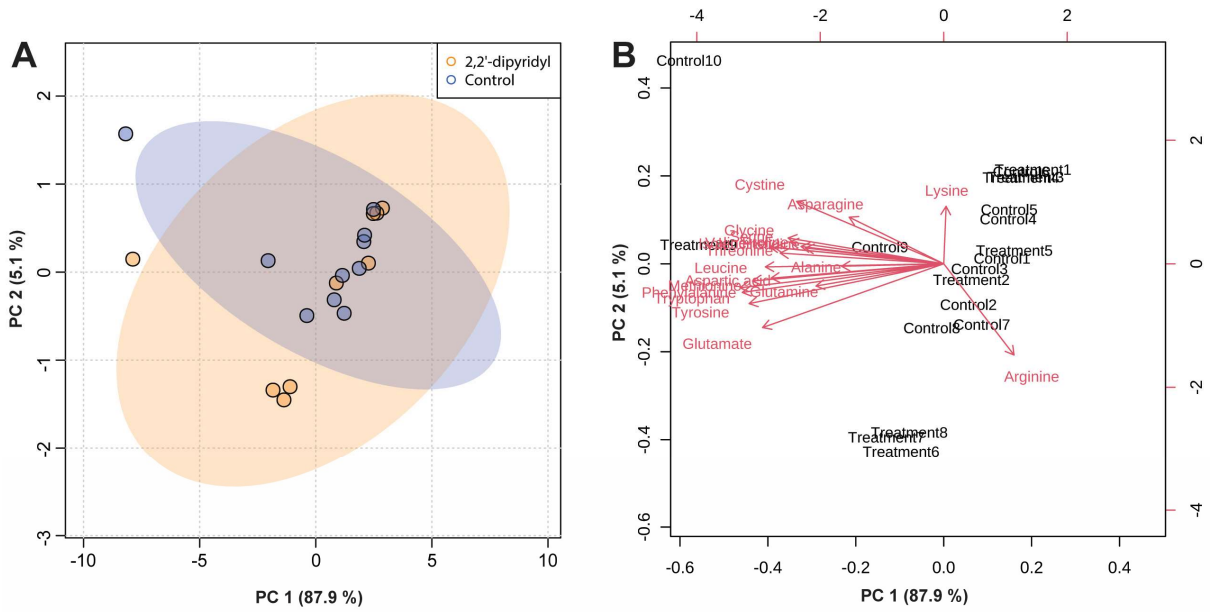

Figure 5.3. Score plot obtained from principal component analysis (PCA) of proteinogenic amino acids in mycelia of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 cultured in a growth medium containing iron (blue) only, or when supplemented with 2,2'-dipyridyl (yellow) to mimic iron-starvation. The percentage of variation of the data explained by PC1 and PC2 is in parentheses (87.9 and 5.1%, respectively) (**A**). The loadings in the PCA plot (biplot) illustrate the variables with largest effect on each sample, whereby the longer the vector, the more influence the variable has on the samples (**B**).

Protein concentration in the mycelia of iron-starved cultures decreased when compared with the control cultures ($p<0.05$) (Fig. 5.4A). The mycelial protein concentration of iron-starved cultures was 24.85 ± 0.55 µg mg^{-1}, compared to 28.15 ± 1.23 µg mg^{-1} in the mycelia of the control cultures (Fig. 5.4A). Ornithine, a non-proteinogenic amino acid, tended to decrease in the mycelia of iron-starved cultures though not different from the control cultures ($p>0.05$) (Fig. 5.4B). The mycelia of iron-starved cultures contained 2.28 ± 0.81 µg mg^{-1} of ornithine compared with 1.34 ± 0.56 µg mg^{-1}, whereas the concentration of ornithine in the mycelia of the controls was 1.34 ± 0.56 µg mg^{-1} (Fig. 5.4B).

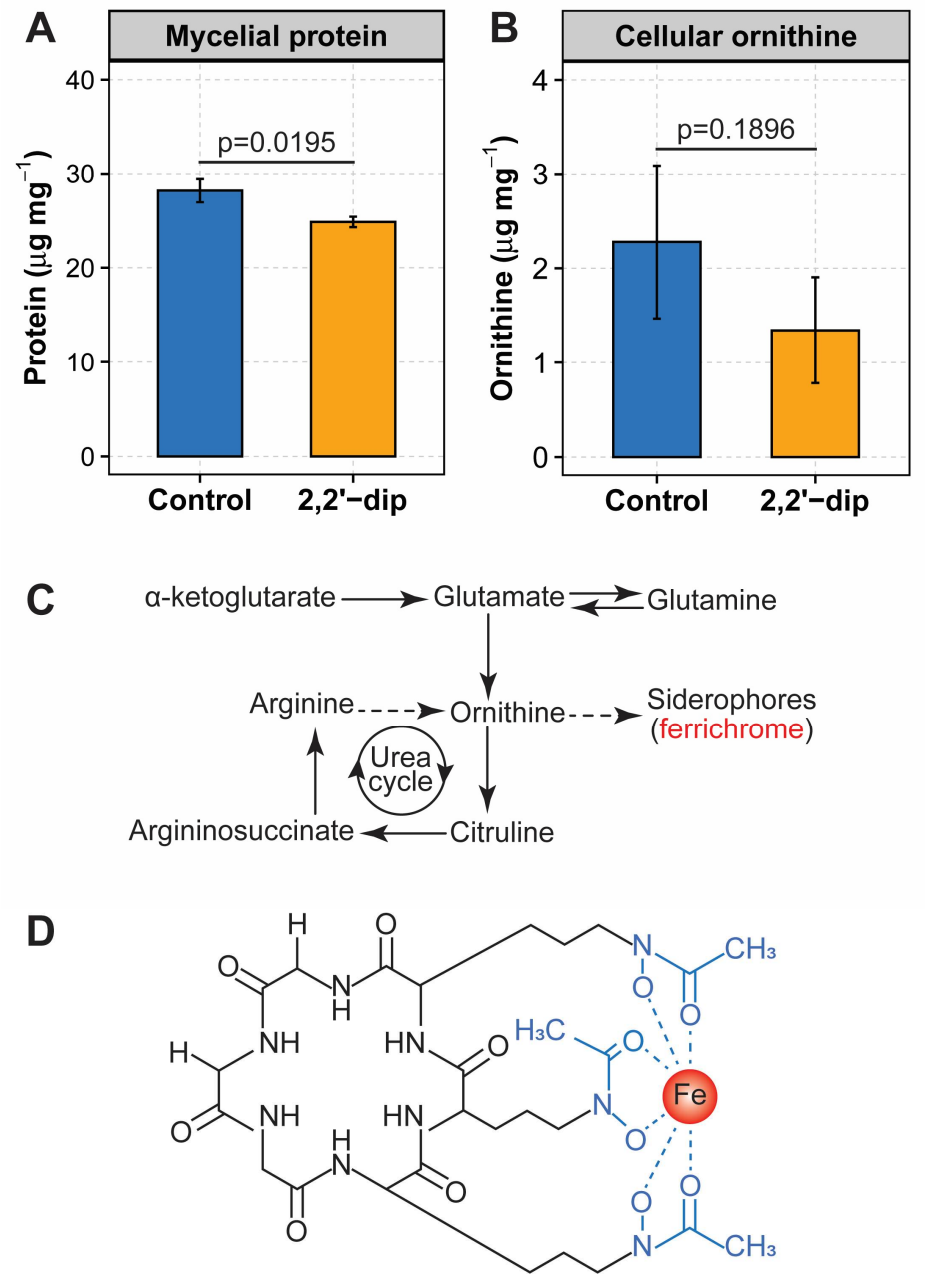

Figure 5.4. Siderophore biosynthesis in *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4. Concentration of total soluble protein in mycelia of Foc cultured in a growth medium containing 10 µM $FeSO_4$ and supplemented with 100 µM 2,2'-dipyridyl (yellow bars) to mimic iron-starvation or controls containing only 10 µM $FeSO_4$ (blue bars) (**A**). Concentration of the non-proteinogenic amino acid, ornithine, in the mycelia of Foc cultured in a growth medium containing 10 µM $FeSO_4$ and supplemented with 100 µM 2,2'-dipyridyl (yellow bars) to induce iron-starvation or controls containing only 10 µM $FeSO_4$ (blue bars) (**B**). Schematic illustrating the link between the siderophore biosynthesis pathway and the urea cycle siderophore-producing fungi. Ornithine, the precursor for siderophore biosynthesis is produced in the mitochondria from glutamate or from arginine in the cytoplasm. Arginine can also be synthesized from ornithine via citrulline in the urea cycle (**C**). Schematic illustrating the molecular structure of the hydroxamate siderophore, ferrichrome which consists of three residues of glycine and ornithine. The terminal nitrogen atoms of ornithine are oxidized to hydroxylamine, -NOH-, and combined with three acetyl groups, CH_3CO-, to yield the hydroxamate moieties (in blue) which chelate iron molecule (red circle) (**D**).

5.5 Discussion

In this study, we have demonstrated that Foc TR4 produces the hydroxamate siderophore, ferrichrome, in response to iron starvation. The accumulation of ferrichrome in the mycelia of iron-starved cultures suggests *de novo* biosynthesis. This is primarily deduced from the absence of ferrichrome in the controls, the increase in arginine, and a decrease in ornithine observed in the mycelia of iron-starved cultures. The decrease in ornithine may suggest that ornithine is withdrawn from the tricarboxylic acid cycle and used for the synthesis of siderophores including ferrichrome (Anke et al., 1973; Beckmann et al., 2013; Misslinger et al., 2021). Arginine, which serves as precursor for ornithine, can thus be regarded as an essential metabolite for the adaptation of Foc to iron starvation, which is similar to the case of *Aspergillus fumigatus* (Dietl et al., 2020).

The absence of ferrichrome in the filtrate of iron-starved cultures suggests that ferrichrome is an intracellular siderophore that serves for iron storage in Foc. This result contrasts the findings in other microorganisms such as *Schizosaccharomyces pombe* (Schrettl et al., 2004) and *Ustilago sphaerogena* (Budde and Leong, 1989), where ferrichrome was reported both in the mycelia and filtrate. The importance of ferrichrome in iron acquisition and storage is partly attributed to the three iron-binding moieties that strongly bind ferric iron, thereby making ferrichrome a super-binding molecule with a stability constant of $10^{29.07}$ (Hider and Kong, 2010). Although this study did not identify the siderophores present in the filtrate of iron-starved cultures of Foc TR4, siderophores such as fusigen and fusarinine were previously reported in the filtrate of Foc cultures (Diekmann and Zähner, 1967).

The concentration of extracellular siderophores in the culture filtrate declined with increasing concentrations of iron, suggesting that siderophore biosynthesis in Foc is tightly linked to iron bioavailability. This suggests that iron deficiency may trigger iron-sensing mechanisms (Gupta and Outten, 2020) in Foc, which, in turn, activate the biosynthesis of siderophores. However, the biosynthesis and secretion of siderophores may require a substantial investment of cellular resources such as ATP (Imperi et al., 2009). Thus, it would

be homeostatically prudent to repress siderophore biosynthesis once iron is abundant. Such a feedback mechanism would prevent a superfluous production of siderophores, but also spare cellular resources such as ATP, especially considering that ATP is synthesized via iron-consuming pathways (Mercier et al., 2008).

The reduction in protein concentration in the mycelia of iron-starved cultures may reflect a decline in protein synthesis, for instance, through the inhibition of translation. Protein synthesis is one of the cellular processes that depends on iron-containing enzymes (Shakoury-Elizeh et al., 2010). Protein synthesis could shift cellular iron into storage proteins, thereby reducing the amount of iron available for the synthesis of essential enzymes required for basic metabolic functions such the production of ATP (Mercier et al., 2008). Circumventing protein synthesis through the inhibition of translation is an iron-sparing mechanism that has been reported in eukaryotic cells and microorganisms such as *U. sphaerogena* (Ecker et al., 1982; Higashida et al., 2021). This could explain why most proteinogenic amino acids were not altered by iron starvation. This data further suggests that iron starvation does not cause a remodelling of amino acid metabolism in Foc TR4, except for arginine, which is required for biosynthesis of ornithine, the precursor for siderophore biosynthesis. The increase in siderophore biosynthesis in Foc TR4 under iron starvation would require that arginine is synthesized and subsequently converted to ornithine. Thus, it is plausible that the level of arginine increased under iron starvation. The findings in this study also raise the intriguing possibility that the cellular components that limit growth are amino acids, because depletion of one or a few individual amino acids would deter protein synthesis (Shakoury-Elizeh et al., 2010). Protein synthesis is carried out by ribosomes, which are a complex ribonucleoprotein assembly. In actively growing cells, biogenesis of ribosomes is a major energy and iron-consuming process that accounts for a significant proportion of total transcriptional output (Kispal et al., 2005).

5.6 Conclusion

The acquisition of iron by phytopathogens, such as Foc, is essential for survival and pathogenesis. This study provides evidence that Foc abundantly produces siderophores when challenged with iron starvation. Specifically, mycelia of iron-starved Foc accumulate the hydroxamate siderophore, ferrichrome. Thus, the siderophore biosynthesis may be a counteractive mechanism to evade iron starvation by Foc TR4. The empirical evidence so far on whether ferrichrome is essential for growth and pathogenicity of Foc under iron starvation is suggestive, and requires further investigations using reverse genetics. Moreover, the reduction in the level of ornithine observed under iron starvation suggests that the rate of arginine-to-ornithine conversion may be higher than the rate of ornithine-to-siderophore conversion, i.e. ornithine is replenished faster than it is used. Therefore, a time-course analysis of arginine, ornithine, and siderophores will be crucial in the future.

Author contributions

FR and AV conceived the project, sourced the funding and provided the resources. EW designed and performed the experiments, curated the data, conducted data analysis and visualisation, and drafted the original manuscript. EW and JS, performed metabolite analysis; FR, JS, and AV critically revised and gave further inputs to the final manuscript.

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Data availability statement

All data generated or analysed during this study are included in this article.

Declaration of competing interest

The authors declare no potential conflict of interest.

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6. General discussion and outlook

6.1 Overview

Globally, banana production is threatened by Fusarium wilt disease caused by the notorious soil-borne, root-infecting fungal pathogen, *Fusarium oxysporum* f. sp. *cubense* (Foc) (Ploetz, 2015; Dita et al., 2018; Viljoen et al., 2020). Three physiological races of Foc are pathogenic to banana, but Foc Tropical Race 4 is considered the virulent and devastating race of Foc (Ploetz, 2015). Fusarium wilt can be effectively managed by the use of genetically resistant banana cultivars with commercially acceptable or desirable fruit and agronomic traits (Dadzie, 1998; Smith et al., 2014; Hwang and Ko, 2004; Dita et al., 2018; Viljoen et al., 2020). However, such cultivars are currently not available for management of Foc TR4 (Dita et al., 2018; Viljoen et al., 2020). Management of Foc TR4 largely relies on cultural practices and containment measures aimed at reducing the inoculum load in soil and control of pathogen spread (Dita et al., 2018; Viljoen et al., 2020). However, these measures are have failed to contain the disease, as evidenced by the inexorable spread and devastating impact of Foc TR4 (Pegg et al., 2019; Drenth and Kema, 2021). Therefore, understanding the ecology and biology of Foc is necessary and represents a continued focus of research for fine-tuning or development of strategies for effective management of Foc TR4 (Dita et al., 2018).

Infection of host roots and disease severity are influenced by complex below-ground interactions between the plant, the soil physical-chemical environment, and microorganisms in the root-soil interface (rhizosphere) (Dita et al., 2018; Viljoen et al., 2020). Accordingly, previous studies have shown that manipulation of the banana rhizosphere, for instance through legume intercropping or application of synthetic iron chelators, can suppress Fusarium wilt and reduce the abundance and/or efficacy of inoculum (Peng et al., 1999; Pattison et al., 2014; Zuo, et al., 2015). Integrating rhizosphere manipulation with existing disease management strategies could provide a prospect for better management of Fusarium

wilt. However, the prospect of rhizosphere manipulation has been compromised largely by inconsistent efficacy in the field due to the complexity of banana below-ground interactions (Peng et al., 1999; Aguilar et al., 2000; Thangavelu & Mustaffa 2010; Wibowo et al., 2013; Orr & Nelson, 2018; Sasse et al., 2018; Orr et al., 2021; Gregory et al., 2022). Understanding the mechanisms of banana below-ground interactions and the physiological response of Foc to perturbations, such as iron starvation that arise through rhizosphere manipulation, is thus key for harnessing the potential of rhizosphere manipulation.

This doctoral thesis contributes to the fundamental ecological understanding of banana below-ground interactions related to Fusarium wilt. First, this doctoral thesis analysed literature from four electronic databases (AGRIS, CAB Direct, SciVerse Scopus, ProQuest) to bring together the relatively scant data available on banana below-ground interactions in relation to Fusarium wilt and to highlight the key knowledge gaps for progressive research (Chapter 2). In Chapter 3, the study underpins phenolic acids from legume cover crop roots as root-secreted metabolites that could suppress Foc TR4 by non-host plants. The roles of pH and iron in the process of Foc chlamydospore germination were then determined *in vitro* (Chapter 4) confirming that iron is essential for growth of Foc TR4. Given the importance of iron to Foc TR4, the production of iron-sequestering microbial secondary metabolites, called siderophores, was assessed as a potential counteractive mechanism for Foc TR4 to evade iron starvation (Chapter 5).

Chapter 6 of this doctoral thesis determines if the evidence generated through the different research chapters is consistent with the study hypotheses proposed in Chapter 1. In doing so, this chapter brings together the lessons learned with respect to the research objectives (section 6.2) and collectively discusses the findings in a wider context (section 6.2 - 6.5). Section 6.6 draws conclusions and provides suggestions for progressive research. The effect of pH on the process of chlamydospore germination, and the *de novo* synthesis of the hydroxamate siderophore, ferrichrome by Foc TR4 in response to iron starvation, were not described before. Therefore, this study is novel and provides empirical evidence that extends the fundamental understanding of the biology and ecology of Foc TR4. Supported by an

extensive review of literature on banana below-ground interactions, this doctoral thesis provides information that could help to realize the potential of rhizosphere manipulation for management of Foc TR4.

6.2 Answers to the research hypotheses

The findings from the different chapters of this doctoral thesis support the hypotheses advanced for objectives 2 and 3, whereas a mixed picture is obtained for objective 1. The respective guiding hypotheses for this research were:

i. The root metabolome of *D. uncinatum* and *M. pruriens* contains phenolic compounds with antimicrobial activity that suppress Foc TR4 by inhibition of pathogen development.
ii. Iron is essential for DNA synthesis in germinating Foc TR4 chlamydospores and scarcity leads to selective optimization of ribonucleotide reductase function by enhanced expression of *rnr1* and *rnr2* genes.
iii. Iron starvation triggers a compensatory increase in siderophore biosynthesis in Foc TR4 to scavenge iron. This counteractive response requires arginine that is synthesized and subsequently converted to ornithine, the biogenic precursor of siderophores.

In agreement with hypothesis (i), crude root exudates of *D. uncinatum* and *M. pruriens* were cytotoxic to Foc TR4 and suppressed mycelial growth *in vitro* (Chapter 3). The toxicity was attributed to the three phenolic acids specifically benzoic, *t*-cinnamic, and *p*-hydroxybenzoic acids, which were consistently common in root exudates of both legumes. However, with exception of benzoic acid, *t*-cinnamic and *p*-hydroxybenzoic were inhibitory at high concentrations. All phenolics significantly suppressed fusaric acid biosynthesis in Foc TR4 at all concentrations tested, whereas low concentrations stimulated the production of beauvericin (Fig. 3.3, Chapter 3). In agreement with hypothesis (ii), iron-starved chlamydospores stalled

at the onset of outgrowth and exhibited polarized-growth arrest characterized by the inability to form a germ tube and reduced metabolic activity (Fig. 4.3, Chapter 4). Moreover, iron-starved chlamydospores exhibited up-regulated expression of *rnr2,* the gene that encodes the small subunit of ribonucleotide reductase (RNR), a key enzyme that regulates cell growth by control of DNA synthesis through the synthesis of dNTPs (Fig. 4.4, Chapter 4). Evidence generated through *in vitro* assays (Chapter 5) are in support of hypothesis (iii), that siderophore production by Foc TR4 may be a counteractive mechanism to evade iron starvation through iron scavenging and storage.

6.3 Root-exuded phenolics contribute to suppression of Foc TR4 by non-host plants

Root exudates of *D. uncinatum* and *M. pruriens* exhibited cytotoxicity against Foc TR4 (Chapter 3). This effect was, in part, attributed to phenolic acids in the root exudates of both legumes. This finding suggests that exudation of antimicrobial metabolites, such as phenolic acids by roots of non-host plants, such as *D. uncinatum* and *M. pruriens*, can potentially suppress Foc TR4 in banana production systems, where such plants are included as rotation crops or intercrops. Crop rotation and intercropping can contribute to a reduction in pathogen inoculum by suppression of the pathogen or by providing an interval without an opportunity for pathogen reproduction (Scott et al. 2012; Ampt et al., 2022).

In Chapter 3, phenolic acids detected in root exudates of the legumes *D. uncinatum* and *M. pruriens* suppressed fusaric acid production in Foc TR4. This finding is novel and interesting, because low concentrations of phenolics did not inhibit fungal growth but strongly inhibited fusaric acid production at all concentrations tested (Fig. 3.3, Chapter 3). It will be interesting to determine the concentration levels of the phenolic acids in soil under field conditions. The mechanism for suppression of fusaric acid production by phenolics remains unclear, but could be linked to dissipation of the intracellular pH in the fungus (Fig. 3.3D, Chapter 3). These findings suggest that phenolics in root exudates of *D. uncinatum* and *M. pruriens* may interfere with the fitness of Foc TR4 through suppression of fusaric acid

production. Besides its role as virulence factor, fusaric acid is a potent non-specific toxin that kills competing soil microorganisms and therefore plays an important role in the ecological fitness of plant pathogenic *F. oxysporum*. For example, Notz et al. (2002) reported that fusaric acid down-regulates the production of the antibiotic compound DAPG, a key factor in the antagonistic activity of the biological control agent, *Pseudomonas fluorescens* CHA0 against this *F. oxysporum*.

Majority of world's banana is produced on subsistence farms, characterized by small plots, cultivar mixtures, or intercropping with other perennial and annual crops (Bellamy, 2013; Karangwa et al., 2016; Kimunye et al., 2020). Moreover, many farms are characterized by minimal or no inputs and intensively or continuously cultivated, which can hasten land degradation and reduce yield (Blomme et al., 2020). From an agro-ecological perspective, there are concomitant benefits to incorporation of legumes in banana production systems. Legume crops fix atmospheric nitrogen (N_2), which could minimize the need for synthetic fertilizer inputs, while improving soil health and reducing costs for production. Moreover, grain legumes, such as cowpea and beans, could help build resilience to abiotic and biotic stresses (including pests and diseases) and provide income streams and ecosystem services (Gogoi et al., 2018; Ocimati et al., 2019; Blomme et al., 2022).

6.4 Physiology of chlamydospore germination in Foc TR4

Chlamydospores (Greek = mantle) are viable, asexually produced accessory spores, resulting from the structural modification of vegetative hyphal segments or conidial cells (Ohara and Tsuge, 2004). Chlamydospores possess a thick wall mainly consisting of newly synthesized cell wall material (Griffiths, 1974; Ohara and Tsuge, 2004). The primary function of chlamydospores is survival in the soil (Griffiths, 1974; Ohara and Tsuge, 2004). Chlamydospores are the source of primary inoculum of Foc (Rishbeth, 1957). Chlamydospores can remain quiescent in Foc-infested soil until activated by nutrients; if these nutrients are from a susceptible host plant, then the pathogen is predisposed to attack the

plant. Thus, root exudates from the host are key in the success or failure of the infection. The mechanisms and conditions that play important roles in the formation, quiescence and germination of chlamydospores in Foc are largely unknown. Such information will be essential for biological control, for instance, through interfering with the life cycle of Foc, in part by targeting inoculum.

Spore germination is considered the first step in the formation of a fungal colony and a crucial step in the infection of a host (Sephton-Clark and Voelz*,* 2018). Chapter 4 of this doctoral thesis investigated the role of iron and pH in the process of chlamydospore germination in Foc TR4 *in vitro*. Chlamydospores exhibited plasticity with pH, i.e. more than 50% germination was noted over a wide pH range of 3 to 11. These findings are in contrast to previous suggestions that acidic soils promote diseases by stimulating chlamydospore germination due to increased iron bioavailability in acidic soils (Domínguez et al., 1996; Orr and Nelson, 2018). The findings of this doctoral thesis confirm that iron bioavailability is essential for chlamydospore germination (Peng et al., 1999) and suggest that pH-mediated reduction of disease may be attributed to effect of pH on other factors, such as host root physiology, the physiology of the pathogen, modulation of the microbiome structure and function as well as the bioavailability of nutrients. For example, actinomycetes and bacterial populations are favoured by high soil pH (Rousk et al., 2009; Wu et al., 2017). Therefore, alkaline soils may promote populations of antagonistic bacteria, including actinomycetes, which have been shown to suppress Foc via diverse mechanisms, such as toxin production, and competition for nutrients (Bubici et al., 2019; Zou et al., 2021; Chen et al., 2022).

Microorganisms capable of growing over a wide pH range require (i) a versatile, efficient pH homeostatic mechanism to protect intracellular processes from extremes of pH and (ii) a means of ensuring that activities undertaken beyond the boundaries of pH homeostasis are only attempted at appropriate ambient pH (Tilburn et al., 1995). Although the pH homeostatic mechanisms of Foc TR4 are largely unknown, studies related filamentous fungi show that survival over such a wide pH range requires the secretion of different sets of enzymes at different ambient pH values, so that their pH optima matches that of the environment. This

phenomenon has been documented in *Aspergillus nidulans*, which secretes acid phosphatases under acidic conditions and alkaline phosphatases in alkaline environments (Nozawa et al., 2003). Interestingly, similar regulatory processes have been reported in other fungi, such as *A. niger* (MacRae et al., 1988), *Botrytis cinerea* (Manteau et al., 2003), *Saccharomyces cerevisiae* (Li and Mitchell, 1997), and *F. oxysporum* (Caracuel et al., 2003).

Fusarium oxysporum relies on the highly conserved zinc finger transcription factor PacC and the PalH/Rim signalling pathway to sense and respond to extracellular pH (Caracuel et al., 2003). At alkaline ambient pH, proteolytic processing of the full-length PacC polypeptide results in a shorter, functional form, which activates genes preferentially expressed at alkaline pH and represses genes expressed under acidic conditions (Caracuel et al., 2003). Genes, whose expression is controlled by PacC, relate to extracellular enzymes, components of secondary metabolite biosynthetic pathways and proteins involved in cell wall biosynthesis (Gielkens et al., 1999; Caracuel et al., 2003).

6.5 Iron homeostasis and evasion of iron starvation in Foc TR4

Suppression of Fusarium wilt by manipulation of iron bioavailability in the banana rhizosphere (Peng et al., 1999) is premised on the previous study by Domínguez et al. (1996), which reported that severity of Fusarium wilt was high in soils where iron bioavailability was high in Canary Islands. Suppression of Fusarium wilt by soil application of synthetic iron chelators with a high stability constant (K), such as HBED ($K = 10^{39.7}$) and EDDHA ($K = 10^{33.9}$), which can both sequester iron, and thereby limit iron bioavailability to the pathogen, was investigated in a pot experiment (Peng et al., 1999) and a field study (Orr et al., 2021). In a pot trial, EDDHA reduced germination of Foc chlamydospores by one-third to one-half and disease severity (Peng et al., 1999). In the field, however, no decrease in the severity of Fusarium wilt was observed with the application of HBED and EDDHA to Foc-infested soils (Orr et al., 2021), suggesting that the iron nutrition of Foc is not effectively altered by chelators.

The findings in Chapter 4 and Chapter 5 verify that iron starvation can lead to suppression of chlamydospore germination and mycelial growth of Foc TR4, which is consistent with the report of Peng et al. (1999), and highlights the importance of iron in the life cycle of Foc. Chapter 5 investigated the production of siderophores, as a potential counteractive mechanism by which Foc TR4 can subvert iron chelators, and thereby evade iron starvation. The production of extracellular siderophores declined with increasing iron bioavailability, which suggests that siderophore biosynthesis in Foc TR4 is tightly regulated by iron bioavailability. Moreover, the hydroxamate siderophore, ferrichrome, was only detected in the mycelia of iron-starved cultures, which suggests *de novo* synthesis (Fig. 5.1, Chapter 5). Siderophore synthesis was followed by a concomitant increase in arginine and a subsequent reduction in ornithine levels in the mycelia of iron-starved cultures. The increase in siderophore biosynthesis in Foc TR4 under iron starvation would require that arginine is synthesized and subsequently converted to ornithine, the precursor for siderophore biosynthesis (Hider and Kong, 2010). Thus, it is plausible that the level of arginine increased under iron starvation.

The mechanism of siderophore-mediated iron acquisition in Foc TR4 is largely unknown. Generally, three mechanisms for siderophore-mediated iron acquisition have been described in model fungi, such as *A. niger* and *S. cerevisiae* (Hilder and Kong, 2010): (i) a shuttle mechanism, where when the iron-siderophore complex enters the cell, iron is offloaded, and the siderophore exits the cell to capture more iron (Emery, 1971); (ii) intracellular hydrolysis of the siderophore after removal of iron without recycling the siderophore; (iii) a taxicab mechanism, where iron enters the cell, whereas the siderophore (the taxi) remains extracellular (Moore et al., 2003). Besides their role in iron scavenging and storage, microbial siderophores have also been reported to play a role in fungal virulence. For example, entomopathogenic fungi from the genus *Metarhizium* produce both extracellular and intracellular cyclopeptide siderophores for iron sequestration and acquisition (Donzelli and Krasnoff, 2016). Compellingly, the intracellular siderophore, ferricrocin, but not the extracellular siderophore metachelin, is required for full virulence (Donzelli and Krasnoff,

2016). While the role of siderophores in host infection by *F. oxysporum* is not clearly established, López-Berges et al. (2012) demonstrated that the regulator of iron homeostasis, HapX is required for virulence on mice and on tomato plants. Moreover, HapX-regulated iron homeostasis is key for rhizosphere competence in *F. oxysporum* (López-Berges et al., 2012). Thus, it will be interesting to establish the role of ferrichrome in the rhizosphere competence and virulence of Foc TR4.

The empirical evidence in Chapter 5 suggests that siderophore biosynthesis could enhance the ecological fitness of Foc TR4, whereby extracellular siderophores scavenge iron from iron-limited environments, particularly the rhizosphere. Competition for iron in the rhizosphere is one of the key mechanisms of disease suppression by rhizosphere microorganisms and biological control agents (Robin et al., 2008; Colombo et al., 2014; Gu et al., 2020; Jarmusch et al., 2021). The ability of Foc TR4 to abundantly produce siderophores in the absence of iron (Fig. 5.1B, Chapter 5) suggests that the pathogen could be well adapted to compete for iron in the rhizosphere, where iron bioavailability is generally limited (~10^{-18} M), and well below the optimal requirement for plants (10^{-9} to 10^{-4} M) and microorganisms (10^{-7} to 10^{-5} M) (Robin et al., 2008; Colombo et al., 2014). Moreover, these findings suggest that siderophore production could be a counteractive mechanism by which the pathogen can subvert iron chelators, and thereby evade iron starvation. Collectively, the findings in Chapter 4 and Chapter 5 could, for instance guide in the selection of biological control agents and microbial consortia that are capable of producing higher affinity siderophores than Foc TR4. Such microorganism could compete for iron with Foc TR4, and thereby suppress the pathogen. For example, bacteria of the *Enterobacteriaceae* family, such as *Serratia* spp., produce high affinity siderophores, including enterobactin, which has a higher binding constant ($K = 10^{52}$) compared to ferichrome ($K = 10^{29.07}$) (Hider and Kong, 2010). Moreover, these findings could guide development of genetically resistant banana plants using RNA interference (RNAi) technology, such as host-induced gene silencing (HIGS) (Ghag, 2017; Lata et al., 2022) to thwart siderophore biosynthesis in Foc TR4.

6.6 Implications of banana below-ground interactions for management of Fusarium wilt TR4

Conflicting observations on the effect of fertilizer, pH, soil amendments, and plant nutrition on Fusarium wilt are frequently encountered in the literature (Chapter 2). This is not surprising in view of the intricacy of the Foc-host-soil complex (Fig. 6.1).

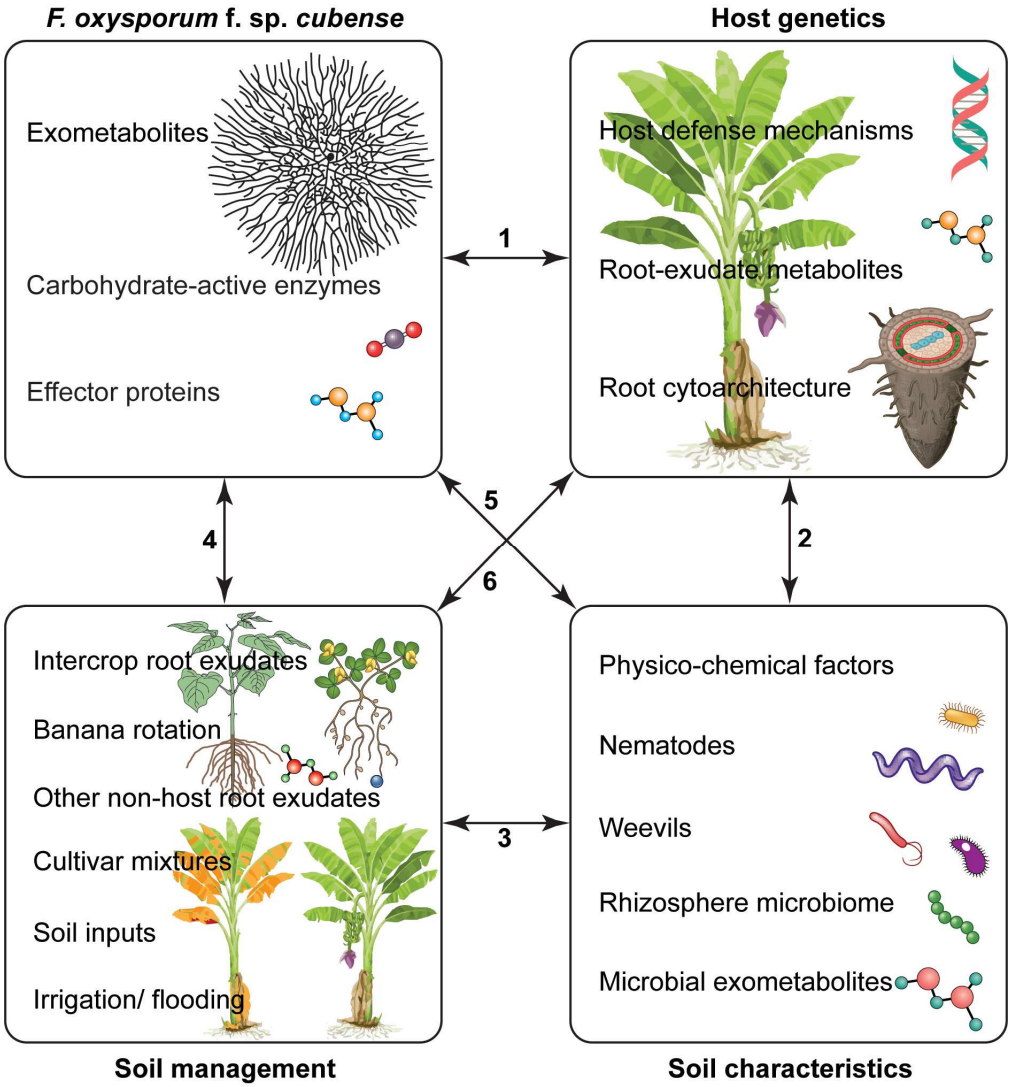

Figure 6.1. Schematic illustration of the perceived web of interactions (1 - 6) between the components of the banana rhizosphere in relation to Fusarium wilt disease caused by *Fusarium oxysporum* f. sp. *cubense* (*Source:* Evans Were).

In some field and greenhouse trials, soil nutrient management contributed to the development of Fusarium wilt (Chapter 2). Similarly, the effect of potassium, phosphorus and nitrogen fertilizers, in various forms, has been evaluated for the impact of disease incidence, but trends remain elusive. For instance, some studies have reported that the effect of nitrogen fertilizers on disease severity is a function of the nitrogen fertilizer rate, but not form (Orr et al, 2022). In contrast, other studies have attributed the severity of Fusarium wilt to the form of available nitrogen and the pH of the soil (Pittaway et al., 1999; Mur et al., 2017; Segura-Mena et al., 2021).

The effect of nitrogen source appears to be three-fold: first, increased, N applications change microbial activities by altering bacterial and fungal communities (Paungfoo-Lonhienne et al., 2015; Yeoh et al., 2016). Second, some observed effects appear only to be related to the rate of plant growth and not disease expression (Chapter 2). Third, NO_3-N increases rhizosphere pH, whereas NH_4-N decreases the rhizosphere pH (Chapter 2). In Chapter 3, the production of fusaric acid was repressed at pH 5, and stimulated at pH 7 (Fig. 3.3, Chapter 3). This finding suggests that soil physico-chemical factors associated with soil pH could profoundly influence pathogen fitness by enhancing production of virulence factors including phytotoxins such as fusaric acid. Besides fertilizers, the use of soil amendments or use of BCA (Chapter 2) can reduce the abundance and/or efficacy of inoculum, or enhance the disease-suppressiveness of soils (Dita et al., 2018). Recognizing the conditions, which reduce the efficacy of BCA, is a key step toward improving the efficacy and performance of BCA in the field (Chapter 2). Moreover, the variable efficacy of biological control.

Understanding the soil moisture dynamics is crucial for disease management, for example in irrigation systems. Foc can grow under anaerobic conditions provided that yeast extract, MnO_2, nitrate, selenite or ferric ions are present (Gunner and Alexander, 1964). Survival under low oxygen tensions is presumably facilitated by the capacity of *F. oxysporum* to utilize both nitrate (Shoun and Tanimoto, 1991) and elemental sulphur (Abe et al., 2007) as terminal electron acceptors to support anaerobic respiration. The stages of growth, decline, or resting of a Foc population in soil depend on the ecological balance and nutrient availability

(Chapter 2). The most critical limitation is chemical energy and the competitive use of the energy sources present in the soil in competition with the rhizosphere microbiome. The mechanisms of disease control by competitive microorganisms seem to be mainly due to their depletion of available carbon sources, but also to antibiotic production (Chapter 2). Thus, systems for management of Fusarium wilt should include an analysis of competition by soil microbiome and fauna as well as methods of favouring competition.

As an auxotroph, *F. oxysporum* requires a carbon source for structure and energy and twelve inorganic compounds including carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, magnesium, sulphur, iron, manganese, molybdenum, and zinc (Chapter 2). Copper has not been shown to be indispensable. Chloride has not been shown to be essential to *F. oxysporum*, but may benefit disease-producing functions in the fungus. Chloride activates a number of pectolytic and amylolytic enzymes (Aghajari et al., 2002). The amounts required are of considerable magnitude and are not in the trace element category. These nutrients are absolute requirements, which, if unsatisfied, will limit growth, sporulation, and survival of the fungus.

The epidemiological outcome of Fusarium wilt is collectively determined by the inherent relationships between plant and Foc and how various environmental factors affect the banana-Foc interactions (Fig. 6.1). These complex interactions and seasonal dynamics of disease can be described by the disease triangle model (Chapter 1, section 1.3), which remains a core principal of plant pathology. Thus, for effective management of Fusarium wilt, a multifaceted systems approach for suppression of Fusarium wilt of banana is needed to maintain field longevity and productivity. The available strategies for disease suppression (Chapter 2) need to be accompanied by proper selection of banana cultivars. Moreover, manipulation of plant nutrition as a means to increase host resistance has shown to be effective (Chapter 2) should be further evaluated in the field. Altering the host and environment through methods to enhance colonization by beneficial microorganisms, such as arbuscular mycorrhizal fungi, along with with incorporation of biochar has great promise in disease management (Mohandas et al., 2010; Pandit, et al., 2020).

Overall, there is increasing evidence that banana below-ground interactions play an important role in host infection and disease severity, and that rhizosphere manipulation has potential for suppression of Fusarium. However, there are still considerable gaps in our understanding of the mechanisms by which banana below-ground interactions collectively influence the rhizosphere ecology of Foc. These knowledge gaps may be bridged by considering a systems approach. Banana plant genotype, pathogen race, banana-associated microbial communities, and soil management practices could be considered as the key components of such a system. Moreover, objectives should be listed as well as the order of priorities on the basis of the disease status of the farm. Components and areas of the system should be subject to research in sequence to maximize productivity on a fundamental or practical basis. Priority should be decided on the basis of the importance of existing problems, the state of development of methodology, and probable ease of solution. Altogether, this doctoral thesis extends the fundamental understanding of the biology and ecology of Foc and provides a base for realizing the potential of rhizosphere manipulation for management of Foc TR4.

6.7 References

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Supplementary material

Table S3.1. Chromatography gradient for analysis of phenolic acids

Time (min)	Mobile Phase (B)%	Mobile Phase (C)%
0	90	10
1	90	10
12	40	60
13	10	90
15	10	90
15	90	10
17	90	10

10 mM Formate buffer (pH 3.7) (B)
Acetonitrile (C)

Table S3.2. Chromatography gradient for analysis of beauvericin

Time (min)	Mobile Phase (B)%	Mobile Phase (C)%
0	50	50
2	50	10
12	0	100
14	0	100
15	0	100
15	50	50
17	50	50

0.2% (*v*/*v*) formic acid (eluent B)
Acetonitrile with 0.2% (*v*/*v*) formic acid (eluent C)

Table S3.3. Chromatography gradient for analysis of fusaric acid

Time (min)	Mobile Phase (B)%	Mobile Phase (C)%
0	95	5
1	95	5
11	10	90
13	10	90
13	95	5
16	95	5

0.2% (*v*/*v*) formic acid in 10% acetonitrile (eluent B)
0.2% (*v*/*v*) formic acid (eluent C)

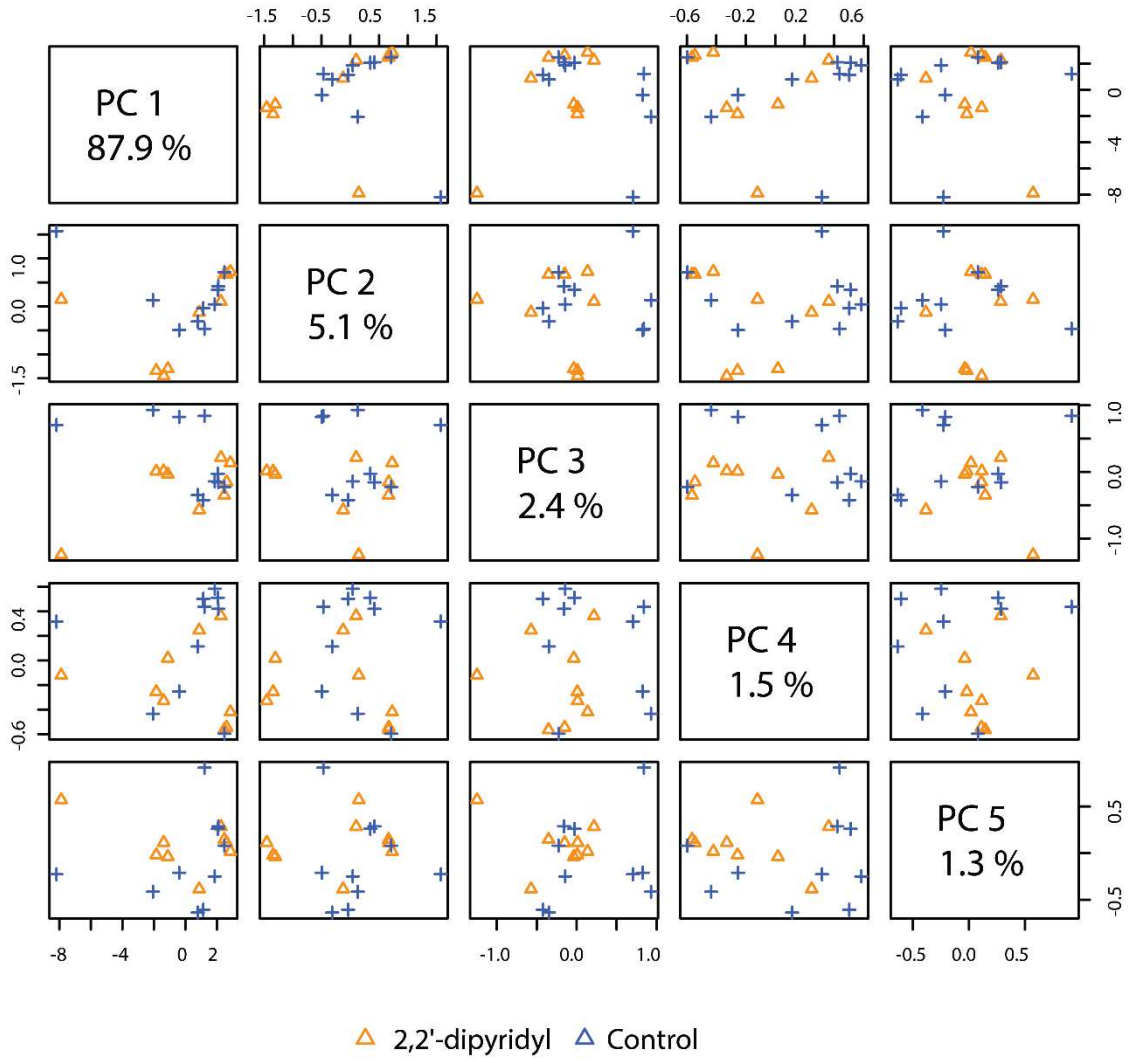

Figure S5.1. Matrix scatter plot obtained from principal component analysis (PCA) of the amino acid profiles in cultures of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) cultures cultured in iron-sufficient and iron starvation conditions.

Table S5.1. Chromatography gradient for analysis of amino acids

Time (min)	Mobile Phase (A)%	Mobile Phase (B)%
0	95	5
3	95	5
3.1	90	10
8	90	10
13	70	30
16	20	80
17	10	90
17.1	95	5
19.5	95	5

10 mM Ammonium formate buffer, pH 6.3 with 2% (*v*/*v*) methanol (A)
Acetonitrile with 0.2% (*v*/*v*) formic acid (B)

Table S5.2. The charge-to-mass ratio (*m*/*z*) and retention time of amino acids

Amino Acid	*m*/*z* of protonated amino acid derivate (M^+)	Retention time (min)
Aspartic Acid	304	2.35
Alanine	260	7.76
Arginine	345	9.4
Asparagine	303	6.3
Cystine	581	7
Glutamic Acid	318	2.6
Glutamine	317	6.5
Glycine	246	6.6
Histidine	326	7.3
Isoleucine	302	13.55
Leucine	302	13.75
Lysine	317	16.8
Methionine	320	12.9
Ornithine	473	13.6
Phenylalanine	336	14.1
Proline	286	8.83
Serine	276	6.35
Threonine	290	7.35
Tryptophan	375	14.2
Tyrosine	352	12.3
Valine	288	12.6

Table S5.3. Chromatography gradient for analysis of ferrichrome

Time (min)	Mobile Phase (A)%	Mobile Phase (B)%
0	98	2
3	98	2
10	80	20
12	10	90
14	10	90
15	98	2
17	98	2

10 mM Ammonium formate buffer, pH 4.9 with 2% (*v*/*v*) methanol (A)

Acetonitrile with 0.2% (*v*/*v*) formic acid(B)

Appendices

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

Mechanistic aspects of the eco-physiology of *Fusarium oxysporum* f. sp. *cubense* TR4

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Stuttgart, 23.5.2022

Place, Date

Signature

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2013.08 – 2016.06	MSc. Molecular Biology, Makerere University, Kampala, Uganda
2010.08 – 2012.05	BSc. Biological Sciences, Kyambogo University, Kampala, Uganda
2007.08 – 2009.05	Diploma, Biological Sciences, Kyambogo University, Kampala, Uganda

RESEARCH EXPERIENCE

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Technical Assistant (T/A) Researcher
Hans-Ruthenberg Institute (490e), University of Hohenheim, Germany
Supervised a molecular biology laboratory and coordinated the planning and execution of research projects for Ph.D. and MSc students. Assessed and procured consumables required for research projects.

2009.11 – 2017.10

Research assistant (Plant Pathology)
International Institute of Tropical Agriculture (IITA), Uganda
Supervised a microbiology laboratory, and coordinated the planning and execution of laboratory, greenhouse, and field experiments. Lead studies to isolate, purify, preserve and characterize plant pathogenic bacteria, fungi and viruses using DNA, RNA such as PCR and ELISA techniques. Developed and validated methods for analysis for isolation of plant pathogenic microorganisms. Curated and analysed data and presented research findings in seminars and authored journal articles. Trained students and guest researchers on molecular plant pathology techniques.

AWARDS

2017	Ph.D. Scholarship Award, German Academic Exchange Service (DAAD)
2013	MSc. Scholarship Award, International Institute of Tropical Agriculture (IITA)
2007	Uganda government excellence Scholarship Award, Undergraduate student

PROFESSIONAL MEMBERSHIP

British Mycological Society (BMS)
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INTERNATIONAL EXPERIENCE

2019.06 –2019.10	Research visit, Jacaranda Agricultura Lda- Nampula, Mozambique and Stellenbosch University, Cape Town South Africa
2015.01	Research visit, International Livestock Research Institute (ILRI)- Addis Ababa, Ethiopia
2014.04	Research visit, International Institute of Tropical Agriculture (IITA), Bukavu, Democratic Republic of Congo
2012.10	Research visit, Institut des Sciences Agronomiques (ISABU), Bujumbura, Burundi

SELECTED CONFERENCES AND PRESENTATIONS

2021	Tropentag, 15th – 17th September, hybrid conference, Germany (Poster presentation)
2020	Banana Fusarium Virtual Symposium 16th – 17th December (Oral presentation)
2020	World FOOD Virtual Colloquium: 16th October, Food Security Center (FSC), University of Hohenheim, Germany (Oral presentation)

MENTORSHIP AND STUDENT SUPERVISION

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PUBLICATIONS INCLUDED IN THE THESIS

1. **Were, E.,** Viljoen, A., Rasche, F. (2022). Back to the roots: understanding banana below-ground interactions is crucial for effective management of Fusarium wilt. Plant Pathol. https://doi.org/10.1111/ppa.13641.
2. **Were, E.,** Schöne, J., Viljoen, A., Rasche, F. (2022). *De novo* synthesis of ferrichrome by *Fusarium oxysporum* f. sp. *cubense* TR4 in response to iron starvation. Fung. Biol. doi.org/10.1016/j.funbio.2022.05.005.
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4. **Were**, **E**., Viljoen, A., Rasche, F (2023). Iron necessity for chlamydospore germination in *Fusarium oxysporum* f. sp. *cubense* TR4. (under peer review, submitted to Biometals)

SELECTED PUBLICATIONS

5. Dar, A., **Were, E**., Hilger, T., Zahir, Z. A., Ahmad, M., Hussain, A., & Rasche, F. (2022). Bacterial secondary metabolites: Possible mechanism for weed suppression in wheat. Can. J. Microbiol. https://doi.org/10.1139/cjm-2022-0181.
6. Ocimati, W., Tazuba, A.F., Tushemereirwe, W.K., Tugume, J., Omondi, B.A., Acema, D., **Were, E.,** Onyilo, F., Ssekamate, A.M., Namanya, P., Kubiriba, J., (2021). First report of banana bunchy top disease caused by Banana bunchy top virus in Uganda. New Dis. Rep. doi.org/10.1002/ndr2.12052.
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8. Kimunye, J., **Were, E.,** Swennen, R., Viljoen, A., Mahuku, G. (2021). Sources of resistance to *Pseudocercospora fijiensis*, the cause of black Sigatoka in banana. Plant Pathol. doi.org/10.1111/ppa.13408.
9. Kimunye, J., Jomanga, K., Tazuba, A. F., **Were, E.,** Viljoen, A., Swennen, R., Mahuku, G. (2021). Genotype × Environment Response of 'Matooke' Hybrids (Naritas) to *Pseudocercospora fijiensis*, the Cause of Black Sigatoka in Banana. Agronomy 11: 1145. doi.org/10.3390/agronomy11061145.
10. Nakato, G.V., Studholme, D.J., Blomme, G., Grant, M., Coutinho, T.A., **Were, E.,** Wicker, E., Mahuku, G., G. (2021). SNP-based genotyping and whole-genome sequencing reveal previously unknown genetic diversity in *Xanthomonas vasicola* pv. *musacearum*, causal agent of banana xanthomonas wilt, in its presumed Ethiopian origin. Plant Pathol. 70: 534-543. doi.org/10.1111/ppa.13308.
11. Kimunye, J.N., **Were, E.,** Mussa, F., Tazuba, A., Jomanga, K., Viljoen, A., Swennen, R., Muthoni, F.K., Mahuku, G. (2020). Distribution of *Pseudocercospora* species causing Sigatoka leaf diseases of banana in Uganda and Tanzania. Plant Pathol. 69: 50-59. doi.org/10.1111/ppa.13105.
12. Nakato, G. V., Christelova, P., **Were, E.,** Nyine, M., Coutinho, T. A., Doležel, J., Mahuku, G. (2019). Sources of resistance in Musa to *Xanthomonas campestris* pv. *musacearum*, the causal agent of banana xanthomonas wilt. Plant Pathol. 68: 49-59. doi.org/10.1111/ppa.12945.
13. Ocimati, W., **Were, E.,** Groot, J. C., Tittonell, P., Nakato, G. V., Blomme, G. (2018). Risks posed by intercrops and weeds as alternative hosts to *Xanthomonas campestris* pv. *musacearum* in banana fields. Front. Plant Sci. 9: 1471. doi.10.3389/fpls.2018.01471.
14. **Were, E.,** Nakato, G.V., Ocimati, W., Ramathani, I., Olal, S., Beed, F. (2015). The banana weevil, *Cosmopolites sordidus* (Germar), is a potential vector of *Xanthomonas campestris* pv. *musacearum* in bananas. Can. J. Plant Pathol. 37:4 427-434. doi.10.1080/07060661.2015.1113444.

Stuttgart, 24.01.2023

[11]Yours, O Lord, is the greatness and the power and the glory and the victory and the majesty, for all that is in the heavens and in the earth is Yours. Yours is the kingdom, O Lord, and you are exalted as head above all. [12]Both riches and honor come from You, and You rule over all. In Your hand are power and might, and in Your hand it is to make great and to give strength to all. [13]And now we thank You, our God, and praise Your glorious name.

1 Chronicles 29:11–13 (ESV)

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