The effect of soil physico-chemical characteristics on the severity of Fusarium wilt of bananas

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Statement on the contribution of others

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Additional chapter specific contributions are stated at the beginning of each chapter.

Abstract

Banana production is under threat globally from Fusarium wilt, caused by the soil-borne pathogen *Fusarium oxysporum* f.sp. *cubense* (Foc) Tropical Race 4. Foc Tropical Race 4 was first discovered in Australia's primary banana production region of North Queensland in 2015 and has slowly spread since. To date there are no agronomically suitable disease resistant varieties and no known treatments, so quarantine has been the primary management tool, though it is not a permanent solution. The objective of this thesis was to increase understanding of how soil physico-chemical characteristics affect the severity of Fusarium wilt of banana.

A review of literature relevant to Fusarium wilt was undertaken to identify abiotic soil properties that hold promise for agronomic management of Fusarium wilt and to understand the mechanisms by which they reduce disease severity (Chapter 2). Across a variety of crops soil temperature, redox potential, and extractable iron and manganese contents are generally positively correlated with disease severity, whereas pH, nitrate : ammonium ratio, organic matter content and extractable calcium, zinc, silicon, potassium, phosphorus and boron contents are negatively correlated, but less consensus exists for bananas. This review identified the interconnectedness of soil characteristics as a challenge for management. Two possible management options were identified that warranted further investigation, namely the manipulation of micronutrient availability and nitrogen fertiliser management.

To identify banana farming soils that were suitable for testing, as well as provide regional context to experimental results, we characterised soil from 28 banana farms in North Queensland on soil types accounting for > 85% of Australia's banana production (Chapter 3). Soil characteristics at these sites covered a similar range to those measured in a large commercial dataset from across the industry (n=1,812), confirming their representativeness. Variation in soil properties and leaf nutrient concentrations were driven largely by site- (principal component 1 in both cases) and management-related factors (principal component 2 in both cases). The most important site characteristics appeared to be soil parent material and climate, with the mostly basaltic and low rainfall Mareeba sub-region differing from the Innisfail and Tully sub-regions. This information on the range of soil characteristics and plant nutrient status could facilitate efficient research, extension, monitoring and regulation regarding production- and environment-related banana issues.

The severity of Fusarium wilt of bananas has long been classified based on visual assessment of necrosis in rhizome or pseudostem cross-sections. We developed a digital image analysis method to

quantify the proportion of necrotic rhizome tissue (Chapter 4). This method agrees well with visual classification, but provides greater reproducibility, precision and statistical power. It was employed in the subsequent work.

Naturally occurring and synthetic chelating ligands can act as suppressants for Fusarium wilt in several crop species, based on their ability to alter micronutrient bioavailability in soil. We tested the ability of chelating ligands to solubilise metals in key banana producing soils over an environmentally relevant pH range (Chapter 5) and reduce Fusarium wilt of banana severity in tropical soils (Chapter 6). Six topsoils from farms in North Queensland were adjusted to pH 5, 6 and 7 and then extracted with a range of chelating ligands and analysed for a range of metals. EDDHA, a highly stable chelator, solubilised iron effectively under all the conditions tested, indicating its likely suitability for disease suppression. The concentration of aluminium in EDDHA extracts was positively correlated with pH, and at pH 7 the concentration of aluminium was far greater than that of iron, with possible environmental implications due to its toxicity.

After identifying a promising chelating ligand two pot trials were undertaken to determine the capacity of this treatment to reduce disease severity in banana plants (cv. Ducasse, *Musa* ABB) grown in pots inoculated with Foc, Race 1 (Chapter 6). Experiment 1 compared amendment with iron chelates (using ligands with differing iron binding stability) with water, plus an uninoculated unamended control, in two tropical Australian soils. Experiment 2 examined the effect of Fe-HBED application rate with high or low calcium addition. Fusarium wilt of bananas was not significantly affected by addition of iron chelates to these soils. The lack of effect was likely due to high iron availability in the soils overwhelming the capacity of the treatments to alter iron availability to the host plant and pathogen. Application of strong chelating ligands increased the concentration of aluminium and decreased the concentration of manganese in plant tissue, with possible detrimental effects. Based on these results, manipulation of micronutrient availability was not examined further.

Inorganic nitrogen fertiliser application is an integral part of commercial banana production. Both the rate of application and its form are thought to play a role in the susceptibility of banana plants to Fusarium wilt. In a pot trial we investigated the effect of nitrogen rate, nitrogen form and inoculation with Foc on disease severity and banana plant growth (Chapter 7). Growth in disease-free plants was positively correlated with nitrogen rate, with no effect of form. In inoculated plants, disease severity was also positively correlated with nitrogen rate, resulting in plant growth being greatest at intermediate nitrogen rates and decreasing at high rates.

To understand the source of the disease effect we examined each member of the disease triangle (pathogen, banana host and environment) separately. Nitrogen rate and form had no effect on the abundance of the pathogen in the rhizosphere, as determined by qPCR. The effect on soil microbial populations was principally due to changes in soil pH. Protein expression rates in banana roots were analysed by proteomic analysis. Inoculation caused enrichment of defence and metabolic pathways in the banana plant. Increased nitrogen rate caused enrichment of nitrogen metabolism, carbon fixation, amino acid metabolism, and importantly defence related pathways. In particular, Pathogenesis-related protein 1, a key endpoint for the salicylic acid defence response to biotrophic pathogens, was strongly negatively correlated with ammonium addition in diseased plants.

In conclusion, of the two examined management approaches to reduce the severity of Fusarium wilt of banana, one appears promising in the Australian banana industry. Management of iron availability appears ineffective for the North Queensland growing area but nitrogen fertilisation rate shows promise, presuming trade-offs in the plant between growth and defence can be optimised through plant genetics or agronomy. The survey work (Chapter 3), in combination with the experimental work (Chapters 5-7), suggests that efficacy of treatments associated with intrinsic soil characteristics such as micronutrient concentration are likely to be more geographically variable than those associated with highly managed characteristics such as nitrogen nutrition. Geographic variability is due to regional differences in soil forming factors such as parent material and climate, and generally appears to rule out manipulation of iron availability as a treatment option in highly weathered tropical soils such as those of North Queensland. On the other hand, management of nitrogen nutrition is likely to be more broadly applicable.

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1 Introduction

Throughout the world, banana production is under threat from Fusarium wilt, a vascular wilt disease caused by the soil-borne pathogen *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4. This pathogen was first discovered in Australia's primary banana production region of North Queensland in 2015 (O'Neill *et al.* 2016) and has since spread to five farms (Biosecurity Queensland 2021). Its spread has been predicted to cause upwards of $138 million per year in industry losses in the near future (Cook *et al.* 2015). With no current treatment or commercially acceptable resistant cultivar for Fusarium wilt of banana the use of management to reduce disease severity and losses is a promising approach (Dita *et al.* 2018).

Banana production in Australia is approximately 365,000 tonnes/year produced from 11,280 hectares, of which greater than 90% is in North Queensland (ABGC 2017). The region is situated between approximately 15°50'S and 18°20'S latitude along the east coast of Queensland. Production is primarily on coastal plains and slopes, with annual rainfall ranging from 1,800 to 4,500 mm (Murtha 1986). Smaller growing areas exist on nearby elevated tablelands, up to 600 m elevation and with annual rainfall of approximately 1,400 mm, supplemented by irrigation (Laffan 1988).

Fusarium oxysporum is a diverse species complex of fungus including both pathogenic and non-pathogenic forms (Leslie and Summerell 2008). The pathogenic strains are largely host specific and are responsible for yield losses in a variety of important crops such as tomato, cucumber, melons, flax, lettuce, strawberry, oil palm, tobacco, carnation, cotton, and banana (Table 2-1) and can even affect humans (Leslie and Summerell 2008). *F. oxysporum* is currently subdivided into formae speciales (f.sp.) based on host plant species rather than taxonomic distinctness (Leslie and Summerell 2008; Kang *et al.* 2014). *Fusarium oxysporum* f.sp. *cubense*, is subdivided into 'races' based on the cultivars of banana affected, with Tropical Race 4 affecting approximately 64% of world production, (Ploetz 2015a) and 97% of Australian production (ABGC 2017). As a hemibiotroph, *F. oxysporum* f.sp. *cubense* attacks susceptible hosts, but it is a facultative saprophyte, able to survive on dead organic material for extended periods of time. It can also live as an endophyte in symptomless host plants, including common weed species (Hennessy *et al.* 2005; Altinok 2013). While the impact of pathogenic strains has been geographically limited in the past, the presence of *F. oxysporum* f.sp. *cubense* Tropical Race 4 has now been confirmed throughout Southeast Asia (Chittarath *et al.* 2017; Hung *et al.* 2017; Mostert *et al.* 2017), and in China, South Asia, the Middle-East, Africa (Qi *et al.* 2008; García-Bastidas *et al.* 2013; Ordonez *et al.* 2015; Ploetz *et al.* 2015; Thangavelu 2016), South

America (García-Bastidas *et al.* 2020)(Acuña *et al.* 2021) and Australia (Cook *et al.* 2015; O'Neill *et al.* 2016). Disease severity, as opposed to the growth of the pathogen, is governed by the three-way relationship between host, pathogen and the environment (Agrios 2005).

Previous studies have demonstrated that soil characteristics and their management affect Fusarium wilt of banana in other parts of the world; however, this approach has not been investigated in North Queensland, Australia. I synthesized the existing knowledge relating Fusarium wilt severity to soil abiotic characteristics, assessed the range of soil abiotic characteristics on banana farms in the North Queensland region, developed a method to assess Fusarium wilt severity, and assessed two approaches for reducing severity of Fusarium wilt of banana (Figure 1-1). The two approaches that were trialled both aimed to alter nutrient availability, exploiting differences in the way the pathogen and host plant take up nutrition to advantage the host. The approaches differed in that available nitrogen is highly managed and relatively consistent between regions whereas iron is largely determined by natural availability and can vary considerably.

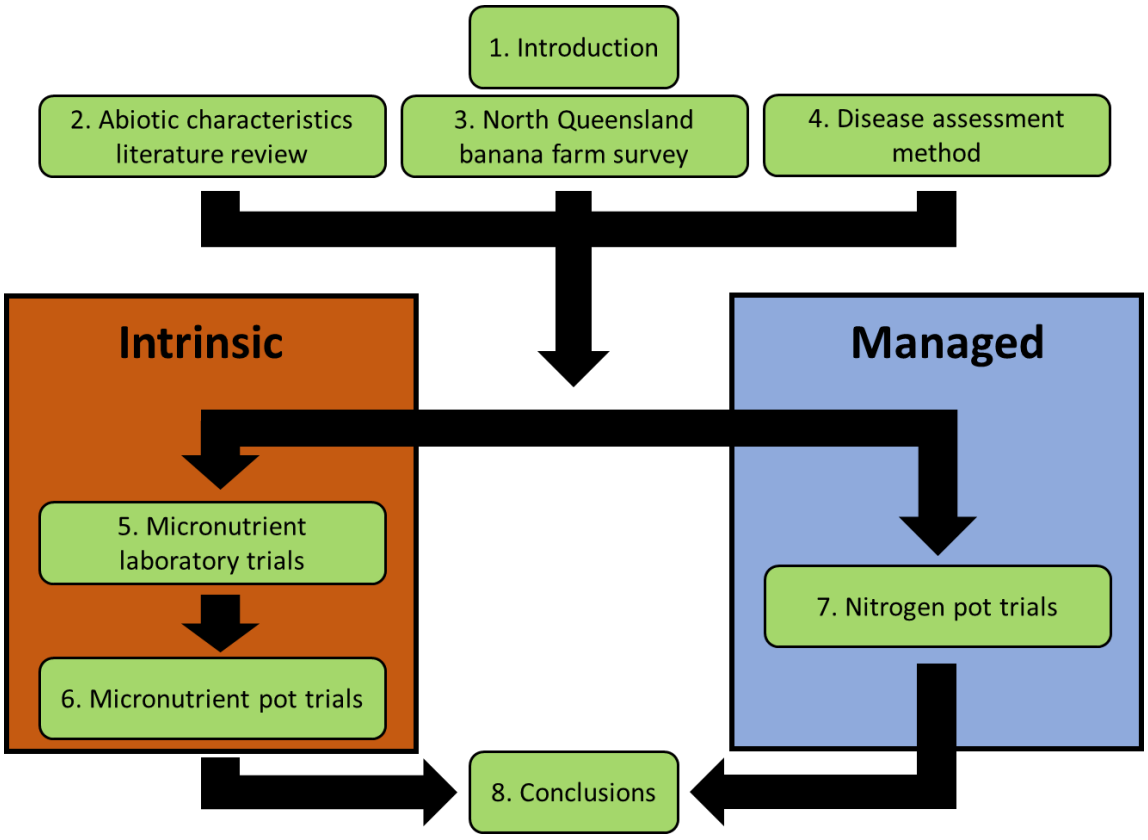

Figure 1-1. Schematic representation of how the chapters connect. Chapters 2, 3 and 4 provided the necessary background information and methodology for testing the effect of manipulating an intrinsic soil characteristic (Chapters 5 and 6) and a highly managed soil characteristic (Chapter 7).

A review of the literature (Chapter 2) identified substantial effects of soil characteristics on Fusarium wilt, but these soil characteristics have not been systematically surveyed in the context of the Australian banana industry. The variation of soils and plant nutrient status in the banana industry in North Queensland has previously been described in general terms. Reports on banana production and management mentioned soil types (Daniells 1984; Pattison *et al.* 2005; Harvey *et al.* 2018) and several surveys have sampled conventional banana farms and compared them with either organic or unfarmed areas (Pattison *et al.* 2008; Geense *et al.* 2015; Pattison *et al.* 2018b). However, this is the first study of the north Queensland banana industry to systematically analyse soil properties and foliar nutrient concentrations based on their soil classification. The study covered intrinsic properties such as parent material and related characteristics, which are difficult or in some cases impossible to manipulate, as well as properties that are essentially determined by management.

Based on the results of the regional survey I helped to select locations and design an honours project to assess the relative conduciveness or suppressiveness to Fusarium wilt of soils from across the Far North Queensland banana growing region (Bowen *et al.* 2019). That research demonstrated that soils differ in their conduciveness to Fusarium wilt and that clay content and metal content are of particular importance. It also highlighted the important role of non-pathogenic soil organism activity in suppressing Fusarium wilt. This allowed for an assessment of the relative risk from Fusarium wilt on different soil types and indicated soil properties that may be manipulated to reduce disease severity, which is the purpose of this thesis. Clay content is difficult to manage, however, related properties such as metal availability can be managed more easily, and iron availability has previously been shown to affect Fusarium wilt severity.

To quantify the outcomes of the treatments an objective method was required. Existing methods for quantifying Fusarium wilt severity visually categorised cross sections based on the level of vascular tissue discoloration of rhizome and sometimes pseudostem or root tissue (Zadoks and Schein 1979; Orjeda 1998; Peng *et al.* 1999; Carlier *et al.* 2003; Smith *et al.* 2008; Viljoen *et al.* 2016; Zuo *et al.* 2018). We developed an improved version of the method that would have two advantages over existing methods: 1) increased precision and thus statistical power through use of a continuous rather than discrete measured variable 2) reduced subjectivity and human error by using computerised image analysis rather than the human eye to determine the proportion of tissue that is discolored (Chapter 4). This method was used in subsequent experimentation.

Iron management was the first strategy tested for its ability to reduce the severity of Fusarium wilt as it has been shown to be effective in a number of other crops. Under certain growing conditions Fusarium wilt of carnation (Duijff *et al.* 1994), chickpea (Saikia *et al.* 2005), cucumber (Scher and Baker 1982), flax (Scher and Baker 1982; Lemanceau *et al.* 1988), radish (Scher and Baker 1982; Leeman *et al.* 1996), tomato (Jones and Woltz 1970; Segarra *et al.* 2010; López-Berges *et al.* 2012) and banana (Peng *et al.* 1999; Dong *et al.* 2016), have all been partially controlled by altering the availability of iron. Iron availability is related to parent material, with our survey (Chapter 3) showing that basaltic soils had higher iron availability than soils developed on other parent materials. The only two studies into the effect of manipulating iron availability on Fusarium wilt of banana (Peng *et al.* 1999; Dong *et al.* 2016) were performed under unique experimental conditions, so the usefulness of this treatment approach in commercial banana cultivation was uncertain. Peng *et al.* (1999) found that application of iron ethylenediaminedi-O-hydroxyphenylacetic acid (Fe-EDDHA) fertiliser halved disease severity in a pot trial with two soils. The chelating ligand used, EDDHA, is highly stable and is thought to make iron available to the plant whilst inducing iron deficiency in *Fusarium oxysporum* f.sp. *cubense*. However, the soils they used had a pH of 8, low micronutrient availability and were from atypical low-rainfall conditions. In a hydroponic trial Dong *et al.* (2016) found increasing iron availability to both the banana plant and Foc, using iron chloride fertiliser, also resulted in a significant decrease in disease severity.

The effect of iron on the severity of Fusarium wilt appears to depend on its relative availability to the host and pathogen. Availability to the two organisms can differ and is dependent upon the chelating ligand and soil considered. The severity of Fusarium wilt of flax was increased by the addition of weakly bound iron, in the form of iron ethylenediaminetetraacetic acid (Fe-EDTA) and iron diethylenetriaminepentaacetic acid (Fe-DTPA), but decreased by addition of stable iron binding chelating ligands such Fe-EDDHA and iron N,N-bis(2-hydroxyphenyl)ethylendiamine-N,N-diacetic acid (Fe-HBED) (Scher and Baker 1982; Lemanceau *et al.* 1988). Such effects may occur only in conditions where iron availability is already limited (Simeoni *et al.* 1987; Segarra *et al.* 2010). Therefore, the effectiveness of chelating ligand application as a treatment for Fusarium wilt is likely to differ between locations and soil types and required testing in the growing conditions of North Queensland, Australia. I first tested the effect of soil type, pH and chelating ligand on metal availability (Chapter 5), followed by testing the effect of manipulating metal availability on Fusarium wilt severity (Chapter 6). The approach was unsuccessful at altering Fusarium wilt severity in the soils tested so was not pursued further.

The second management strategy focused on a characteristic that is more highly managed and so more easily manipulated. In North Queensland iron was determined to be naturally too plentiful for successful reduction whereas nitrogen must be added to cropping systems for optimum productivity. Environmental factors such as a higher rate, or different form of nitrogen fertiliser, can alter the susceptibility of a host organism, the growth and virulence of a pathogen and the interactions of both with the surrounding microbiota. In plants, greater nitrogen availability has been proposed to cause a growth-defence tradeoff to maximize plant fitness (Herms and Mattson 1992). Plants that grow more rapidly, resulting in greater productivity, due to an abundance of nitrogen must sacrifice defensive capabilities due to a metabolic incompatibility, leaving them more susceptible to disease (Huot et al. 2014; Neuser et al. 2019).

Increased nitrogen fertiliser use has recently been found to increase Fusarium wilt of banana severity (Segura-Mena *et al.* 2021). The specifics of this relationship are complicated by the indirect effects of the fertiliser input, such as soil pH change, which is dependent on the form that the nitrogen fertiliser takes. Generally, nitrate fertiliser reduces Fusarium wilt disease severity while ammonium increases it (Woltz and Engelhard 1973; Jones *et al.* 1975; Morgan and Timmer 1984; Woltz *et al.* 1992; Wang *et al.* 2016; Zhou *et al.* 2017) though there are exceptions (Jarvis and Thorpe 1980). Ammonium fertilisers, on the other hand, generally increase Fusarium wilt severity. Ammonium fertiliser use decreases the soil pH, which increases Fusarium wilt severity, whereas nitrate fertilisers increase soil pH. Understanding the effects of nitrogen fertiliser form and dose, the indirect effect of pH change, and the effect of both of these on the members of the disease triangle is integral in explaining the role of nitrogen in disease management. These changes were assessed in a pot trial (Chapter 7).

Finally, the results of the experimental work were synthesised to advance our general understanding of how key soil abiotic characteristics and their management can affect the severity of Fusarium wilt of banana. The implications for management strategies to reduce losses from Fusarium Wilt of banana in Far North Queensland and globally are discussed.

2 Impacts of soil abiotic attributes on Fusarium wilt, focusing on bananas

Chapter No.	Details of publication (s) on which chapter is based	Nature and extent of the intellectual input of each author, including the candidate
2	This chapter is identical (apart from formatting, and incorporation of the reference list into one combined list at the end of the thesis) to the published paper: Orr, R. and P. N. Nelson (2018). "Impacts of soil abiotic attributes on Fusarium wilt, focusing on bananas." Applied Soil Ecology **132**: 20-33.	The authors co-developed the idea for this literature review. Orr collected and synthesised the literature with assistance from Nelson. Data analysis was performed by Orr with assistance from Dr. Tobin Northfield. Orr wrote the first draft of the paper which was revised with editorial input from Nelson. Orr developed the figures and tables with assistance from Nelson.

2.1 Abstract

Production of many crops, including bananas, is threatened worldwide by the spread of pathogenic strains of *Fusarium oxysporum*, the causal agent of Fusarium wilt. Severity of the disease is related to soil biotic and abiotic attributes, which influence the plant, the pathogen and the other soil organisms. Across a variety of crops, soil temperature, redox potential, and extractable iron and manganese contents are generally positively correlated with disease severity, whereas pH, nitrate : ammonium ratio, organic matter content and extractable calcium, zinc, silicon, potassium, phosphorus and boron contents are negatively correlated, but less consensus exists for bananas. There are numerous incompletely understood interactions between soil abiotic attributes and disease severity, including those between pH- and redox-controlled micronutrient availability, buffering by organic matter and clay, and effects of nutrients on plant defence mechanisms. Though not all soil attributes can be managed, pH, organic matter content and availability of nutrients show promise for manipulation to reduce disease severity and mitigate risk.

2.2 Introduction

'Fusarium wilt', a vascular wilt disease, is caused by soil-borne pathogenic strains of *Fusarium oxysporum*, a diverse species complex of fungus including both pathogenic and non-pathogenic forms (Leslie and Summerell 2008). The pathogenic strains are largely host specific and are responsible for yield losses in a variety of important crops such as tomato, cucumber, melons, flax, lettuce, strawberry, oil palm, tobacco, carnation, cotton, and banana (Table 2-1) and can even affect humans (Leslie and Summerell 2008). *F. oxysporum* is currently subdivided into formae speciales based on host plant species rather than taxonomic distinctness (Leslie and Summerell 2008; Kang *et al.* 2014). As a hemibiotroph, *F. oxysporum* attacks susceptible hosts, but it is a facultative saprophyte, able to survive on dead organic material for extended periods of time. It can also live as an endophyte in symptomless host plants, including common weed species (Hennessy *et al.* 2005; Altinok 2013). While the impact of pathogenic strains has been geographically limited in the past, the rise of global transport has vastly increased their spread and importance (Desprez-Loustau *et al.* 2007). The relationship between soil properties and disease severity may differ between species and strains of *Fusarium* (Jarvis and Thorpe 1980).

Table 2-1. Previous studies correlating Fusarium wilt severity with soil physicochemical variables, bioavailability of essential elements and amendment with various materials. 'Positive correlation' refers to an increase in disease severity with increasing value of the variable. Underlining indicates hydroponic studies and superscripts indicate host plant species: [1]banana; [2]asparagus; [3]carnation; [4]chickpea; [5]chrysanthemum; [6]cotton; [7]cucumber; [8]flax; [9]lettuce; [10]lime; [11]muskmelon; [12]oil palm; [13]radish; [14]strawberry; [15]tomato; [16]watermelon; [17]wheat.

Variable	Positive Correlation* (manipulated)	Positive Correlation (observed)	Negative Correlation (observed)	Negative Correlation* (manipulated)
Temperature	Peng et al. (1999)[1], Ferrocino et al., 2013, Scott et al., 2010[9], Fang et al. (2011)[14], Larkin and Fravel (2002)[15]			
Reduction potential (eH)	Huang et al., 2015b, Peng et al., 1999, Wen et al., 2015[1], Blok et al.			

Variable	Positive Correlation* (manipulated)	Positive Correlation (observed)	Negative Correlation (observed)	Negative Correlation* (manipulated)
	(2000)[2], Bhatti and Kraft (1992)[4], Yao et al. (2016)[8], Jorge-Silva et al. (1989)[11], Oritsejafor (1986)[12], Ebihara and Uematsu (2014)[14], Ghaemi et al. (2011)[15], Cao et al. (2016)[16]			
pH	Peng et al. (1999)[1], Cao et al. (2016)[16]		Alvarez et al., 1981, Deltour et al., 2017, Domínguez et al., 1996, Domínguez et al., 1995, Rishbeth, 1957, Román Jerí, 2012, Stover, 1956[1], Yuen et al. (1983)[3]	Fan and Li, 2014, Shen et al., 2015a[1], Duuff et al. (1995)[3], Jones et al. (1975)[7], Höper et al., 1995, Scher and Baker, 1980, Senechkin et al., 2014[8], Gatch and du Toit (2016)[9], Fang et al. (2012)[14], Borrero et al., 2004, Jones and Woltz, 1969, Jones and Woltz, 1968, Jones and Woltz, 1970, Woltz and Jones, 1973[15], Everett and Blazques, 1967, Jones et al., 1975[16]
Organic Matter Content			Alvarez et al. (1981)[1]	Pérez Salas et al., 2013, Shen et al., 2015a[1], Markakis et al. (2016)[7], van Bruggen et al. (2015)[8], Abadie et al. (1998)[12], Akhter et al.,

Variable	Positive Correlation* (manipulated)	Positive Correlation (observed)	Negative Correlation (observed)	Negative Correlation* (manipulated)
				2016, Castaño et al., 2011[15], Cao et al., 2016, Yogev et al., 2011[16]
Nitrate:Ammonium ratio	Zhang et al. (2013)[1], López-Berges et al. (2010)[15], Cao et al. (2016)[16]			Jones et al., 1975, Wang et al., 2016, Zhou et al., 2017[7], Morgan and Timmer (1984)[10], Borrero et al., 2012, Duffy and Défago, 1999, Woltz and Jones, 1973, Woltz et al., 1992[15], Jones et al. (1975)[16]
Calcium content			Alvarez et al. (1981)[1]	Peng et al. (1999)[1], Höper et al., 1995, Keane and Sackston, 1970[8], Gatch and du Toit (2016)[9], Spiegel et al. (1987)[11], Jones and Woltz (1969)[15]
Iron & Manganese content	Peng et al. (1999)[1], Duijff et al., 1994, Duijff et al., 1993[3], Saikia et al. (2005)[4], Scher and Baker (1982)[7], Höper et al., 1995, Kloepper et al., 1980, Lemanceau and Alabouvette, 1993,	Domínguez-Hernández et al., 2010, Domínguez et al., 1996, Domínguez et al., 1995[1]		Dong et al. (2016)[1], Duffy and Défago (1999)[15]

Variable	Positive Correlation* (manipulated)	Positive Correlation (observed)	Negative Correlation (observed)	Negative Correlation* (manipulated)
	Lemanceau et al., 1988, Scher and Baker, 1982[8], Leeman et al., 1996, Scher and Baker, 1982[13], Jones and Woltz, 1969, Jones and Woltz, 1970, Segarra et al., 2010[15]			
Zinc content	Gatch (2013)[9], Duffy and Défago (1999)[15]		Gutierrez Jerez et al. (1983)[1]	Fernández-Falcón et al., 2004, Hecht-Buchholz et al., 1998[1], Saikia et al. (2009)[4], Duffy and Défago (1997)[15]
Silicon content	Smith et al. (2005)[6], Huang et al. (2011)[15]			Fortunato et al., 2014, Fortunato et al., 2012a, Fortunato et al., 2012b, Jones, 2013, Kidane and Laing, 2008, Wibowo et al., 2014[1]
Sodium content & Electrical Conductivity	Triky-Dotan et al., 2005, Woltz et al., 1992[15]		Dominguez et al., 2001, Domínguez et al., 1996[1]	
Potassium content		Domínguez-Hernández et al. (2010)[1]		Ollagnier and Renard, 1976, Renard and Franqueville, 1991[12] Cao et al. (2016)[16]

Variable	Positive Correlation* (manipulated)	Positive Correlation (observed)	Negative Correlation (observed)	Negative Correlation* (manipulated)
Phosphorus content	Woltz and Jones (1973)[15]			Shen et al., 2015a, Shen et al., 2015b[1]
Boron content				Dong et al. (2016)[1], Keane and Sackston (1970)[8]

Several reviews (Höper and Alabouvette 1996; Janvier *et al.* 2007; Dordas 2008) outlined the effects of soil abiotic characteristics on severity of soil-borne diseases but did not focus on Fusarium wilts. Höper and Alabouvette's (1996) review did discuss Fusarium wilt, but subsequent research has furthered the understanding of several mechanisms. A recent review of management of Fusarium wilt of banana by Ploetz (2015b) only briefly addressed manipulation of soil properties. This review focuses on bananas due to the recent spread of Panama disease, but it also includes relevant results from a variety of other crops affected by *F. oxysporum*. We review the effects of soil abiotic attributes on Fusarium wilt, based on field surveys, manipulative trials and laboratory experiments.

This review focusses on soil chemical characteristics, with special emphasis on micronutrient availability. The effects of soil physical characteristics are only briefly examined due to the limited number of studies. Most physical properties are best measured in the field, but most research has focused on soil characteristics amenable to analysis in the laboratory. Many soil characteristics influence availability of micronutrients to organisms, and manipulation of micronutrient availability has been proposed as a method to reduce the severity of Fusarium wilt (Lemanceau 1989). The relationships between Fusarium wilt severity and soil attributes are complex and far from being fully understood. However, a rapidly improving understanding of the mechanisms involved suggests that management interventions to mitigate effects of the disease may be possible in the future.

2.3 Fusarium wilt of bananas (Panama disease)

Bananas and plantains are a major source of food and income for people in many tropical and subtropical regions (Lescot 2015). Nearly 50% of world production is of the Cavendish cultivar

(*Musa* AAA), which is susceptible to a currently spreading pathogenic strain of *F. oxysporum*. Similarly susceptible cultivars cover a large proportion of remaining production (Lescot 2015).

The causal agent of Panama disease, *Fusarium oxysporum* f.sp. *cubense* (Foc), is subdivided into 'races' based on the cultivars of banana affected (Ploetz 2015a). Race 1 (R1) affects Maqueno, Silk, Pome, Pisang Awak and Gros Michel cultivars, Race 2 affects cooking bananas, Race 3 does not affect bananas, and Race 4 affects Race 1- and 2-susceptible cultivars as well as Cavendish (Ploetz 2015a). Race 4 is subdivided into Subtropical Race 4 (SR4), that affects susceptible cultivars under stressful growing conditions in the subtropics, and Tropical Race 4 (TR4), that affects susceptible cultivars in all conditions (Ploetz 2015a).

Over the past hundred years Panama Disease has had devastating effects on global banana production (Ploetz 2015a). The disease was originally described in 1874 in Brisbane, and again in 1890 in Central America. In the first half of the 20th century over 40,000 ha of commercial Gros Michel (*Musa* AAA) bananas, plus lesser amounts of other susceptible cultivars, were destroyed by Foc R1 (Pegg *et al.* 1996). The spread of Foc R1, subsequent production losses of Gros Michel, and associated economic drivers caused a large-scale transition to Cavendish, the most agronomically successful resistant cultivar. The clonal nature of bananas and reliance upon a single cultivar in large plantations means the industry is vulnerable to rapid spread of diseases.

Foc TR4, which was first identified in the 1990's, now poses a serious threat to global banana production due to its continual spread, and the widespread cultivation of susceptible cultivars (Ploetz and Pegg 1997). Foc TR4 has now been confirmed throughout Southeast Asia (Chittarath *et al.* 2017; Hung *et al.* 2017; Mostert *et al.* 2017), and in China, South Asia, the Middle-East, Africa (García-Bastidas *et al.* 2013; Ploetz *et al.* 2015) (Ordonez *et al.* 2015) (Qi *et al.* 2008) (Thangavelu 2016) and Australia (Cook *et al.* 2015; O'Neill *et al.* 2016). Future spread to growing regions in Latin America would make the impact global.

There is not yet a commercially effective method of controlling Panama disease (Ploetz 2015b). Quarantine measures have slowed, but are unlikely to stop the spread of Foc TR4. Selection and breeding of Foc TR4-resistant varieties has generated some promising cultivars such as Goldfinger and Taiwanese GCTCV somaclones (Hwang and Ko 2004), but a suitably productive alternative to Cavendish has not yet been found. Fungicides will not be able to control this soil-borne pathogen in

the field, for a variety of reasons, but they have shown promise for disinfection of farm implements contaminated by *F. oxysporum* of tomato (Song *et al.* 2004; Amini and Sidovich 2010), watermelon (Everts *et al.* 2014) and banana (Nel *et al.* 2007). The rotation of crops, or fallowing of fields is ineffective as Foc commonly persists in soil beyond 20 years (Stover 1962). Recently, addition of microorganisms (Cotxarrera *et al.* 2002; Wu *et al.* 2013; Shen *et al.* 2015a; Cha *et al.* 2016; Fu *et al.* 2017) and chemical amendments (Peng *et al.* 1999; Fortunato *et al.* 2012a; Liu *et al.* 2016) have shown promise for reducing disease severity. In case of only partially resistant cultivars being found, continued large-scale production of bananas would rely upon agronomic management of the disease.

2.4 Mechanisms of soil-borne disease suppression

Suppressiveness or conduciveness is the tendency of a soil to inhibit or enhance soil-borne disease. Suppressive soils are those in which disease impact is low despite the presence of a susceptible host, pathogen and a suitable environment (Baker and Cook 1974). This involves inhibition of the pathogen's growth or virulence, enhancement of plant defence mechanisms, enhancement of the abundance or activity of microorganisms antagonistic or competitive to the pathogen, or a combination of these mechanisms (Figure 2-1).

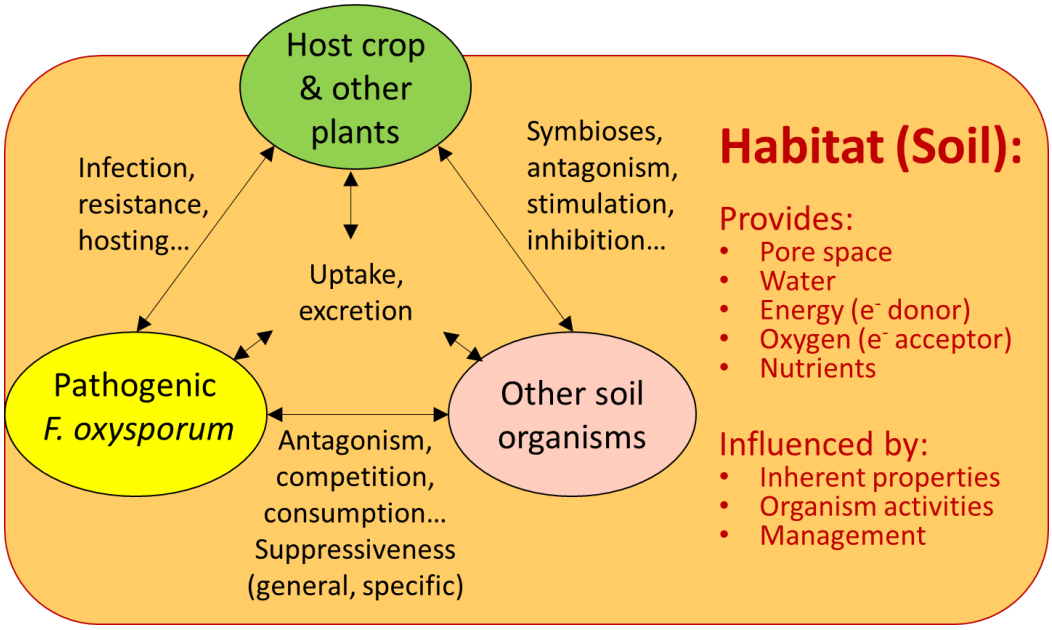

Figure 2-1. Soil is the habitat for all organisms involved in soil-borne diseases such as Fusarium wilt. Soil physicochemical conditions govern the resources available for those organisms and can thereby influence severity of the disease.

Suppression is primarily a function of soil microbiology and can be classed as general or specific, though also involves the effectiveness of plant defence. General suppression involves competition between the pathogen and other soil microorganisms for limited resources (Janvier *et al.* 2007). Edaphic, climatic and management factors affect these microbial interactions (Weller *et al.* 2002). Specific suppression involves the direct effects of an antagonistic organism on the pathogen (Janvier *et al.* 2007). Specific suppression can be induced or enhanced through the introduction of a specific organism (Nel *et al.* 2006; Xue *et al.* 2015; Fu *et al.* 2017). General suppression is typically considered non-transferrable and must be enhanced in situ (Weller *et al.* 2002). General and specific suppression are not mutually exclusive; they can operate simultaneously and in varying degrees. The size and diversity of the soil microbial community strongly influences suppressiveness (Shen *et al.* 2015b), and soil microbial community composition depends on soil abiotic properties. Similarly, the susceptibility and effectiveness of the host plant defence is dependent on soil characteristics. Therefore, alteration of the soil properties can influence suppression and disease severity. By shifting environmental conditions to favour organisms competitive with, or antagonistic to, Foc, or enhancing host plant defence, the suppressiveness of the soil may be increased.

2.5 Impacts of soil abiotic attributes

A wide variety of abiotic soil attributes have been correlated with suppression of Fusarium wilt in a range of crops, including bananas (Figure 2-2, Table 2-1). Effects on suppression are generally consistent across susceptible crops but there are exceptions. The effect of soil attributes on suppression is usually described in terms of a particular attribute, but it should be kept in mind that attributes and their associations with suppression are interrelated (Figure 2-3). Studies may have manipulated a particular soil attribute and report results accordingly, but it is certain that other attributes would also have been altered, and the effect of the measured attribute may have been indirect. It is impossible to test the effect of individual attributes, due to the complexity of the soil environment. For example, manipulation of pH alters bioavailability of most nutrients. Furthermore, as suppressiveness is a relative measure, it is important to understand the differences between test and control treatments, as many factors may influence responses. Finally, translating results from one environment to another may not be wise because such conditions may influence the treatment effects. Nevertheless, examining multiple studies can give insights into the importance of various attributes. To explore the studied effects of soil attributes, each of the following sections identifies, as far as possible, the mechanisms of suppression, as well as links to related attributes, gaps in understanding, and possible implications for management.

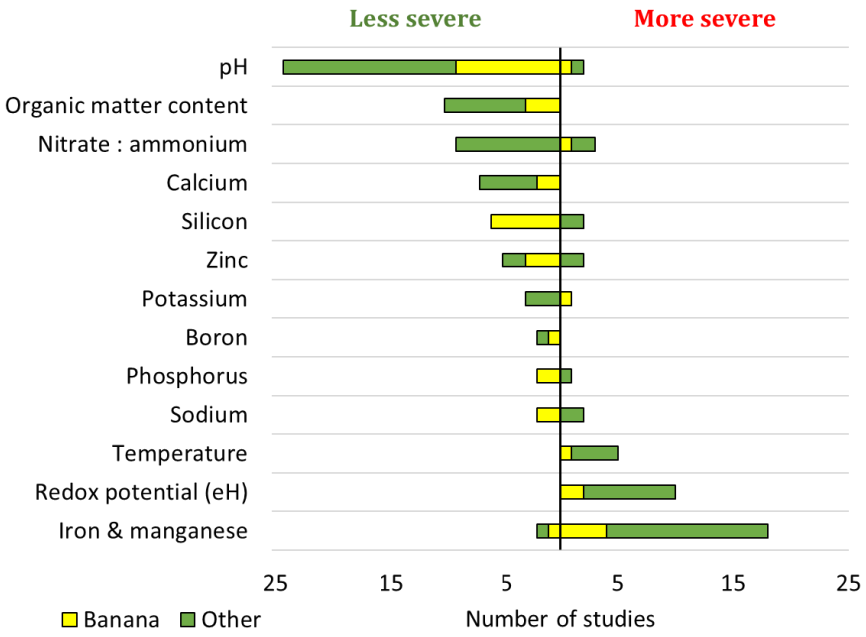

Figure 2-2. Summary of the studies describing relationships between soil characteristics and severity of Fusarium wilt. More or less severe correspond to an increase in the listed characteristic. See Table 2-1 for references.

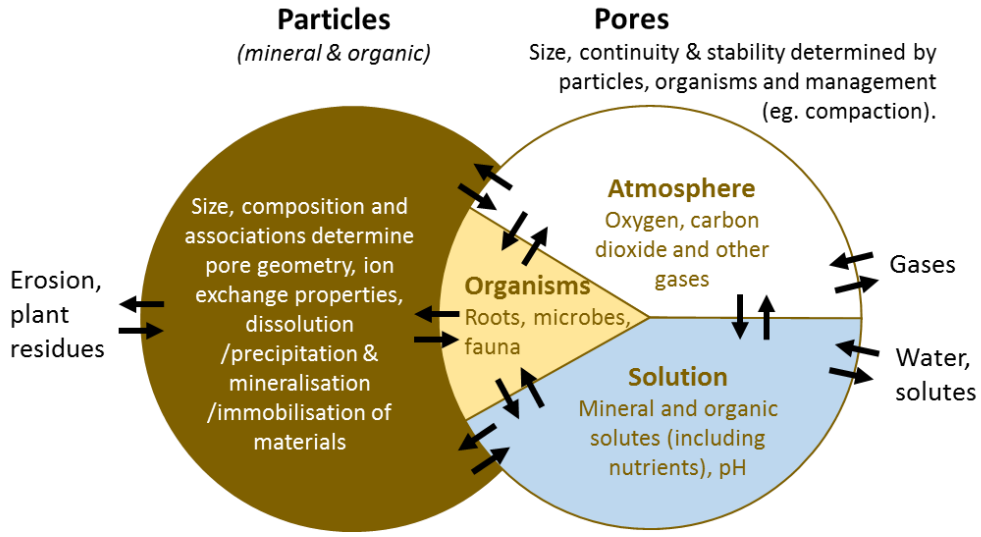

Figure 2-3. Soil physical, chemical and biological properties and processes interact in many ways to influence severity of soil-borne diseases. Arrows indicate influences and fluxes.

2.5.1 Temperature

Fusarium wilt severity is positively correlated with soil temperature in pot trials of banana (Peng *et al.* 1999), tomato (Larkin and Fravel 2002) and lettuce (Scott *et al.* 2010; Ferrocino *et al.* 2013). For example, an increase from a high/low diurnal temperature regime of 26/18°C to 28/20°C

substantially increased disease severity in a pot trial with lettuce (Scott *et al.* 2010). Similarly, an increase from 24 to 34°C significantly increased disease severity in both conducive and suppressive soils in a pot trial with banana (Peng *et al.* 1999).

Controlling growing temperature for banana is difficult due to its perennial growth and large land coverage. Unlike short-season crops, whose growing season can be shifted to align with optimum seasonal temperature, banana is cultivated year-round. Soil temperature can be reduced by shading, with a denser planting or cultivation of ground cover. Ground cover has been shown to reduce Foc incidence and severity in a field trial with bananas (Pattison *et al.* 2014) and the effect may have been partially due to reduced soil temperature. Covering the ground with plant residues or mulch has also been shown to modulate daily temperature variation (Horton *et al.* 1994). Temperature-controlled greenhouses are not practical for large scale commercial cultivation of banana due to the large areas required.

2.5.2 Water and Oxygen

Reductive soil disinfestation has been found effective as a potential means of controlling Foc in field trials in China *(Huang et al. 2015b).* The procedure, initially developed in Japan and the Netherlands, was based on observations of soil-borne disease suppression in irrigated rice paddies, which are regularly flooded. A suppressive anaerobic environment is created by consumption of oxygen during decomposition of soil organic matter, as replenishment by diffusion is minimal in saturated soil (Momma *et al.* 2013; Huang *et al.* 2015b; Shrestha *et al.* 2016). This anaerobic decomposition generates antifungal acids and volatile organic compounds and shifts microbial community composition to being bacteria-dominated and suppressive to Fusarium wilt (Hewavitharana *et al.* 2014; Huang *et al.* 2015a; Momma 2015; Liu *et al.* 2016). Once aerobic conditions resume, the suppressive community remains (Goud *et al.* 2004). It is worth noting that suppression did not occur when soil was saturated without organic matter addition in laboratory experiments (Wen *et al.* 2015), nor when oxygen was available to surface soil in field trials (Stover 1955). This indicates that suppression is unlikely to occur during natural flood events that are typically short lived, with aerated water and variable addition of organic matter. Reductive soil disinfestation by flooding is generally not feasible for banana production due to the constraints of water availability, topography and fallow time required; waterlogging is detrimental to banana root development and functioning (Rishbeth 1957; Aguilar *et al.* 1998).

Although reducing conditions have been shown to control Fusarium wilt, opposing processes may also occur. A reducing soil environment tends to increase the solubility and bioavailability of redox-sensitive micronutrients. Increased micronutrient bioavailability from reduced pockets within the crop root zone has been theorised to increase *F. oxysporum* pathogenicity (Dominguez *et al.* 2001). Furthermore, a reducing environment inhibits nitrification, increasing the concentration of soil ammonium, which is generally conducive to Fusarium wilt development. In the trials showing disinfestation by waterlogging, such effects were presumably overcome by the negative effects on the pathogen, such as an inability to function anaerobically, the release of antifungal bacterial exudates and the absence of a host crop to infect (Stover 1954).

Low soil water content has been shown to be conducive to Fusarium wilt in several crops. In pot trials, soil water content below field capacity was conducive to Fusarium wilt of banana (Peng *et al.* 1999), tomato (Ghaemi *et al.* 2011), chickpea (Bhatti and Kraft 1992) and melon (Jorge-Silva *et al.* 1989), possibly due to water stress of the host plant.

2.5.3 Mineralogy, Texture and Structure

A correlation between Fusarium wilt suppression and soil mineralogy was first identified in Central and South America (Rishbeth 1957; Stotzky *et al.* 1961; Stotzky and Torrence Martin 1963). As Foc R1 moved through the region, banana plantations that remained in production longer than 10 years before abandonment due to disease losses were all observed to have montmorillonite-like clays present (Stotzky and Torrence Martin 1963). Pot trials with wheat and flax have also found montmorillonite more suppressive to Fusarium wilt than other clays (Amir and Alabouvette 1993; Höper *et al.* 1995). Deltour et al. (2017) identified a positive correlation between clay content and suppression of Fusarium wilt of banana in a field survey in Brazil, but did not identify the types of clay involved.

Clay may influence suppression by altering oxygen diffusion (Stotzky and Rem 1967; Dominguez *et al.* 2001), pH buffering (Rosenzweig and Stotzky 1979) and availability of nutrients (Lavie and Stotzky 1986). Based on early research Stotzky and Rem (1967) suggested that reduced oxygen diffusion is harmful to pathogen growth. More recently however, it has been suggested that the formation of anaerobic micropores in soil aggregates promotes Foc SR4 pathogenicity, by increasing iron availability (Dominguez *et al.* 2001). Such anaerobic micropores are more likely to form in soils with higher clay content. The ability of clay to buffer pH and ion concentrations may reduce the impact of pH change on suppression (Siebner-Freibach *et al.* 2004). Smectite has been demonstrated

to reduce the micronutrient acquisition effectiveness of siderophores produced by pathogenic fungi, through adsorption of the ferrisiderophore complex (Figure 2-4) (Lavie and Stotzky 1986; Siebner-Freibach *et al.* 2004). Conversely, *Pseudomonas* siderophores and the synthetic chelate EDDHA, both of which suppress Fusarium wilt (Scher and Baker 1982; Lemanceau and Alabouvette 1993), are highly successful at iron acquisition in the presence of smectite clays (Ferret *et al.* 2014). Siderophores are typically organism-specific, though some micronutrient piracy does occur (Harrington *et al.* 2015). Greater adsorption of fungal siderophores than competing chelates by clay could be expected to reduce micronutrient uptake by *F. oxysporum*. Thus, the impacts of clay on suppression of fungal diseases may involve interactions with soil characteristics such as pH, redox state and micronutrient bioavailability.

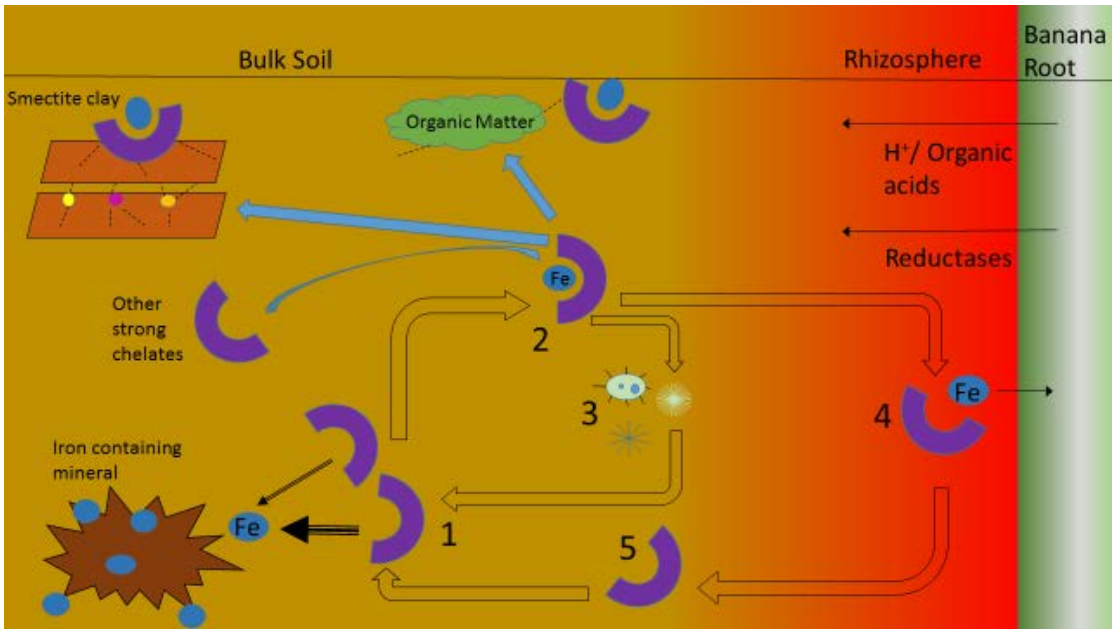

Figure 2-4. Micronutrient acquisition by chelating ligand (purple 'c's). Step 1: Competition between microbially-produced chelating ligand (siderophores) of different stability (the more stable the greater the amount of iron chelated). Step 2: Bound iron is transported by diffusion, which is hampered by adsorption to organic matter and clay, and piracy of iron by more stable chelating ligands. Step 3: Microorganisms take up their specific siderophores with bound iron and siderophore is regenerated. Step 4: Loss of stability in acidic / reducing rhizosphere, freeing iron cation. Step 5: Chelating ligand leaves rhizosphere and regains structure and chelating ability.

Analysis of soil physical characteristics in suppression studies has almost exclusively focused on those that can be measured in the laboratory, such as texture and aggregation (Dominguez *et al.* 2001; Deltour *et al.* 2017). Only limited field research on the effects of hydraulic conductivity, porosity and structural stability on disease has been carried out. A field survey of bananas and Foc SR4 in the Canary islands found that hydraulic conductivity and macroporosity were negatively correlated with

disease severity, and that structural stability was also important (Gutierrez Jerez *et al.* 1983). Further research is required to investigate the effect of soil structural characteristics on disease severity.

2.5.4 pH

Soil pH is often referred to as a master variable due to its substantial effect on biotic and abiotic processes (Brady and Weil 2000), and effects on Fusarium wilt have been shown in several studies. A negative relationship between Fusarium wilt severity and soil pH has been demonstrated for banana and a variety of other hosts (Figure 2-5). However, there have also been contradictory results demonstrating a positive relationship, with both banana (Peng *et al.* 1999) and other crops (Hopkins and Elmstrom 1976; Huang *et al.* 2012; Cao *et al.* 2016). One possible reason for the difference may be related to the nature of the soils studied. Peng et al. (1999) studied a soil with pH 8, which they acidified, whereas most other studies examined mildly acidic soils, which they limed. Soil pH is a major determinant of microbial community composition (Rousk *et al.* 2010) and the soil organisms acclimatized to the naturally high pH in Peng et al.'s (1999) pot trial with banana may have responded differently to those found in acidic soils. In the case of the other anomalous studies, pH varied as a result of various treatments but was not deliberately manipulated; Cao et al. (2016), in pot and field experiments with watermelon, and Huang et al. (2012), in a pot trial with cucumber, varied organic amendment type, whereas Hopkins and Elmstrom (1976), in a field trial with watermelon, varied ammonium fertiliser rates. Although a correlation between pH and suppression was observed, it may have been coincidental. The inconsistency amongst results demonstrates the complexity of the response to changes in pH and the importance of directly versus indirectly manipulated variables.

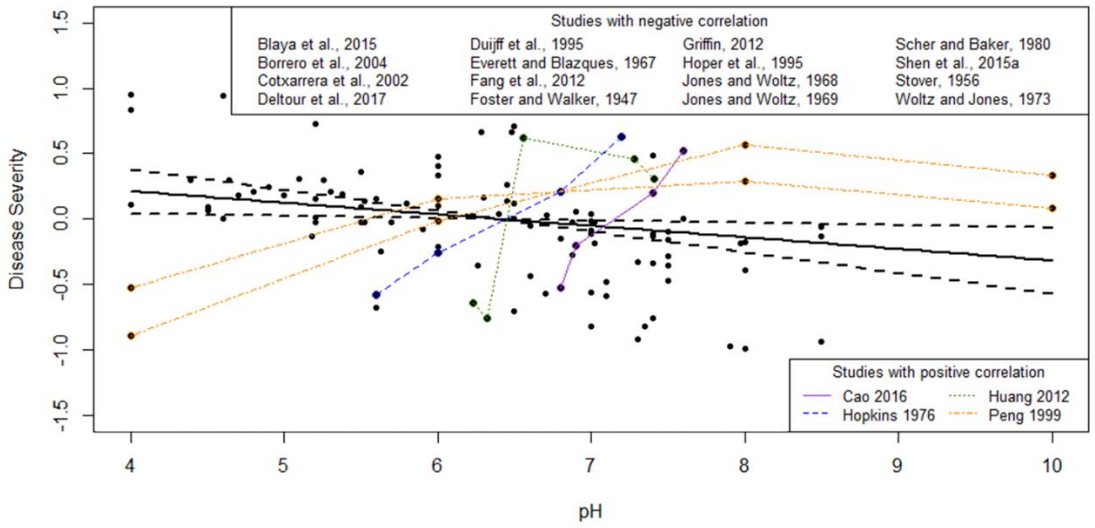

Figure 2-5. Generalised additive model for the meta-analysis of pH and disease severity from 21 studies of Fusarium wilt across a variety of crops and regions (see Table 2-1 for crops). In the studies included, soil pH was measured but not necessarily deliberately manipulated. Disease severity values reported in studies were normalised for comparison, with 0 representing the average disease severity for all studies. The overall trend is negative (full line, $p = 0.013$), as is the trend for most of the individual studies. The four studies demonstrating a positive correlation have been individually identified. Dashed lines represent the 95% confidence interval.

Soil pH, in combination with redox state, modulates the bioavailability of essential elements, particularly redox-sensitive micronutrients such as iron, manganese, zinc and copper (Collins and Buol 1970). Availability of nutrients influences health of the host plant, *F. oxysporum* and other soil microorganisms. To obtain micronutrients many organisms produce siderophores with stability constants differing in magnitude and pH dependence. Therefore, the relative ability of different species to obtain essential micronutrients differs with pH, due to the effect of pH on solubility of the metals and stability of the chelated forms (Boukhalfa and Crumbliss 2002; Dhungana and Crumbliss 2005).

Soil pH is also strongly positively correlated with bacterial diversity and abundance, with a doubling of bacterial diversity between pH 4 and 8 (Rousk *et al.* 2010), which encompasses the ideal soil pH range for most crops. Increased diversity of the bacterial population enhances general suppression and broadens the array of nutrient acquisition strategies, increasing the likelihood of a strong competitor to *F. oxysporum* being present. Increased competition, combined with reduced availability of nutrients, means *F. oxysporum* is less likely to meet its metabolic requirements at higher pH.

Soil pH can be altered by inputs and losses of materials, including cycling of nitrogen and carbon; this is particularly important in agricultural systems in which nitrogen fertiliser and organic matter are applied. Application of ammonium-based fertilisers or urea, or generation of ammonium from organic matter breakdown, leads to acidification of the rhizosphere due to excretion of protons by roots when ammonium is taken up, and to nitrification and leaching loss of nitrate. On the other hand, application of nitrate-based fertilisers tends to increase soil pH (Tinker and Nye 2000). Organic matter, either produced in situ or imported from elsewhere, has a large cation exchange capacity, which buffers soil pH through adsorption of ions (Baldock and Nelson 2000). These changes to pH then initiate other changes in the soil environment, such as nutrient availability, influencing suppression.

Although there have been numerous studies on the effects of pH on *F. oxysporum,* our understanding of the relationship is far from complete. The optimum soil pH_{water} for growing bananas is approximately 5.0 to 7.5 (Weinert and Simpson 2016). If pH modification were to be used as a means of reducing disease severity in the field, then the possible detrimental effect of raising pH above the optimum for bananas must be outweighed by the effect on disease suppression. For each combination of soil conditions and management regime there is presumably an optimum pH for plant growth and disease suppression. The relationship between pH and suppression requires in-field research to better understand these complex interactions.

2.5.5 Organic Matter

Organic matter strongly affects plant health, the chemical, physical and biological characteristics of soil, and disease suppression. It is also one of the most commonly added (as organic amendment applications or via exudation and death of plant roots) and compositionally diverse soil amendments. Furthermore, the amount and composition of organic matter within soil depend upon the inputs, climate, mineralogy, organisms and management. It is difficult to identify the mechanisms of suppression attributable to organic matter versus what are unimportant or confounding factors. This, in combination with limited analytical characterisation of organic matter, and variability of results, makes it difficult to identify consistent trends. Reviews linking organic matter characteristics to disease suppression have had mixed success at identifying unifying trends (Hoitink and Fahy 1986; Bonanomi *et al.* 2007; Janvier *et al.* 2007; Griffin 2012; Baum *et al.* 2015). That said, a meta-analysis has found at least some suppression from organic matter application in 74% of cases involving *Fusarium* across more than 150 studies (Bonanomi *et al.* 2007).

Suppression of Fusarium wilt associated with organic matter is generally attributable to a combination of interlinked biotic and abiotic characteristics (Baum *et al.* 2015). The biotic controls include total microbial community size, which creates competition for organic carbon, nutrients and space, and microbial antagonism by specific organisms (Baum *et al.* 2015). The importance of microbial populations to disease suppression has been demonstrated through a loss of suppressiveness when soil is sterilised (Cotxarrera *et al.* 2002; Reuveni *et al.* 2002; Noble and Coventry 2005). The abiotic characteristics of the organic matter and soil influence the microbial population composition, both directly and indirectly affecting the pathogen (Figures 2-1, 2-3). Physically, the organic matter impacts on the structure and structural stability of the soil, including pore spaces (Baldock and Nelson 2000). Chemically, organic matter impacts the soil pH, pH buffering capacity, amounts, forms and bioavailability of carbon and nutrients, and concentrations of important molecules such as siderophores (Baum *et al.* 2015).

The decomposability of organic matter appears of principal importance for disease suppression, as it affects the balance between the pathogen and competitive microorganisms (Figure 2-6) (Bonanomi *et al.* 2010). As decomposability declines, from fresh plant residues, through composts to different forms of peat, suppression tends to increase initially then decline steadily (Bonanomi *et al.* 2010). Application of compost has been demonstrated to generally increase suppression of Fusarium wilt of flax, melon and chrysanthemum in pot trials, though the effect varied with the type of compost (Chef *et al.* 1983; van Rijn *et al.* 2007; Saadi *et al.* 2010).

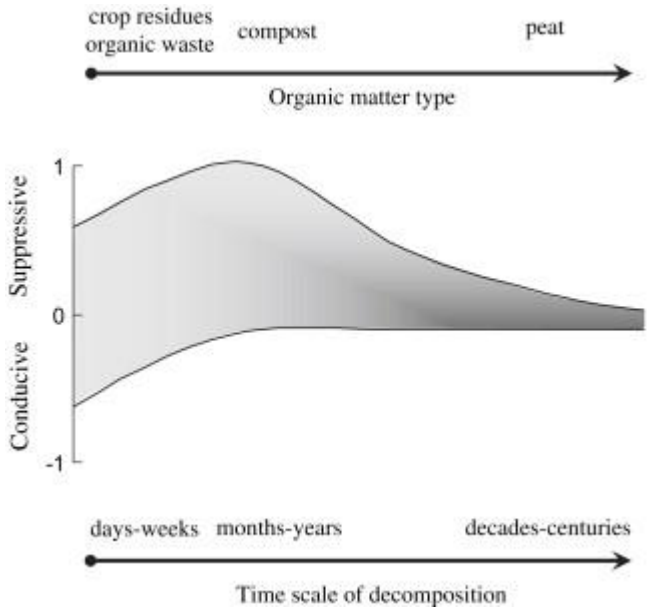

Figure 2-6. Schematic representation of disease suppression dynamics during organic matter decomposition (Bonanomi *et al.* 2010).

During the process of decomposition labile carbon sources are preferentially converted to carbon dioxide by microbes, increasing the overall recalcitrance of the remaining organic matter. This progressive loss of carbon and increased competition has been demonstrated to play a central role in the suppressiveness of some soils to *F. oxysporum* (Elad and Baker 1985b; Lemanceau 1989; Mazurier *et al.* 2009). High concentrations of labile carbon sources such as sugars in fresh plant residues mean there is little competition for carbon when they are added and thus no suppression. Increasing levels of glucose addition increased Fusarium wilt (*F. oxysporum*) severity in a pot trial with flax (Lemanceau 1989). An increased proportion of more complex compounds such as carboxylic and amino acids in amendments, has been found to increase suppression in pot trials with tomato and field trials with flax (Borrero *et al.* 2006; Senechkin *et al.* 2014). Castaño et al. (2011)

correlated hemicellulose concentration in organic amendments with suppression of *F. oxysporum* in tomato and carnation. Eventually, when decomposition has reduced carbon availability below that required to support a suppressive microbial community, suppression declines (Hoitink *et al.* 1997). In summary, if there is little competition and carbon is readily available to *F. oxysporum*, disease appears to increase, whereas if competition is strong, or carbon sources are more difficult for *F. oxysporum* to metabolise, suppression occurs.

The carbon-to-nitrogen (C:N) ratio of organic matter appears to be related to suppression. C:N ratio is a commonly measured characteristic of organic matter associated with the extent of decomposition (Brady and Weil 2000), and it differs substantially amongst organic amendments and soils. C:N ratio is positively correlated with *F. oxysporum* suppression, primarily due to its control on microbial community dynamics and ammonium generation during decomposition (Griffin 2012). A very high C:N ratio (>70:1) generally lowers mineral nitrogen concentration as microorganisms scavenge nitrogen; this reduces ammonium production, which can otherwise mitigate compost suppressiveness (Cotxarrera *et al.* 2002; Yogev *et al.* 2006). A C:N ratio above 20:1 also reduces plant-available nitrogen stressing the plant and likely increasing susceptibility to disease (De Ceuster and Hoitink 1999). A full understanding of the relationship between C:N ratio and suppression will require further research and a better understanding of the relative importance of the effects on the host plant, pathogen and competing microorganisms.

Soil organic matter greatly affects pH and nutrient availability due to its cation exchange capacity and wide range of acidic and basic functional groups (Brady and Weil 2000). Soil pH was found to strongly positively correlate with suppression in a field trial with flax where varying combinations of plant residues and animal dung were added (Senechkin *et al.* 2014), as well as in pot trials with tomato and carnation where various plant residues were added (Borrero *et al.* 2004; Borrero *et al.* 2009). In addition to supplying energy to heterotrophs, nutrients in organic matter can be important for plants and soil organisms (Brady and Weil 2000). In addition, siderophores, molecules produced by microorganisms to acquire micronutrients, can adsorb to organic matter, affecting their acquisition effectiveness (Figure 2-4) (Ahmed and Holmström 2014). In summary, the substantial effects of organic matter on suppression are complex, varied and incompletely understood.

2.5.6 Nitrogen

In addition to nitrogen in organic matter being important for disease severity and management, so is the nature of mineral nitrogen compounds in soil. Typically, the addition of nitrate fertiliser increases

the suppressiveness of soils to *F. oxysporum*, whereas addition of ammonium decreases suppressiveness (Table 2-1), although results vary. Studies have shown ammonium to be disease conducive relative to nitrate in field trials with melon (Jones *et al.* 1975), in pot trials with lime, cucumber, tomato and chrysanthemum (Woltz and Engelhard 1973; Morgan and Timmer 1984; Woltz *et al.* 1992; Wang *et al.* 2016), and in hydroponics trials with tomato and cucumber (Duffy and Défago 1999; Borrero *et al.* 2012; Wang *et al.* 2016; Zhou *et al.* 2017). On the contrary, ammonium application has been found suppressive relative to nitrate application in a pot trial with tomato (López-Berges *et al.* 2010), and a hydroponics trial with banana (Zhang *et al.* 2013). Also, soil ammonium concentration was negatively correlated with disease severity of watermelon in field and pot experiments with piggery waste (following anaerobic biogas generation) application (Cao *et al.* 2016). Finally, ammonium application has been reported to have no impact on disease severity on a field trial with cucumber (Jones *et al.* 1975) and pot trial with tomato (Jarvis and Thorpe 1980). The effects of mineral nitrogen form on disease severity presumably occur through their effects on rhizosphere chemistry, plant defence mechanisms and fungal pathogenicity.

Ammonium and nitrate differ in their impact on rhizosphere chemistry. Nitrogen fertiliser is predominantly applied as ammonium, nitrate or urea, which rapidly hydrolyses to generate ammonium. When organic matter with a low C:N ratio decomposes ammonium is also generated, as nitrogen is surplus to the needs of the decomposers (Brady and Weil 2000). Ammonium skews plant ion uptake to cations, hence altering the uptake of other ions such as potassium, chloride and nitrate (Hawkesford *et al.* 2012). Also, as the plant takes up ammonium, protons are excreted into the rhizosphere, acidifying the soil. Conversely, nitrate uptake increases relative cation uptake, and protons are removed from the rhizosphere for plant reduction of nitrate to ammonia (Tinker and Nye 2000). This increases the pH of the rhizosphere (Neumann and Römheld 2012), which has been shown to decrease Fusarium wilt severity.

Nitrogen supply and form affect plant defence, via the regulation of intracellular concentrations of cell sugars and amino acids, which provide nutrients for invading fungi upon cell apoptosis; polyamines, which act as defence signals; and nitric oxide (Mur *et al.* 2017). For an in-depth review of the role of nitrogen in plant defence against pathogens, see the recent work by Mur et al. (2017). The reduction of nitrate (NO_3^-) to nitrite (NO_2^-) generates nitric oxide (NO) through the nitrate reductase pathway (Gupta *et al.* 2005). A high concentration of ammonium inhibits this reaction, thereby reducing the concentrations of nitric oxide in the plant (Gupta *et al.* 2013). Nitric oxide is involved in plant defence both initially, as part of the pathogen-triggered immunity response (Zeidler *et al.* 2004), and upon cell death as a scavenger, reacting with superoxide (O_2^-) as part of the pathogen-induced hypersensitivity

response (Delledonne *et al.* 1998; Delledonne *et al.* 2001; Swarupa *et al.* 2014). This scavenging function is particularly important in limiting cell death in relation to *F. oxysporum* infection. As a facultative saprophyte, *F. oxysporum* can survive on living or dead tissue and so can benefit from cell apoptosis due to the discharge of nutrient-rich cytoplasm (Anthony *et al.* 2017).

Nitrogen supply also affects pathogenicity of *F. oxysporum.* Most experiments have shown increased severity of Fusarium wilt with increasing supply of ammonium (Figure 2-2), but López-Berges et al. (2010) found the opposite in a pot trial with tomato. Invasive growth, hyphal fusion and host adhesion were all suppressed by ammonium and increased in the presence of nitrate (López-Berges *et al.* 2010). When deprived of nitrogen in their preferred form (organic nitrogen or ammonium), saprophytic fungi entered a nutrient exploration mode resulting in rapid infection of the host (Walters and Bingham 2007). Zhou et al. (2017) tested suppression both *in vitro* and *in vivo* to differentiate between the suppression of the pathogen and the disease. They determined that both the plant and pathogen grew better with addition of nitrate but it was the balance of the *F. oxysporum* pathogenicity mechanism against the opposing plant defence mechanism that determined infection success and disease severity (Zhou *et al.* 2017).

Nitrogen amount, as well as form, is important for Fusarium wilt suppression. Nitrogen deficiency stress in the plant is likely to increase susceptibility to disease, and increased nitrogen application has been shown to increase suppression in a field trial with fava bean (Dong *et al.* 2013), and a hydroponic trial with cucumber (Zhou *et al.* 2017) . Despite this, research has shown no correlation between plant tissue nitrogen concentrations and suppression of Fusarium wilt of tomato in a pot trial (Hoffland *et al.* 2000). This may indicate that above the concentration that the plant can take up, further nitrogen fertilising has no benefit. Further research is needed to identify the optimal level of nitrogen in each system, as well as understand better the effects of nitrogen form on *F. oxysporum*, particularly Foc TR4.

2.5.7 Calcium

Addition of calcium to soil has been shown to suppress Fusarium wilt across a wide variety of host plants and soil conditions (Table 2-1). Calcium supply has often been manipulated together with pH (Everett and Blazques 1967; Jones and Woltz 1969; Jones and Woltz 1970; Jones and Overman 1971; Gatch and du Toit 2016). Soil pH is typically manipulated with calcium hydroxide ($Ca(OH)_2$) or limestone ($CaCO_3$). To manipulate calcium supply without altering pH, calcium sulphate has been

used. An additive benefit from increasing both pH and calcium concentration has been identified in pot trials with tomato (Jones and Woltz 1969).

Calcium, like silicon, stabilises the cell structure, accumulating at the cell wall and the middle lamella (Hawkesford *et al.* 2012). Increased calcium concentration in the cell wall helps to reduce the loss of cytoplasmic compounds in the case of cellular penetration by fungi. Higher concentrations of calcium in the intercellular space can also inhibit attack by parasitic fungi (such as *F. oxysporum*) by inhibiting enzymes designed to degrade the middle lamella (Huber *et al.* 2012).

Calcium is also involved in intracellular mechanisms regulating plant defence and pathogen growth. In the plant cytosol, calcium concentration change has been identified as a key secondary messenger in defence against both biotic and abiotic stressors (Lecourieux *et al.* 2006). Plant cytosol calcium concentrations modulate response to both parasitic and symbiotic fungi, such as arbuscular mycorrhizal fungi (Navazio and Mariani 2008; Johnson and Oelmüller 2013; Sherameti *et al.* 2014). Inoculation with arbuscular mycorrhizal fungi has shown promise for Foc R1 suppression in bananas by increasing nutrient availability, and high calcium supply enhances this symbiosis (Smith 2006). In the cytosol of *F. oxysporum,* calcium is involved in sporulation and in signalling pathways regulating hyphal growth rate and morphology (Hoshino *et al.* 1991; Kim *et al.* 2015) indicating that *F. oxysporum* may be controlled by limiting calcium. However, there is no evidence to suggest that calcium supply could be regulated at a level that benefits the host plant but not the pathogen, so future research would best focus on maximising the relative benefit for the host plant compared to the pathogen.

2.5.8 Iron and Manganese

Iron and manganese are here discussed together, as they are similar with respect to environmental effects on their availability and mechanisms of suppression. The lower the supply of bioavailable iron or manganese in soil, the more successful host plants and beneficial bacteria appear to be in the presence of *F. oxysporum* (Table 2-1). Micronutrient requirements differ amongst organisms, and *F. oxysporum* has a particularly high requirement (Woltz and Jones 1981). The bioavailability of metals essential for microorganisms and plants, particularly iron and manganese, is determined largely by their oxidation state, which is driven by soil pH and redox potential. These metals change from soluble, mobile and plant-available forms with low oxidation state to insoluble, immobile forms with high oxidation state as soil pH and redox potential increase (Lindsay and Schwab 1982). The ability

of soil organisms to obtain bioavailable micronutrients can be altered by manipulating the soil pH and redox state (Jones and Woltz 1970; Höper and Alabouvette 1996).

Organisms have developed specific acquisition strategies to compete for micronutrients, as naturally occurring concentrations of available forms in soil are typically below those required for continued growth (Figure 2-4). Strategy 1 plants, such as bananas, excrete protons and organic acids to acidify the rhizosphere, and reduce insoluble Fe^{3+} to soluble Fe^{2+} with reductases (Marschner and Römheld 1995). Strategy 2 plants, comprised entirely of grasses, excrete phytosiderophores that chelate Fe^{3+}, increasing its bioavailability (Marschner and Römheld 1995). This acquisition strategy is similar to that of fungi and bacteria, which also produce siderophores (Marschner and Römheld 1995; Lemanceau *et al.* 2009). Plants are able to take up iron bound by bacterially produced siderophores either directly, by taking up the entire siderophore-metal complex (Vansuyt *et al.* 2007; Xiong *et al.* 2013), or indirectly, by acidifying and reducing the rhizosphere, destabilising the complex (Bienfait 1986). Once free of the siderophore, diffusion moves iron ions to the root, where they are taken up. Fungal and bacterial siderophores must compete with each other to make iron available. The higher the stability of a metal-chelate complex, the greater the proportion of available metal cations captured by the chelate.

Chelating agents with high chelate stability have long been shown to induce suppression of *F. oxysporum*, as shown with application of 8-quinolinol to Fusarium wilt of cotton by Subramanian (1956). The strong artificial chelating agent ethylenediaminedi-*O*-hydroxyphenylacetic acid (EDDHA, $K=10^{33.9}$) (Lindsay 1979) outcompetes the weaker *F. oxysporum* siderophore fusarinine ($K=10^{29}$) for iron (Scher and Baker 1982). Pseudobactin ($K=10^{32}$) (Meyer and Abdallah 1978), produced by *Pseudomonas fluorescens*, also outcompetes fusarinine for iron, leading to *F. oxysporum* suppression (Scher and Baker 1982; Van Peer *et al.* 1990; Saritha *et al.* 2015). Both EDDHA, which binds free iron, and Fe-EDDHA have been shown to suppress *F. oxysporum* growth and Fusarium wilt severity in rhizosphere soil (Scher and Baker 1982). The plant is able to take up iron from Fe-EDDHA and release the EDDHA to bind free iron (Figure 2-4) (Elad and Baker 1985a). In bulk soil, away from the roots, EDDHA decreases *F. oxysporum* chlamydospore germination, but not Fe-EDDHA (Elad and Baker 1985a). EDDHA in bulk soil, unlike saturated Fe-EDDHA, sequesters available iron, decreasing *F. oxysporum* germination. EDDHA has also been shown to shuttle iron from clay-adsorbed fungal siderophores to the plant due to its low adsorption affinity, further enhancing plant growth at the expense of soil fungi (Siebner-Freibach *et al.* 2004). Lemanceau and Alabouvette (1993) provided a comprehensive (to that date) review of suppression by addition of chelate and production of siderophores by *Pseudomonas*.

Effects of iron availability on suppression of Fusarium wilt (*F. oxysporum)* have been attributed to the effect of the pathogen's iron status on virulence. Iron is essential for many processes in fungi, so it must be acquired at an adequate rate for growth and pathogenicity. Iron deficiency and toxicity can both occur, so organisms have complex processes to maintain specific internal concentrations. The deletion of Hap-X, a bZIP protein in *F. oxysporum* responsible for iron homeostasis, substantially reduces the virulence and growth of the pathogen under iron-depleted conditions (López-Berges *et al.* 2012; López-Berges *et al.* 2013; Gsaller *et al.* 2014). Virulence is further reduced if competition for iron is increased by the presence of siderophore producing antagonistic bacteria (López-Berges *et al.* 2013). The environmental concentration of iron required for *F. oxysporum* f. sp. *Cucumerinum* chlamydospore germination has been identified in laboratory studies as between 10^{-22} and 10^{-27} M, and concentrations in the rhizosphere may fall below this value (Simeoni *et al.* 1987). The link between pathogenicity, propagation and iron status points to possible future management strategies. Reducing bioavailable micronutrient concentrations below the levels required by the pathogen may provide a means of reducing disease severity.

Manganese has similar effects to iron. It is essential for photosynthesis, nitrogen metabolism and the formation of variety of enzymes in plants (Millaleo *et al.* 2010). Manganese is also involved in the production of lignin, an important component for plant defence against fungal invasion (Dordas 2008; Broadley *et al.* 2012; Gatch 2013). Like iron, manganese is acquired by fungi and bacteria through chelation, and has similar vulnerabilities to treatment with chelates. Low available manganese has been shown to reduce Fusarium wilt disease severity in a pot trial with tomato (Jones and Woltz 1970) and *F. oxysporum* chlamydospore germination in laboratory trials (Sneh *et al.* 1984).

2.5.9 Zinc and other trace elements

Zinc, like iron and manganese, is a redox-sensitive essential micronutrient, but it tends to have the opposite effect on Fusarium wilt. In plants, zinc is involved in protein and starch synthesis, membrane stability and defence mechanisms, and as an enzyme cofactor (Siddiqui *et al.* 2015). Studies have shown zinc is important in the oxidative burst defence mechanism of plants (Cakmak 2000; Kawano *et al.* 2002) which reduced disease severity of *F. solani* in wheat, except if zinc was deficient (Khoshgoftarmanesh *et al.* 2010).

Increased zinc bioavailability reduces production of fusaric acid by *F. oxysporum* (Duffy and Défago 1997). Fusaric acid has been identified as a cause of phytotoxicity, a trigger for plant defence

mechanisms, and a virulence factor in pathogenic *F. oxysporum*, but its effect is suppressed by the addition of zinc, copper and iron (López-Díaz *et al.* 2017). This indicates that fusaric acid is both a phytotoxin and a chelator, produced as part of a feedback loop to provide necessary micronutrients to pathogenic *F. oxysporum* (López-Díaz et al. 2017). However, increased bioavailability of zinc has shown mixed effects on Fusarium wilt in pot trials with tomato (Jones and Woltz 1970), except in high concentrations where phytotoxicity is likely to be involved (Duffy and Défago 1997). While most studies suggest that zinc has a suppressive effect on Fusarium wilt, its requirement for so many biological processes makes it difficult to identify mechanisms for the effects.

Other essential micronutrients also appear to influence Fusarium wilt severity. A molybdenum concentration below 20 mg/L decreased Fusarium wilt severity in hydroponic studies of tomato (Duffy and Défago 1999). Molybdenum is necessary for the conversion of nitrate nitrogen to amino acids in plants (Siddiqui *et al.* 2015) and plant molybdenum requirements are highly dependent upon the nitrogen fertiliser form supplied (Broadley *et al.* 2012). However, molybdenum's role in plant defence and Fusarium wilt suppression is unclear. Copper suppressed Fusarium wilt of tomato at concentrations >20 mg/L in hydroponic trials (Duffy and Défago 1999). Copper is important for formation of a physical lignin barrier to disease, and for production of phenolic compounds, part of the plant response to pathogens (Siddiqui *et al.* 2015). Boron deficiency has also been demonstrated to increase Fusarium wilt disease severity in flax (Keane and Sackston 1970), though no change was found in a hydroponic study of bananas (Dong *et al.* 2016). Like copper, boron plays a role in cell wall structure and the production of defence-related compounds. Other trace elements may also influence Fusarium wilt, but their effects have not been reported.

2.5.10 Silicon

Although not qualifying as an essential element for plants (Arnon and Stout 1939), silicon has long been identified as an important constituent in plant defence (Huber *et al.* 2012). Various pot trials have concluded that the addition of soluble silicon reduces Fusarium wilt severity (Foc R1 and TR4) in bananas (Kidane and Laing 2008; Fortunato *et al.* 2012a; Fortunato *et al.* 2012b; Jones 2013; Wibowo *et al.* 2014). The mechanisms of defence in bananas are not entirely clear but have been hypothesized to encompass both physical and biochemical factors (Sakr 2016). For an in-depth review of silicon in plant defence against pathogens, see the recent reviews by Sakr (2016) and Wang et al. (2017).

Silicon fertiliser increases plant silicon uptake, allowing formation of a barrier in root cell walls that resists fungal penetration and ingress (Fortunato *et al.* 2012a; Jones 2013). Research has shown silicon is most effective for defence, and is most efficiently absorbed, when applied to the roots (Liang *et al.* 2005), triggering both the biochemical and cell wall physical mechanisms (Dallagnol *et al.* 2015).

Biochemical defence mechanisms involving silicon include local and whole-plant responses to attack. A whole-of-plant response is evident from enhanced resistance to both root and foliar diseases when silicon supply to the roots is increased (Dallagnol *et al.* 2015). Plant defence responses include activation of the phenylpropanoid pathway (Fortunato *et al.* 2014) and silicon-mediated accumulation of phenolics and lignin at infection sites (Fortunato *et al.* 2012a; Whan *et al.* 2016).

Silicon is a major constituent of most soil minerals but little is available in soluble form. Accelerated mineral weathering by root exudates has been shown to increase bioavailable silicon in the rhizosphere, compared to bulk soil (Gattullo *et al.* 2016). This enhanced weathering was linked to phytosiderophore production in graminacious plants suffering iron deficiency (Gattullo *et al.* 2016). Bananas, as iron acquisition strategy 1 type plants (Marschner and Römheld 1995), do not produce phytosiderophores, but they do exude protons, reductases and organic acids, and this is likely to increase mineral weathering and silicon availability. Bananas are also likely to benefit from fungal and bacterial siderophore-mediated weathering in the rhizosphere.

2.6 Conclusions

Fusarium wilt severity is influenced by numerous soil abiotic attributes and their interactions. Several promising avenues for mitigating Fusarium wilt of banana are suggested by previous research with a variety of crops, however this research is mostly pot trials and field observations. These avenues have yet to be tested in field conditions and commercial production systems. We hypothesise that suppression will be maximised by: increasing soil pH to the highest value possible without impacting banana plant growth; maintaining soil water content, which largely controls the soil redox state, consistent and high to avoid stress on the plant; maintaining high concentrations of bioavailable calcium, silicon and zinc; keeping concentrations of bioavailable iron and manganese low to disadvantage the pathogen; and, when mineral fertiliser is added, applying nitrate rather than ammonium. Addition of partially decomposed organic matter with C:N ratio between 20:1 and 70:1, amended with suppressive organisms, also appears to decrease disease severity. To assess their effectiveness these approaches need to be tested in manipulative field experiments, individually and in

combination, in different regions, soil types, cultivars and management systems. Due to the semi-perennial nature of banana production, trials must be long term to examine possible accumulation or dissipation of effects over time. Despite the complexities and challenges involved, further elucidation of the mechanisms discussed here appears well worthwhile, with the ultimate goal of developing commercially feasible cultural techniques to reduce severity of Fusarium wilt.

3 Soil physicochemical characteristics and leaf nutrient contents on banana farms of North Queensland, Australia

Chapter No.	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author, including the candidate
3	This chapter is unpublished at the time of thesis submission, but the full data set collected has been published as: Orr, R. and P. Nelson (2021). North Queensland banana farm survey 2017. J. C. University. This chapter has also contributed to the following publications that are not included in this thesis: Bowen, A., Orr, R., McBeath, A.V., Pattison, A., and Nelson, P.N. (2019). "Suppressiveness or conduciveness to Fusarium wilt of bananas differs between key Australian soils." Soil Research 57(2): 158-165. Oliver, D. P., Li, Y., Orr, R., Nelson, P.N., Barnes, M., McLaughlin, M.J., and Kookana, R.S. (2019). "The role of surface charge and pH changes in tropical soils on sorption behaviour of per- and	Ryan Orr, Dr. Paul Nelson and Dr. Tony Pattison co-developed the idea for this chapter. Site selection and sample collection was carried out by Orr, with help from Tegan Kukulies, Stewart Lindsay and Shanara Veivers from the Queensland Department of Agriculture. Data analysis was performed by Orr with assistance from Nelson. Orr wrote the first draft of the paper which was revised with editorial input from Nelson, Pattison and Dr. Tobin Northfield. Orr developed the figures and tables with assistance from Nelson.

polyfluoroalkyl substances (PFASs).” Science of The Total Environment 673: 197-206.

Oliver, D. P., Li, Y., Orr, R., Nelson, P.N., Barnes, M., McLaughlin, M.J., and Kookana, R.S. (2020). “Sorption behaviour of per- and polyfluoroalkyl substances (PFASs) in tropical soils.” Environmental Pollution 258: 113726.

3.1 Abstract

Bananas are an important crop in tropical Australia and the industry faces a variety of challenges including costs of production, disease and pests, and environmental impacts. North Queensland produces more than 90 % of Australia’s bananas in three primary sub-regions, two coastal with very high rainfall (Tully and Innisfail) and one on an elevated tableland with less rainfall (Mareeba). The range of soil characteristics and banana nutrient status on banana farms has not previously been systematically described. In this work we characterised chemical and physical characteristics of soil and banana leaf tissue from 28 banana farms on soil types accounting for >85 % of Australia’s banana production. Soil characteristics at these sites covered a similar range to those measured in commercial tests from across the industry (n=1,812).

Variation in soil properties and leaf nutrient concentrations were driven largely by site- (principal component 1 in both cases) and management-related variables (principal component 2 in both cases). The most important site characteristics appeared to be soil parent material and climate, with the mostly basaltic and low rainfall Mareeba sub-region differing from the other two sub-regions. Foliar leaf nitrogen concentrations were mostly below the level that allows farmers to apply fertiliser nitrogen at rates above the regulated limit. This information on the range of soil characteristics and plant nutrient status could facilitate efficient research, extension, monitoring and regulation regarding production- and environment-related issues.

3.2 Introduction

Banana production in Australia is approximately 365,000 tonnes/year produced from 11,280 hectares, of which greater than 90 % is in North Queensland (ABGC 2017). The region is situated between approximately 15°50'S and 18°20'S along the east coast of Queensland. Production is primarily on coastal plains and slopes, with annual rainfall ranging from 1,800 to 4,500 mm (Murtha 1986). Smaller growing areas exist on nearby elevated tablelands, up to 600 m elevation and with annual rainfall of approximately 1,400 mm, supplemented by irrigation (Laffan 1988).

The variation of soils and plant nutrient status in the banana industry in North Queensland has previously been described in general terms. Soils of the region have been classified and mapped (Murtha 1986; Laffan 1988; Murtha 1989; Cannon *et al.* 1992; Murtha *et al.* 1996; Enderlin *et al.* 1997; Morrison *et al.* 2021), the location of banana farms is known (2015; Biosecurity Queensland 2016; Clark and McKechnie 2020), and several reports on banana production and management mentioned soil types (Daniells 1984; Pattison *et al.* 2005; Harvey *et al.* 2018). Additionally, several surveys have sampled conventional banana farms in tandem with either organic or unfarmed areas (Pattison *et al.* 2008; Geense *et al.* 2015; Pattison *et al.* 2018b). However, to our knowledge this is the most extensive survey of North Queensland banana farms, both geographically and in the number of characteristics analysed, and is the first to sample based on maximising soil map unit diversity.

Understanding the characteristics of soils used for banana production is important for applications such as nutrient and disease management, minimising off-site environmental impacts, and future expansion of the industry. For example, the severity of Panama disease, a current threat to the industry, is related to soil properties (Orr and Nelson, 2018). Panama disease, or Fusarium wilt of banana, is caused by a soil-borne fungal pathogen (*Fusarium oxysporum* f. sp *cubense*) and the potential cost of a strain first detected in North Queensland in 2015 is greater than $138 million per year (Cook *et al.* 2015; O'Neill *et al.* 2016). Fusarium wilt severity differs between key soils of North Queensland (Bowen *et al.* 2019). Nutrient management, which is affected by soil type, has also been of particular concern in this region as it drains to the environmentally sensitive Great Barrier Reef (Rasiah *et al.* 2010; Armour *et al.* 2013; Armour 2018). Banana cultivation requires high inputs of potassium, moderate inputs of nitrogen and relatively low inputs of phosphorus (Armour 2018). Regulations were recently introduced limiting application of nitrogen and phosphorus fertiliser in banana production to a maximum of 400 kg N / ha / year, unless foliar nitrogen content is below 3.5 % dry weight, and 60 kg P / ha / year, unless foliar phosphorus content is below 0.22 % dry weight (DES 2019). These rates consider neither the effect of soil characteristics on the availability and retention of nutrients nor the range of soil types currently under cultivation. The aim of this research

was to characterise the range of soil characteristics and plant nutrient status across the banana farms of North Queensland.

3.3 Materials and methods

3.3.1 Site Selection

Sampling locations were selected on the basis that they were used for banana production and to maximise the variability of soil characteristics as well as geographic distribution within the North Queensland banana growing region. Geology is primarily granite and other acid igneous rocks, metamorphosed sediments, alluvium and basalt (Table 3-1). The region was divided geographically into three sub-regions (Figure 3-1).

A total of 28 sampling locations were selected on soil map units representing > 94 % of the area under banana production in North Queensland, and > 88 % of that in Australia (Table 3-1). Geographic data sets were obtained from the Queensland Government spatial catalogue and analysed in ArcGIS version 10.3.1. The area under banana production was obtained from the "Commercial banana production areas for Panama disease tropical race 4 program – North Queensland (2016)" data set and soil types were obtained from the "Soil and agricultural land suitability series (2016)" data set. Maps of banana production and soil types were not available for the two northernmost sites (25 and 26) in the Lakeland agricultural region, but soil types chosen were representative of banana growing conditions in the region (Morrison *et al.* 2021). For the purposes of analysis the Lakeland locations have been allocated to the "Mareeba" sub-region, to which they are geographically and climatically most similar. Soil survey layers were clipped to banana growing areas, merged to a single layer and subdivided based on primary soil type. Soil types were ranked by total area used for banana cultivation in this region and those comprising >0.4 % of the area were sampled. Three classifications were excluded; "Stream Channel" as it is based on proximity to streams rather than soil characteristics, and "Jarra" and "Dingo" soil types due to their small area and restricted access due to presence of Fusarium wilt of banana Tropical Race 4. The soil series chosen have been described by Murtha (1986), Laffan (1988), Cannon *et al.* (1992), Murtha *et al.* (1996) Enderlin *et al.* (1997), and Morrison *et al.* (2021).

Table 3-1: Sites sampled and their proportion of the banana growing area in North Queensland (NQ). Soil series without production area are from unclassified production areas or are combination soil types.

Site	Soil sample sub-region	Soil map unit	Parent material	Australian Soil Classification	Proportion of NQ banana area (%)
1	Innisfail	Innisfail	Allu.(w)	Brown Dermosol	14.8
2	Innisfail	Pin Gin	Basalt	Red Ferrosol	14.5
3	Innisfail	Tully	Allu. (w)	Brown Dermosol	13.7
4	Mareeba	Tolga	Basalt	Red Ferrosol	9.3
5	Innisfail	Liverpool	Allu.(w)	Orthic Tenosol	6.3
6	Innisfail	Mundoo	Basalt	Red Ferrosol	4.9
7	Tully	Thorpe	Granitic	Brown Kandosol	4.6
8	Tully	Virgil	Allu.(w)	Red Kandosol	3.1
9	Innisfail	Eubenangee	Basalt	Red Ferrosol	3.1
10	Innisfail	Coom	Allu.(p)	Redoxic Hydrosol	2.7
11	Tully	Mossman	Allu.(w)	Yellow Dermosol	2.7
12	Innisfail	Galmara	Metam.	Red Dermosol	2.3
13	Tully	Utchee	Granitic	Red Dermosol	2.1
14	Mareeba	Walkamin	Basalt	Brown Ferrosol	2.1
15	Innisfail	Tolga, Rocky	Basalt	Red Ferrosol	1.4
16	Innisfail	Tyson	Granitic	Red Kandosol	1.3
17	Innisfail	Timara	Allu.(p)	Redoxic Hydrosol	0.9
18	Mareeba	Morganbury	Granitic	Red Kandosol	0.8
19	Innisfail	Garradunga	Basalt	Red Ferrosol	0.8
20	Mareeba	Cobra	Metam.	Red Kandosol	0.8
21	Innisfail	Mission	Metam.	Red Kandosol	0.6
22	Mareeba	Ray	Basalt	Grey Ferrosol	0.5
23	Tully	Hillview	Granitic	Red Kandosol	0.5
24	Innisfail	Bulgun	Allu.(p)	Grey Dermosol	0.4
25	Mareeba	Laura	Basalt	Red Ferrosol	-
26	Mareeba	Bullhead	Basalt	Brown Dermosol	-
27	Innisfail	Coom-Tully	Allu.(p)	Redoxic Hydrosol/Brown Dermosol	-
28	Tully	Galmara – Mission	Metam.	Red Dermosol/Red Kandosol	-

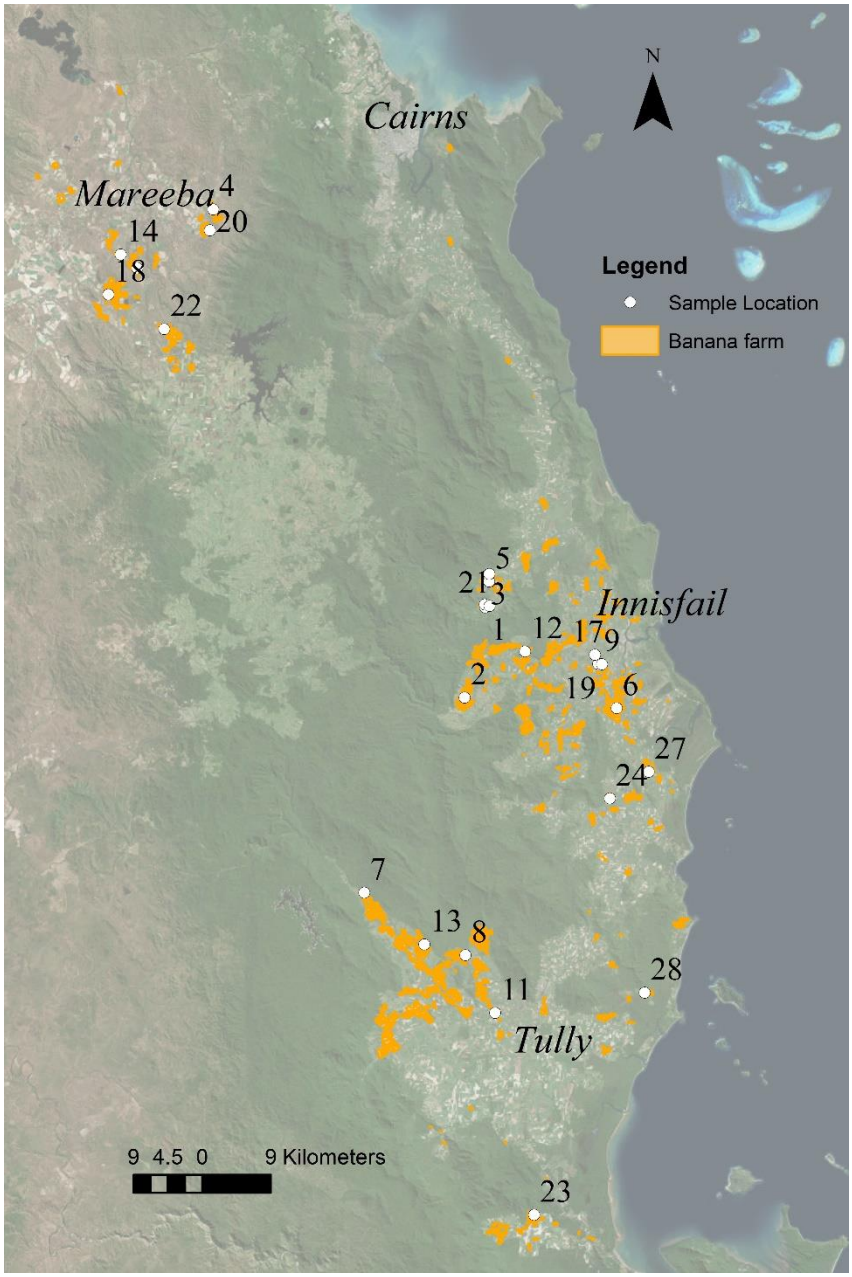

Figure 3-1: Sampling locations in North Queensland, Australia centred on the three towns of Mareeba, Innisfail and Tully. Sites 25 and 26, at Lakeland, 150 km NW of Cairns, are not shown.

3.3.2 Sampling

Composite soil samples, each comprised of 12 samples, were taken at each location in February – April 2017. Each sampling area was 20 m long and 4 rows (approximately 35 m) wide. As plant species and crop duration have both been shown to affect the soil microbiome, sampling areas were restricted to fields in which Cavendish bananas (*Musa* AAA) had been grown continuously for at least two years (Smalla *et al.* 2001; Garbeva *et al.* 2004; Shen *et al.* 2018) ; limiting the growing time further was not deemed practical. At each site the samples were combined, homogenised and

subsampled. Soil samples were taken 0.4 m from in front of the leading banana plant pseudostem, at 0.0 – 0.1 and 0.1 – 0.25 m depths. Plants sampled were mature, but not flowering or bunched, as development stage can influence soil and plant nutrition (Garbeva *et al.* 2004). Banana foliar samples were taken from the banana plant associated with each soil sample. Foliar samples were a 0.20 m wide strip from the centre of the third completely emerged leaf, from each side to the midrib (Broadley *et al.* 2004). Samples were rinsed with deionised water, the 12 individual samples were combined, and the sample was stored at 4°C until drying.

The fields sampled were managed in a variety of ways. Most had received applications of lime to neutralise soil pH prior to planting, with small doses annually. Most also had fertiliser blends applied regularly, with nitrogen primarily in the form of urea or ammonium, potassium as potassium chloride, phosphorus as phosphate, and some calcium, magnesium and micronutrients. Sites 4, 14 and 15 (from Table 3-1) had nitrogen applied as calcium and potassium nitrate. In addition to inorganic fertiliser blends, sugarcane millmud, a by-product from sugar mills, chicken manure or various 'biological' products were added to some fields. Site 5 was 'Ecoganic', applying less fertiliser, pesticides and nematicides than conventional and site 21 was certified Organic, applying no synthetic nitrogen fertiliser, pesticides or herbicides.

3.3.3 Analysis

All analyses were performed on samples from both 0 - 0.1 m and 0.1 - 0.25 m depths, except for mineralogy, which was measured only on the 0.1 - 0.25 m samples. Chemical analyses were carried out by Nutrient Advantage Laboratory, Werribee, Victoria. Analysis included (with method codes from Rayment and Lyons (2011)): ammonium and nitrate nitrogen 7C2b; chloride (1:5 water) 5A2b; boron 12C2; electrical conductivity (1:5 water) 3A1; exchangeable cations (calcium, magnesium, potassium, sodium) (1M ammonium acetate) 15D3; organic carbon (Walkley and Black) 6A1; pH (1:5 water) 4A1; phosphorus buffer index 9I2b; phosphorus (Colwell) 9B2; silicon ($CaCl_2$) (Haysom and Chapman 1975); sulphur (MCP) 10B3; total nitrogen (combustion) 7A5; total carbon (combustion) 6B2b; total (acid digest) phosphorus, calcium, copper, iron, magnesium, manganese, potassium, sulphur, zinc 17B1 and sand, silt and clay (Gee and Or 2002). Water holding capacity of each soil was determined using a 1-bar ceramic pressure plate with -10 kPa pressure applied to a blended, dried sample. After equilibration on the pressure plate, soils were weighed, dried for 24 hours at 105°C and reweighed, to determine water content.

Soil mineralogy was analysed by CSIRO Land and Water, Urrbrae, South Australia. Due to possible dehydration of the montmorillonite (smectite) interlayer samples were dispersed in 0.25 M calcium chloride, centrifuged at 5150 x *g* (Eppendorf Centrifuge 5810, Australia) for 10 minutes, calcium saturated again, washed with water, then ethanol (centrifuging between each step), and oven dried at 60°C. X-ray diffraction patterns were recorded with a PANalytical X'Pert Pro Multi-purpose Diffractometer using iron filtered cobalt Kα radiation, automatic divergence slit, 2° anti-scatter slit and fast X'Celerator Si strip detector. The diffraction patterns were recorded from 3 to 80° in steps of 0.017° 2 theta with a 0.5 second counting time per step for an overall counting time of approximately 35 minutes.

Qualitative analysis was performed on the X-ray diffraction data using in-house XPLOT and HighScore Plus (from PANalytical) search/match software. Quantitative analysis was performed on the X-ray diffraction data using the commercial package SIROQUANT from Sietronics Pty Ltd. Results are presented as a percentage of soil, as opposed to a percentage of the clay fraction alone.

Foliar samples were dried at 70°C, ground to a fine powder and analysed by Nutrient Advantage Laboratory, Werribee, Victoria for calcium, magnesium, phosphorus, potassium, sodium, sulphur, boron, copper, iron, manganese and zinc using a nitric acid and hydrogen peroxide digest followed by analysis with inductively coupled plasma atomic emission spectroscopy (ICP-AES). Ammonia, nitrate and chloride were extracted in a 1: 125 water extract and analysed by flow injection analysis (Kalra 1997). Total nitrogen was analysed by combustion (Kalra 1997). Two elemental ratios commonly used in diagnosis of nutrient deficiencies, N/P and N/K, were also included as variables.

To assess whether the samples collected were representative of North Queensland banana growing soils, the soil characteristics were compared to two anonymised soil data sets from North Queensland banana farms over the period 2012 - 2017 provided by Incitec Pivot (n = 738) and Total Grower Services (n = 1074). Soil characteristics chosen for comparison were those in which analysis methodology was consistent across all three data sets, i.e. pH (calcium chloride), electrical conductivity, cation exchange capacity, and extractable phosphorus (Colwell), sulphur (MCP), calcium, magnesium and potassium (ammonium acetate), iron, manganese, copper and zinc (DTPA), and boron (hot calcium chloride).

Rainfall and minimum temperature data were obtained from the Australian historical climate database SILO (Scientific Information for Land Owners) (Jeffrey *et al.* 2001). Minimum temperature was selected, as growth reduction at low temperature, principally due to reduced photosynthesis, cannot be avoided, whereas high temperature impacts are related to water deficiency, which can be countered by irrigation (Turner and Lahav 1983). Monthly rainfall and minimum temperature values were obtained for each point location spanning from June 2016 to February 2017 and averaged. Nine months was chosen as it represented the likely growing period of the plants sampled, based on growth rates and plant development stage.

3.3.4 Data analysis

Seventy-nine foliar nutrient and soil characteristics were analysed, and there was a great deal of covariance between them. Thus, principal components analysis was performed (R Core Team 2017) on soil characteristics and foliar nutrients to reduce the data sets to fewer orthogonal variables. Soil characteristics were excluded from the principal components analysis if they were redundant analytical methods for the same characteristic to avoid overweighting certain components due to multiple inclusions of the same information. The reduced list of characteristics used for principal components analysis was: water holding capacity, pH ($CaCl_2$), electrical conductivity, chloride, nitrate, ammonium (KCl), phosphorus (Colwell), phosphorus buffer index (Colwell), calcium (ammonium acetate), potassium (ammonium acetate), magnesium (ammonium acetate), sodium (ammonium acetate), cation exchange capacity (calculated from ammonium acetate), copper (DTPA), iron (DTPA), manganese (DTPA), zinc (DTPA), boron (Hot $CaCl_2$), sulphur (MCP), organic carbon, silicon ($CaCl_2$), total carbon, total nitrogen, carbon-to-nitrogen ratio, total cadmium, total calcium, total chromium, total copper, total iron, total lead, total magnesium, total manganese, total nickel, total phosphorus, total potassium, total sodium, total sulphur, total zinc, clay, sand, silt. The foliar nutrient concentration data were not reduced prior to principal components analysis.

Data were centred and scaled prior to running principal components analysis. Five principal components were retained for both soil and foliar analysis, based on their explained variance being greater than the average contribution of a single variable (Kaiser's method). Comparisons between sub-regions based on principal component values were calculated with a one-way analysis of variance followed by Tukey Post-Hoc analysis to determine specific group differences at $\alpha = 0.05$.

3.4 Results

The soils sampled were diverse, with a wide range of values for most properties measured (Figure 3-2). The full dataset, including soil characteristics not reported here have been published separately (Orr and Nelson 2021). The range of values for soil characteristics in this study was close to that in the much larger data sets obtained from Incitec Pivot and Total Grower Services (Figure 3-2).

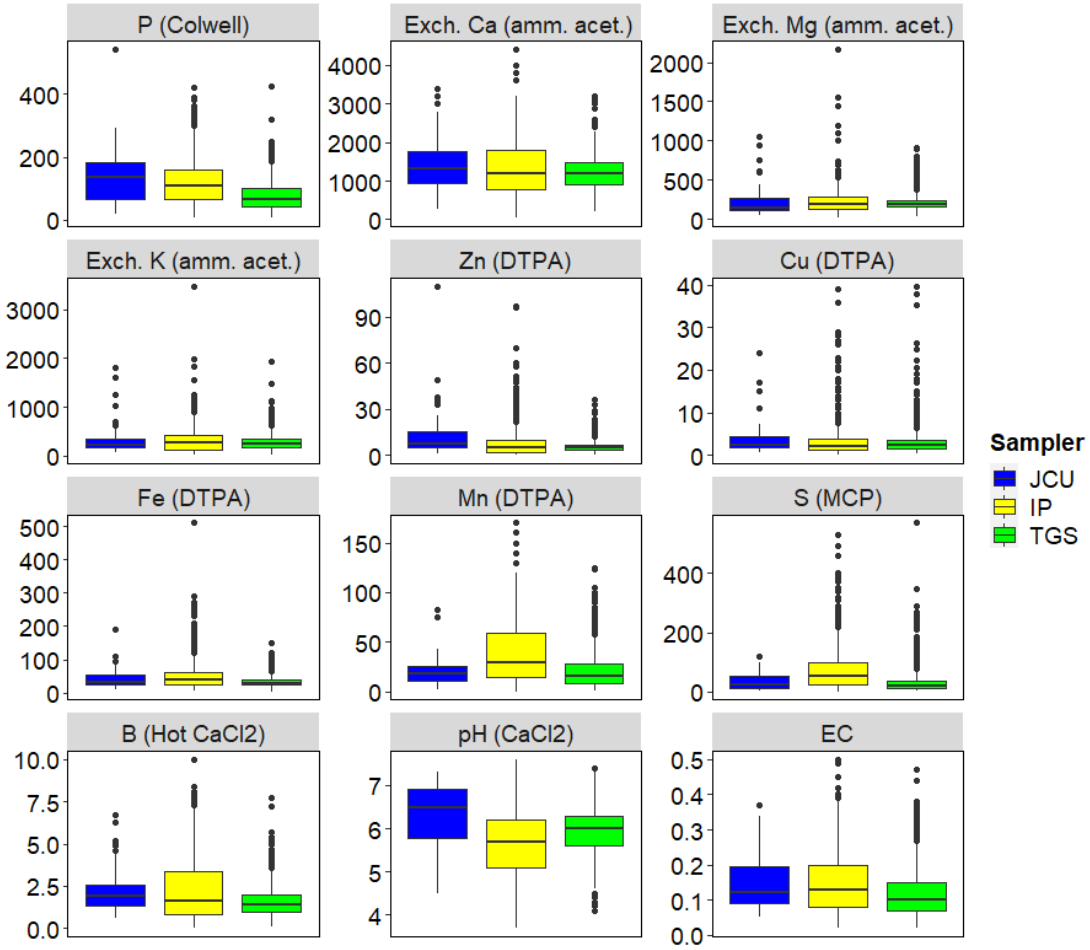

Figure 3-2: Comparison of selected soil characteristics between samples collected for this work (JCU, n = 28) and data sets provided by Incitec Pivot (IP, n = 738) and Total Grower Services (TGS, n = 1074). Units are mg / kg except for pH (unitless) and EC (dS / m). Lines represent the median value, boxes represent the interquartile range, tails represent the most extreme observation within the median ± twice the interquartile range and points represent outliers.

Due to the interrelated nature of soil characteristics many were highly correlated. Principal components analysis was useful in identifying those characteristics that represented the greatest variability within the dataset. For the soil characteristics, principal components 1 and 2 explained 39 % and 12 % of the variability, respectively (Table 3-2). Principal component 1 was most strongly

associated with electrical conductivity, total cadmium, total iron, cation exchange capacity and total nickel concentration, which were largely related to clay content and mineralogy. Principal component 2 was most strongly associated with silt content, total nitrogen, total sulphur, total carbon and organic carbon concentration, which were broadly related to organic matter content (Table 3-2).

Water holding capacity of the soils ranged from 12.3 – 36.6 %, with a median value of 31.0 % and was principally determined by the clay content of the soil ($R^2 = 0.453$, $t_{26} = 4.832$, $P < 0.001$). Kaolinite was the dominant clay mineral, accounting for > 50 % in most soils. Sampling locations with < 50 % kaolinite (with major other clay types) were (with numbers from Table 3-1): 5 (K-Feldspar, Albite, Mica/ Illite and Chlorite), 9 (Gibbsite and Hematite), 23 and 27 (K-feldspar) and 28 (Mica/Illite). Hematite was also common amongst soils of basaltic origin.

Table 3-2: The top five highest loadings for the considered principal components of the soil and foliar characterisation. While five principal components were retained only two were logically interpretable.

Soil

PC1 (39 %)	Loadings	PC2 (12 %)	Loadings
EC	0.228	Silt content	-0.312
Cadmium (Total)	0.225	Nitrogen (Total)	-0.287
Iron (Total)	0.218	Sulphur (Total)	-0.267
Cation exch. Capacity	0.218	Carbon (Total)	-0.267
Nickel (Total)	0.215	Carbon (Organic)	-0.264

Foliar

PC1 (31 %)	Loadings	PC2 (21 %)	Loadings
Sulphur	0.392	N/P Ratio	0.483
Calcium	0.338	N/K Ratio	0.432
Magnesium	0.326	Nitrogen	0.350
Iron	0.309	Phosphorus	-0.341
Manganese	0.288	Potassium	-0.326

Foliar nutrients, like soil characteristics, were highly intercorrelated, though less of the variance in the dataset was explained by the first principal component (Table 3-2). The foliar first principal

component appears related to particular proteins (sulphur), chlorophyll and chloroplasts (magnesium and iron), cell walls (calcium) and enzyme cofactors (magnesium, iron and manganese). The second principal component was primarily dependent on the concentration of nitrogen, alone and relative to the other macronutrients phosphorus and potassium (Table 3-2).

The soil characteristics differed significantly between Mareeba and the other growing sub-regions based on principal components 1 and 2 (Figure 3-3). The Mareeba sub-region had a greater range in values for principal component 1 (clay content and mineralogy), whereas the Innisfail and Tully sub-regions had greater variation in principal component 2 (organic matter). Soil principal component 1 strongly differentiated between basalt soils and those of other parent material and was significantly affected by rainfall but not by temperature (Figure 3-4). The foliar principal component 1 also significantly separated the Mareeba sub-region from the others (Figure 3-3). Foliar principal component 1 was significantly related to average monthly rainfall and average minimum temperature for the nine months preceeding sampling but did not differ between soil parent materials (Figure 3-4). Foliar principal component 2, primarily indicative of the macronutrients nitrogen, potassium and phosphorus, did not differ significantly between sub-regions.

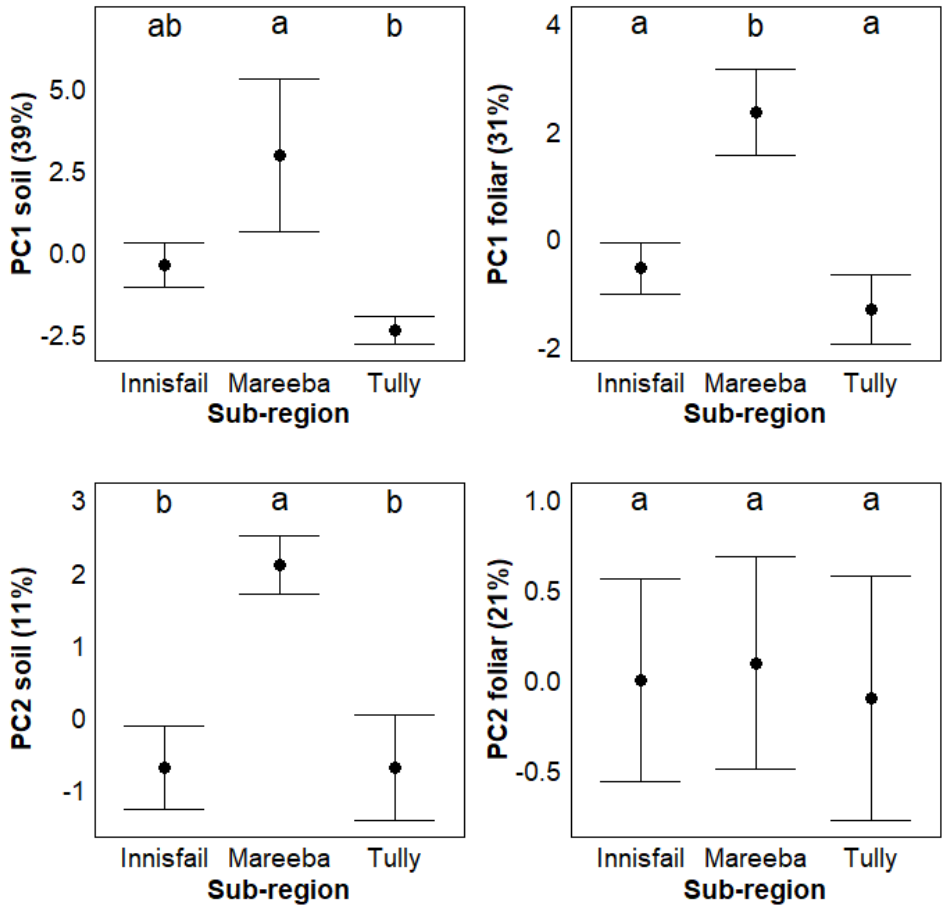

Figure 3-3: Principal component 1 and 2 for soil (left) and foliar nutrition (right) compared between the banana producing sub-regions of Far North Queensland. Letters indicate groupings based on Tukey Post-hoc assessment ($\alpha = 0.05$).

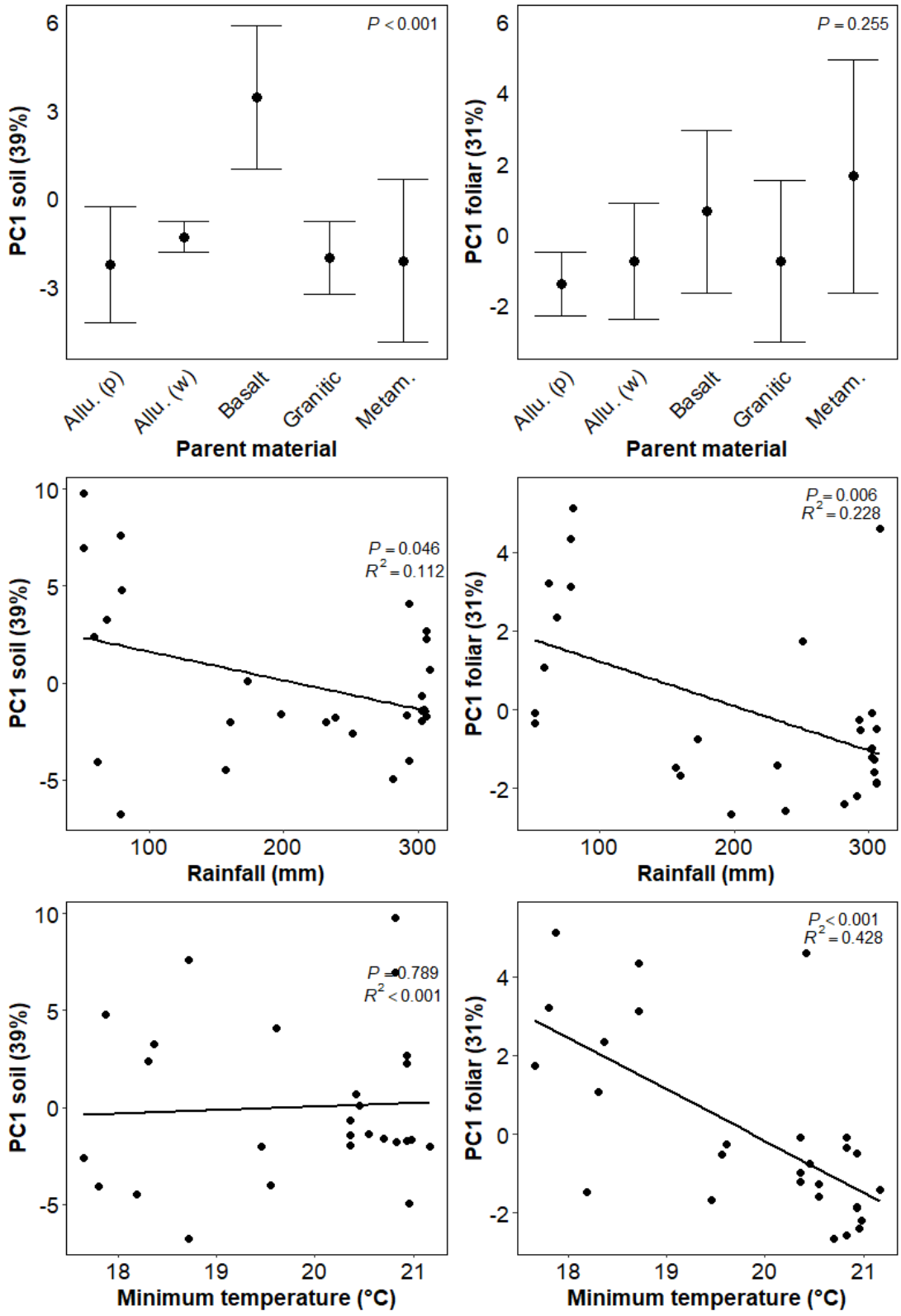

Figure 3-4: Soil (left) and foliar (right) principal component 1 compared with soil parent material, the average monthly rainfall and average minimum temperature at each sampling location over the nine months preceeding sampling. For parent material, points indicate mean values, error bars indicate standard error.

The range of foliar nutrients and soil characteristics measured here was important for several issues currently facing the banana industry. Suppressiveness of North Queensland soils to Panama disease, measured using a subset of the soils examined here, was positively correlated with clay and total iron content (Bowen *et al.* 2019). We found that clay content did not differ significantly between the sub-regions ($F(2, 25) = 1.348$, $P = 0.278$) but that soil total iron content did ($F(2, 25) = 4.493$, $P = 0.022$) (Figure 3-5), so risk of disease severity may differ between the sub-regions. Total foliar nitrogen and phosphorus content are important for determining if a grower may increase fertiliser use above the regulated maximum rate. Total foliar nitrogen content did not significantly differ between banana farms in the sub-regions of North Queensland ($F(2, 25) = 0.878$, $P = 0.428$) and was below the regulated limit for nearly all sites tested (Figure 3-5). Total foliar phosphorus content also did not significantly differ between sub-regions ($F(2, 25) = 0.219$, $P = 0.805$) but many of the samples collected exceeded the regulated limit of 0.22 % dry weight (Figure 3-5).

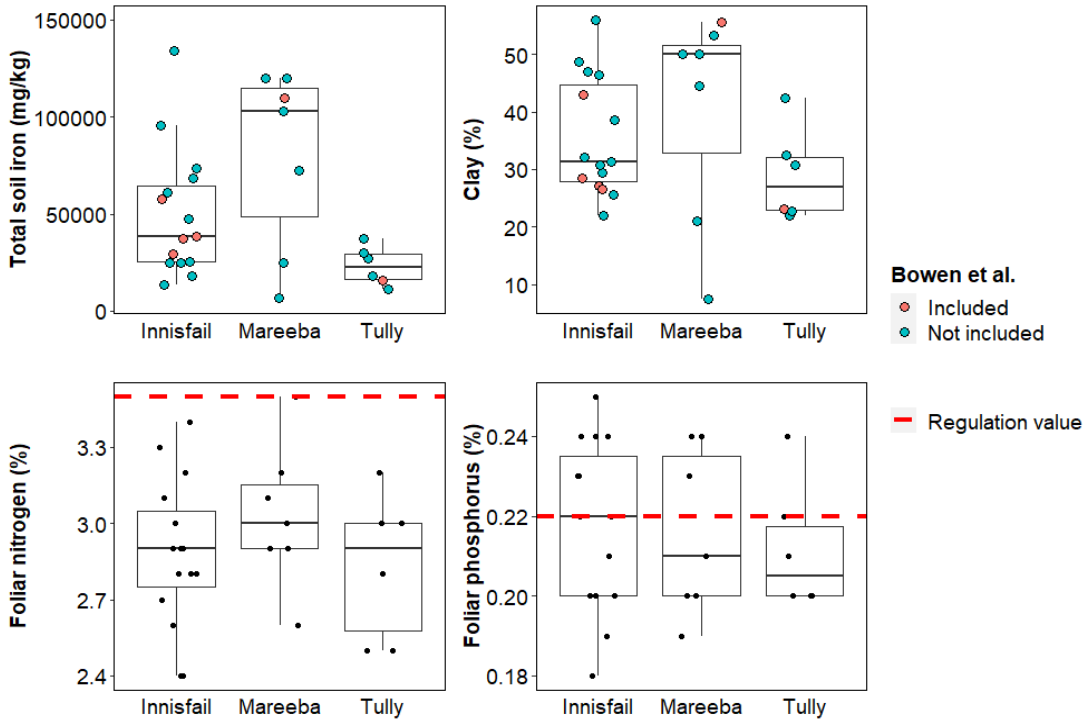

Figure 3-5: Top) Total soil iron and clay content from banana farms in the three sub-regions, highlighting the 6 soils studied for suppressiveness to Panama disease by Bowen *et al.* (2019). In that study the Mareeba sub-region soil (Tolga series) was most suppressive and the Tully sub-region soil (Virgil series) was least suppressive. Bottom) Foliar nitrogen and foliar phosphorus concentrations, grouped by sub-region and compared with regulation guideline values. Points represent individual data values, tails represent minimum and maximum values, boxes represent the interquartile range and lines represent the median value.

3.5 Discussion

This is the first study of the north Queensland banana industry to systematically analyse soil and foliar properties based on soil types. Intrinsic soil forming factors such as parent material and climate and managed factors such as fertiliser application are both known to influence soil characteristics (Singh and Schulze 2015; Armour 2018). We have determined that a farm's soil and plant nutrient status, relative to the larger growing region, was predominantly determined by location and intrinsic factors (principal component 1), with management factors such as fertiliser being of secondary importance (principal component 2). Despite the large diversity in soil types in the Far North Queensland growing region, most research on bananas has been carried out on a very limited number of soil types (Rasiah and Armour 2001; McKergow *et al.* 2004; Rasiah *et al.* 2009; Rasiah *et al.* 2010; Armour *et al.* 2013). Pattison *et al.* (2018b) sampled from a wide range of soil types throughout the region but concluded that farm management affected soil biological indices more than physical and chemical characteristics and they did not consider the effect of soil type on management or biological indices. We have shown that variation in the Far North Queensland region is principally determined by intrinsic soil forming factors and secondarily by management, therefore future research should be structured to span key parent materials and other variables.

The soil characteristics that explained most variation in principal component 1 were largely related to parent material and weathering (Table 3-2, Figure 3-4). Electrical conductivity can be related to rainfall or how recently fertiliser was applied, as soluble salts are flushed away in high rainfall areas like Innisfail and Tully, but accumulate in low rainfall irrigated areas such as Mareeba (Figure 3-4) (Brady and Weil 2000). Clay and total metal content were highly correlated and were largely determined by the parent material of the soil. Most of the soils from the area surrounding Mareeba had basalt parent material whereas the soils of the Innisfail and Tully regions did not (Table 3-1) (Murtha 1986; Cannon *et al.* 1992; Enderlin *et al.* 1997). Soil principal component 2 was largely based on managed characteristics such as nitrogen and organic matter-related properties. While some variation exists due to inherent site variability, many of these characteristics are controlled by growers through the application of agri-chemicals and management practices such as cover cropping.

The foliar first principal component was related to nutrients with relatively low concentrations (<1 %) and generally not applied in fertilisers for the purpose of crop nutrition (magnesium, calcium, sulphur, iron and manganese). Considerable quantities of calcium, magnesium and sulfur are applied, but calcium and magnesium are primarily applied in liming materials for pH adjustment and sulfur is

primarily applied as the balancing ion in nitrogen and phosphorus fertilisers. Values were generally higher on banana farms in Mareeba than the other sub-regions, presumably due to the same variables explaining the difference in soil characteristics, i.e. soil parent material and rainfall (Anderson 1988). The second foliar principal component, which also explained a large proportion of the variation (21 %), included the major nutrients applied in fertiliser; nitrogen, phosphorus, potassium and their ratios. It is perhaps not surprising that there was little separation of the sub-regions based on these characteristics, as the application of nitrogen, phosphorus and potassium is actively managed on most farms, typically based on foliar concentration, to within a narrow range (Reuter and Robinson 1997; Armour 2018).

The results could be used to guide future sampling and experimentation to ensure adequate representation of banana farms. Differences exist between banana farms in the sub-regions, largely attributable to parent material, with basalt soil separating from the other materials, and climate variables such as temperature and rainfall (Figure 3-4). Therefore, if time and resources permit, it would be beneficial to incorporate at least one location in the Mareeba sub-region and one in either the Innisfail or Tully sub-region. Additionally, at least one site should be on basalt soils and one on another parent material, as conclusions drawn from one sub-region, or parent material, may not be applicable to another.

Our findings also provide context to research at particular sites or on particular soil types. Bowen *et al.* (2019) determined the disease severity of Fusarium wilt of banana in a pot trial using soils from six sites that were a subset of those collected in this work. Disease severity was negatively correlated with clay content, field capacity water content, extractable boron and the concentration of total iron, copper and cadmium (Bowen *et al.* 2019) in agreement with previous work in Australia (Peng *et al.* 1999) and elsewhere (Stotzky *et al.* 1961; Stotzky and Torrence Martin 1963; Dominguez *et al.* 2001). Although only 6 soils were studied by Bowen *et al.* (2019), this work shows they covered most of the range of clay and total iron concentration on North Queensland banana farms (Figure 3-5). Additionally, we can see that the soil types chosen represent 61.7 % of the growing area, including the five most prominent soil types (Table 3-1). When considering specific applications such as Fusarium wilt of banana it may be beneficial to consider addition characteristics such as calcium : nitrogen ratio as this has previously been shown to impact Fusarium wilt severity (Teixeira *et al.* 2021).

Our findings are relevant to recently introduced fertiliser regulations for nitrogen and phosphorus (DES 2019). All but one location had a foliar total nitrogen concentration below the regulated value of

3.5 % (Figure 3-5) and would therefore be allowed to apply nitrogen fertiliser above the regulated maximum rate. Conversely, a large number of locations had a foliar phosphorus concentration higher than the regulated value of 0.22 %. This means that if farmers were already at the maximum annual phosphorus fertiliser rate of 60 kg / ha / yr only some farms would be allowed to increase their application rate. Also, it is interesting to note that there was little difference in leaf nitrogen or phosphorus concentrations between the sub-regions (Figure 3-5). This is likely due to the extent to which phosphorus and nitrogen concentration are optimised by the farmer, irrespective of location and soil type (Reuter and Robinson 1997; Armour 2018). It would thus appear desirable to have different nutrient management recommendations for farms with different soil types (basalt versus other) and different climates (Mareeba versus other); such soil- or location-specific recommendations do not yet exist for the industry.

Nitrogen responses and losses are likely to differ substantially between soil types and locations but those effects are not yet known. Experiments to determine optimal nitrogen rates for banana cultivation have been undertaken in the Innisfail or Tully sub-region as that is representative of the largest proportion of production (Prasertsak *et al.* 2001; Armour *et al.* 2013). One nitrogen rate trial has been performed on a basaltic soil (Pattison *et al.* 2018a) and the optimal application rate differed from a trial performed on non-basalt soils (Armour *et al.* 2013) although the trials occurred over different years and using different forms of nitrogen. To our knowledge, the only published study of nutrient management and cycling that compared basalt and non-basalt soils of North Queensland is that of Masters (2019). They found that nitrous oxide emission differed considerably between the two locations due to rainfall and soil permeability (Masters 2019). Presumably other cycling pathways for nitrogen and other nutrients would also differ. Our results suggest that it would be beneficial for future nutrient research and regulations to take into account sub-regional variability to ensure broad applicability.

3.6 Data availability statement

The data that support this study are available in Research Data JCU at https://doi.org/10.25903/mvp4-2759.

4 Image-based quantification of Fusarium wilt severity in banana

Chapter No.	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author, including the candidate
4	This chapter is identical (apart from formatting) to the publication: Orr, R., Pattison, A., East, D., Warman, N., O'Neill, W., Czislowski, E., and Nelson, P.N (2019). "Image-based quantification of Fusarium wilt severity in banana." Australasian Plant Disease Notes **14**(1): 14.	Orr developed the idea for this manuscript. Orr, Pattison, East and Warman collected the data. O'Neill and Czislowski assisted with genetic analysis and interpretation. Data analysis was performed by Orr with assistance from Nelson. Orr wrote the first draft of the paper which was revised with editorial input from Nelson. Orr developed the figures and tables with assistance from Nelson.

4.1 Abstract

The severity of Fusarium wilt of bananas has long been classified based on visual assessment of necrosis in rhizome or pseudostem cross-sections. The improved method proposed here uses digital image analysis to quantify the proportion of rhizome tissue that is necrotic. It agrees well with visual classification, but provides greater reproducibility, precision and statistical power.

4.2 Main body

Fusarium wilt of banana, or Panama disease, is rapidly spreading throughout the world. As the impact of this disease increases, so too does the need to quantify disease severity in research projects assessing prevention and treatment options. The method proposed here has been designed for

Fusarium wilt of banana, but it could also be used to assess Fusarium wilt of other crops, or other plant diseases resulting in tissue discoloration.

Existing methods for quantifying Fusarium wilt severity visually categorise cross sections based on the level of vascular tissue discoloration of rhizome and sometimes pseudostem or root tissue (Zadoks and Schein 1979; Orjeda 1998; Peng *et al.* 1999; Carlier *et al.* 2003; Smith *et al.* 2008; Viljoen *et al.* 2016; Zuo *et al.* 2018). *Fusarium oxysporum* f. sp *cubense* (Foc), the causal organism of Fusarium wilt, initially infects the roots of bananas, moves through the rhizome, causing rhizome necrosis and discoloration, before moving into the pseudostem and leaves (Warman and Aitken 2018). Therefore, rhizome tissue colour or necrosis is a suitable proxy for disease severity (Ploetz 2015a), however the causative organism should still be confirmed using traditional methods (Puhalla 1985). The method presented here has two advantages over current methods: 1) precision and thus statistical power are increased as the variable measured is continuous rather than discrete 2) subjectivity and human error are reduced by using computerised image analysis rather than the human eye to determine the proportion of tissue that is discolored.

Existing methods typically use a 6–class scale with the first class for no disease presence. Each rhizome cross section is scored and then scores are averaged across all sections from an individual plant. The classification system from Viljoen et al. (2016) is: 1 = No symptoms; 2 = Few internal spots; 3 = <1/3 Discoloured tissue; 4 = 1/3-2/3 Discoloured tissue; 5 = >2/3 Discoloured tissue; 6 =Entire inner rhizome discoloured. Thus, samples with slightly different discoloration can have severity scores differing by 33% and samples with the same score may differ by up to 32%. By using a continuous scale that has high reproducibility the precision can be improved.

The method proposed here quantifies discoloration of the central region of vascular tissue in the rhizome as follows. Three transverse sections of the rhizome are taken, one quarter, half, and three quarters up, after the removal of all soil and roots. A photograph is taken of the sections, including a gray scale reference, and converted to a binary image based on the differentiation of healthy and diseased tissue (white and black pixels respectively). Conversion to binary values removes any variations in colour unrelated to disease. The gray scale reference allows for comparison between photos taken under different light conditions. The pixel values for the area of interest in each rhizome section are averaged. These section values are then averaged to determine an average pixel value for all sections of rhizome which is divided by the pixel value for diseased tissue to determine the proportion of necrotic tissue for each plant rhizome. This analysis is possible using a variety of image analysis programs, but we provide a step-by-step guide for the open source image analysis program ImageJ (see Appendix 1).

We compared the digital image analysis method with an existing visual rating method from Viljoen et al. (2016) in four banana pot experiments using either Ducasse (*Musa* ABB, synonym Pisang Awak) inoculated with Foc Race 1 (Vegetative Compatibility Group (VCG) 0124, BRIP 43996 and 61873) or Williams (*Musa* AAA, Cavendish subgroup) inoculated with Foc Subtropical Race 4 (VCG 0120, BRIP 40272 and 42107), so that internal disease symptoms spanned the entire range of disease severity. Samples of discoloured tissue were previously confirmed positive with initial Foc inoculum, either VCG 0124 or VCG 0120, using purified isolates of the fungus re-isolated from banana rhizomes (Puhalla 1985). Furthermore, a 660 base pair region of the translation elongation factor (EF1-α) from each isolate was amplified, sequenced and compared with Foc sequences from previous studies with 100% sequence identity (O'Donnell *et al.* 1998; Fourie *et al.* 2009; Czislowski *et al.* 2018). The sequences generated in this study are available on GenBank (accessions MK767022-MK767025). Finally, tissue discolouration was absent in plants unless they received Foc inoculum. There was close correlation between the methods, and the absence of disease was well classified in both methods (Figure 4-1). The quadratic best-fit (Figure 4-1) indicated that compared with the proposed method, severity scores between 4.3 and 6 were classified too high, and values between 2.3 and 1.3 were classified too low using the Viljoen et al. (2016) method (Figure 4-1). This may result from researcher's inadvertently increasing variability in the classification of their samples despite the presence of a limited range of disease severity. It can also be observed that at high disease severity there was greater differentiation amongst samples using the imaging method compared to use of disease severity classes, indicating the imaging method was more effective at differentiation between samples with high levels of tissue discolouration.

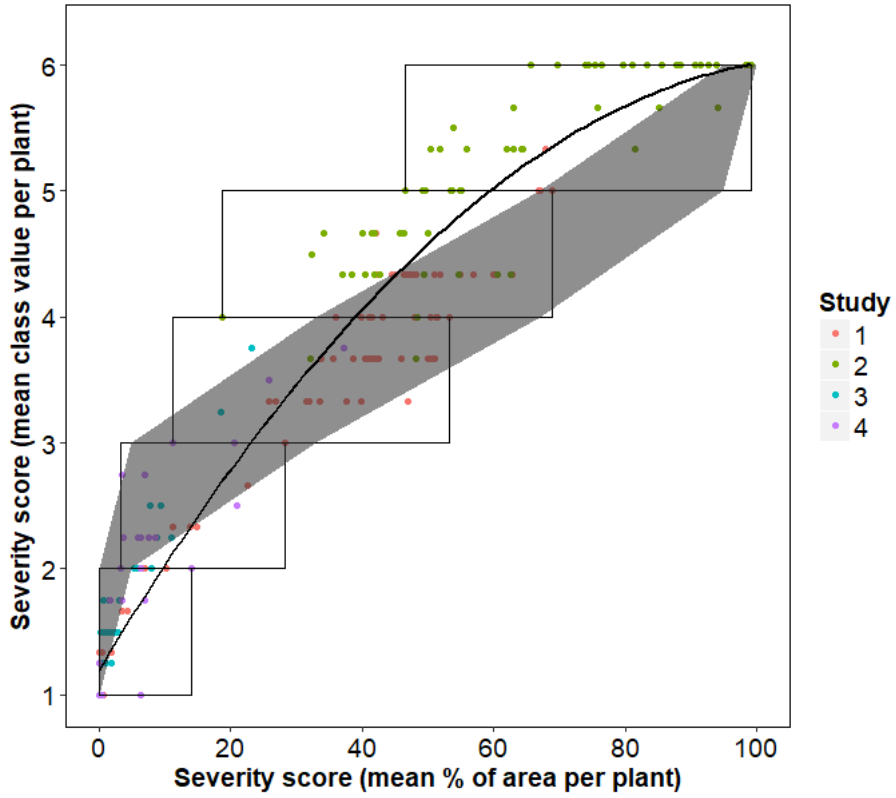

Figure 4-1. A comparison of disease severity as determined using the 6-class scale of Viljoen et al. (2016) and the method proposed here (proportion of area), across four pot experiments ($y = -0.0004x^2 + 0.0876x + 1.1358$, $R^2 = 0.9641$) . Rectangles show the range of values using the new method for five ranges of the class method and the shaded region shows the theoretical zone of correspondence between the two methods.

By increasing the precision of disease severity rating, the new method increases statistical power, allowing greater confidence in experimental results, or reducing the number of samples required for a given level of confidence. Furthermore, by decreasing the requirement for human decision making and classification it is likely that better comparison between studies can be made.

4.3 Appendix 1

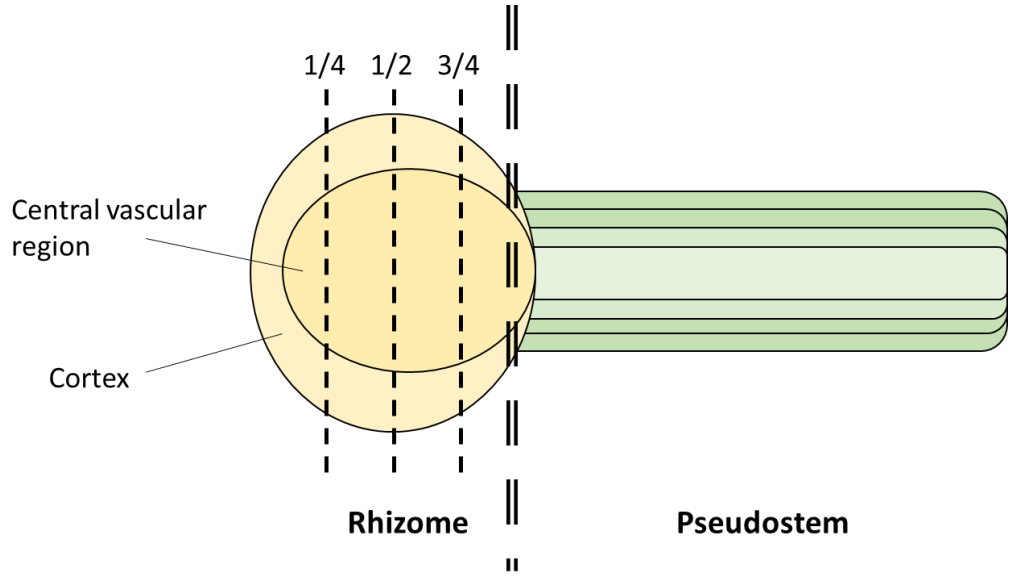

Figure 4-2. Following removal of roots, the rhizome should be separated from the pseudostem and transverse sections of the rhizome taken at ¼, ½ and ¾ of the distance from the bottom of the rhizome.

Notes for photographing sections:

- Good focus is required for maximum effectiveness
- Avoid image distortion by taking photos from directly overhead
- Consistent light level simplifies analysis
- Avoid partial shadows or light variation within the image
- Inclusion of an 8-bit grayscale calibration allows for consistent comparison between photos
- Rhizome sections must be free from dark material such as soil, leaf litter.

All image processing can be performed using ImageJ v1.51t, as follows.

1. Open photo. *File>Open*.
2. Create duplicate. *Image> Duplicate*. Keep both visible on your screen, this makes for easy comparison between the processed and unprocessed image.
3. Convert image duplicate to 8-bit black and white. *Image> Type> 8-bit*.

4. Convert duplicate to binary. *Image>Adjust>Threshold>Apply*. Set threshold value so that areas of disease are red, and areas of non-disease are not red.

   Note: Threshold value may change between images due to variance in lighting, use the in-photo grayscale to establish value, typically differentiation between 108 and 128 pixel value on the calibration strip.

5. Setup for measurement. *Analyze>Set Measurements*> Ensure "*Mean gray value"*, "*Display label"* and "*Add to overlay"* are checked. Set *Redirect to:* Binary image.
6. Measure values: On the non-binary image use the "*Freehand selections"* tool (fourth from left on toolbar) to outline central vascular region of rhizome in one polygon. The oval or elliptical selector is not suitable as the relevant tissue is rarely a perfect oval.
7. Measure value for one section: *Analyze>Measure (Ctrl+M)*. Record the mean pixel value for the selected area for further calculation.

8. Repeat outlining and measurement steps for other sections.
9. Data can then be transposed for analysis. As the mean value is measured on a binary image with white (0) and black (255) values, the mean for the selection is the ratio of discoloured to non-discoloured tissue. The rhizome section values can be averaged and then divided by 255 (value if all pixels are black) to obtain the proportion of necrosis for each plant.

5 Extraction of metals from mildly acidic tropical soils: interactions between chelating ligand, pH and soil type

Chapter No.	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author, including the candidate
5	This chapter is identical (apart from formatting) to the publication: Orr, R., Hocking, R. K., Pattison, A., and Nelson, P. N. (2020). "Extraction of metals from mildly acidic tropical soils: Interactions between chelating ligand, pH and soil type." Chemosphere **248**: 126060.	Orr developed the idea for this manuscript in collaboration with Nelson and Hocking. Orr designed and carried out the experiment and analysed the data. Orr wrote the first draft of the paper which was revised with editorial input from Nelson and Pattison. Orr, Nelson and Hocking developed the figures and tables with assistance from Pattison.

5.1 Abstract

Naturally occurring and synthetic chelating ligands can act as suppressants for fungal pathogens, nematodes and weeds, based on their ability to alter micronutrient bioavailability in soil. Chelators are also used as detergents, for remediation of heavy metal contamination and for supplying metals as fertiliser. The aim of this work was to test the ability of chelators to solubilise metals, in particular iron, in tropical soils over an environmentally relevant pH range. Six topsoils from farms in North Queensland, Australia were adjusted to pH 5, 6 and 7 and then extracted with $CaCl_2$, EDTA, DTPA, EDDHA and mimosine. The extracts were analysed for concentrations of aluminium, copper, iron, magnesium, manganese, potassium, strontium and zinc. EDDHA solubilised iron effectively under all of the conditions tested, indicating its likely suitability for pest suppression. The concentration of aluminium in EDDHA extracts was positively correlated with pH, and at pH 7 the concentration of aluminium was far greater than that of iron. An increase in the mobility of aluminium from EDDHA

application to soil may lead to aluminium toxicity in plants, which should be considered further in any practical application of EDDHA. Mimosine, which is also a strong chelator, was a poor extractor of all metals, possibly due to adsorption to the soil.

5.2 Introduction

Chelating ligands, which are produced by plants, microorganisms and chemical synthesis, are commonly used as fertiliser, extractants for estimating plant-available micronutrients and for remediation of contaminated sites (Wallace 1963; Menzies *et al.* 2007; Leštan *et al.* 2008). Additionally, they have been used as suppressants of pests including fungi, nematodes and weeds (Wilson and Arthur Bell 1978; Scher 1986; Prasad and Subhashini 1994; Peng *et al.* 1999; Nguyen and Tawata 2016). Chelating ligands bind to ions through multiple bonds, changing the reactivity and solubility of ions and the availability of ions to organisms. In the case of fertilisers, chelating ligands are most commonly bound to iron to increase its availability to plants, which can separate iron from the iron-ligand complex. If an organism is unable to free the iron from a ligand complex the organism may become deficient. This induced deficiency makes chelating ligands attractive for suppression of pests poor at competing for micronutrient uptake. Two chelating ligands that have shown promise for suppressing soil-borne plant diseases are EDDHA (ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)) and mimosine (β-N (3-hydroxy-4-pyridone)-α-amino propionic acid).

EDDHA has shown promise for suppression of organisms that are poor competitors for micronutrient uptake, such as pathogenic strains of *Fusarium*, which affect many agriculturally important crops, including banana (Scher 1986; Lemanceau *et al.* 1988; Peng *et al.* 1999). EDDHA is generally considered to be iron-selective and to form stable iron complexes, acting as a transporter of iron from insoluble compounds to plant roots (Lindsay and Schwab 1982; Schenkeveld *et al.* 2014b). Diffusion transports mobilised, chelate-bound iron to the plant roots, where the acidic, reducing conditions of the rhizosphere release it. The free chelate ligand then diffuses back into the bulk soil. It is important to note that copper, manganese and aluminium have all been shown to compete with iron for chelation by unsaturated EDDHA in solution, to varying degrees (Gil-Ortiz and Bautista-Carrascosa 2004; Schenkeveld *et al.* 2007; Schenkeveld *et al.* 2015). The ability of EDDHA to select for and mobilise iron will affect its suitability for suppression of pest organisms.

Mimosine, extracted from legumes in the Mimosoideae family, is effective as a fungicide, nematicide and herbicide in direct application (Prasad and Subhashini 1994; Anitha *et al.* 2005; Williams and Hoagland 2007; Ahmed *et al.* 2009; Xuan *et al.* 2013; Nguyen *et al.* 2015; Chen *et al.* 2018).

Mimosine inhibits ribonucleotide reductase, interrupting cellular replication by chelating essential metal ions; particularly iron (Perennes *et al.* 1993; Lamberth 2016). Mimosine has been effective when incorporated in agar and hydroponic mediums or applied directly to target organisms, but not necessarily when applied to soil (Xuan *et al.* 2006; Hiradate *et al.* 2010). A loss of allelopathic capacity in soil was identified by Hiradate et al. (2010) but the cause was not determined. Xuan et al. (2006) noted a relationship between adsorption and decreased allelopathic capacity of mimosine, and found that approximately 60% of mimosine was adsorbed.

Soil characteristics and chelate structure strongly affect the stability of ligand-ion complexes in soil. Important soil characteristics include pH, redox state, relative concentrations of competing ions, and the amount and nature of clay and organic matter (Lindsay 1979; Katyal and Sharma 1991; Schenkeveld *et al.* 2007). EDTA and DTPA have similar structures, and form iron complexes with relatively low stability compared to complexes with other ions (Figure 5-1). Conversely, EDDHA and mimosine form highly stable complexes with, and are selective for iron due to binding through the phenolate or α-ketohydroxy group (Figure 5-1) (Yunta *et al.* 2003b; Schenkeveld 2010; Kontoghiorghe *et al.* 2015). The phenolate group in EDDHA causes a greater basicity of the ligand compared with EDTA and DTPA and results in a more favourable interaction with "hard", charge-dense cations such as Al^{3+} and Fe^{3+} (Figure 5-1) (Pearson 1963; Martell *et al.* 1996). The α-ketohydroxy group on mimosine has a similar effect but the requirement of three ligands to fill the binding sites, and the charged, unbound ligand tails may reduce the concentration of cations mobilised by mimosine. The comparison of EDDHA and mimosine should provide insight into the importance of hexadentate binding for the mobilisation of metals from soil by chelates. The effect of soil properties on chelate stability has been modelled (Norvell 1991) but the effects of soil pH, soil type and chelate type on ion extraction have been only cursorily examined (Menzies *et al.* 2007; Schenkeveld *et al.* 2007; López-Rayo *et al.* 2015). Biological activity, changing mineral and organic matter stability, and elemental availability over micro and macro scales all further complicate chelate stability and are unaccounted for in modelling. It is important to examine the action of these chelating ligands in a variety of soils under environmentally relevant conditions to validate and improve models predicting their behaviour.

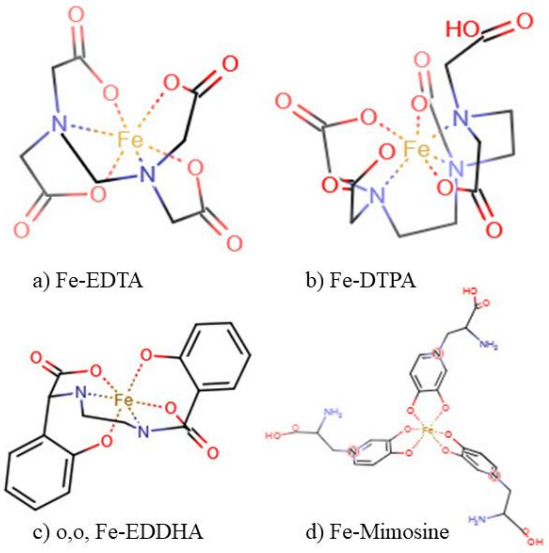

Figure 5-1. The chemical structures of iron complexed with ligands, in order of increasing iron complex stability from a to d.

The aims of this study were threefold: first, to investigate the effectiveness of EDDHA and mimosine at chelating iron and other metals in soil, as an indication of their potential effectiveness for pest suppression; second, to determine the effect of soil characteristics, especially pH, on metal extraction by various chelating ligands; and finally, to identify any metals competitive or co-extracted with iron by EDDHA and mimosine.

5.3 Methods

A full factorial design was used in this experiment, with six soils, five extractants and three levels of pH as factors and three replicates of each treatment combination.

5.3.1 Soil sampling and preparation

Soils used were from the 0-25 cm layer of profiles in banana farms in North Queensland classified as follows: Innisfail series (Brown Dermosol), Liverpool series (Orthic Tenosol), Pingin series (Red Ferrosol), Tolga series (Red Ferrosol), Tully series (Brown Dermosol) and Virgil series (Red Kandosol) (Table 5-1, (Murtha 1986; Cannon *et al.* 1992; Enderlin *et al.* 1997)). Soil samples were dried at 40°C then sieved to <2 mm. Soil chemical analyses were conducted by Nutrient Advantage Laboratory, Werribee, Victoria using the methods described by Rayment and Lyons (2011), unless

otherwise noted. Total content of phosphorus, aluminium, calcium, copper, iron, magnesium, manganese, potassium, sulphur, zinc was measured by overnight digestion of samples in aqua regia followed by inductively coupled plasma optical emission spectroscopy (ICPOES, PerkinElmer Avio 500, Australia) (Method 17B1). DTPA-extractable content of copper, iron, manganese, and zinc was measured using a 2-hour digest in a solution of 0.005 M DTPA, 0.01 M calcium chloride and 0.10 M triethanolamine followed by analysis by ICPOES (PerkinElmer Avio 500, Australia) (Method 12A1). Total carbon content, equivalent to organic carbon for these soils, was measured using the Dumas method (Method 6B2b). Soil pH was measured by mixing the soil at a 1:5 ratio with 0.01 M calcium chloride for 15 minutes before measurement of the unstirred supernatant with a pH probe (Method 4B2). Exchangeable magnesium, potassium and aluminium were extracted using a barium and ammonium chloride with no prewash to remove soluble salts followed by a magnesium exchange and analysis with ICPOES (Method 15E1). Sand, silt and clay content was measured by the hydrometer method (Gee and Or 2002). Oxalate-extractable aluminium, iron and manganese were measured by CSIRO Land and Water, Glen Osmond, South Australia. Soil samples (1.0 g) were shaken end-over-end in the dark for 4 hours with 100 mL of solution containing 0.114 M ammonium oxalate and 0.086 M oxalic acid, adjusted to pH 3. Samples were centrifuged and analysed using ICPOES (Varian Vista-pro, Australia) (Method 13A1). Water holding capacity was determined using a 100 kPa ceramic pressure plate with -10 kPa pressure applied to a blended, wet sample. After equilibration on the pressure plate, soils were weighed, dried for 24 hours at 105°C and reweighed, to determine water content.

Table 5-1. Selected characteristics of soils used in this study; units are g/kg, except for pH.

Characteristic	**Innisfail**	**Liverpool**	**Pingin**	**Tolga**	**Tully**	**Virgil**
pH (1:5, $CaCl_2$)	6.4	6.6	6.8	5.5	5.7	5.6
Carbon, Total	16	15	20	19	12	33
Clay	286	266	430	556	272	232
Sand	528	540	450	372	582	680
Hematite	20	10	20	120	10	20
Goethite	0	0	60	0	0	0
Gibbsite	10	20	30	10	20	50
Iron, Total	29.60	38.40	57.40	110.00	37.00	15.80
Aluminium, Total	29.40	36.60	49.40	63.80	29.40	47.40
Manganese, Total	0.92	0.72	1.48	2.14	0.68	0.11
Zinc, Total	0.14	0.11	0.23	0.10	0.12	0.05

Copper, Total	0.04	0.04	0.08	0.12	0.05	0.03
Magnesium, Total	2.40	2.70	1.96	1.00	2.16	0.62
Potassium, Total	1.44	1.66	1.14	1.68	1.56	0.36
Iron, Oxalate	3.45	4.41	4.17	8.50	2.56	2.26
Aluminium, Oxalate	2.15	1.85	2.58	3.73	1.68	5.72
Manganese, Oxalate	0.79	0.49	1.36	1.56	0.47	0.05
Magnesium, Exchangeable	0.25	0.13	0.38	0.31	0.18	0.36
Potassium, Exchangeable	0.35	0.16	0.39	0.59	0.20	0.16
Aluminium, Exchangeable	<0.01	<0.01	<0.01	0.02	<0.01	<0.01

Soil mineralogy was analysed by CSIRO Land and Water, Urrbrae, South Australia. Due to possible dehydration of the montmorillonite (smectite) interlayer samples were dispersed in 0.25 M calcium chloride, centrifuged at 5150 x *g* (Eppendorf Centrifuge 5810, Australia) for 10 minutes, calcium saturated again, washed with water then ethanol (centrifuging between each step) and oven dried at 60°C. XRD patterns were recorded with a PANalytical X'Pert Pro Multi-purpose Diffractometer using Fe filtered Co Kα radiation, automatic divergence slit, 2° anti-scatter slit and fast X'Celerator Si strip detector. The diffraction patterns were recorded from 3 to 80° in steps of 0.017° 2 theta with a 0.5 second counting time per step for an overall counting time of approximately 35 minutes.

Qualitative analysis was performed on the XRD data using in-house XPLOT and HighScore Plus (from PANalytical) search/match software. Quantitative analysis was performed on the XRD data using the commercial package SIROQUANT from Sietronics Pty Ltd.

To determine the amount of acid or alkali necessary to adjust soils to the three desired levels of pH (5, 6 and 7), the pH buffering capacity of each soil was established (Nelson and Su 2010; Wang *et al.* 2015a). Six-gram (oven-dry equivalent weight) soil samples had 0, 0.33, 0.66 or 0.99 mL aliquots of either 0.55 M hydrochloric acid (VWR Chemicals, Australia) or 0.55 M sodium hydroxide added (Univar, Australia). Calcium chloride (VWR Chemicals, Australia) was added to equalise the ionic strength of the samples. Deionised water was then added to make samples up to 95% of water holding capacity before homogenization and incubation at 26°C for one week. After incubation, samples were extracted with 30 mL of 0.01M calcium chloride and pH of the extract was measured (Ionode pH

probe IJ44C, Australia) (Method 4B1) (Rayment and Lyons 2011). The buffering capacity was the linear relationship between pH and addition of OH^- (positive) or H^+ (negative) added (Table 5-2).

For the experiment, 8-g (oven-dry equivalent weight) soil samples were adjusted to pH 5, 6 and 7 using either hydrochloric acid or sodium hydroxide based on the pH buffering capacity of the soils. Calcium chloride was added to samples to equalise ionic strength and water was then added up to 90% of water holding capacity. The samples were incubated at 26°C for one week prior to extraction, as pre-equilibrium of soils has previously been shown to affect chelate extraction efficiency (Schenkeveld *et al.* 2017).

Table 5-2. Parameters describing pH buffering capacity of the six soils, where y = a(pH) + b and y = amount of H^+ or OH^- added in mmol / g, with H^+ addition expressed as a negative value.

	Innisfail	Liverpool	Pingin	Tolga	Tully	Virgil
a	0.0455	0.0416	0.0600	0.0635	0.0374	0.0566
b	-0.246	-0.237	-0.356	-0.300	-0.199	-0.317
R^2	0.992	0.978	0.938	0.966	0.994	0.990

5.3.2 Extraction by chelators

The compounds used for extractions were calcium chloride (≥ 99%,VWR Chemicals, Australia), EDTA (≥ 99%, Univar, Australia), DTPA (≥ 98%, Sigma-Aldrich, Australia), *o,o* - EDDHA (≥ 86.3%, Wallace Labs, USA) and mimosine (≥ 98%, Sigma-Aldrich, Australia). Each of the six soils were extracted with each of the five extractants at pH 5, 6 and 7. Three blanks per extractant were also run using deionised water and one of hydrochloric acid, sodium hydroxide or calcium chloride solution in place of soil to determine concentrations of ions in the solutions.

Extraction methods were adapted from established methods for EDTA, DTPA and EDDHA (Johnson and Young 1973; Lindsay and Norvell 1978; Rayment and Lyons 2011). Extractant : soil ratio (40 mL: 8 g), calcium chloride concentration (0.01 M) and shake time (120 min.) were uniform across treatments but extractant concentration differed. EDHA and DTPA concentrations were 0.005 M, but EDDHA and mimosine concentrations were 0.001 M due to low solubility at 0.005 M, as previously noted by Johnson and Young (1973). After addition of the extractant, samples were shaken end-over-end for 120 minutes to allow for equilibration while limiting ligand degradation. Samples were then centrifuged for 5 minutes at 1811 x *g* (Eppendorf Centrifuge 5810, Australia) and the supernatant was withdrawn for analysis. Supernatant samples of Tolga soil type at pH 6 and 7 were filtered (0.45µm

Millipore MF-membrane filter, Australia) to remove visible suspended solids. Supernatant solutions were analysed for aluminium, calcium, copper, iron, magnesium, manganese, potassium, sodium, strontium and zinc by ICPOES (Varian 725 ES ICP-OES, Australia) by the Queensland Department of Environment and Science (Brisbane). For each extract, the average concentration of each element in the blanks was subtracted from sample values. Calcium and sodium concentrations are not reported because they were used in extractants and to adjust pH. Strontium concentrations are also not reported as they were $<5.11 \times 10^{-5}$ mmol / kg in all cases.

5.3.3 Data analysis

All data analysis and graphing was performed using the R platform (R Core Team 2017). Data initially reported as below the reporting limit were replaced with the actual measured values, despite a lower confidence.

Treatment and interaction effects were analysed for significance with a 3-way ANOVA and general linearised models using a Wald t-test with fixed effects of pH, soil type and extractant. In the cases of significance being established this was followed by a Tukey analysis using the agricolae package in R to determine individual differences at $\alpha = 0.05$ (de Mendiburu 2017). Summary statistics were generated using the plyr package in R (Wickham 2011). All graphs were generated using the ggplot2 data visualisation package in R (Wickham 2016).

Ionization potential or charge density of each element was calculated as the ion charge divided by the ion radius for the most commonly chelated ion of each element. Selectivity for iron or aluminium were calculated as iron or aluminium molar concentration divided by the sum of the molar concentrations of aluminium, copper, iron, magnesium, manganese, potassium and zinc.

5.3.4 Pourbaix Diagrams

Pourbaix diagrams were calculated using a combination of the Act2 module in Geochemist's workbench V8.0, (2011) and the published stability constants for aluminium (Rajan *et al.* 1981) and iron (Norvell 1991) complexes of EDTA and EDDHA. Pourbaix diagrams were calculated with activity levels of Fe^{3+} and Al^{3+} 10^{-6} M.

5.4 Results

Soil type, pH and extractant, as well as the interaction of these factors, significantly affected the concentration of metals extracted from soil (P <0.01, Figure 5-2). Under the majority of tested conditions, the amount of metals extracted decreased in the order: magnesium > potassium > aluminium > manganese > iron > zinc > copper (Figure 5-2). For each soil, the chelate-extracted concentrations (averaged across EDTA, DTPA, EDDHA and mimosine) were significantly correlated with total concentrations for copper (P = 0.033), iron (P = 0.001), manganese (P = 0.002) and zinc (P < 0.001) but not aluminium (P = 0.853), magnesium (P = 0.504) or potassium (P = 0.950). Extracted concentrations of magnesium and potassium were significantly related to their exchangeable forms (P = 0.010 and 0.008, respectively). Aluminium concentration was primarily dependent upon the pH, extractant used, and the concentration of competing metals rather than the concentration of either its total or exchangeable forms.

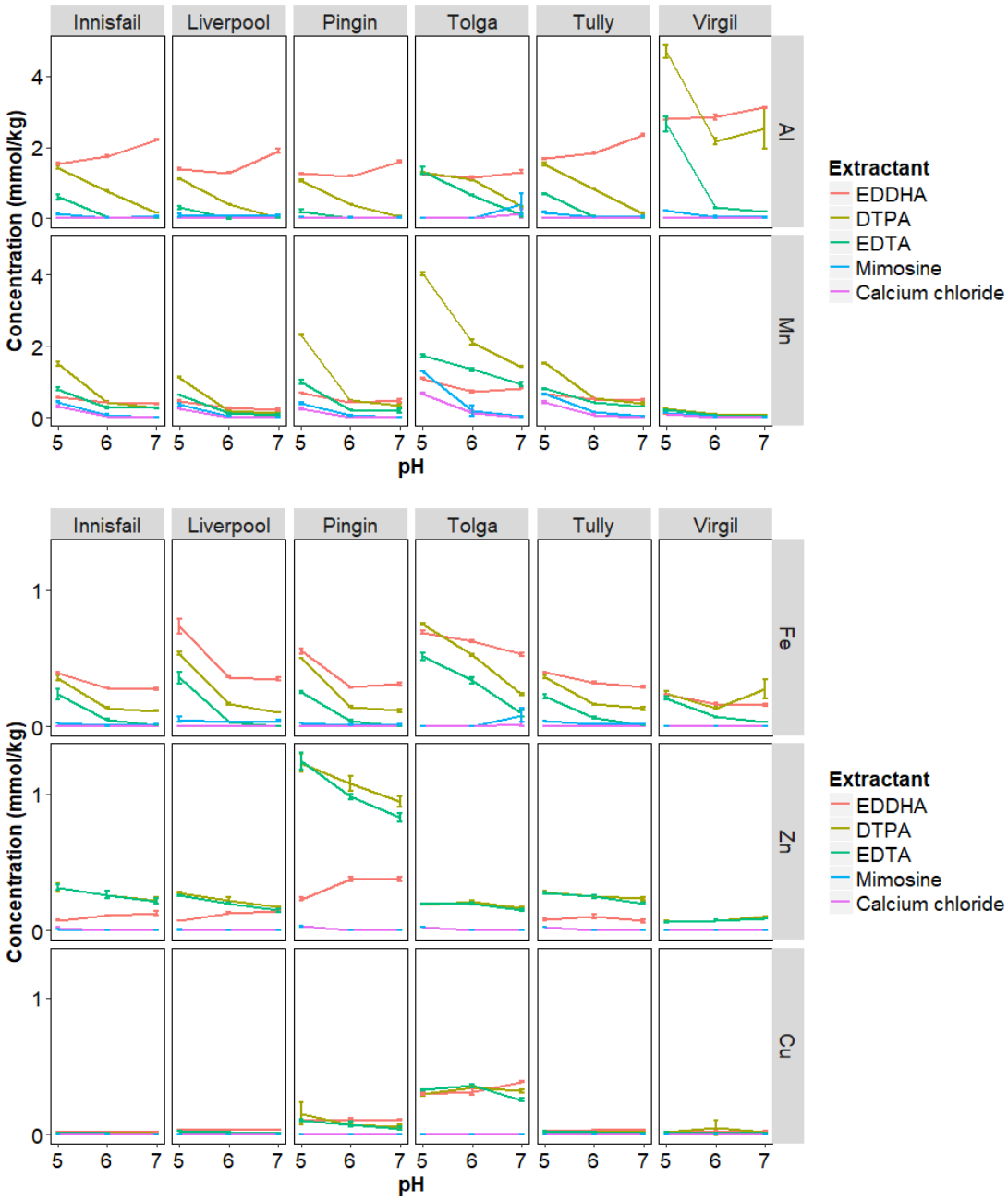

Figure 5-2. Concentration of extracted metals for each combination of extractant, pH and soil type. The lines join mean values for treatments at pH 5, 6 and 7 and error bars represent standard error of the mean. The top panel (aluminium (Al) and manganese (Mn)) has a different y-axis scale than the bottom panel (iron (Fe), zinc (Zn) and copper (Cu)). Potassium and magnesium are excluded due to their much greater concentration (see supplementary information for the figure for potassium and magnesium).

In general, the concentration of the metals in extracts decreased as the extractant pH increased (Figure 5-2). The exception to this was the concentration of aluminium extracted with EDDHA, which in all soils increased as pH increased (Figure 5-5). When considered independently of the pH effect, EDDHA preferentially extracted the metals that favour the oxidation state of three and have the

greatest ionization potential (aluminium and iron). Geochemical modelling suggests that the EDDHA complexes of aluminium and iron are more thermodynamically stable than their main mineral forms in soil in the pH range considered (Figure 5-3). EDTA most effectively extracted the three elements (copper, potassium and zinc) that favour the oxidation state of two and have the lowest ionization potential. Mimosine did not correspond with the above trends due to the extremely low concentrations of metals extracted. Mimosine did not effectively extract metals though the reasons for this were not clear.

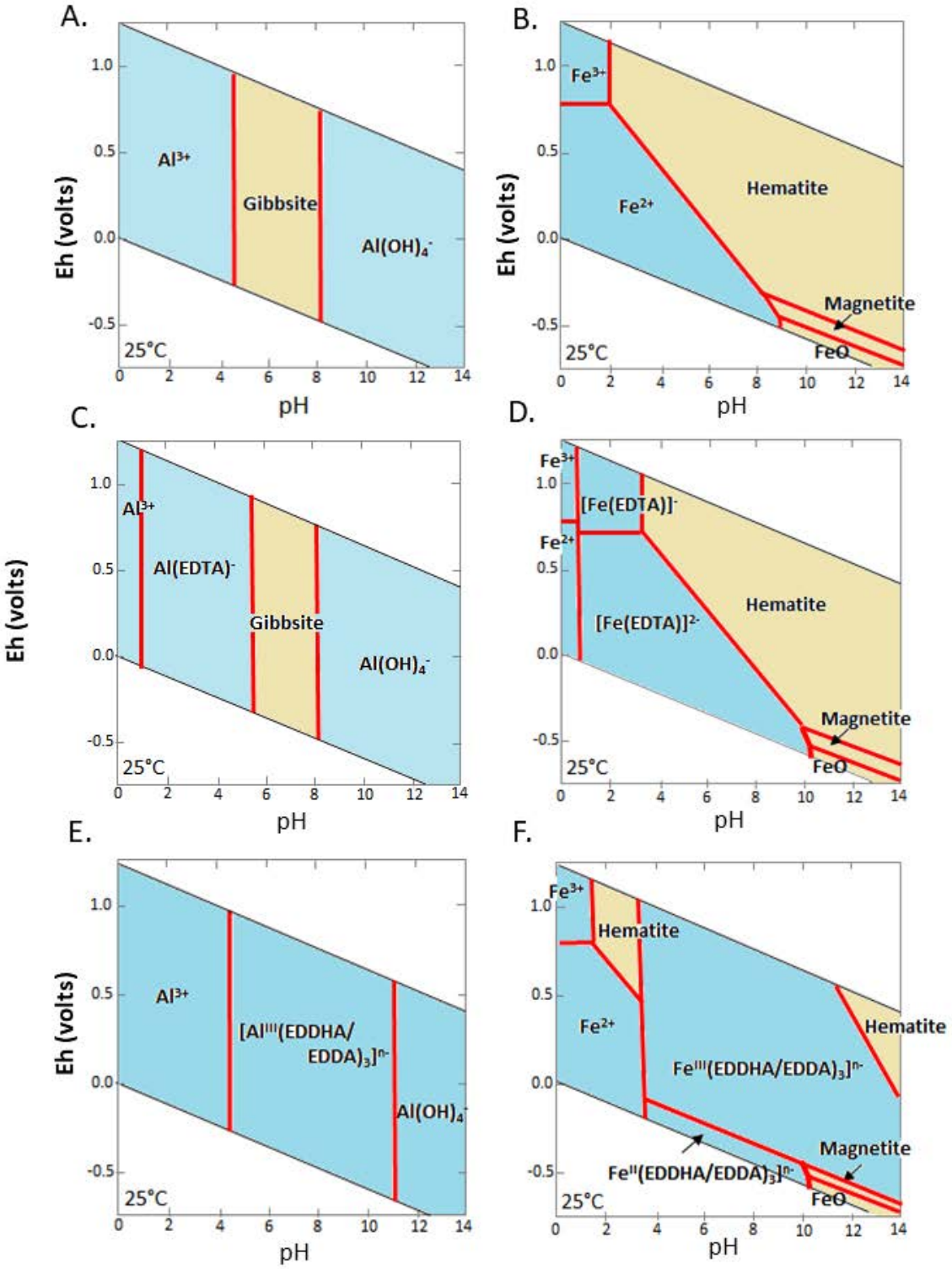

Figure 5-3. Pourbaix diagrams for aluminium (Al) (A, C, E) and iron (Fe) (B, D, F) in the absence of a chelating ligand (A, B), with EDTA (C, D) and EDDHA (E, F) at an activity level of 10^{-6} M. Red lines indicate phase interfaces, a blue background indicates the phase is aqueous whilst brown indicates a solid and labels indicate the primary phase for a given redox and pH condition. For the iron diagrams, goethite is metastable with respect to hematite so hematite is depicted as the

thermodynamically stable phase under these conditions. The diagrams for EDDHA are further complicated by the redox chemistry of EDDHA and its possible protonation states.

The selectivity of the chelating ligands for specific metals (concentration of the element divided by the sum of all extracted metals) was influenced by the treatments. The effect of pH on chelate selectivity differed between iron and aluminium (Figure 5-4). Though the concentration of iron in EDDHA and DTPA extracts decreased as pH increased the iron selectivity was relatively unchanged. In contrast, the iron concentration and selectivity of EDTA, the chelator that forms the weakest iron-ligand complex, decreased significantly when pH was increased. Aluminium concentration in EDDHA solution, and the selectivity of EDDHA for aluminium, both increased significantly when pH was increased (Figure 5-2 and 5-4). The concentration of aluminium in EDDHA extracts was positively correlated with the ratio of oxalate-extracted aluminium to oxalate-extracted iron and manganese, irrespective of soil type and pH (Figure 5-5). Mimosine was not selective for iron or aluminium at any value of pH (Figure 5-4).

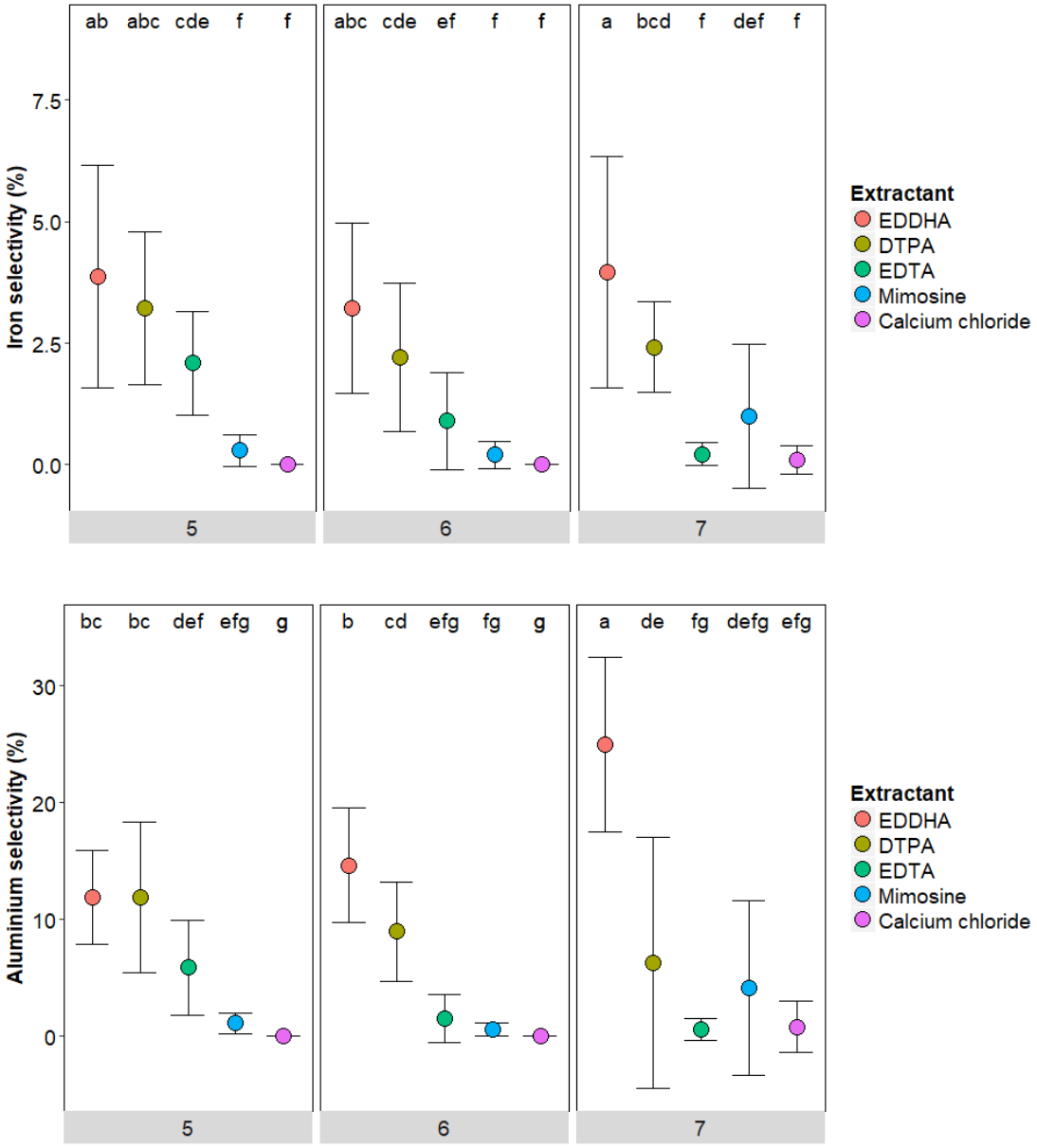

Figure 5-4. Ratio of the mean concentration of iron (top) and aluminium (bottom) to the sum of all measured metal concentrations (aluminium, copper, iron, magnesium, manganese, potassium and zinc) at pH 5, 6, and 7, across all soils. Error bars indicate standard deviation. Letters indicate groupings based on Tukey post-hoc analysis ($\alpha = 0.05$).

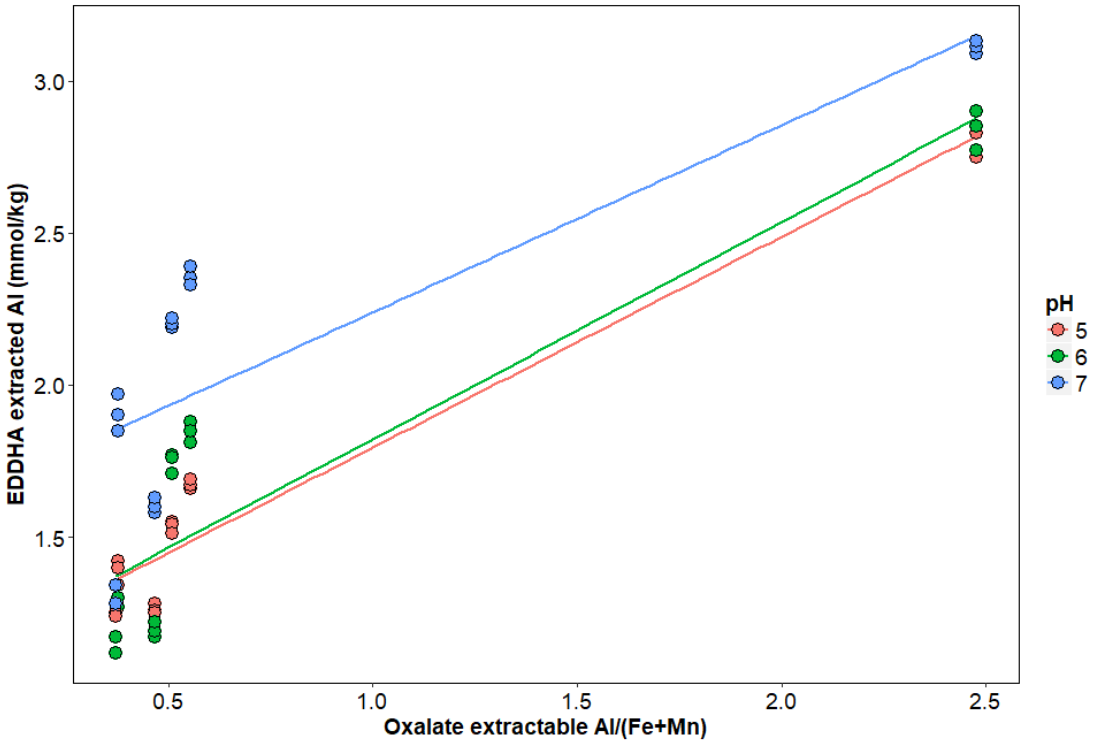

Figure 5-5. Extracted aluminium (Al) concentration as a function of the molar ratio of oxalate-extracted aluminium to iron (Fe) and manganese (Mn) for each of the soils at each pH tested (P<0.001). Extracted aluminium concentration at pH 7 differed significantly from that at pH 5 or 6 (P<0.001).

5.5 Discussion

The concentration of metals extracted from soils in this study depended upon the extractant, pH, and the concentration of the metals in the soil (Figure 5-2). The concentration of extracted copper, iron, manganese and zinc were proportional to the total concentration of these metals in the soil. The concentration of extracted magnesium and potassium, was strongly correlated with the DTPA exchangeable form, and was likely displaced by the calcium and sodium salts in the extractant solutions.

Soil pH affects metal solubility directly and also affects chelating ligand selectivity, so the effect of pH depended upon the extractant used and the metal being extracted (Brady and Weil 2000). The iron selectivity of EDTA was most affected by the pH change as its relative acid dissociation constant (pKa) of 6.2 fell within the range tested (Chauhan *et al.* 2015). The most relevant pKa of the other chelates were outside of the pH range examined in this study: DTPA 8.6, EDDHA 8.66, and mimosine 7.2 (Yunta *et al.* 2003b; Chauhan *et al.* 2015; Nguyen and Tawata 2016). The binding mechanism of EDTA changes at pH above the pKa, reducing chelate-metal complex stability and the

concentration of extracted metals (Figure 5-4). The decrease in EDTA-extracted iron from pH 5 to 6 corresponds with the modelled phase boundary between the iron-EDTA complex and hematite (Figure 5-3). In a similar pH range, the higher concentration of EDDHA-extracted iron (Figure 5-2) corresponded with a modelled EDDHA-iron complex that remains stable rather than forming hematite (Figure 5-3).

The concentration of less charge-dense metals, magnesium, manganese, potassium and zinc was greatest in EDTA or DTPA extracts (Figure 5-2), though this may not have been so if the concentration of EDDHA used was equal to that of EDTA and DTPA. This result agrees with modelling by Norvell (1991), who showed a greater mole fraction of these metals chelated by EDTA or DTPA than EDDHA. Norvell (1991) also predicted that aluminium chelation would occur in acidic conditions only when there was no competition from iron. With the exception of one investigation of phytosiderophore mobilisation (Schenkeveld *et al.* 2014a) more recent modelling of strong chelates in soil generally has overlooked aluminium, instead focusing on plant essential nutrients such as copper, iron, manganese and zinc (Yunta *et al.* 2003a; Yunta *et al.* 2012; López-Rayo *et al.* 2015; Schenkeveld *et al.* 2015). One of the challenges in modelling these systems is that the relevant phases of metal oxides and oxy-hydroxides can be nanoparticulate, which affects their thermodynamic stability, making the metal oxides more soluble with respect to chelates (Navrotsky *et al.* 2008). We found that DTPA and EDDHA in particular solubilised a considerable amount of aluminium despite competition from iron (Figure 5-2). The average ratio of extracted aluminium to iron found in our study (EDTA = 2.3, DTPA = 4.4, EDDHA = 6.2) agrees well with ratios found in previous research, though our maximum ratio of 20.1 (EDDHA, pH 7, Virgil soil type) far exceeds previously published values (Manouchehri *et al.* 2006; Schenkeveld *et al.* 2007). Schenkeveld et al. (2014b), using 0.0895 mM *o,o* – EDDHA in a pH 8.0 soil, found a maximum aluminium-to-iron ratio of approximately 1.1 and Manouchehri et al. (2006), using 0.002 M EDTA in a pH 5.7 soil, found a ratio of approximately 3.25. The differences between study results are likely due to differences in chelate type, concentration, pH and the ratio of competitive metals.

The concentration of aluminium in unsaturated EDDHA extracts significantly correlated with the availability of aluminium relative to iron and manganese, as represented by the oxalate extractable concentrations, in agreement with previous research by Schenkeveld (2007) (Figure 5-5). When more aluminium is available, relative to competing ions of iron, manganese and protons, more is extracted by EDDHA. Thus, while competition from other metals is important it is not prohibitive as suggested by Norvell (1991) (Figure 5-5). The selectivity of EDDHA for aluminium also increased with pH in all of the soils tested (Figures 5-2 and 5-4). This was the result of both increased concentration of

aluminium and decreased concentration of other metals. Presumably, greater aluminium solubilisation resulted from reduced competition with iron and other metals. Iron (III) has been shown to bind to organic matter more strongly than aluminium (III), and this difference increases when pH changes from 6 to 8 (Jones *et al.* 2009). As the total chelated metal concentration is likely to vary little over this pH range, less competition from iron or manganese ions (due to strong binding with organic matter) would result in an increase in selectivity for aluminium ions. Furthermore, an increase in pH would reduce proton competition with aluminium ions for hard bases such as EDDHA. Proton competition is an important contributor to aluminium-EDDHA instability in acidic conditions, but is negligible when pH is neutral (Martell *et al.* 1996; Dalla Torre *et al.* 2018). However, whether a reduction in proton competition for EDDHA favours chelation of aluminium preferentially over iron is unclear.

Plants cycle chelating ligands such as EDDHA, leading to an accumulation of elements like aluminium in the rhizosphere and soil pore water (Lindsay and Schwab 1982; Schenkeveld *et al.* 2014b). This indicates that EDDHA application may eventually lead to increased aluminium in groundwater or aluminium phytotoxicity in susceptible plants such as bananas (Rufyikiri *et al.* 2001). This potentially detrimental side effect of EDDHA application warrants further investigation.

The ability of chelating ligands to create an iron deficiency in target organisms is a key requirement for the suppression of undesirable organisms in soil. To induce a deficiency, iron must be either permanently immobilised by the chelating ligand or be bound, solubilised and removed from the vicinity of the organism. Our findings show that EDDHA may perform this role well through its ability to complex available iron in soil. Mimosine showed little ability to extract iron or other essential metals, but its ability to complex metals in situ and thereby make them unavailable was not tested. The ability of EDDHA to suppress undesirable organisms such as pathogenic strains of *Fusarium* is yet to be tested in these acidic tropical soils.

The low concentration of metals in mimosine extracts suggests adsorption of mimosine to organic matter and clay minerals is occurring. Such adsorption is unsurprising considering that the bi-dentate binding of mimosine to iron creates a molecule with multiple unbound carboxylate and amino groups (Figure 5-1). Adsorption limits the ability of mimosine to induce Fe deficiency and may account for its reduced allelopathic capacity in soil and minimal impact on metal phytoaccumulation (Chou and Kuo 1986; Hiradate *et al.* 2010; Nguyen and Tawata 2016). Adsorption of approximately 60% of mimosine to soil was noted in a previous study, but the allelopathic capacity of the mimosine was not

inhibited (Xuan *et al.* 2006). Xuan et al. (2006) did not report the clay and organic matter content of their soil and it is possible that there was greater adsorption in our study due to the high clay and organic matter content of the soils we used. This is in contrast with EDDHA, for which adsorption is negligible (Siebner-Freibach *et al.* 2004). The difference in metal mobilising capacity between EDDHA and mimosine demonstrates the importance of chelate-soil interactions in addition to chelate – ion stability.

5.6 Conclusions

We identified that EDDHA was effective at extracting iron and other micronutrients from soil, whereas mimosine was not, likely due to adsorption to soil particles. The amount of copper, iron, manganese and zinc extracted was significantly related to total soil concentrations. The amount of magnesium and potassium extracted were more strongly related to exchangeable concentrations than total concentrations, and differed little between extractants. There was a greater effect of pH on extraction by EDTA and DTPA than by EDDHA and mimosine, due to the less stable binding of carboxylate groups in the former compared to phenolate and α-ketohydroxy groups in the latter.

A large amount of aluminium, relative to iron, was extracted under the slightly acidic to neutral soil conditions tested, and aluminium concentration in EDDHA extracts increased with pH, contrasting with predictions from chelate extraction models. The concentration of aluminium extracted with EDDHA was strongly related to the ratio of oxalate-extractable aluminium to iron, indicating that competition between the two is based on their availability rather than differences in stability of their complexes. Complexation of aluminium by strong synthetic or naturally occurring chelates may impede the effectiveness of iron shuttling to plants, increase the concentration of aluminium in soil pore water and increase the risk of aluminium toxicity. Future study of strong chelates, both naturally occurring and synthetic, should consider aluminium as an element of interest in addition to the essential nutrients.

5.7 Supplementary Information

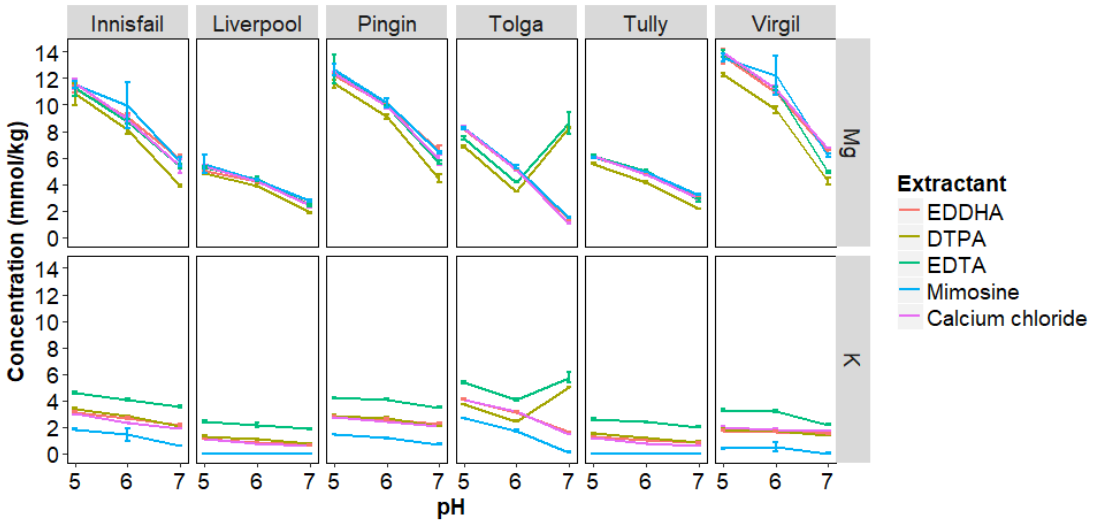

Figure 5-6 supp. Concentration of extracted metals magnesium (Mg) and potassium (K), for each combination of extractant, pH and soil type relating to figure 5-2 in the manuscript. The lines join mean values for treatments at pH 5, 6 and 7 and error bars represent standard error of the mean.

6 Iron chelates have little to no effect on the severity of Fusarium wilt of bananas in soils of the humid tropics

Chapter No.	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author, including the candidate
6	This chapter is identical (apart from formatting) to the publication: Orr, R., Pattison, A., Northfield, T., and Nelson, P. N. (2021). "Iron chelates have little to no effect on the severity of Fusarium wilt of bananas in soils of the humid tropics." Journal of Plant Pathology 103(2): 595-604.	Orr developed the research question in consultation with the other authors. Orr designed and executed the experiments and performed the data analyses with assistance from Nelson. Orr wrote the first draft of the paper which was revised with editorial input from Nelson, Northfield and Pattison.

6.1 Abstract

Previous research has shown that application of iron chelates to soil reduces Fusarium wilt in several crop species. The aim of this work was to test the effect for bananas grown in tropical soils.

Disease severity and plant characteristics were measured in banana plants (cv. Ducasse, *Musa* ABB) grown in pots inoculated with *Fusarium oxysporum* f.sp. *cubense*, Race 1 in two experiments. Experiment 1 compared amendment with iron chelates (with ligands of differing iron binding stability) with water, plus an uninoculated unamended control, in two tropical Australian soils. Experiment 2 examined the effect of Fe-HBED application rate with high or low calcium addition.

In Experiment 1, iron-saturated chelate application did not significantly affect disease severity or plant tissue iron concentration, irrespective of the iron binding stability of the chelate. In Experiment 2, disease severity was not affected by Fe-HBED or calcium application rate. The concentrations of iron and aluminium in plants were both somewhat affected by the addition of Fe-HBED, calcium and their interaction. The lack of effect was likely due to high iron availability in the soils overwhelming the capacity of the treatments to alter iron availability to the host plant and pathogen. Application of strong chelating ligands increased the concentration of aluminium and decreased the concentration of manganese in plant tissue, with possible detrimental effects.

6.2 Introduction

Fusarium wilt of banana, caused by the hemibiotrophic soil fungi *Fusarium oxysporum* f.sp. *cubense* (Foc), is rapidly spreading throughout the banana growing regions of the world, and farming practices must adapt to this threat. The disease causes a reduction in transpiration by blocking the vascular tissue of the plant, resulting in a characteristic yellowing and leaf wilting (Dita *et al.* 2018). Currently, there are no effective means of controlling the disease, removing the pathogen once it has colonised a field, nor is there an agronomically suitable resistant cultivar. Therefore, the reduction of disease severity through management practices warrants investigation (Dita *et al.* 2018). The severity of disease depends upon relationships between the host, pathogen and environment. To reduce disease severity, agronomic management must modify the growing environment to advantage the host and disadvantage the pathogen (Orr and Nelson 2018).

Tropical North Queensland accounts for 94% of Australian banana production and is under threat from Fusarium wilt caused by Foc Tropical Race 4 (Foc TR4). Foc TR4 affects the banana cultivars Cavendish, Ducasse and Ladyfinger, which together make up over 99% of Australian production (ABGC 2017). The disease was confirmed in tropical North Queensland in 2015, causing widespread industry interest in management options for suppressing the disease (O'Neill *et al.* 2016). North Queensland, like many banana production regions throughout the world, has high rainfall and highly weathered, acidic soils with moderate to high micronutrient availability (FAO 2018).

Under certain growing conditions Fusarium wilt of some crop species, such as carnation (Duijff *et al.* 1994), chickpea (Saikia *et al.* 2005), cucumber (Scher and Baker 1982), flax (Scher and Baker 1982; Lemanceau *et al.* 1988), radish (Scher and Baker 1982; Leeman *et al.* 1996), tomato (Jones and Woltz 1970; Segarra *et al.* 2010; López-Berges *et al.* 2012) and banana (Peng *et al.* 1999; Dong *et al.* 2016) , has been partially controlled by altering the availability of iron. The only two studies into the effect of

manipulating iron availability on Fusarium wilt of banana (Peng *et al.* 1999; Dong *et al.* 2016) were performed under unique experimental conditions, so the usefulness of this treatment approach in commercial banana cultivation is uncertain. Peng *et al.* (1999) found that application of iron ethylenediaminedi-O-hydroxyphenylacetic acid (Fe-EDDHA) fertiliser halved disease severity in a pot trial with two soils. The chelating ligand used, EDDHA, is highly stable and is thought to make iron available to the plant whilst inducing iron deficiency in Foc. However, the soils they used had a pH of 8, low micronutrient availability and were from atypical low-rainfall conditions. Dong *et al.* (2016) found in a hydroponic trial that increasing iron availability to both the banana plant and Foc, using iron chloride fertiliser, also resulted in a significant decrease in disease severity.

The effect of iron on the severity of Fusarium wilt appears to depend on its relative availability to the host and pathogen. Availability to the two organisms can differ and is dependent upon the chelating ligand and soil considered. The severity of Fusarium wilt of flax was increased by the addition of weakly bound iron, in the form of iron ethylenediaminetetraacetic acid (Fe-EDTA) and iron diethylenetriaminepentaacetic acid (Fe-DTPA), but decreased by addition of stable iron binding chelating ligands such Fe-EDDHA and iron N,N-bis(2-hydroxyphenyl)ethylendiamine-N,N-diacetic acid (Fe-HBED) (Scher and Baker 1982; Lemanceau *et al.* 1988) (Figure 6-1). Such effects may occur only in conditions where iron availability is already limited (Simeoni *et al.* 1987; Segarra *et al.* 2010). The iron binding stability of chelating ligands, as well as the inherent iron availability are affected by the concentration of competing cations such as protons, calcium and aluminium (Norvell 1991). Therefore, the effectiveness of chelating ligand application as a treatment for Fusarium wilt is likely to differ between locations and soil types. The ability of chelating ligands to mobilise iron and other metals in micronutrient-rich tropical soils typically used for banana production has recently been demonstrated (Orr *et al.* 2020) but the effect of manipulating iron availability on the severity of Fusarium wilt of banana in such soils has not yet been tested.

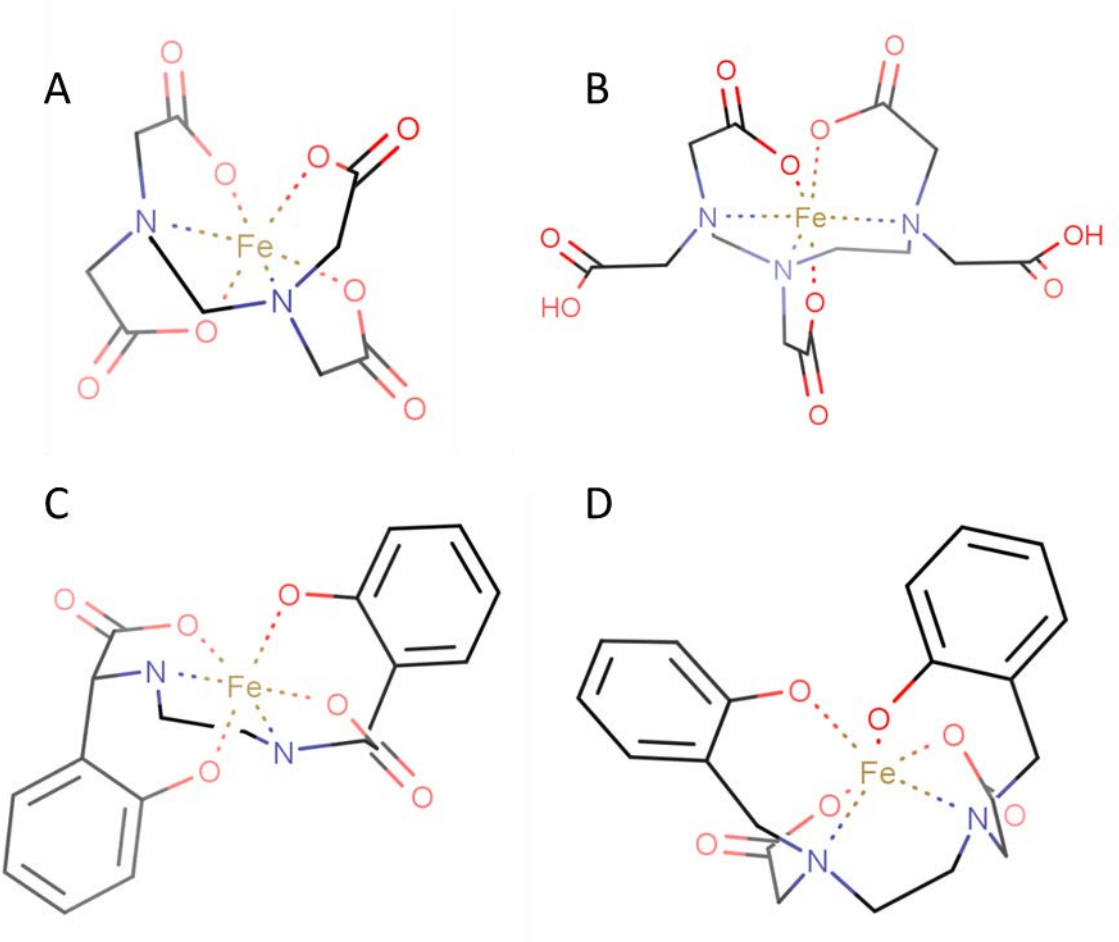

Figure 6-1. The chemical structures of A) Fe-EDTA, B) Fe-DTPA, C) o,o-Fe-EDDHA, and D) Fe-HBED in order of increasing iron complex stability.

The aim of this study was to test the effect of chelating ligands on Fusarium wilt of bananas in soils of tropical North Queensland. In Experiment 1 we tested if iron availability affected the severity of Fusarium wilt in two soils differing in disease suppressiveness and micronutrient availability. Iron availability was manipulated by application of chelating ligands differing in iron stability. In Experiment 2 we determined the relationship between Fe-HBED dose, plant nutrition and severity of Fusarium wilt, at low and high rates of calcium application, an ion that may compete with iron for chelation.

6.3 Methods

6.3.1 Experiment 1

6.3.1.1 Experimental design

Two soils (Liverpool, Tolga) and six combined chelate and Foc R1 inoculation treatments were used (water and no inoculation, water and inoculation, Fe-EDTA and inoculation, Fe-DTPA and inoculation, Fe-EDDHA and inoculation, Fe-HBED and inoculation). Treatments were replicated six times for a total of 72 plants. Liverpool, a Dermasol, is developed in well-drained alluvium, and Tolga, a Ferosol, is developed from weathered basalt. Both were sourced from banana farms (see Supplementary information). In a previous study, Liverpool was found significantly more conducive to Fusarium wilt of banana than Tolga, as well as having a lower concentration of available micronutrients (Bowen *et al.* 2019). The four chelating ligands (Oligo Fe-EDTA 13% ™, Oligo Fe-DTPA 11%™, Oligo Fe-EDDHA 6% (5.2% ortho-ortho) ™, and Iperen Fer Marathon Fe-HBED 9%™) were provided by Van Iperen International.

Each soil was a composite collected from 0.00 – 0.25 m depth, 10, 20 and 30 m from the end of a row, in fields that had been planted to the Cavendish cultivar for at least two years. The soils were sieved to <10 mm and homogenized for each replicate. For each pot an equivalent dry mass of 9.98 kg soil was thoroughly mixed with either a solution of dissolved iron chelate or water, placed in a sealed 7 L pot, uniformly compacted (1.2 kg/L dry bulk density) and wetted to 80% of water holding capacity. The appropriate mass of chelating ligand was fully dissolved in 2 L of deionized water and 80 mL of chelate solution or water was mixed into the soil of each pot to give the desired concentration. The mass ratio of chelated iron-to-soil was equal across all chelate solutions (24.1 mg/kg), however the mass of chelating ligand used varied based on the ratio of iron to chelating ligand. Tissue culture Foc R1-susceptible bananas (*Musa* ABB, Ducasse) were obtained from the Department of Agriculture and Fisheries' tissue culture facility in Maroochydore, Queensland. Plants were grown for 85 days in potting mix before being cut 5 cm above the rhizome and planted into the treated soil. 35 days after planting, 10 mL of millet, either inoculated with Foc R1 (vegetative compatibility group 0124, a mixture of isolates BRIP43996 and 61873) or autoclave-sterilized, was mixed into the top 2 cm of soil (Elmer 2002).

6.3.1.2 Management

Other than the chelating ligand and inoculation treatments, management was uniform across plants. Soil water content was maintained between 50 and 80% of soil water holding capacity for both soil types. Every second week each pot received urea (8.3 mg N/kg soil), guano phosphorus fertiliser (1.0

mg P/kg soil), and potassium chloride (12.5 mg K/kg soil and 11.4 mg Cl/kg soil). A calcium deficiency was identified in week 10, affecting Liverpool soil in the inoculated control and Fe-EDTA treatment groups most severely and all other treatments slightly. Gypsum (Eco-flo Gypsum, Organic Crop Protectants Pty Ltd) was added as a one-off treatment (3.5 mg Ca/kg soil) to all pots to correct this. Treatments were randomized within blocks in case of environmental gradients within the greenhouse. During the experimental period the average weekly maximum air temperature was 42.1°C and the average weekly minimum air temperature was 22.0°C. The average weekly maximum soil temperature was 37.0°C and the average weekly minimum soil temperature was 23.0°C.

6.3.1.3 Harvest

The plants were harvested 120 days after inoculation. At harvest, plants were cleaned of any soil before being segmented into leaves, pseudostem, rhizome and roots. Each segment was weighed to obtain a fresh weight and then a representative sample from each segment was weighed and dried at 60°C to constant weight to obtain a dry weight. Using these results, dry weight and water content of each plant and plant segment were calculated. Leaf lamina segments 0.2 m wide were collected from the central portion of the third fully opened leaf, cleaned with distilled water and dried at 60°C for nutrient analysis (Weinert and Simpson 2016). They were analysed by Nutrient Advantage Laboratories, Werribee, Victoria for calcium, magnesium, phosphorus, potassium, sodium, sulphur, boron, copper, iron, manganese and zinc using a nitric acid and hydrogen peroxide digest followed by analysis with inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Internal disease severity was determined using the method of Orr *et al.* (2019). Briefly, after removal of the roots, the rhizome was sectioned into quarters and the upper side of each section was photographed before classifying each pixel of the images as either diseased or not diseased using the image analysis program ImageJ. For sections where the upper side was no longer in the corm a value was not recorded. The proportion of the total rhizome pixels classified as diseased was used as a percentage disease severity for each rhizome section. These values were then averaged for each rhizome to provide a disease severity value for that plant.

6.3.2 Experiment 2

6.3.2.1 Experimental design

Fe-HBED, the most promising chelating ligand from Experiment 1, was applied at a range of rates to determine the maximum effect and associated dose. Experimental treatments were Fe-HBED application at ten initial rates of chelated iron (0, 3.1, 6.3, 9.4, 12.6, 15.7, 18.8, 22.0, 25.1 and 28.3

mg/kg dry soil), calcium fertiliser as gypsum, applied every 5 weeks at two rates (4.65 and 46.5 mg/kg), and inoculation (7.5 mL of millet inoculated with Foc R1, VCG 0124 BRIP 43996 and BRIP61873) (Elmer 2002). Fe-HBED solutions of appropriate concentration were made every 5 weeks and 40 mL of the correct concentration was applied to each pot to achieve the described treatment rates. Uninoculated plants received autoclave sterilised millet. Two treatment combinations were replicated 6 times: Fe-HBED rate of 0 mg/kg, both rates of calcium application and both inoculation treatments, and Fe-HBED rate of 15.7 mg/kg, both calcium rates and no inoculation. The treatment combinations of Fe-HBED rates of 3.1 to 28.3 mg/kg, both calcium rates and inoculation were replicated 3 times each. In total there were 66 inoculated and 24 uninoculated pots. The Liverpool soil was used for all pots, prepared as in Experiment 1. The calcium treatment was included to identify the effect of cation competition on chelate effectiveness. The low calcium rate aimed to approximately balance loss through plant uptake and the high rate approximated calcium application in pre-plant field liming. Sealed pots (4 L) were filled with 4.3 kg equivalent dry weight of soil. After the initial dose, Fe-HBED was reapplied every 5 weeks to the soil surface at half the initial dose to account for chelate deactivation through adsorption and degradation.

6.3.2.2 Management

Water and fertiliser management was uniform across treatments, apart from Fe-HBED and calcium rates. Other fertilisers applied were urea (12.4 mg N/kg), guano phosphorus fertiliser (1.55 mg P/kg), and potassium chloride (18.5 mg K/kg and 16.9 mg Cl/kg) every second week. Plants were watered through drip irrigation and watered to weight every second week in the later stages of the trial to account for unequal transpiration rates as plant sizes diverged. Treatments were randomised within blocks to account for any environmental gradients in the greenhouse. During the experiment average maximum air temperature was 36.8°C and minimum of 17.1°C. Soil temperatures averaged a maximum of 31.2°C and minimum of 19.8°C.

Banana tissue culture plantlets (*Musa* ABB, Ducasse) were planted and allowed to grow for 41 days before initial application of chelating ligand and calcium treatments. A further 14 days elapsed before inoculation and the trial then proceeded for a further 63 days before harvest.

6.3.2.3 Harvest

At harvest, plants were cleaned of any soil before being segmented into leaves, pseudostem, rhizome and roots. Each segment was weighed for wet weight, dried in entirety at 60°C to constant weight and weighed for dry weight. The entire dried, above-ground portion of the plant was sent for nutrient

analysis. Tissue samples were ground and analysed by Nutrient Advantage Laboratories for calcium, magnesium, phosphorus, potassium, sodium, sulphur, boron, copper, iron, manganese and zinc using a nitric acid and hydrogen peroxide digest followed by analysis with ICP-AES. Aluminium concentration in plant tissue was analysed by the Queensland Department of Environment and Science, Dutton Park, Queensland using a nitric acid digest followed by analysis with ICP-AES. The roots were not analysed for nutrients due to the likelihood of soil contamination. Internal disease severity was determined in the same manner as for Experiment 1.

6.3.3 Statistical analysis

Results from experiment 1 were log transformed [$\log_e(x+1)$] to address issues of unequal variance between treatments. $\text{Log}_e(x+1)$ rather than $\text{Log}_e(x)$ was used for the transformation of the disease severity results to account for values of 0. The transformed values were then analysed by a Tukey test for each soil type using a linear model with Gaussian distribution. Treatments were compared to a baseline of inoculated water. In Experiment 2 fixed effects were Fe-HBED rate, calcium rate and inoculation, with the exception of the response variable aluminium concentration, for which inoculation was not considered. When examining the response of disease to Fe-HBED all rates of application were considered and Fe-HBED was considered quantitative, however when considering the effects of Fe-HBED, calcium and inoculation treatments and their interactions only 0 and 0.75 g/pot rates of Fe-HBED were used, to provide a more balanced design and were considered categorical variables. All graphs were produced using the ggplot2 package in R (Wickham 2016). In Experiment 1 all data was included for analysis with no outlier removal. In Experiment 2 one plant was removed prior to model calculation as it had no disease present despite being in the inoculated treatment group. It is likely that this plant was mistakenly given sterile millet rather than inoculated millet.

Plant tissue dry weights were used for calculations and graphing. Total nutrient uptake for each plant was calculated by multiplying the plant tissue nutrient concentration by the plant tissue dry weight.

6.4 Results

6.4.1 Experiment 1

In Experiment 1 disease severity was significantly affected by inoculation (Figure 6-2; Table 6-1). The treatment that reduced disease severity most was the application of Fe-HBED to Liverpool soil, which decreased the mean disease severity from 53.3% with water to 39.7%, though this decrease was not significant at $\alpha = 0.05$ (Table 6-1).

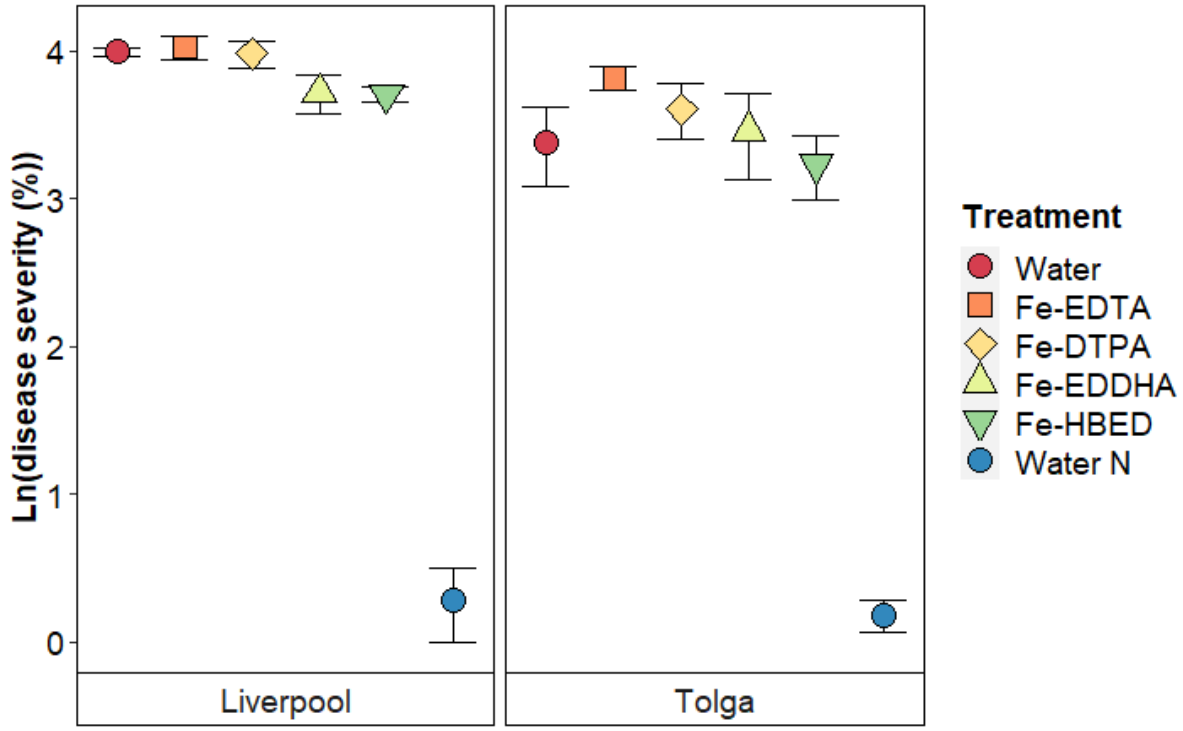

Figure 6-2. Log$_e$ transformed internal disease severity of banana plants grown in two soils amended with iron-saturated chelates or water in Experiment 1. "Water N" represents treatment with water and without inoculation, all other treatments were inoculated with *Fusarium oxysporum* f.sp. *cubense*. Points indicate mean values and error bars indicate standard error.

Table 6-1. The effects of chelate treatments in either soil type in Experiment 1. Treatments are compared to a baseline of inoculated water (Water), all data was log transformed and analysed by a Tukey test using a linear model with Gaussian distribution. The comparison of "Water N – Water" demonstrates the effect of inoculation without treatment.

	Disease severity	**Plant dry weight**	**Iron content**
Liverpool			
Fe-EDTA – Water	$T = 0.05$, $P = 1.000$	$T = 1.45$, $P = 0.900$	$T = 0.64$, $P = 1.000$
Fe-DTPA – Water	$T = -0.09$, $P = 1.000$	$T = 2.66$, $P = 0.186$	$T = -0.71$, $P = 1.000$
Fe-EDDHA – Water	$T = -0.98$, $P = 0.993$	$T = 1.38$, $P = 0.927$	$T = 0.20$, $P = 1.000$
Fe-HBED – Water	$T = -0.83$, $P = 0.998$	$T = 0.79$, $P = 0.999$	$T = -0.29$, $P = 1.000$

Water N – Water	T = - 10.79, P = <0.001	T = 2.34, P = 0.343	T = - 1.21, P = 0.969
Tolga			
Fe-EDTA – Water	T = 1.72, P = 0.765	T = 1.90, P = 0.645	T = 1.50, P = 0.880
Fe-DTPA – Water	T = 0.62, P = 1.000	T = 0.77, P = 0.999	T = 1.27, P = 0.956
Fe-EDDHA – Water	T = - 0.22, P = 1.000	T = 0.23, P = 1.000	T = 0.04, P = 1.000
Fe-HBED – Water	T = - 0.86, P = 0.998	T = 0.84, P = 0.998	T = - 0.09, P = 1.000
Water N – Water	T = - 8.60, P = <0.001	T = 0.41, P = 1.000	T = 1.96, P = 0.599

Plant tissue iron concentration was not significantly affected by chelate addition but did differ between the two soils (Figure 6-3, Table 6-1). Total plant uptake of iron was significantly higher in Tolga soil than in Liverpool, but this was principally due to greater growth in the Tolga soil than the Liverpool soil (Table 6-1), though there was some difference in concentration between the two soils (Figure 6-3).

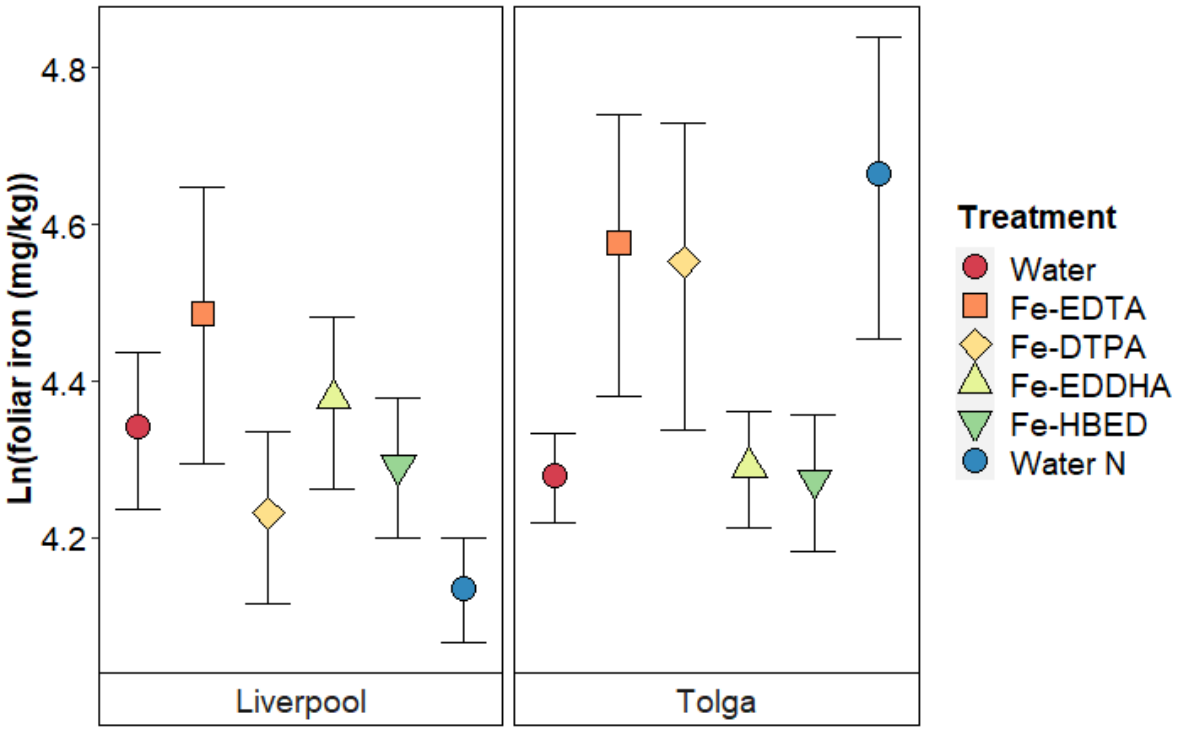

Figure 6-3. Log$_e$ transformed foliar concentration of iron in banana plants grown in two soils amended with iron-saturated chelates in Experiment 1. Points represent mean values and error bars represent standard deviation. No treatments had a significant effect.

6.4.2 Experiment 2

In Experiment 2 with Fe-HBED rate, calcium rate, inoculation and their interactions, only inoculation had a significant effect on disease severity at $\alpha = 0.05$ (Figure 6-4; Table 6-2). Fe-HBED rate did significantly affect the disease severity when the zero rate values were excluded (Figure 6-4), but disease severity did not differ significantly between the individual rates and the zero rate so the difference was considered to be an artefact of the number of treatments tested and not to be meaningful.

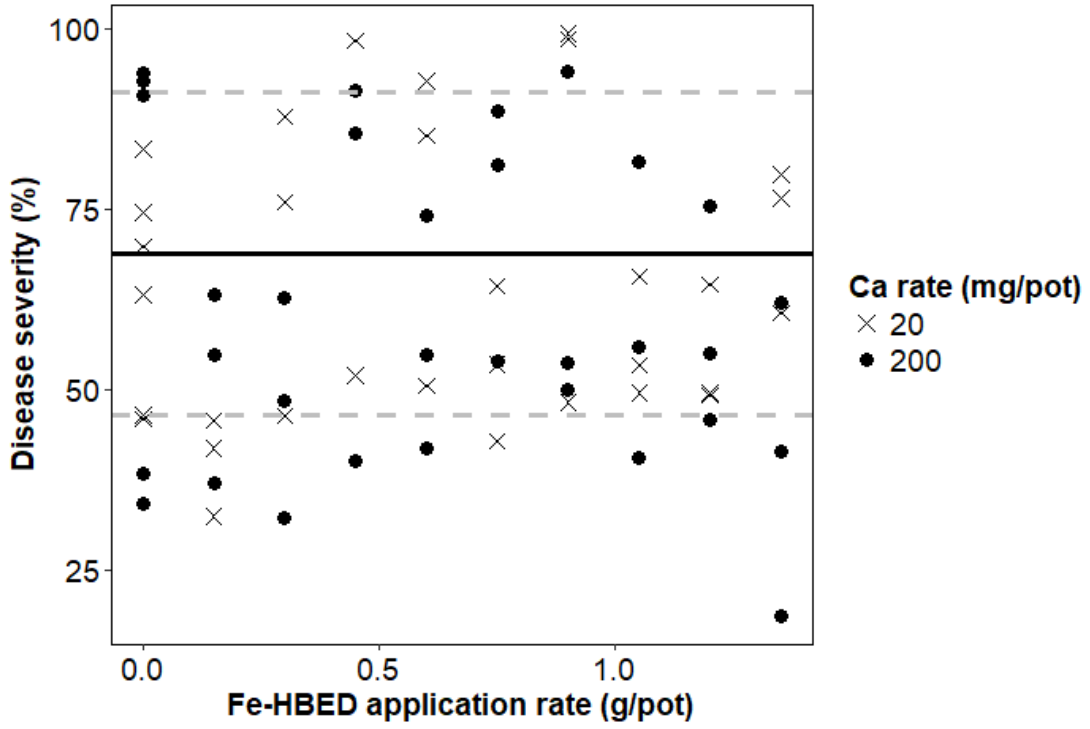

Figure 6-4. Internal disease severity of inoculated plants as a function of Fe-HBED chelate and calcium, as gypsum, application rates in Experiment 2. The solid line indicates the mean disease severity of inoculated plants with 0 g/pot Fe-HBED application, the dashed lines indicate the first and third quartile range. Treatment effects are not significant at α= 0.05.

Table 6-2. The effects of inoculation, Fe-HBED rate, calcium rate and their interactions on plant variables in Experiment 2 calculated using a generalised linear model. The comparison was limited to individuals receiving either 0 or 0.75 g/pot of Fe-HBED. Only disease-free plants were analysed for aluminium content so values relating to inoculation were not calculated.

	Disease severity	Plant dry weight	Iron concentration	Manganese concentration	Aluminium concentration
Inoculation	T_{34} = 7.16, P = <0.001	T_{34} = -4.46, P = <0.001	T_{34} = 1.56, P = 0.128	T_{34} = -2.66, P = 0.012	-
Fe-HBED rate	T_{34} = -0.01, P = 0.992	T_{34} = 0.42, P = 0.679	T_{34} = 1.86, P = 0.072	T_{34} = -2.69, P = 0.011	T_{20} = 1.90, P = 0.072
Ca rate	T_{34} = -0.01, P = 0.991	T_{34} = 1.02, P = 0.314	T_{34} = 1.93, P = 0.062	T_{34} = -0.57, P = 0.570	T_{20} = 1.81, P = 0.086

Inoculation * Fe-HBED rate	T_{34} = -0.83, P = 0.412	T_{34} = 0.34, P =0.734	T_{34} = -1.13, P = 0.267	T_{34} = -0.02, P = 0.982	-
Inoculation * Ca rate	T_{34} = 0.90, P = 0.374	T_{34} = -0.96, P = 0.346	T_{34} = 0.23, P = 0.822	T_{34} = 1.83, P = 0.077	-
Fe-HBED rate * Ca rate	T_{34} = 0.01, P = 0.990	T_{34} = -0.98, P = 0.336	T_{34} = -2.17, P = 0.037	T_{34} = -0.25, P = 0.801	T_{20} = -1.78, P = 0.091
Three-way interaction	T_{34} = 0.62, P = 0.538	T_{34} = 0.42, P = 0.678	T_{34} = 0.92, P = 0.363	T_{34} = -0.42, P = 0.679	-

Plant weight was significantly lower in inoculated plants than in uninoculated (Table 6-2, Figure 6-5). Other treatments, and their interactions, did not have a significant effect. The concentration of iron and aluminium in above-ground plant tissue were mildly affected by the rate of Fe-HBED, calcium and the interaction of the two rates, and iron concentration was not affected by inoculation (Table 6-2). Fe-HBED addition increased the concentration of iron and aluminium in uninoculated plants when the rate of calcium application was low, but when the calcium rate was high application of Fe-HBED actually decreased the mean concentration of both iron and aluminium in plant tissue (Figure 6-6). In inoculated plants the application of Fe-HBED and calcium had little effect on plant iron concentration. The concentration of manganese in plant tissue was significantly decreased by both inoculation and the addition of Fe-HBED, but not by the rate of calcium application (Table 6-2; Figure 6-6). Inoculation significantly increased the concentration of boron, copper, phosphorus, potassium and sulphur in the plant tissue, significantly decreased the concentration of magnesium and did not significantly alter the concentration of calcium or zinc. Calcium and Fe-HBED rate did not significantly affect the other element concentrations tested.

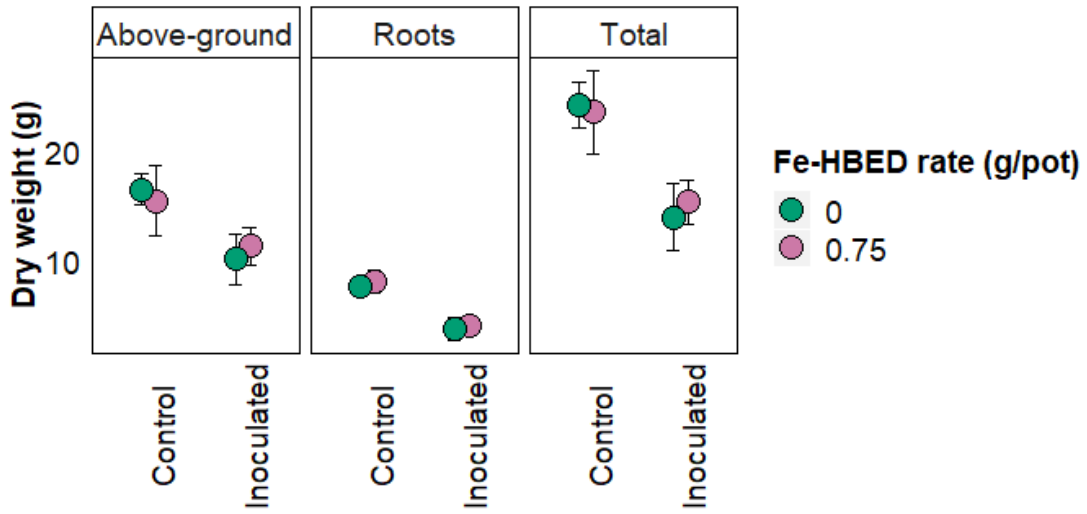

Figure 6-5. Plant section and total plant dry weight with and without inoculation with *Fusarium oxysporum* f.sp. *cubense* in Experiment 2. The effect of Fe-HBED application rate was not significant for the above-ground portion ($P = 0.962$), root ($P = 0.166$) or for total dry weight ($P = 0.679$). Points represent mean values and error bars represent standard deviation.

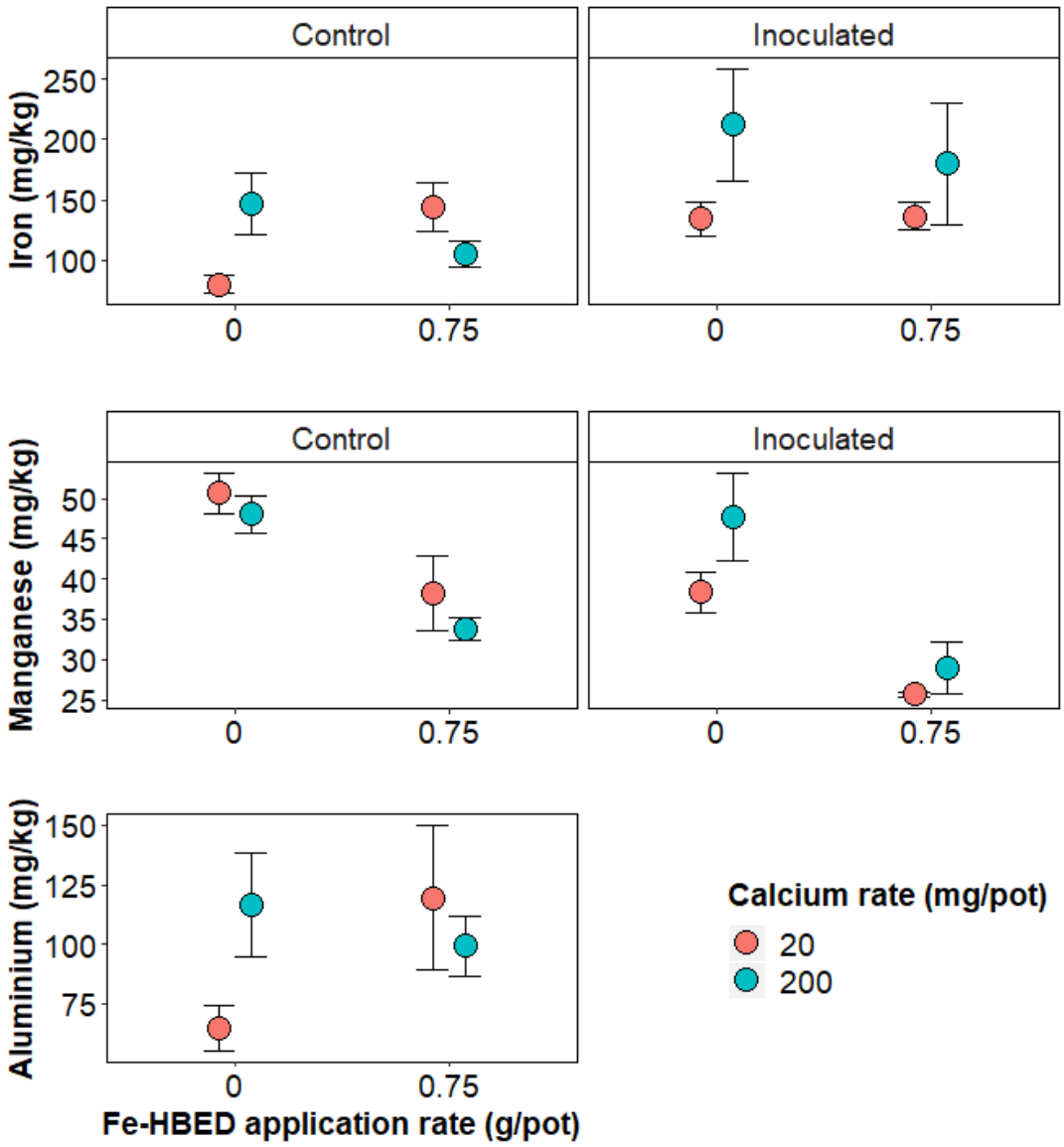

Figure 6-6. Plant iron, manganese and aluminium concentrations as affected by Fe-HBED, calcium and inoculation treatments in Experiment 2. Inoculated plants were not analysed for aluminium, so no inoculated aluminium results are presented. Points represent mean values and error bars represent standard error.

6.5 Discussion

Our findings, that the addition of a strong iron chelate such as Fe-EDDHA or Fe-HBED does not significantly reduce the severity of Fusarium wilt contrast with the findings of Peng *et al.* (1999) and Scher and Baker (1982). In addition, in Experiment 1 we found that the addition of Fe-EDTA did not significantly reduce Fusarium wilt severity, in contrast with the findings of Dong *et al.* (2016). The intention of Experiment 1 was that availability of iron to the pathogen would be increased by Fe-EDTA and Fe-DTPA and decreased by Fe-EDDHA and Fe-HBED due to their iron binding strength,

and that this would increase disease severity in the former and decrease it in the latter, as has been found with other crops (Lemanceau *et al.* 1988). Banana nutrition was expected to be unaffected by the treatments because bananas are able to acquire iron from both weak and strong chelating ligands (Marschner and Römheld 1995). The fact that the expected effect was not observed suggests that the strength of the chelated ligand did not significantly affect iron nutrition of the pathogen.

It is possible that iron supplementation in the studies by Peng *et al.* (1999) and Dong *et al.* (2016) alleviated iron deficiency in the plants and thereby enhanced their resistance to Foc. Foliar application of EDDHA to Arabidopsis activated the salicylic and jasmonic acid defence pathways in addition to iron scavenging mechanisms, suggesting that iron deficiency results in defence downregulation (Aznar *et al.* 2014). Similarly, an adequate supply of iron in maize suppressed the hemibiotrophic fungal pathogen *C. graminicola* by increasing production of reactive oxidative species as part of a defensive oxidative burst (Ye *et al.* 2014). A sufficient iron supply is essential to plant defence against fungal pathogens, and in the hydroponic setting of the study by Dong *et al.* (2016) and the low micronutrient soils used by Peng *et al.* (1999) it is possible the plants without supplemental iron were deficient. Plants in Experiment 1 and 2 of our work were generally in the adequate range for iron concentration in banana plant tissue of 70-200 mg/kg (Reuter and Robinson 1997) so deficiency was unlikely.

The application of chelated iron did not significantly affect plant uptake of iron in Experiment 1, though it did marginally increase it in uninoculated plants in Experiment 2 (Table 6-2, Figure 6-6). The small increase in tissue iron concentration from Fe-HBED addition in Experiment 2 was much less than that found in previous studies and may further explain the lack of disease suppression. In the hydroponic study by Dong et al. (2016) iron supplementation treatments that resulted in the suppression of disease dramatically increased banana leaf and root iron concentrations relative to low-iron treatments. In Experiment 1, despite only 0.240 g of iron being applied per plant in each chelating ligand treatment, plants took up 9.175 ± 2.537 g of iron from the Liverpool soil and 16.305 ± 6.911 g of iron from the Tolga soil. In Experiment 2 plants took up 2.747 ± 1.409 g of iron from the soil on average, compared with a maximum application of 0.182 g of iron. The mass of iron applied was a greater proportion of the total iron taken up by the plant in Experiment 2 than Experiment 1, which may have caused the treatment effect in Experiment 2. It is also possible that the effect was due to the reapplication of Fe-HBED in Experiment 2. Reapplication likely compensated for deactivation of the chelate, which resulted in greater chelate-facilitated iron uptake by the plant in Experiment 2 than Experiment 1. Strong chelates such as Fe-EDDHA and Fe-HBED have been theorised to shuttle bound iron to the plant so they can facilitate considerably more iron uptake than the amount of iron

added, inducing deficiency in surrounding microorganisms (Schenkeveld *et al.* 2014b). Despite this, it appears that the application of strong chelating ligands did not induce an iron deficiency in the pathogen, nor greatly increase the iron uptake by the host plant, because the plant was not iron deficient without addition of iron chelates.

The application of gypsum was expected to interfere with plant uptake of iron from chelating ligands due to an increase in calcium availability, and this did occur when disease was absent, as shown by the interaction between these two treatments (Figure 6-6; Table 6-2). The selectivity of chelating ligands for particular ions depends on the relative concentrations and stabilities of their chelate complex. Curiously, when only gypsum was added, the concentration of iron in plant tissue was increased (Figure 6-6). It is possible that the extra calcium relieved a deficiency in the plants and facilitated greater uptake of iron. As only the above-ground portion of the plant was analysed for nutrient concentrations it is also possible that the added calcium facilitated better transportation of iron to the above-ground portion of the plant. These two explanations seem unlikely, however, as the average calcium tissue concentration with low calcium addition was 1.13%, within the recommended range of 0.5 – 1.2% (Reuter and Robinson 1997).

Aluminium and manganese have also been shown to compete with iron for mobilisation by strong chelating ligands in soil (Schenkeveld *et al.* 2007; Orr *et al.* 2020). As a charge-dense cation, aluminium is much more likely to displace iron from strong chelates such as HBED than calcium or manganese is. The application of Fe-HBED in Experiment 2 increased the plant tissue concentration of aluminium in uninoculated plants with a low rate of calcium addition and decreased the concentration of manganese (Figure 6-6). It is possible that the preferential shuttling of iron and aluminium to the plant rhizosphere displaced plant uptake of manganese resulting in higher plant tissue concentrations of aluminium and iron and lower concentrations of manganese. It is likely that if the growing period under the experimental conditions was longer, the effect of Fe-HBED application on plant aluminium, iron and manganese concentration would have become more significant. This raises the prospect that application of Fe-HBED and Fe-EDDHA may increase plant tissue aluminium concentrations under certain cropping conditions, with possible negative effects on plant health or the suitability of affected crops for human consumption. This unintended possible side-effect of Fe-HBED application bears further investigation.

In both Experiment 1 and 2 there was a high degree of variability between replicates, and this may be masking treatment effects (Figures 6-2, 6-3, 6-4, 6-6). Despite our best efforts to limit variability

between plants there will always be natural heterogeneity when dealing with soil and plant systems and the combination of several treatments resulted in large cumulative variability. Despite being clonal the banana plants themselves grew at different rates and their root systems accessed nutrition in the soil at different rates. The application of the chelate and calcium treatments may not have been evenly distributed throughout the soil column and may have been different distances from plant roots, resulting in variation in plant uptake and plant growth rates. Application of Fe-HBED increased the standard deviation of the uninoculated plant dry weight from 5.02 g for the control to 5.38 g for the Fe-HBED treated plants. Application of the pathogen compounded experimental variability as the distance between the inoculated millet, treatments and plant roots may not have been consistent. The addition of the pathogen increased the standard deviation of the plant dry weight in Experiment 2 from 5.36 g for the uninoculated plants to 5.91 g for inoculated plants.

Overall, it appears that in soils with a high concentration of available micronutrients the effect of strong chelating ligands on Fusarium wilt severity is minimal. This contrasts with the previous findings of suppression of Fusarium wilt of banana by application of iron to a micronutrient-deficient soil or solution (Peng *et al.* 1999; Dong *et al.* 2016). In our experiment, when Foc was present, any effect from the chelating ligand was masked by the greater disease effect. Presumably the effectiveness of this treatment decreases in inverse proportion to the concentration of available micronutrients and further delineation of this relationship is warranted. It would also be valuable to understand to what extent the effect of iron on disease, where it occurs, is due to effects on plant defence or pathogen virulence. Finally, the increase in aluminium concentration in plant tissue when Fe-HBED was applied bears further investigation to ensure that application of this chelating ligand will not negatively affect plant health or suitability of produce for human consumption. We hope that by publishing the non-significant findings of our work we provide greater context to the significant results already present in the literature.

7 Nitrogen fertiliser rate but not form affects the severity of Fusarium wilt of banana

Chapter No.	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author, including the candidate
7	This chapter, in an edited form, is published as: Orr, R., Dennis, P. G., Wong, Y., Browne, D. J., Cooper, M., Birt, H. W. G., Lapis-Gaza, H. R., Pattison, A. B., and Nelson, P. N. (2022). "Nitrogen fertilizer rate but not form affects the severity of Fusarium wilt in banana". Frontiers in Plant Science. 13.	Ryan Orr developed the research question in consultation with Dr. Paul Nelson, Dr. Anthony Pattison and Dr. Paul Dennis. Ryan Orr collected the data with assistance from Dr. Yide Wong, Daniel Browne and Dr. Henry Birt and performed the data analyses with assistance from Dr. Martha Cooper, Dr. Paul Dennis, Daniel Browne and Dr. Paul Nelson. Ryan Orr wrote the first draft of the paper which was revised with editorial input from Dr. Paul Nelson and the other co-authors.

7.1 Abstract

Nitrogen (N) fertilisers are routinely applied to bananas (*Musa* spp.) to increase production, but they may exacerbate plant diseases like Fusarium wilt of banana – the most economically important disease of banana. Here, we characterised the effects of N rate and form (ammonium or nitrate) on plant growth, Fusarium wilt of banana severity, the banana root proteome, the abundance of *Fusarium oxysporum* f.sp. *cubense* (Foc) (the causal agent of Fusarium wilt of banana) in the rhizosphere, and the diversity of rhizosphere bacterial and fungal communities. Banana plants (*Musa* ABB) were grown under greenhouse conditions in soil with ammonium or nitrate applied at five N rates, and with or without inoculation with Foc. The growth of non-inoculated plants was positively correlated with N rate. In bananas inoculated with Foc, disease severity increased with N rate, resulting in Foc-inoculated plant growth being greatest at intermediate N rates. The abundance of Foc in the soil was

weakly related to the treatment conditions and was a poor predictor of disease severity. Fungal diversity was consistently affected by Foc inoculation, while bacterial diversity was associated with changes in soil pH resulting from N addition, in particular ammonium. N rate altered the expression of host metabolic pathways associated with carbon fixation, energy usage, amino acid metabolism, and importantly stress response signalling, irrespective of inoculation or N form. Furthermore, in diseased plants, Pathogenesis-related protein 1, a key endpoint for biotic stress response and the salicylic acid defence response to biotrophic pathogens, was negatively correlated with the rate of ammonium but not nitrate. As expected, inoculation with Foc altered the expression of a wide range of processes in the banana plant including those of defence and growth. In summary, the severity of Fusarium wilt of banana was negatively associated with host defences, which were influenced by N application (particularly ammonium), and shifts in microbial communities associated with ammonium-induced acidification.

7.2 Introduction

Application of synthetic nitrogen (N) fertiliser has enabled humans to dramatically increase agricultural productivity (Erisman *et al.* 2008). Worldwide use of synthetic N fertiliser is projected to increase to 112 million tonnes in 2022 (FAO 2019). Use of N fertiliser has substantial benefits, but also some detrimental effects. Excessive N use has been found to increase the severity of diseases such as Fusarium wilt of banana (Pittaway *et al.* 1999; Mur *et al.* 2017; Segura-Mena *et al.* 2021). Disease severity is governed by the three-way relationship between host, pathogen and the environment (Agrios 2005), with N supply an especially important environmental aspect in the context of Fusarium wilt of banana.

Banana farmers apply N fertiliser to maximise productivity, potentially rendering their plants vulnerable to pathogens such as *Fusarium oxysporum* f.sp. *cubense* (Foc), the soil-borne, hemibiotrophic, fungal pathogen responsible for Fusarium wilt of banana. The mechanism behind the association between N fertilisation and increased Fusarium wilt of banana severity remains unclear, as N fertilisers have multiple effects on soils, including changes to nutrient availability, and pH; which also change depending on the form of N fertiliser applied (Marinari *et al.* 2000; Neumann and Römheld 2012). The main forms of N fertiliser used are ammonium, urea, which converts to ammonium, and nitrate, with ammonium and urea being most common due lower price. Current evidence indicates that nitrate fertiliser reduces Fusarium wilt severity, whereas ammonium increases it (Woltz and Engelhard 1973; Jones *et al.* 1975; Morgan and Timmer 1984; Woltz *et al.* 1992; Wang *et al.* 2016; Zhou *et al.* 2017) though there are exceptions (Jarvis and Thorpe 1980). This effect of N form on disease may be due to the acidifying effect of ammonium fertiliser and alkalising effect of

nitrate fertiliser, as soil acidification increases Fusarium wilt of banana severity (Orr and Nelson 2018; Segura *et al.* 2021; Teixeira *et al.* 2021). Indeed, field trials have shown a greater influence of N fertiliser form on Fusarium wilt of banana severity at low pH than at high (Jones *et al.* 1975). Segura-Mena *et al.* (2021) found that the addition of ammonium nitrate fertiliser increased Fusarium wilt of banana severity, but less so at pH 5.1 than at 6.1. Only one soil-based study, performed on tomato, maintained similar soil pH between different N fertiliser forms and found nitrate to be significantly more suppressive to disease than ammonium (Woltz *et al.* 1992), suggesting a mechanism independent of pH. To understand the role of N in management of Fusarium wilt of banana it is thus important to consider the effects of N fertiliser form and dose and the resulting change of pH.

Greater N availability has been proposed to cause a growth-defence trade-off in plants to maximize fitness (Herms and Mattson 1992). Plants that grow more rapidly due to an abundance of N must sacrifice defensive capabilities due to greater metabolic expenditure, leaving them more susceptible to disease (Huot *et al.* 2014; Neuser *et al.* 2019). Pittaway *et al.* (1999) examined the relationship between Fusarium wilt of banana severity and pathogen invasion and activity under N-fertilised conditions. They hypothesised that the observed increase in disease severity was likely due to a predisposition of the host plant to disease, through a reduction in defence response, rather than changes in the pathogen. If the growth-defence trade-off is responsible for increased banana susceptibility to Fusarium wilt of banana in future it may be possible to mitigate the effect through gene manipulation (Wang *et al.* 2021).

The diversity and biomass of soil bacterial and fungal communities, excluding the pathogen, generally are negatively correlated with N fertiliser rate (Wang *et al.* 2018; Yang *et al.* 2020; Zhou *et al.* 2020). A reduction in microbial diversity and biomass with increased N fertiliser application is largely attributable to changes in soil pH (Wang *et al.* 2018; Yang *et al.* 2020). Fusarium wilt of banana is less severe in fields with high microbial diversity possibly due to increased competition (Shen *et al.* 2015b; Fu *et al.* 2017; Rames *et al.* 2018), so application of N, that reduces microbial diversity of the soil may increase disease severity. Therefore, it is important to consider both the rate of N applied and the form of fertiliser used.

The aim of this work was to determine the effects of inoculation with Foc, N rate and form on plant growth, the severity of Fusarium wilt of banana and the components of the Fusarium wilt of banana disease triangle. In controlled conditions we examined the response of each component of the disease

triangle: the pathogen (abundance in rhizosphere by real-time qPCR), environment (rhizosphere soil chemistry and soil fungal and bacterial population diversity using ITS2 and 16S rRNA gene amplicon sequencing), and host (above- and below-ground growth, tissue N content and isotopic composition, and functional protein content of roots, using SWATH quantitative proteomics).

7.3 Materials and methods

7.3.1 Soil collection

A Liverpool series soil (Dermosol (Murtha 1986) was collected (0-30 cm depth) from a commercial banana (*Musa* [AAA Group, Cavendish Subgroup] 'Williams') farm that had applied relatively low rates of fertiliser N (maximum of 160 kg / ha / year) for the previous seven years. This location was selected to limit the presence of residual N fertiliser. Abiotic characterisation of this soil (Site 5, Liverpool) has been published previously as part of a regional survey (Orr and Nelson 2021). Buckets of soil were sealed for transport and moved to the greenhouse where the experiment was conducted, to prevent the spread of soil-borne disease. Transport was at ambient temperature and took approximately 2 h. Soil was sieved to 10 mm to remove rocks, sticks and large roots and then homogenized before potting.

7.3.2 Greenhouse set-up

To each 4 L pot, 4 kg (dry weight equivalent) of soil was added. Foc Race 1-susceptible banana plantlets (*Musa* [ABB Group, Pisang Awak Subgroup] 'Ducasse'; Maroochy Research Facility, Maroochydore, Queensland), were decanted and planted directly into soil. After 36 days, ammonium (ammonium sulphate) or nitrate (potassium and calcium nitrate) fertiliser were each applied at five rates (3.0, 6.0, 12.1, 30.2 and 48.3 mg N / pot / fortnight; equivalent to field rates of 25, 50, 100, 250 and 400 kg / ha / yr) to each pot. Other fertilisers were applied at uniform rate across the treatments (potassium = 88.1, phosphorus = 6.64, calcium = 36.0, chloride = 72.3 and sulphur = 84.1 mg / pot / fortnight), except for sodium (0 – 139 mg / pot / fortnight).

Fertilisers were applied in dissolved form by saturating the soil and then draining off solution in excess of the soil's water holding capacity. This method allowed the experiment to be carried out in soil, while mimicking some of the benefits of hydroponic systems by redistributing solutes between rhizosphere and bulk soil (which deviated between fertiliser applications due to root activity), equilibrating soil water content between pots (which deviated between pots due to differential transpiration) and removing nitrate (generated by nitrification) from ammonium-treated pots. The water holding capacity of each pot was determined by saturating the known dry weight of soil in each

pot with water and then allowing it to drain freely for 24 hours (until it was no longer dripping) before reweighing. Prior to each fertiliser application, each pot was watered to 200 mL below water holding capacity (by weight) and then placed in a larger container. The pot was then soaked in a fertiliser bath with the required amount of fertiliser being contained in 200 mL of the fertiliser solution. The fertiliser solution entering the pot through small holes at the bottom over a period of approximately 5 minutes, until the solution level was above the soil surface. The pot was then removed from the fertiliser solution and allowed to freely drain for 24 h before weighing. The difference between the weight prior to immersion, and that after freely draining was used to confirm the amount of fertiliser added. The pot weight after free draining was also used to calculate the water required prior to fertiliser addition for the next fertiliser addition 14 days later.

After 61 days, 7.5 mL of Foc-inoculated (Race 1, VCG 0124 [BRIP 43996 and BRIP61873]; Queensland Plant Pathology Herbarium) millet was added to the inoculated treatment pots. Autoclave-sterilised millet was added to the remaining pots, resulting in 20 unique treatment combinations. The millet was mixed into the top 20 mm of soil in each pot, ensuring not to cross contaminate the treatments. Fertiliser-inoculated treatments were replicated 5 times, to account for the greater variability of incorporating an extra treatment, and fertiliser-non-inoculated 4 times (n = 90). All other soil conditions, including temperature and water supply, were consistent between treatments.

7.3.3 Sampling

Soil samples for pH, nitrate, and ammonium analysis were collected 1, 36, and 52 days after inoculation. Samples for root proteome and rhizosphere soil qPCR and amplicon sequencing were collected 52 and 53 days after inoculation by gently removing the plant and roots from the pot and taking the sample (as described below) before replacing plant and remaining roots in the pot. These samples were taken prior to plant harvest to ensure the plant stress response was not due to harvest. Plants were harvested over a two-day period, 56 and 57 days after inoculation, after severe leaf yellowing and pseudostem splitting had been observed in some plants.

At harvest the roots were separated from the rhizome and kept separate to minimize soil contamination. The rhizome was sectioned for determination of disease severity (see below). The aboveground (including rhizome) and root sections were weighed for fresh weight, dried at 60°C until a constant dry weight was achieved, and reweighed to determine dry weight and water content of the tissue. The entire dried aboveground section of the plant was then ground to a fine powder and

homogenized prior to analysis of total N and carbon (C) concentration, and stable isotope composition.

7.3.4 Disease severity

Internal disease severity was determined using the method of Orr *et al.* (2019). Briefly, after removal of the roots, the rhizome was laterally sectioned into quarters and the upper side of each section was photographed. Each pixel of the section photographs was classified as either diseased or not diseased using the image analysis program ImageJ v1.52a (Schneider *et al.* 2012) and a reference grayscale. For sections where the upper side of the lateral section was in the pseudostem tissue rather than the rhizome a value was not recorded. The proportion of the total rhizome pixels classified as diseased was used as a percentage disease severity for each rhizome section. The values for each rhizome section were then averaged to provide a disease severity value for that plant.

7.3.5 Soil pH, nitrate, and ammonium analysis

Soil samples were collected periodically using a small corer. A 10 mm internal diameter, 300 mm length, stainless steel tube was inserted to the complete depth of the pot to collect a core. Each core was homogenized and 4 g (dry weight equivalent) of sample was end-over-end shaken for 1 h with 40 mL of 0.1 M potassium chloride before centrifuging at 3000 × *g* for 5 min. After centrifuging the supernatant pH was measured (Ionode IH series probe; Ionode Pty Ltd. Australia) and subsamples of the supernatant were taken for nitrate and ammonium analysis.

Nitrate analysis followed Hach method 8171 (Hach 2017), using a linear six point calibration curve from analytical grade standards (Hach, Australia). Ammonium was determined using Hach method 8038 (Hach 2017), also using a linear six point calibration curve from analytical grade standards (Hach Australia).

7.3.6 Plant N and C concentration, and isotopic composition

Total N concentration of the plant (aboveground portion) was measured to determine N uptake, and $\delta^{15}N$ was measured to determine if the treatments influenced the proportion of N taken up directly from fertiliser versus soil or microbially processed N (Denk *et al.* 2017). The $\delta^{13}C$ of the same samples was measured to determine effects on water use efficiency (Cernusak *et al.* 2013). C and N concentrations, and $\delta^{13}C$ and $\delta^{15}N$ values of the plant, soil and fertiliser samples were determined using a Costech Elemental Analyzer fitted with a zero-blank auto-sampler coupled via a ConFloIV to

a ThermoFinnigan DeltaVPLUS using Continuous-Flow Isotope Ratio Mass Spectrometry (EA-IRMS) at James Cook University's Advanced Analytical Centre (Cairns). Stable isotope results are reported as per mille (‰) deviations from the VPDB and AIR reference standard scale for $\delta^{13}C$ and $\delta^{15}N$ values, respectively. Precisions (S.D.) on internal standards were better than 0.1% and 0.2% for C and N, respectively.

7.3.7 Amplicon sequencing and qPCR

Sample collection and extraction: Approximately 70 mm long sections from the apex of three healthy roots of each plant, with rhizosphere soil attached, were cut with methanol-sterilised scissors and stored at 4°C until processing. Samples were sonicated in 40 mL phosphate-buffered saline solution to loosen soil and fine roots. Large roots were then removed and the remaining soil suspension was centrifuged for 15 min at 400 × *g* before removal of the supernatant. The remaining soil pellet (representing the rhizosphere) was frozen at -18°C until DNA extraction.

DNA was extracted using a Qiagen DNeasy Powersoil kit (Qiagen, Australia) according to manufacturer recommendations. The amount of soil extracted for each sample was weighed precisely for use in calculating DNA concentrations in soil. The extracted rhizosphere soil DNA sample was then used for both Foc R1 qPCR and amplicon sequencing.

7.3.7.1 PCR amplification and sequencing

To survey bacterial communities, 16S rRNA genes were amplified by PCR using the primers 926wF (5′-AAA CTY AAA KGA ATT GRC GG-3′) and 1392R (5'- ACG GGC GGT GWG TRC -3') (Lane 1991). Fungal communities were surveyed using the ITS2 rRNA gene primers gITS7 (5'- GTG AAT CAT CGA ATC TTT G-'3) (Ihrmark *et al.* 2012) and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-'3) (White *et al.* 1990). Thermocycling conditions were as follows for all reactions: 98°C for 45 s; then 35 cycles of 98°C for 5 s, 56°C for 5 s, 72°C for 6 s; followed by 72°C for 1 min. Each PCR reaction contained 2 µL DNA of the sample in 5X Phire Green Reaction Buffer (Thermo Fisher), 0.4 µL Phire Green Hot Start II DNA Polymerase (Thermo Fisher), 100 µM of each of the dNTPs (Invitrogen), and 10 mM of each primer. The remaining volume was made to 20 µL with molecular grade water. A Simpliamp® 96-well Thermocycler (Applied Biosystems) was used to perform PCR reactions. Gel electrophoresis was used to verify blank extraction controls and negative amplification controls.

Magnetic beads (Rohland and Reich 2012) were used to purify amplicons, which were then dual indexed using the Nextera XT Index Kit (Illumina) according to the manufacturer's instructions. Indexed amplicons were again purified using magnetic beads and quantified using a PicoGreen dsDNA Quantification Kit (Invitrogen). Samples were pooled in equal concentrations and sequenced on an Illumina MiSeq using 30% PhiX Control v3 (Illumina) and a MiSeq Reagent Kit v3 (600 cycles, Illumina) according to the manufacturer's instructions.

Sequence data were processed using a modified UPARSE approach (Edgar 2013). Firstly, reads were demultiplexed and barcodes removed using the cutadapt tool in QIIME2 (v2017.9.0; (Zhang *et al.* 2000). ITS2 or 16S sequences were then processed according to separate protocols. For 16S sequences, primers were removed and trimmed to 250 bp using fastx_truncate of USEARCH (Edgar 2010). Trimmed reads were then quality filtered using fastq_filter of USEARCH with a maxee score of 1.0. For ITS2 sequences, ITSx (Bengtsson-Palme *et al.* 2013) was used to extract the ITS2 region with fungi as the specified profile. Chimaeras were then removed from the extracted sequences using uchime2_ref of USEARCH against the UNITE 8.2 database (Nilsson *et al.* 2019). ITS2 and 16S sequences then resumed with the same pipeline after these steps had completed. Representative sequences were generated using fastx_uniques and cluster_otus of USEARCH with a sequence similarity of 0.97. These representative sequences were used to create an operational taxonomic unit (OTU) table by mapping the remaining reads using the otutab function in USEARCH. Taxonomy was assigned to each OTU using blastn from QIIME2 against the SILVA 128 (Quast *et al.* 2013) database for 16S sequences and UNITE 8.2 database for ITS2 sequences. Sequences that were not either bacteria or fungi were removed using taxa filter-table from QIIME2. The 16S sequences were then aligned using MAFFT (Katoh and Standley 2013) and masked using QIIME2 to generate phylogenetic distance. A midpoint-rooted phylogenetic tree was generated from the alignment using FastTree (v2.1.9) (Price *et al.* 2010). 16S samples were rarefied to 6950 reads, ITS samples were rarefied to 13500. Alpha diversity metrics were produced using QIIME2.

7.3.7.2 qPCR analysis

Foc abundance was quantified with absolute quantification-based qPCR as previously published (Matthews *et al.* 2020), except for the use of SsoAdvanced SYBR® SuperMix (BioRad), which has been shown to increase reaction efficiency compared to other qPCR master mixes (Browne *et al.* 2020). Briefly, Foc-specific DNA sequences were targeted using the forward priming sequence (5'-GACATTTGACGACTTTCTGA-3'), the reverse sequence (5'-GACATTTGACGACTTTCTGA-3') (Matthews *et al.* 2020). Each 10 µL total-volume reaction contained 5 ng of extracted DNA, 0.3 µM of desalt-grade primers (Sigma-Aldrich), 5 µL of SsoAdvanced SYBR® SuperMix, and 2 µL

molecular grade Ultra-Pure $H_2O^{™}$ (Invitrogen) water. Samples were amplified in a reaction including an initial hot start of 10 min. at 95°C, followed by 40 cycles of 10 s at 95°C and 15 s at 66°C. Reactions were followed by a melt curve analysis to ensure primer specificity. Furthermore, technical triplicates were run alongside no template negative controls as well as a sample, extraction, and analysis blank. The entire dilution and technical triplet analysis was also run twice to check dilutions. Values from the two dilution sets were consistent and values were averaged. The reaction amplification efficiency was calculated from the gradient of the standard curve titration in accordance with MIQE guidelines (Bustin *et al.* 2009). Cycle threshold (Ct) scores were converted to DNA copy numbers using a standard curve constructed from a pGEM-T plasmid carrying the Foc specific DNA directed RNA polymerase subunit III gene (*i.e.* $10^8 – 10^1$ copies plasmid/reaction). The target amplicon was 98 base pairs long, reaction efficiency was 98.1%, and standard curve R^2 was 0.99. Reactions were measured by QuantStudio 5 Real-Time PCR Machine running QuantStudio Design and Analysis Software (v1.4.3, Applied Biosystems).

7.3.8 Plant proteomics

7.3.8.1 Sample collection and extraction

When sampling each plant, three healthy root ends (~50 mm long) were cleaned with deionised water to remove soil while attached to the plant, cut, immersed in liquid N, and ground to a fine powder in a liquid-N-cooled mortar and pestle. Samples were then stored at -80°C until processing. Between samples, all surfaces were thoroughly cleaned with methanol to prevent cross-contamination. Peptides were extracted using an acid digestion method adapted from the work of Doellinger *et al.* (2020). For the full method used, see Appendix 1.

7.3.8.2 LC-MS/MS analysis

All samples were analysed with a Ekspert nano-LC415 (Eksigent, USA) liquid chromatography (LC) system running on water with 0.1% (v/v) formic acid in water (Solvent A) and 0.1% (v/v) formic acid in Acetonitrile (Solvent B), coupled to a TripleTOF 6600 (Sciex, USA) mass spectrometer (MS). Digested peptides were first loaded on a C18 10 mm by 300 µm trap column (Trajan, Australia) under 10 µL / min of Solvent A and separated on a C18 250 mm by 300 µm column (Trajan, Australia) with linear gradient of 3-35% solvent B over 75 min at 5 µL / min. Mass spectrometer experimental parameters (Sciex software units) were as follows: curtain gas = 35, ion source gas 1 = 25, ion source gas 2 = 30, ionspray voltage floating = 500, and turboheater temperature = 300°C.

A 0.2 µL aliquot of a replicate from each treatment was pooled into two samples and loaded into the LC-MS system. Information Dependent Acquisition mode was set to capture ions with a charge state between 2 and 5 and at a window of 350-750 Da and 745-1250 Da respectively. A single spectral library was generated from the MS data from both windows for all conditions with the ProteinPilot (Sciex, USA) Paragon method identification workflow under the following settings: Cysteine Alkylation = Iodoacetamide w other Cys mods possible, Digestion = Trypsin, Instrument = TripleTOF 6600, Search effort = Through, ID Focus = Biological modifications, and Unused ProtScore = 0.05. The MS data was matched against a *Musa acuminata* subsp. *malaccensis* protein library containing 45,856 protein sequences, obtained from the Banana genome hub in January 2020 (Droc *et al.* 2013).

The same physical set-up was used in 'Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra' (SWATH) mode to acquire quantitative data for all 90 samples. The SWATH method used 100 windows with 6-50 Da of isolation width, an overlap of 1 Da, and a collision energy spread of 5-10 through the mass range of 399.5 Da to 1249.5 Da. Data was collected over 1847 cycles with an accumulation time of 25 ms for each window.

SWATH data was processed using the SWATH microapp on the PeakView software (Sciex, USA). Retention time for all SWATH samples were normalized with 5 selected peptides from the iRT calibrant (Biognosys, Switzerland) ranging from 487.26 to 699.34 *m/z*. All samples were processed with the previously generated spectral library according to the following SWATH microapp settings: number of peptides per protein = 9, number of transitions per peptide = 9, peptide confidence threshold = 99%, false discovery rate threshold = 1%, XIC extraction window = 20 minutes, and XIC width = 50 ppm.

7.3.9 Statistical analyses

7.3.9.1 Univariate analyses

Unless otherwise specified, data were analysed using an ANCOVA with the ANOVA function in the *car* library in R studio (R Core Team 2017; Fox and Weisberg 2019). This method was selected to address the unbalanced study design with respect to inoculation, incorporating type 3 sum of squares error. The predictors included N rate, considered as a continuous variable, N form, a factor with two levels (ammonium and nitrate), and sometimes inoculation, when considering both inoculated and non-inoculated plants. The normality of the model residuals was tested using the Shapiro Wilks test and data transformed if needed. Soil nitrate concentration and fungal Chao1 index were $\log_e(x+1)$

transformed, plant N tissue concentration and rhizosphere Foc DNA concentration were square root transformed, and the fungal Shannon index was cubed. Soil ammonium concentration was not normally distributed (Shapiro – Wilks $P = 0.014$); however, it was not improved by any standard transformation, so it was used untransformed and should be treated with caution. Both the bacterial and fungal Simpson indexes had highly non-normal distributions even with extreme transformations and so the results should be treated with caution. Though it is worth noting that the results were unchanged for this assessment when using a generalised linear model, which is generally considered more robust for non-normal distributions. Untransformed data are presented in figures for ease of interpretation. For determining a significant effect in the tested models $\alpha = 0.05$ was used, unless otherwise specified.

The effects of the treatments on disease severity were analysed using a beta regression analysis with a logit link in R using the *betareg* package (Cribari-Neto and Zeileis 2010). This was selected due to disease severity being a percentage, with a maximum of 100%. Effects on inoculated plant dry weight were analysed using an ANOVA, but a second order polynomial function was incorporated for N rate. For both disease severity and inoculated plant weight a single outlier value was excluded. The replicate was not an outlier in other analysis possibly indicating an analytical error.

7.3.9.2 Multivariate analyses

To calculate the treatment effects on microbial beta diversity a PERMANOVA analysis was performed using the adonis command in the *vegan* R package (Oksanen *et al.* 2019). For determining a significant effect in the tested models $\alpha = 0.05$ was used, unless otherwise specified.

Proteomic data was quantile normalised and $\log_2(x+1)$ transformed for analysis (Ritchie *et al.* 2015). PERMANOVA was used to determine the significance of treatments and soil pH on protein expression composition. Redundancy analysis was performed using the *vegan* package in R (Oksanen *et al.* 2019), constrained by the treatments of N rate and inoculation as these were found to be significant. Differentially expressed proteins were identified and enrichment was calculated in the *limma* package in R with a false discovery rate cut-off of 0.10 incorporating a Benjamini-Hochberg correction for multiple testing (Ritchie *et al.* 2015; Law *et al.* 2020) using a full model incorporating the treatments of inoculation, N rate and form as well as all interactions. Pathway enrichment was then explored on each main or interaction treatment protein set. Further testing was performed on the expression rates of key, individual defensive genes using a full treatment model. Due to concerns regarding the assumption of linearity for protein expression changes, N was also considered as a

multilevel factor; however, the results were simply a subset of the linear regression analysis due to the reduction in statistical power so were not reported here.

Gene ontology enrichment was calculated using the *topGO* package in R (Alexa and Rahnenfuhrer 2020). Protein IDs were mapped to gene ontology using the Banana Genome Hub (Droc *et al.* 2013). Fisher's exact test was used to determine enrichment using conservative cut offs ($\alpha = 0.01$ and > 3 proteins per ontology). KEGG pathway enrichment was calculated by annotating the full list of inferred genes analysed in this experiment using the FASTA sequence blast function in BlastKoala as part of the KEGG mapping service (Kanehisa *et al.* 2016). KEGG k values from the full experimental protein set were mapped to pathway ko numbers to indicate the protein universe within the samples. Pathway enrichment was determined using a hypergeometric test for each KEGG pathway. This test compared the number of k values (indicating individual proteins or genes) in the differentially expressed protein set with the number of k values in the protein universe. Here, the protein universe was limited to proteins analysed in this experiment, rather than the entire banana proteome (Bessarabova *et al.* 2012). Cut offs for enrichment were the same as for gene ontology.

7.4 Results

7.4.1 Plant growth and disease severity

The rate of N fertiliser application significantly affected plant biomass as well as Fusarium wilt of banana severity (Figure 7-1, Table 7-1). Plant weight in non-inoculated plants responded linearly to N rate (Figure 7-1). Disease severity was significantly related to N rate in inoculated plants, the rate of increase being constrained by the maximal (100%) internal disease. The combined effects on plant growth and disease severity resulted in a second order polynomial relationship between N rate and plant weight in inoculated plants (Figure 7-1).

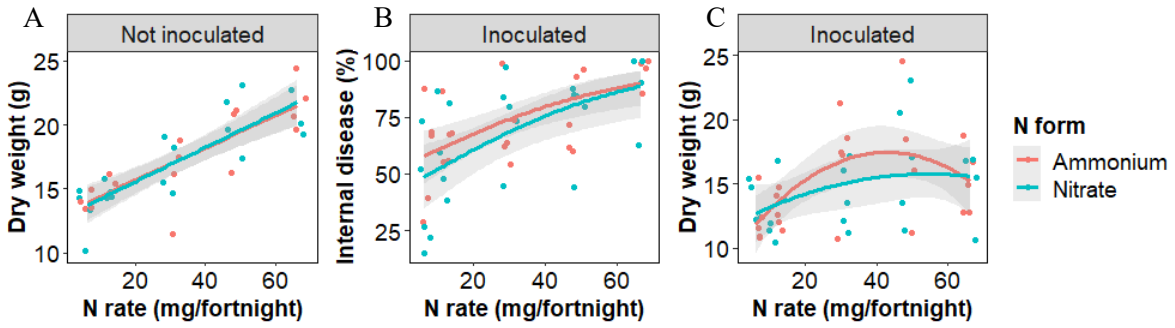

Figure 7-1. The effect of N fertiliser rate on total plant dry weight in plants that were not inoculated (A), disease severity in inoculated plants (B) and dry weight in inoculated plants (C). For regression

model statistics see Table 7-1. Points have been jittered in the x dimension to avoid overplotting. Shaded regions indicate the 95% confidence interval.

Table 7-1. The model outputs for the relationship between plant weight and treatments for inoculated (quadratic) and not inoculated plants (linear) as shown in figure 7-1.

Response variable	Predictor variable	Inoculated		Not inoculated	
Plant weight		*F*	*P*	*F*	*P*
	Intercept	35.4	< 0.001	259.4	< 0.001
	N rate	5.5	0.008	37.1	< 0.001
	N form	0.7	0.394	0.3	0.610
	N rate * N form	0.7	0.483	0.8	0.378
Disease severity		z_{43}	*P*		
	Intercept	0.4	0.676		
	N rate	3.8	< 0.001		
	N form	-1.1	0.286		
	N rate * N form	0.6	0.520		

7.4.2 Soil chemistry

The soil concentration of ammonium was unaffected by the rate, form, or interaction of rate and form of N fertiliser used, whereas the soil concentration of nitrate was positively correlated with the N application rate (F (1, 86) = 44.9, $P < 0.001$), but not the form or the interaction of rate and form (Figure 7-2). Soil pH was significantly affected by the interaction of rate and form (F(1, 86) = 51.4, $P < 0.001$) with ammonium addition decreasing soil pH (Figure 7-2).

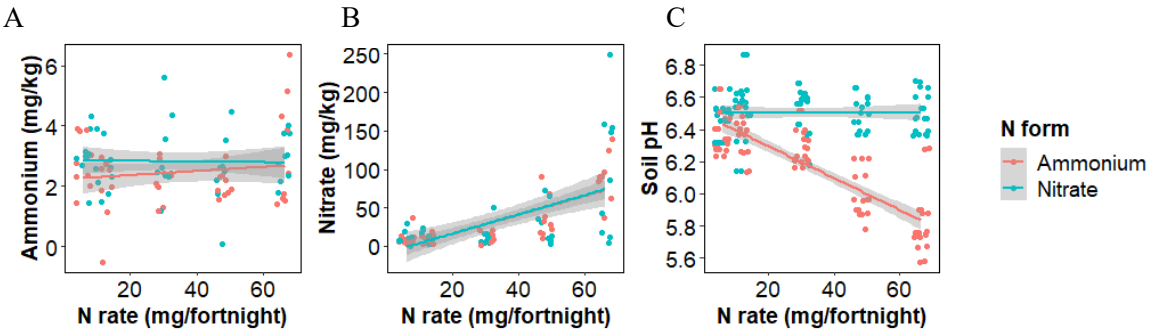

Figure 7-2. The effect of ammonium and nitrate fertiliser addition on the concentration of soil ammonium (A), nitrate (B), and pH (C) at the time of harvest. Shaded regions indicate the 95% confidence interval.

7.4.3 Plant N uptake and water use efficiency

The N concentration of aboveground plant tissue increased significantly with N fertiliser application rate (0.87 - 3.28 %, $F(1, 82) = 128.7$, $P < 0.001$), but was not significantly affected by N fertiliser form, inoculation, or any interactions. The difference between $\delta^{15}N_{Plant}$ and $\delta^{15}N_{Fert}$ was significantly affected by N rate ($F(1,82) = 228.7$, $P < 0.001$), N form ($F(1,82) = 100.2$, $P < 0.001$), and the interaction of N rate and form (Figure 7-3, $F(1,82) = 31.7$, $P < 0.001$), but not inoculation or any other treatment combinations. When fertiliser rates were low, $\delta^{15}N_{Plant}$ values were similar to that of unfertilised soil, but as fertiliser rates increased $\delta^{15}N_{Plant}$ approached that of the fertiliser (difference of zero). $\delta^{13}C$ was significantly increased by N rate (Figure 7-3, $F(1,82) = 62.7$, $P < 0.001$), but no other treatments or combinations.

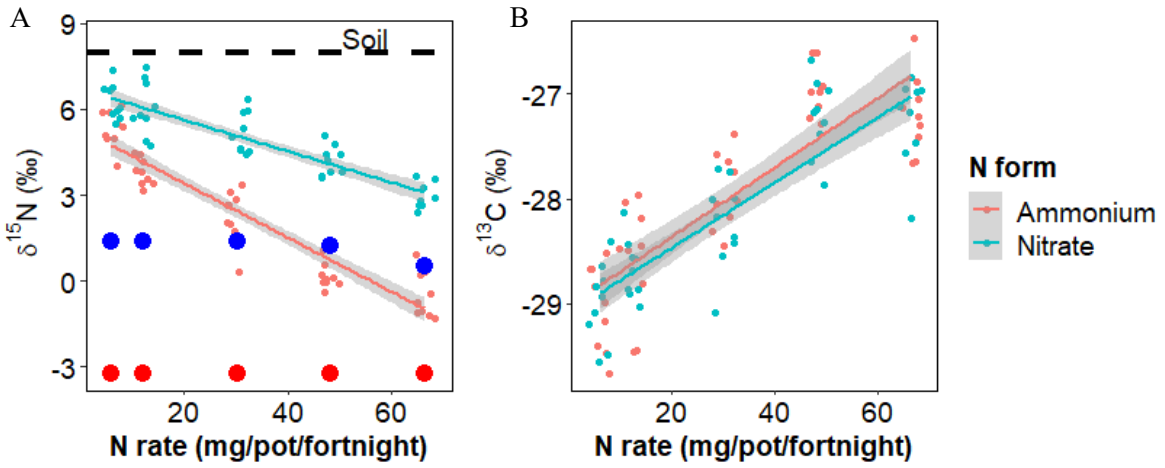

Figure 7-3. A) The effect of nitrate and ammonium fertiliser addition on the $\delta^{15}N$ enrichment in aboveground plant tissue, with reference to the original soil (dotted line) and fertiliser composition (large coloured points for each fertiliser type and rate, red = Ammonium, blue = nitrate). B) $\delta^{13}C$ of aboveground plant tissue. Points have been jittered in the x dimension to avoid overplotting. Shaded regions indicate the 95% confidence interval.

7.4.4 Foc abundance

The concentration of Foc DNA in rhizosphere soil of inoculated pots had no relationship with banana internal disease severity (Figure 7-4). Foc DNA concentration was not significantly affected by N

rate, form, or their interaction but was marginally negatively related to soil pH ($F(1, 48) = 2.8$, $P = 0.098$).

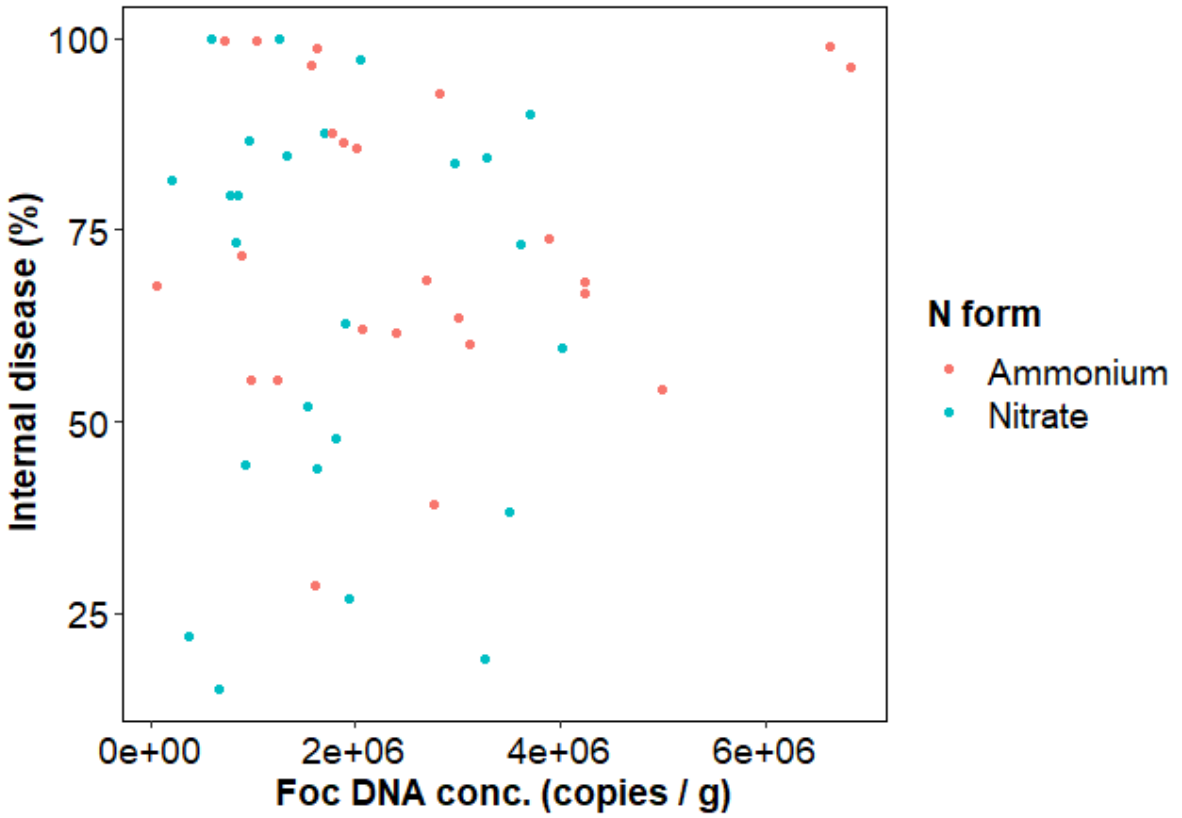

Figure 7-4. *Fusarium oxysporum* f.sp. *cubense* DNA concentration in banana rhizosphere soil in relation to internal disease severity of banana plants.

7.4.5 Soil bacterial and fungal diversity

Bacterial alpha diversity was significantly decreased by N rate, but not significantly affected by N form or inoculation at $\alpha = 0.05$ (Sobs, Chao1, Shannon, Simpson, and PD indices) (Figure 7-5, Table 7-2, appendix 2). An ANOVA with N form and either soil pH or N rate was run to test the relative influence of soil pH and N rate on Shannon diversity, as well as the importance of the interaction of these terms. Both soil pH and N rate were significant predictors of Shannon diversity, but soil pH was stronger. In addition, there was a significant interaction between N rate and N form ($F = 4.3$, $P = 0.042$) but not between soil pH and N form ($F=1.4$, $P = 0.234$). Bacterial beta diversity, as tested with a PERMANOVA, was significantly affected by the main effects of inoculation ($F = 1.5$, $R^2 = 0.017$, $P < 0.001$), N rate ($F = 2.2$, $R^2 = 0.024$, $P < 0.001$) and form ($F = 1.9$, $R^2 = 0.020$, $P < 0.001$), as well as the interactions of N rate and form ($F = 1.7$, $R^2 = 0.018$, $P < 0.001$) and N rate and inoculation ($F = 1.2$, $R^2 = 0.012$, $P = 0.042$). Soil pH and N form both significantly affected bacterial beta diversity,

but their interaction was not significant (F = 1.1, R^2 = 0.012, *P* = 0.265), whereas when N rate and N form were considered, the interaction was significant (F = 1.7, R^2 = 0.018, *P* < 0.001).

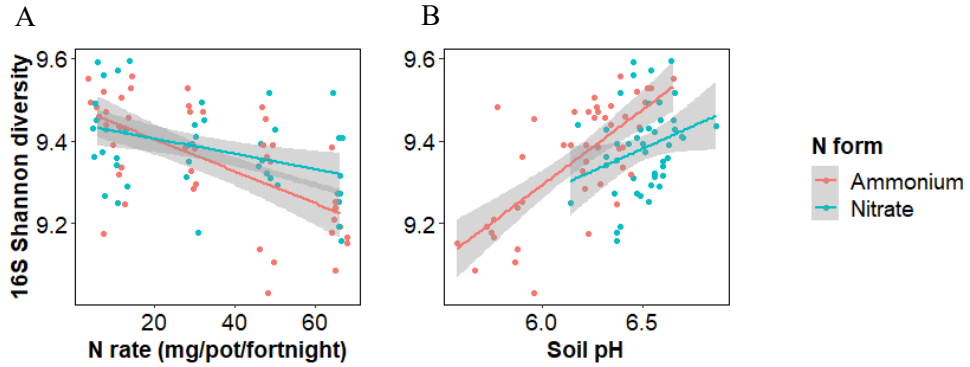

Figure 7-5. Bacterial alpha diversity (Shannon index) as affected by the rate and form of N fertiliser addition (A) and soil pH (B). Points in the left panel have been jittered in the x dimension to minimise overplotting. Shaded regions indicate the 95% confidence interval.

Fungal beta diversity was affected by inoculation (F = 6.8, R^2 = 0.072, p < 0.001; Figure 7-6), but was not significantly affected by N rate, N form, or any interactions. The species *Fusarium solani* was most representative of uninoculated samples while the fungal OTU most affected by inoculation was unsurprisingly the genus *Fusarium*, which encompasses both *Fusarium solani* and Foc, the organism that was used for inoculation (Figure 7-6). Fungal alpha diversity measures that considered only richness (Chao1 and Sobs) were not affected by inoculation; however, those that also considered evenness (Shannon and Simpson) were, indicating that some OTUs were reduced in abundance but not removed in entirety (see Appendix 2).

Table 7-2. Alpha diversity measures of bacteria and fungi in rhizosphere soil. The three main treatments and their interaction effects were tested. For the full list of alpha diversity measures see Appendix 2.

16S

Predictor variable	Sobs		Shannon	
	F (1, 82)	*P*	*F (1, 82)*	*P*
N rate	27.6	< 0.001	14.1	< 0.001
N form	0.2	0.665	0.4	0.554
Inoculation	0.9	0.361	0.9	0.343
N rate * N form	4.9	0.030	1.6	0.212

N rate * Inoculation	0.6	0.446	0.0	0.987
N form * Inoculation	0.2	0.677	0.0	0.900
3 way interaction	0.1	0.779	0.0	0.858

ITS

Predictor variable	Sobs		Shannon	
	F(1, 82)	*P*	*F(1, 82)*	*P*
N rate	0.0	0.926	0.0	0.977
N form	0.0	0.998	0.1	0.722
Inoculation	0.0	0.981	4.9	0.029
N rate * N form	0.0	0.859	0.1	0.805
N rate * Inoculation	0.4	0.538	0.0	0.872
N form * Inoculation	0.2	0.634	0.0	0.945
3 way interaction	0.0	0.940	0.3	0.589

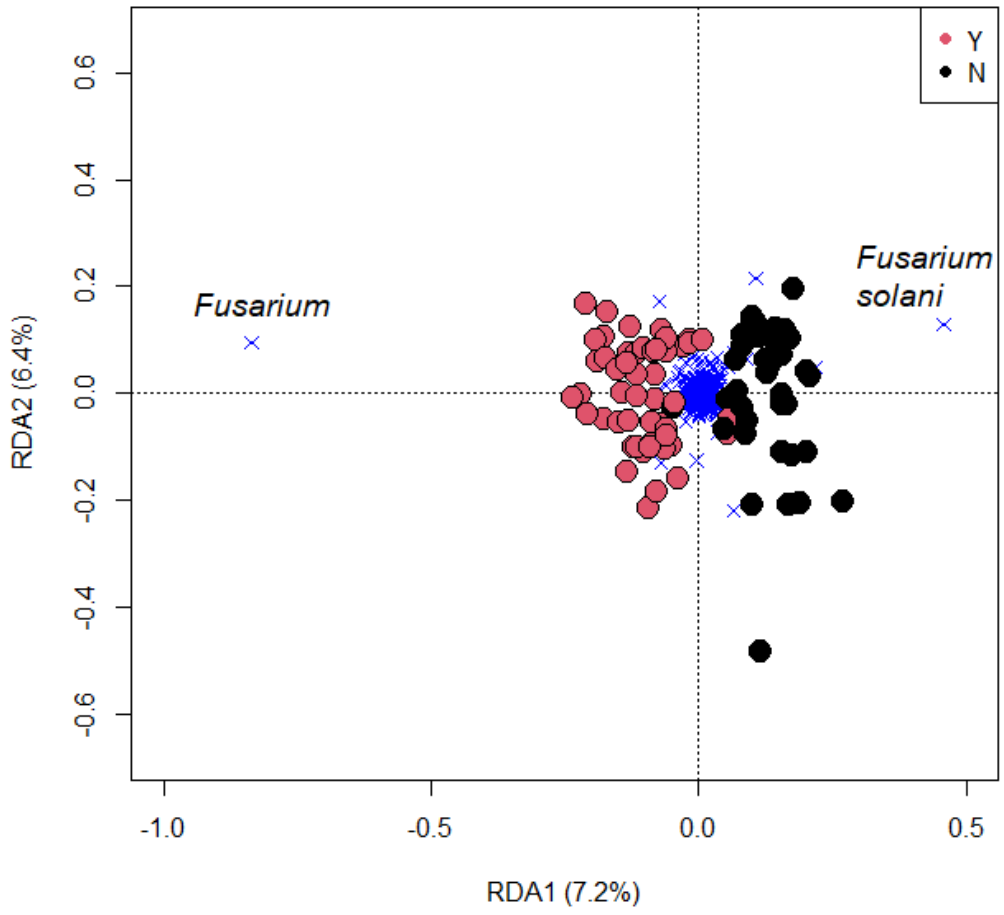

Figure 7-6. A redundancy plot of the fungal community with inoculation of *Fusarium oxysporum* f.sp. *cubense* (Y) or not (N) constrained by inoculation. Circles represent samples and crosses OTUs. The far-left cross represents the genus *Fusarium,* which exerts considerable influence on the significance of the treatment effect.

7.4.6 Plant proteomics

A full factorial PERMANOVA analysis of the proteome dataset considering inoculation, N rate and form demonstrated a large effect of inoculation ($F = 6.0$, $R^2 = 0.059$, $p < 0.001$), and N rate ($F = 6.0$, $R^2 = 0.060$, $p < 0.001$) and the interaction of all three treatments was significant ($F = 2.0$, $R^2 = 0.019$, $p < 0.027$). N form and the other interactions were not significant (Figure 7-7). When soil pH was included in the model it was not significant, nor were any interactions with it.

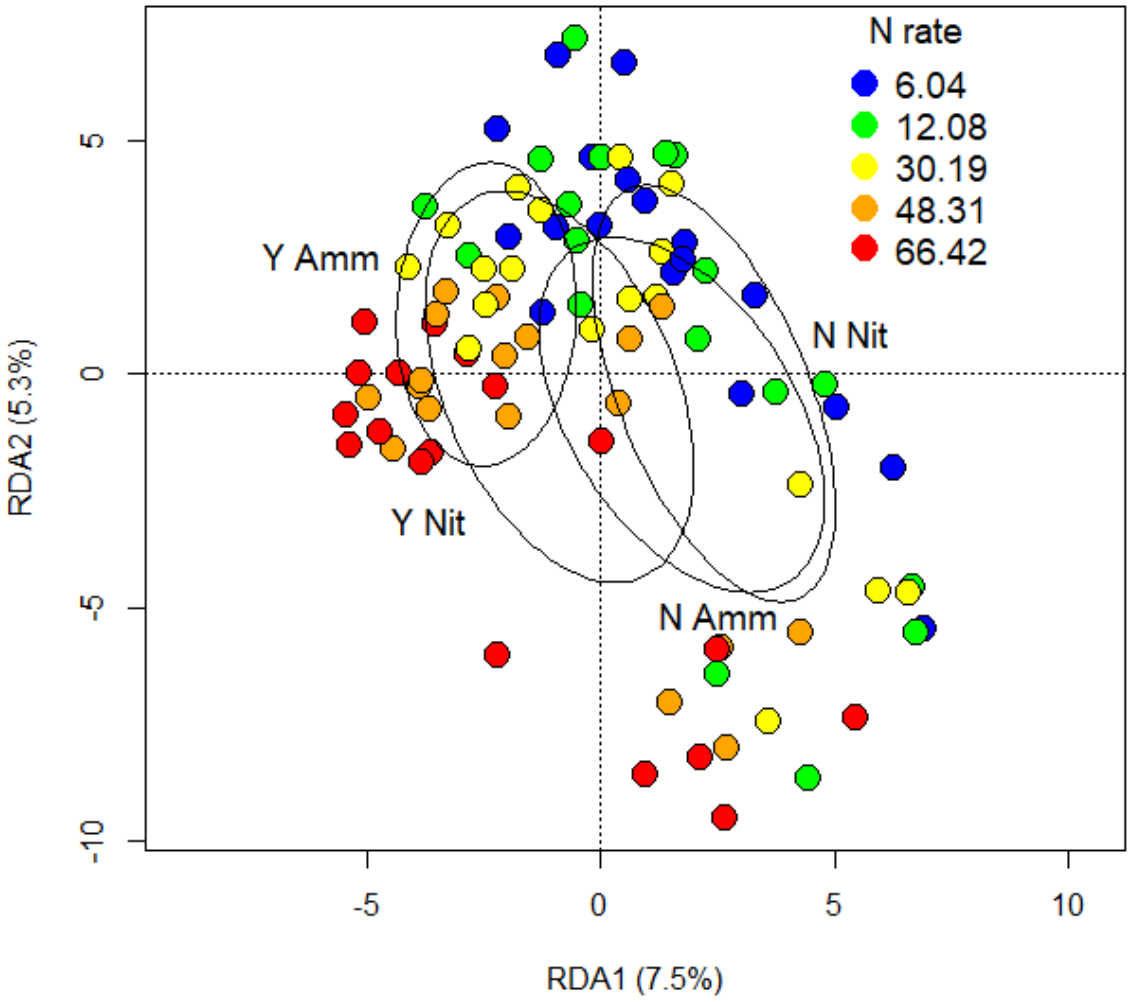

Figure 7-7. Redundancy analysis of the proteomic output constrained by factors of inoculation, N form and N rate. Point colours indicates N rate and ellipses with text labels indicate combinations of inoculation (Y or N) with N form (Ammonium or Nitrate).

Table 7-3. Quantitative proteomics/Differential protein expression with up or down regulation based on the combined treatments of N rate, N form and inoculation. The reference level for inoculation is not inoculated and for N form is ammonium.

	Up	No change	Down
N rate	88	1566	61
N form	0	1715	0
Inoculation	137	1437	141

N rate * N form	0	1715	0
N rate * Inoculation	0	1715	0
N form * Inoculation	7	1707	1
3-way interaction	0	1715	0

Examination of differential expression on the individual protein level showed that the main effects of N rate and inoculation had the greatest effect, but there was no main effect of N form (Table 7-3). N form and inoculation had the sole significant interaction though none of the identified proteins were related to growth or defence. Therefore, further investigation was focused on those proteins differentially expressed under the main effects of N rate and inoculation. It is worth noting that the false discovery rate used for differential expression was extremely conservative due to the large number of tests performed and the multiple testing adjustments required, so subtle treatment effects are likely to have been missed. A separate investigation of key defensive proteins included all treatments and their interactions.

Inoculation with Foc resulted in differential expression of the proteins associated with the "Defense response" process as well as a wide range of other molecular functions (Table 7-4). KEGG pathway analysis identified differentially expressed groups of proteins primarily related to metabolism of sugars (Table 7-5).

Table 7-4. Gene ontologies that were significantly differentially expressed following inoculation with Foc.

GO ID	Term	Annotated	DE	*P*	Sub ontology
GO:0004864	Protein phosphatase inhibitor activity	5	5	<0.001	MF
GO:0038023	Signalling receptor activity	5	5	<0.001	MF
GO:0010427	Abscisic acid binding	5	5	<0.001	MF
GO:0030246	Carbohydrate binding	17	8	0.004	MF
GO:0030145	Manganese ion binding	11	6	0.005	MF

GO:0004553	Hydrolase activity, hydrolyzing O-glycos...	40	14	0.016	MF
GO:0008061	Chitin binding	4	3	0.018	MF
GO:0004448	Isocitrate dehydrogenase activity	4	3	0.018	MF
GO:0004190	Aspartic-type endopeptidase activity	11	5	0.027	MF
GO:0016614	Oxidoreductase activity, acting on CH-OH...	29	9	0.029	MF
GO:0004866	Endopeptidase inhibitor activity	7	4	0.029	MF
GO:0009738	Abscisic acid-activated signalling pathwa...	5	5	<0.001	BP
GO:0006952	Defense response	10	7	<0.001	BP
GO:0006511	Ubiquitin-dependent protein catabolic pr...	19	7	0.039	BP
GO:0005840	Ribosome	112	25	0.014	CC

Table 7-5. Significantly differentially expressed KEGG pathways, based on proteins that are differentially expressed with inoculation. Pathways are ordered based on KEGG pathway number, inferring functional similarity.

K	Function	Measured	DE	*P*
03010	Ribosome	71	22	0.014
00500	Starch and sucrose metabolism	14	6	0.015
00520	Amino sugar and nucleotide sugar metabolism	21	8	0.019
00051	Fructose and mannose metabolism	9	4	0.024

Gene ontology and KEGG pathway enrichment affected by N rate were primarily processes and functions associated with energy fixation and use (Tables 7-6, 7-7). The KEGG MAPK signalling pathway was also affected, indicating that the addition of N may have altered the plants' ability to respond to biotic and abiotic stressors (Table 7-7).

Table 7-6. Significantly differentially expressed gene ontologies based on N rate.

GO ID	Term	Annotated	DE	*P*	Sub ontology

GO:0033897	Ribonuclease T2 activity	5	3	0.006	MF
GO:0008270	Zinc ion binding	18	5	0.018	MF
GO:0008964	Phosphoenolpyruvate carboxylase activity	3	2	0.023	MF
GO:0003735	Structural constituent of ribosome	111	16	0.035	MF
GO:0005471	ATP:ADP antiporter activity	4	2	0.043	MF
GO:0004448	Isocitrate dehydrogenase activity	4	2	0.043	MF
GO:0004634	Phosphopyruvate hydratase activity	4	2	0.043	MF
GO:0015977	Carbon fixation	3	2	0.024	BP
GO:0006412	Translation	152	19	0.036	BP
GO:0006099	Tricarboxylic acid cycle	15	4	0.042	BP
GO:0005840	Ribosome	112	16	0.020	CC
GO:0016272	Prefoldin complex	7	3	0.023	CC
GO:0000015	Phosphopyruvate hydratase complex	4	2	0.049	CC

Table 7-7. Significantly differently expressed KEGG pathways, based on proteins that were differentially related to N rate.

K	Function	Measured	DE	*P*
00710	Carbon fixation in photosynthetic organisms	16	6	0.003
03018	RNA degradation	8	3	0.016
04016	MAPK signalling pathway - plant	9	3	0.026
03010	Ribosome	71	15	0.030
00270	Cysteine and methionine metabolism	19	5	0.038
00230	Purine metabolism	10	3	0.039

Pathogenesis related protein 1 (PR1) expression rates were examined due to their importance as late-stage biotic infection response proteins. Inoculation significantly increased expression of all three PR1

proteins (Table 7-8, Figure 7-8). All three forms of PR1 showed strong, complex interactions between the three treatments so the N rate response was examined separately for each combination of inoculation and N form. Increased N rate significantly reduced PR1 expression in inoculated plants treated with ammonium but there was not a significant effect of N rate on inoculated plants treated with nitrate, nor on disease-free plants fertilised with either form of N (Figure 7-8).

Table 7-8. Effects of inoculation, N rate and N form on the three forms Pathogenesis related protein 1 measured.

Predictor variable	Ma02_p15060.1		Ma02_p15080.1		Ma04_p29640.1	
	F(1, 82)	*P*	*F(1, 82)*	*P*	*F(1, 82)*	*P*
N rate	2.3	0.133	1.5	0.227	0.8	0.377
N form	0.1	0.788	0.4	0.524	0.0	0.967
Inoculation	13.6	<0.001	13.3	<0.001	6.9	0.01
N rate * N form	0.0	0.994	2.8	0.098	0.0	0.995
N rate * Inoculation	4.1	0.047	7.3	0.008	3.2	0.076
N form * Inoculation	9.0	0.004	9.2	0.003	1.6	0.214
3 way interaction	3.4	0.068	9.0	0.004	1.7	0.193

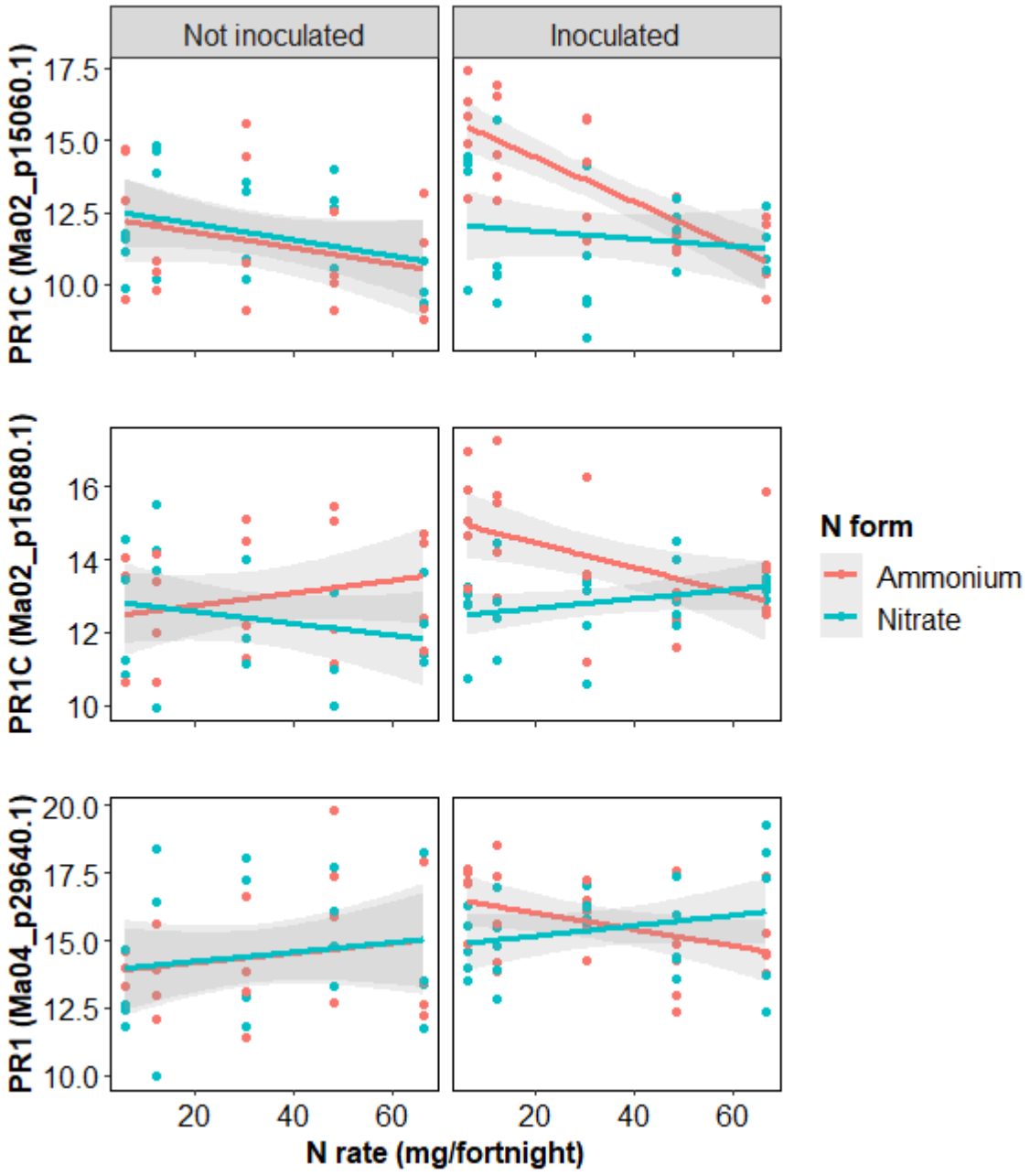

Figure 7-8. Log_2 transformed expression rates of measured forms of pathogenesis related protein 1 (PR1), shown with gene locations, with treatments of inoculation, N rate and form. Shaded regions indicate the 95% confidence interval.

7.5 Discussion

Disease severity, determined here by the proportion of plant tissue that was necrotic, was examined in response to Foc infection, N fertiliser rate and form and the interaction of these treatments. In Foc inoculated plants, disease severity was positively correlated with N fertiliser rate in either N form irrespective of changes to soil pH, resulting in a curvilinear growth response to increased N (Figure 7-

1). This result agrees with previous findings that N is positively correlated with disease severity of both Foc Race 1 and Tropical Race 4 (Pittaway *et al.* 1999; Segura-Mena *et al.* 2021; Teixeira *et al.* 2021), though disagrees with the general trend that acidification associated with ammonium use increases Fusarium wilt and Fusarium wilt of banana severity, whereas nitrate use is protective (Orr and Nelson 2018). $\delta^{13}C$ was also positively associated with N rate in both inoculated and disease free plants indicating that water use efficiency of photosynthesis, of which $\delta^{13}C$ is a proxy (Farquhar *et al.* 1982), was affected by N availability as expected (Evans 1989) but not by the presence of water restricting wilt symptoms, which in inoculated plants are affected by N availability.

Disease is the result of the interactions between the three components of the disease triangle: plant, pathogen, and environmental influences (Agrios 2005). We found that soil Foc load was a poor predictor of disease severity and only marginally related to N rate or soil pH, in agreement with Pittaway *et al.* (1999). Soil bacteria and fungi populations were more consistently affected by soil pH and the introduction of the pathogen than by N rate per se as the N rate effect differed between N forms (Figure 7-4, Table 7-2). High rates of N fertiliser, and associated increased plant growth, are often linked with increases in disease due to a trade-off between growth and defence (Herms and Mattson 1992; Huot *et al.* 2014). Expression of proteins associated with the "MAPK signalling pathway – plant", a plant stress and defence pathway, was significantly changed by increased N supply, independent of the presence of the pathogen. PR1 expression, a key endpoint for the "MAPK signalling pathway – plant" and salicylic acid defence response was negatively affected by ammonium fertilisation in diseased plants. N fertilisation enriched the "Carbon fixation in photosynthetic organisms" pathway that is a key contributor to growth. This change was likely associated with an increase in photosynthetic efficiency shown by the measured enrichment in $\delta^{13}C$ with N fertilisation. Taken together, these findings support the growth-defence trade-off hypothesis that suggests that an increase in disease severity results from a reduction in host plant defence resulting from an increase in growth, and a pH effect on the soil microbial community.

Auxin induced plant growth, triggered by the addition of N, suppresses defensive processes such as salicylic and jasmonic acid signalling (Yaeno and Iba 2008; Guo *et al.* 2018; van Butselaar and Van den Ackerveken 2020). Jasmonic acid production is the primary response to necrotrophic pathogens, whereas salicylic acid production is the primary response to biotrophic and hemibiotrophic pathogens such as *Fusarium oxysporum* f.sp. *cubense*, though there is important crosstalk between the two responses (Di *et al.* 2016; Sun *et al.* 2020; Fernandes and Ghag 2022). The induction of auxin and target of rapamycin pathways, which are associated with plant growth, has been shown to suppress the salicylic acid response, PR1, and plant resistance (van Butselaar and Van den Ackerveken 2020). The

trade-off between auxin synthesis (growth) and salicylic acid accumulation (defence) is particularly marked in root tissue (Denancé *et al.* 2013), which was the focus of this study. The salicylic acid response and the production of PR1 has been previously identified as a key defence indicators differentiating Fusarium wilt resistant and susceptible banana cultivars (Van Den Berg *et al.* 2007; Wang *et al.* 2015b; Ramu *et al.* 2016; Li *et al.* 2017; Zhang *et al.* 2019; Zhang *et al.* 2020). The exogenous application of salicylic acid has also been demonstrated to induce partial resistance to Fusarium wilt in banana (Wang *et al.* 2015b; Emilda *et al.* 2020). Systemic acquired resistance, in parallel with salicylic acid defence is likely controlled by the production of nitric oxide, itself controlled by N nutrition (Sun *et al.* 2020). Mur *et al.* (2017) suggest nitric oxide dependent defence is enhanced by nitrate fertilisation and reduced by ammonium. Our findings agree that PR1 expression decreased as more ammonium fertiliser was added to the system, though we did not find a significant increase from nitrate fertilisation, nor an N form effect on the "MAPK signalling pathway – plant" pathway. Further examination of the proteins that contribute to PR1 expression would be useful to understand how the full signalling pathways respond to N fertilisation.

The expression of PR1 at low fertilisation rates is greater with ammonium than nitrate and the decrease of PR1 in response to ammonium is much greater than the increase in response to nitrate causing the expression rates based on the two N forms to intersect at high rates (Figure 7-8). This is despite the $\delta^{15}N$ findings indicating that at low fertilisation rates all plants are likely taking up nitrate (Figures 7-2 and 7-3) and there is minimal pH difference between the two fertiliser forms (Figure 7-2). There was no statistically significant N form effect on either disease severity or inoculated plant dry weight at low N rates despite the difference in PR1 expression between the two N forms. This may indicate additional important factors not considered here.

Initiation of defensive signalling in bananas as a response to Foc exposure has been shown to affect the level of banana resistance to Foc (Zhang *et al.* 2019). The group of proteins categorised under the gene ontology of "Defense response", which was differentially regulated in inoculated compared with uninoculated samples, is the primary response to the presence of a foreign body or injury (Gene Ontology 2021). The "Chitin binding" gene ontology also could be a defence associated response to the fungal pathogen, due to the importance of chitin in fungal cell walls (Zhang *et al.* 2019). The "Signalling receptor activity" pathway, which includes immune receptor activity, was also affected by Foc inoculation possibly indicating a reduction in the transmission of the defence response (Table 7-4). In agreement with previous studies, inoculation with Foc caused a substantial upregulation of PR1, demonstrating the importance of PR1 to the banana's defence against Foc (Li *et al.* 2015; Zhang *et al.* 2019).

The effect of N fertiliser application on disease severity is complicated by a change in soil pH, which effects pathogen abundance and soil bacteria. Soil pH was reduced by the addition of ammonium and slightly increased, though statistically nonsignificant, by nitrate addition. Nitrate fertiliser has been previously shown to provide protection to plants from Fusarium wilts whereas ammonium typically increases disease severity (Morgan and Timmer 1984; Wang *et al.* 2016; Mur *et al.* 2017; Zhou *et al.* 2017). However, we found that increased application of both nitrate and ammonium was significantly positively correlated with internal disease severity, suggesting that in our experiment soil pH change may not be the principal determinant of disease severity (Figure 7-1). Morgan and Timmer (1984) found an ammonium and nitrate treated soil had a final pH of 5.8 and 6.9 respectively. The soils we used had a greater pH buffering capacity, possibly limiting the suppressive potential of pH increase and explaining the disparity between our findings and that of previous researchers.

Our $\delta^{15}N$ findings provide further insight into the uptake of the two N forms (Figure 7-3). At low N fertiliser rates the nitrate-fertilised plants took up less than half of their N as nitrate, directly from the fertiliser ($\delta^{15}N$ of plants nearer to that of soil than fertiliser) whereas the ammonium-fertilised plants did not take up the equivalent amount of fertiliser-derived ammonium N. Either they took up all their N from the soil N pool or from fertiliser-derived N that had nitrified to nitrate (Figure 7-2). Therefore, at low N fertiliser rates, plants appeared to take up mostly nitrate, irrespective of the form applied. Conversely, at high fertiliser rates plants drew N from the fertiliser supplied but in a combination of forms despite nitrate concentrations being far higher than ammonium concentrations irrespective of the form applied (Figure 7-2).

Separating the effect of N rate from the effect of pH decline caused by ammonium on Fusarium wilt disease symptoms and the growth of Foc is difficult (Orr and Nelson 2018; Segura-Mena *et al.* 2021). Organisms differ in their capacities to function at low pH. Generally, plants are regarded as having greater tolerance to low pH, fungi moderate tolerance, and bacteria are intolerant to reductions in pH (Rousk *et al.* 2009; Rousk *et al.* 2010). Soil pH was negatively correlated with soil bacterial alpha diversity (Figure 7-5), and to a minor extent Foc DNA concentration, indicating that the effect of N on the soil microbiome may be occurring at least partially via pH change. However, changes in Foc DNA concentration were unrelated to banana plant disease severity (Figure 7-4) indicating that Foc abundance was not a driver of disease severity in this case. Our results agree with those of Pittaway *et al.* (1999) who found pathogen activity was unrelated to disease severity. A limitation of our approach is that we did not measure virulence, which may or may not be linked to abundance; virulence factors may have responded to N supply irrespective of abundance (Divon *et al.* 2006; Bolton and Thomma

2008; López-Berges *et al.* 2010; Ding *et al.* 2018). Li *et al.* (2019) recently applied simultaneous transcript profiling of Foc and the banana plant during infection. This approach may offer further insight into the effect of N on pathogenicity in future by directly coupling the response of the plant and pathogen.

Inoculation with Foc was significantly associated with changes in the beta diversity of the bacterial (16S) and fungal (ITS) rhizosphere communities and inoculated samples had lower fungal alpha diversity. Contrary to the findings of Effendi *et al.* (2019), we found inoculation was not associated with a reduction in the alpha diversity of the bacterial community. Ou *et al.* (2019) determined that inoculation with Foc increased bacterial alpha diversity in disease suppressive soils and decreased it in conducive soils. The soil we used was relatively disease conducive compared to other soils cultivated to banana in Australia (Bowen *et al.* 2019) though there is no way to compare our soils to those of Ou *et al.* (2019) as conduciveness is a relative measure. It is worth noting that by measuring rhizosphere soil DNA we inherently included residual DNA from organisms that were present prior to inoculation possibly artificially increasing the diversity of the inoculated samples (Carini *et al.* 2016).

The controlled growing conditions in this work differed from commercial situations, and the effects demonstrated the need for field investigation across soil types. However, several previous studies suggest that our findings should be applicable to field situations. For example, a meta-analysis of the effect of N addition on fungal plant pathogens identified no difference between field and pot trials or when the pathogen was naturally or artificially introduced (Veresoglou *et al.* 2013). Additionally, the effect of N on Fusarium wilt severity caused by Race 1 and Tropical Race 4 are similar (Segura-Mena *et al.* 2021) and infection with these two races initiates a similar defence response (Li *et al.* 2013). The similarities between Foc Races and agreement between field and pot experiments suggest that the results of our experiment may be applicable to wider conditions though it would be valuable to test this.

7.6 Conclusions

Our results demonstrated that a high rate of N fertiliser led to an increase in the severity of Fusarium wilt of banana and suggest this may be partially due to a reduction in the defensive capabilities of the plant. The effect of N form requires further investigation, as PR1, a key defensive compound was not affected by nitrate, despite nitrate fertiliser being positively correlated with disease severity. The increase in disease severity may also have been partially due to a reduction in bacterial diversity caused by a decrease in soil pH following ammonium addition. The abundance of the pathogen in the

rhizosphere appeared to have no effect on disease severity, but was weakly related to soil pH. It would appear there is a threshold of N application, above which elevated internal disease severity is detrimental to plant dry weight. It may in future be possible to apply fertiliser to maximise banana plant growth while avoiding the penalty of increased internal Fusarium wilt severity, by enhancing expression of PR1 or related defence genes, decoupling the plant growth – defence trade-off, and by preventing soil pH decline.

7.7 Appendix 1

TFA proteomics extraction method for banana roots

1. Weigh approximately 0.016 g of dried sample into 2 mL Eppendorf tube, record exact sample weight.
2. Freeze dry samples at -60℃.
3. Add 300 uL 100% TFA (enough to cover the sample).
4. Agitate at room temperature at ~800 × *g* for 30 minutes.
5. Add 300 uL of saturated Tris base (same volume as TFA).
6. Centrifuge at 13,000 × *g* for 5 minutes.
7. Avoiding the pellet transfer supernatant to new 2 mL centrifuge tube. Repeat steps 6 and 7 twice to remove all particulate matter from the extract.
8. Add 50 uL of stock protein solution into 1.5 mL centrifuge tube.
9. Add 85 uL saturated Tris base, check pH is between 7.5 and 8.5.
10. Make 0.5 M stock solution of TCEP reducing agent for use in samples.
11. Dilute protein solution 1:50 with TCEP for final TCEP concentration of 0.010 M. If using volumes listed above need ~2.7 uL.
12. Agitate at 40℃ at ~800 × *g* for 20 minutes. Add 1:60 ratio of 15% Acrylamide: protein solution for final concentration of 0.025 M Acrylamide.
13. Agitate at 90℃ at 800 × *g* for 3 minutes with weight on the sample caps to prevent popping of caps due to heat.
14. Leave for 20 minutes at room temperature for solutions to cool with weight on lid.
15. Centrifuge for 0.5 minutes at >1000 × *g* to remove precipitation on lid.
16. Test pH of samples is between 7.5 and 8.5.
17. Add 1 ug SOLu-Trypsin (Sigma) to digest proteins. Mix 40 uL Trypsin with 760 uL 50 mM TEAB, then add 20 uL to each sample.
18. Agitate overnight at 39℃. Alternatively can digest for 1 hour with a Barocycler.
19. Add 0.5 ug more SOLu-trypsin (same solution as above).
20. Centrifuge for 0.5 minutes at >1000 × *g* to remove precipitation on lid.
21. Agitate for 120 minutes at 37 ℃.
22. Add TFA to protein solution for 1:5 ratio to give final TFA concentration of 4%.
23. Check pH to make sure it is between 3 and 4.
24. Solid-phase stage tips were prepared by stacking two layers of Empore™ Octadecyl C18 (Supelco/Sigma-Aldrich, USA) disc punches in a 200 uL pipette tip. Stage tips

were supported over a 2 mL tube and washed via centrifugation at 1300 × *g* for 2 minutes with 100 uL of methanol followed by 100 uL of 0.2% TFA.

25. Centrifuge samples at 13,000 × *g* for 2 minutes and transfer the supernatant to the stage tip.

26. Centrifuge at 1300 × *g* for 30 minutes until solution has passed through tip into fresh centrifuge tube. Reload the solution back onto the same stage tip and centrifuge at 1300 × *g* for 30 minutes.

27. Wash with 200 uL 0.2% TFA. Centrifuge at 1300 × *g* for 30 minutes 3 times. Take tips to fresh **1.5mL Eppendorf tubes**
28. Centrifuge at 1500 × *g* to wash the stage tips into fresh centrifuge tubes with 100 uL 60% Acetonitrile, 0.2% formic acid.
29. Dry on speed vac at room temperature for approximately 1hour, until completely dry.
30. Store the sample at -80℃ until use.

Quantifying protein concentration

31. Re-suspend peptides in 15 uL of 2% Acetonitrile, 0.1% formic acid.
32. Add Biognosys IRT internal standard as per manufacturer specifications and mix the sample.
33. Place 1 uL on Nanodrop (Thermofisher) to quantify protein concentration using method 205
34. Dilute samples with 2% Acetonitrile, 0.1% formic acid and internal standard to the minimum sample peptide concentration or 0.1 ug/uL whichever is higher**.**
35. Transfer samples to 0.5 mL tubes, placed inside a 1.5 mL tube and centrifuge at 13,000 × *g* for 5 minutes.
36. Transfer supernatant into a mass spec vial for analysis.

7.8 Appendix 2

The assessment of alpha diversity measures of bacteria (16S) and fungi (ITS) in rhizosphere soil as affected by the three main treatments and interactions.

16S

Predictor variable	Sobs		Shannon		Simpson		Chao1		Faith's PD	
	F (1, 82)	*P*	*F (1, 82)*	*P*	*F (1, 82)*	*P*	*F (1, 82)*	*P*	*F (1, 82)*	*P*
N rate	27.6	< 0.001	14.1	< 0.001	3.8	0.055	13.7	< 0.001	32.9	< 0.001
N form	0.2	0.665	0.4	0.554	0.2	0.688	0.2	0.649	0.3	0.606
Inoculation	0.9	0.361	0.9	0.343	0.4	0.513	0.9	0.347	0.0	0.890
N rate * N form	4.9	0.03	1.6	0.212	0.1	0.723	2.4	0.124	5.6	0.021
N rate * Inoculation	0.6	0.446	0.0	0.987	0.0	0.838	0.5	0.502	2.0	0.163
N form * Inoculation	0.2	0.677	0.0	0.900	0.0	0.941	0.5	0.476	0.0	0.969
3 way interaction	0.1	0.779	0.0	0.858	0.1	0.723	0.0	0.906	0.4	0.544

ITS

Predictor variable	Sobs		Shannon		Simpson		Chao1	
	F(1, 82)	*P*	*F(1, 82)*	*P*	*F(1, 82)*	*P*	*F(1, 82)*	*P*
N rate	0.01	0.926	0	0.977	0.01	0.932	4.66	0.034

N form	0	0.998	0.13	0.722	0.02	0.897	2.08	0.153
Inoculation	0	0.981	4.93	0.029	8.8	0.004	3.43	0.068
N rate * N form	0.03	0.859	0.06	0.805	0.11	0.739	0.96	0.33
N rate * Inoculation	0.38	0.538	0.03	0.872	0.38	0.539	4.67	0.034
N form * Inoculation	0.23	0.634	0	0.945	0.17	0.678	2.28	0.134
3 way interaction	0.01	0.94	0.29	0.589	0.23	0.629	1.31	0.256

8 Synthesis and Conclusions

This work has addressed several concepts and questions at the intersection of plant pathology, plant nutrition, soil physicochemistry and soil microbiology, relevant to the devastating disease Fusarium wilt of banana, or Panama disease. All three components of the disease triangle (host, pathogen and environment) were examined, with a focus on manipulating the environment to reduce disease severity. The relative importance of inherent and managed soil characteristics to pathology was also explored, and is the focus of this synthesis.

A wide variety of abiotic soil attributes have been associated with suppression of Fusarium wilt in a range of crops, including bananas. Effects of these attributes on suppression are generally consistent across susceptible crops but there are exceptions. The effects are usually described in terms of a particular attribute, but it should be kept in mind that soil attributes are interrelated. Studies may have manipulated a particular soil attribute and report results accordingly, but it is certain that other attributes would also have been altered, and so the reported effect may be indirect. It is impossible to test the effect of individual attributes in isolation, while still involving the constituents of the disease triangle, due to the complexity of the soil environment. For example, manipulation of pH alters bioavailability of most nutrients, and conversely the addition of nitrogen fertiliser, a nutrient, alters pH. Due to the interrelated nature of soil, translating results from one environment to another is complex, and results may not be consistent, because environmental conditions interact with the treatment effects. Therefore, it is essential to test solutions in multiple field environments and not overextend findings from one region to another.

Disease severity is determined by the host's ability to resist or tolerate infection; the pathogen's abundance and pathogenicity; and the diversity and abundance of other microorganisms in the soil. This thesis focused on treatments that changed nutrient availability to advantage the host and disadvantage the pathogen. The relative ability of iron to host and pathogen can be altered by chelation. In chelated form, iron is available to host plants due to their ability to destabilise chelates in the rhizosphere, whereas if the iron is chelated by ligands more stable than Fusarium siderophores, it is unavailable to the fungal pathogen. There is also a difference in the way in which the host plant and pathogen acquire nitrogen. The host plant primarily takes up inorganic forms, either nitrate or ammonium. The fungal pathogen however is either saprophytic or pathogenic, dependent largely upon live or dead organic matter as a nitrogen source. Manipulation of nitrogen and iron availability were two means of altering disease severity explored in this work. It is likely that availability of other nutrients with acquisition differences between host and pathogen also alter suppression.

The context of this work, in terms of geography and management practices, is important, as both affect the spread and severity of disease. Like many banana growing areas of the world, the North Queensland region has warm temperatures, high annual rainfall (1,800 to 4,500 mm) and highly weathered soils. However, there is a large range of soil parent materials, texture, drainage status and associated properties. Unlike many regions it also incorporates elevated tablelands with lower annual rainfall (1,400 mm), a more temperate climate, and mostly basaltic soils.

The most variable soil characteristics in the growing region were electrical conductivity, clay and total metal contents. Electrical conductivity can be related to the amount of rainfall a location receives or how recently fertiliser has been applied, as the soluble salts that cause conductivity are flushed away in high rainfall areas like Innisfail and Tully, but accumulate in low rainfall irrigated areas such as Mareeba. Clay and total metal content were highly correlated and largely determined by parent material. Most of the soils from the Mareeba region had basalt parent material whereas near Innisfail and Tully other parent materials predominated. After the effects of parent material on overall soil properties, characteristics more closely related to management, such as pH and organic matter-related properties were important. While some variation in these characteristics exists due to inherent site variability, many are controlled by growers through the application of agri-chemicals. This work showed that, in soils used for banana production in North Queensland, the severity of Fusarium wilt of banana could not be altered by manipulating availability of iron but could be with nitrogen.

In some situations, managed reduction of iron availability to the pathogen has been a highly successful approach to reduce Fusarium wilt disease severity but it was unsuccessful in this work. Based on this range of evidence we hypothesise that the effectiveness of this approach is inversely proportional to the natural availability of iron in the soil, as the treatment is unable to induce an iron deficiency for the pathogen in soils with high iron availability. Thus, this approach has the potential to be high impact but is also highly specific in its application and requires adaptation to each individual farming location.

Conversely, the approach of manipulating soil nitrogen concentration, which is closely controlled to agronomically recommended values in nearly all production areas, is likely to have less regional variability in its effectiveness. Nitrogen supply is naturally limited, unlike iron, which may vary depending on region, but is generally plentiful where bananas are grown in Australia. Nitrogen limitation is relieved by fertilisation, the amount varying from farm-to-farm. Additionally, the

concentration of plant available nitrogen declines rapidly if it is no longer applied, meaning that farmers can decrease the available concentration for disease suppression if desired.

For banana producers and researchers this research may provide an early step in the development of a long-term management strategy for Fusarium wilt of banana in Far North Queensland. The evidence presented in Chapter 7 shows that high rates of nitrogen fertiliser application makes disease more severe. It is now necessary to do further research, including long-term field trials, to determine the rate of fertiliser to optimise the growth-defence trade-off in the banana plants and the diversity of non-pathogenic soil microorganisms.

In conclusion, of the two examined management approaches to reduce the severity of Fusarium wilt of banana, one appears promising in the Australian banana industry. Management of iron availability appears ineffective for the North Queensland growing area but nitrogen fertilisation rate shows promise, presuming trade-offs in the plant between growth and defence can be optimised through plant genetics or agronomy. The survey work (Chap 3), in combination with the experimental work (Chaps 4-7), suggests that efficacy of treatments associated with intrinsic soil characteristics such as micronutrient concentration are likely to be more geographically variable than those associated with highly managed characteristics such as nitrogen nutrition. Geographic variability in the former is due to regional differences in soil forming factors such as parent material and climate, and generally appears to rule out manipulation of iron availability as a treatment option in highly weathered tropical soils such as those of North Queensland. On the other hand, management of nitrogen nutrition is likely to be more broadly applicable.

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Chapter 7: Nitrogen fertilizer rate but not form affects the severity of Fusarium wilt in banana.

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