Nematode pests of soybean and associated microbial communities with potential as biocontrol agents

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“The more I study nature, the more I stand amazed at the work of the Creator.”

Louis Pasteur

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To my Heavenly Father, thank you for giving me the strength and ability to complete this thesis. **“For nothing will be impossible with God.” Luke 1:37.**

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ABSTRACT

The expansion of soybean production in recent years lead to increased land requirements for growing the crop and the increased risk of exposing this valuable crop to various pests and diseases. Of these pests, plant-parasitic nematodes (PPN), especially *Meloidogyne* and *Pratylenchus* spp., are of great concern. Several *Meloidogyne* and *Pratylenchus* spp. have been listed for South Africa causing substantial damage to various economically important crops, such as grain and oilseed. However, recent reports suggest that a more pathogenic species of RKN, *M. enterolobii*, and the lesion nematode *P. brachyurus* are becoming more prevalent in soybean-maize rotation schemes in South Africa. The increase in the population densities of these nematodes can cause significant damage to valuable crops. Furthermore, the use of crop rotation and cultivars (cvs.) with genetic resistance traits might not be effective for *Meloidogyne* and *Pratylenchus* management, resulting in increased attention towards biocontrol research. Although very little is known about the soil microbial communities associated with soybean in relation to different levels of *Meloidogyne* and *Pratylenchus* infestations, as well as the interaction(s) between them, molecular techniques such as Next Generation Sequencing (NGS) can be implemented to identify endemic rhizosphere bacteria associated with the rhizosphere of a certain crop. This information can then be used to identify potential microbial organisms such as *Bacillus* spp. that can be used in biocontrol product research. Downstream analyses of NGS data can also aid in the identification of microbial genera that are considered having significantly different abundances using linear discriminant analysis (LDA) Effect Size (LEfSe). Moreover, these microbial genera can then possibly be associated with decreased abundance of *Meloidogyne* and *Pratylenchus* species. Therefore, this study aimed at identifying the PPN community composition associated with soybean-maize rotations in the Mpumalanga Highveld while using species-specific PCR and sequencing to identify the presence and distribution of especially *M. enterolobii* and *P. brachyurus* while using NGS to characterise the bacterial community in the rhizosphere of these crops. Moreover, to demonstrate the nematicidal effect of naturally occurring bacteria, several *Bacillus* spp were isolated from the rhizosphere of soybean producing localities and tested against a mixed *Meloidogyne* spp. community. Results suggest that *M. enterolobii* and *P. brachyurus* occur at several localities as mixed communities with several other species from these genera. The use of NGS identified several bacterial species associated with decreased *Meloidogyne* and *Pratylenchus* abundance while *in vitro* assays of isolated *Bacillus* spp. mixtures demonstrated the potential to suppress mixed PPN communities. This suggests that endemic rhizosphere bacteria can provide a good platform for biocontrol research toward PPN management.

Keywords: Biocontrol; Crop protection; *Meloidogyne*; *Pratylenchus*; Soybean

PREFACE

This thesis is written according to the article format prescribed by North-West University. Chapters are written as manuscripts in the style of the relevant journal in which they were published or submitted to. As required by North-West University, contributions of all authors for each article/chapter as well as their consent for use as a part of this thesis are provided.

This thesis contains the following chapters:

Chapter 1 – Introduction

Chapter 2 – Literature review (1st Article) (Published): **Agriculture.** https://doi.org/10.3390/agriculture10060242

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Chapter 6 – Research article (3rd Article) (Published): **Rhizosphere**. https://doi.org/10.1016/j.rhisph.2022.100528

Chapter 7 – Conclusion and future perspectives

The reference lists for Chapter 2, 3 and 6 is according to the instructions to authors of the journals *Agriculture, Microorganisms* and *Rhizosphere* respectively. The title pages of the published articles are included as Appendix A, B and C.

AUTHOR CONTRIBUTIONS

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Author	Article	Contribution	Consent
G Engelbrecht	1	Principal investigator: Responsible for study design, sampling, data analyses as well as interpretation. First author and responsible for writing of manuscripts.	
H Fourie	1	Promotor: Supervised the study design and progress. Provided intellectual input during the practical work and writing of articles and thesis	
S Claassens	1	Co- promoter: Supervised the study design and progress. Provided intellectual input during the practical work and writing of articles and thesis.	
CMS Mienie	1	Co- promoter: Supervised the study design and progress. Provided intellectual input during the practical work and writing of articles and thesis.	

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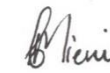
Author	Article	Contribution	Consent
G Engelbrecht	2	Principal investigator: Responsible for study design, sampling, data analyses as well as interpretation. First author and responsible for writing of manuscripts.	
H Fourie	2	Promotor: Supervised the study design and progress. Provided intellectual input during the practical work and writing of articles and thesis	
S Claassens	2	Co- promoter: Supervised the study design and progress. Provided intellectual input during the practical work and writing of articles and thesis.	
CMS Mienie	2	Co- promoter: Supervised the study design and progress. Provided intellectual input during the practical work and writing of articles and thesis.	

Engelbrecht, G., Claassens, S., Mienie C.M.S. & Fourie, H. 2022. Filtrates of mixed *Bacillus* spp. inhibit second-stage juvenile motility of root-knot nematodes. Rhizosphere, 22:100528. doi: 10.1016/j.rhisph.2022.100528.

Author	Article	Contribution	Consent
G Engelbrecht	3	Principal investigator: Responsible for study design, sampling, data analyses as well as interpretation. First author and responsible for writing of manuscripts.	
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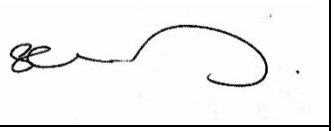
S Claassens	3	Co- promoter: Supervised the study design and progress. Provided intellectual input during the practical work and writing of articles and thesis.	
CMS Mienie	3	Co- promoter: Supervised the study design and progress. Provided intellectual input during the practical work and writing of articles and thesis.	

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LIST OF ABBREVIATIONS

ASV	amplicon sequence variants
BCS	*Bacillus* ChromoSelect agar
bp	base pairs
cvs	cultivar
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic acid
FO	Frequency of occurrence
g	grams
h	hours
ha	hectare
HIV/AIDS	human immunodeficiency virus/acquired immunodeficiency syndrome
IPM	Integrated Pest Management
J2	second stage juvenile
J3	third stage juvenile
J4	fourth stage juvenile
LB	Luria-Bertani
LEfSe	Linear discriminant analysis Effect Size
LDA	Linear discriminant analysis
m	meters
min	minutes
MT	Metric ton
MPD	Mean Population Density
ng	nanograms
NGS	Next Generation Sequencing
nM	nanomolar
NMDS	Non-metric multidimensional scaling
PCR	polymerase chain reaction
PGPEB	plant growth-promoting endophytic bacteria
pM	picomolar
PPN	plant parasitic nematode
PV	prominence value
QIIME2	Quantitative Insights into Microbial Ecology 2
RKN	root-knot nematode
RR	Roundup ready

rRNA	Ribosomal ribonucleic acid
SA	South Africa
SCAR-PCR	Sequence Characterised Amplified Region-polymerase chain reaction
spp	species
SRC	sequence read count
SSA	sub-Saharan Africa
µL	microliter
µm	micrometres
µM	micromolar
USA	United States of America
USD	United States dollar
USDA	United States Department of Agriculture
UNDESA	United Nations Department of Economic and Social Affairs
UN	United Nations
V	volts
ZAR	South African rand

CHAPTER 1: GENERAL INTRODUCTION

“To know that we know what we know, and to know that we do not know what we do not know, that is true knowledge.”

Nicolaus Copernicus

1.1 Introduction

This study focused on determining the abundance and diversity of nematode communities associated with soybean (*Glycine max* (L.) Merr.) production in the second largest soybean production area in South Africa (SA) (Grain, 2021), namely the Highveld region of Mpumalanga. Ultimately, attempts were made to identify (using advanced molecular methods) microbial communities associated with soybean rhizospheres that can be used as biocontrol agents to combat the predominant nematode pests identified. This introductory chapter will focus on certain aspects of the soybean crop, the economically most important nematode genera that threaten crop productivity, as well as strategies used to manage them. The chapter concludes with information about the aim and objectives of the study.

1.2 History of soybean

Estimations made by the United Nations placed the world human population at 7.6 billion in 2017 while expecting that it would increase to 8.6 billion in 2030 (UNDESA, 2017), thus making the need for global food security greater than ever before. Soybean is considered as an important summer legume crop worldwide that serves as a very vital source for dietary protein and oil for both animal and human consumption (Hartman *et al*., 2011). Soybean cultivation can be traced back to the south-eastern region of Asia approximately 6 000-9 000 years ago (Kim *et al*., 2012). The value of this crop was soon realised, and cultivation spread to other continents. It is now produced on large scale in countries such as the United States of America (USA), Brazil, Argentina, China, India, Paraguay, Canada, Turkey, Italy, Egypt and SA (Yadava *et al*., 2011; Fourie *et al*., 2017). In SA, soybean production dates to the 1960s with production estimated at only 2,631 metric tonnes (MT) (Shurtleff & Aoyagi, 2009).

1.3 Soybean plant and life cycle

The height of a mature plant ranges from 40-100 cm with a well-developed root system. Each plant can produce several pods containing seeds. The seeds are usually round and vary in colour. Individual pods can each contain up to four seeds. Certain seed colours such as a pale-yellow colour will usually be accepted for commercial use. The stem is hairy and branched while the stem colour depends on the cultivar. Mature soybean plants are categorised as either determinate or indeterminate. Determinate type soybean plants are short and have relatively short growth periods as compared to the indeterminate types which are usually longer plants (DAFF, 2010). The root system of a soybean plant has a tap root with lateral roots. The root system also has several nodules containing species specific *Rhizobium* bacteria that contribute to the nitrogen fixation abilities of the plant (Clúa *et al*., 2018). The growth and development of a soybean plant

can be divided into two distinct phases: the vegetative stage and the reproductive stage (Figure 1.1).

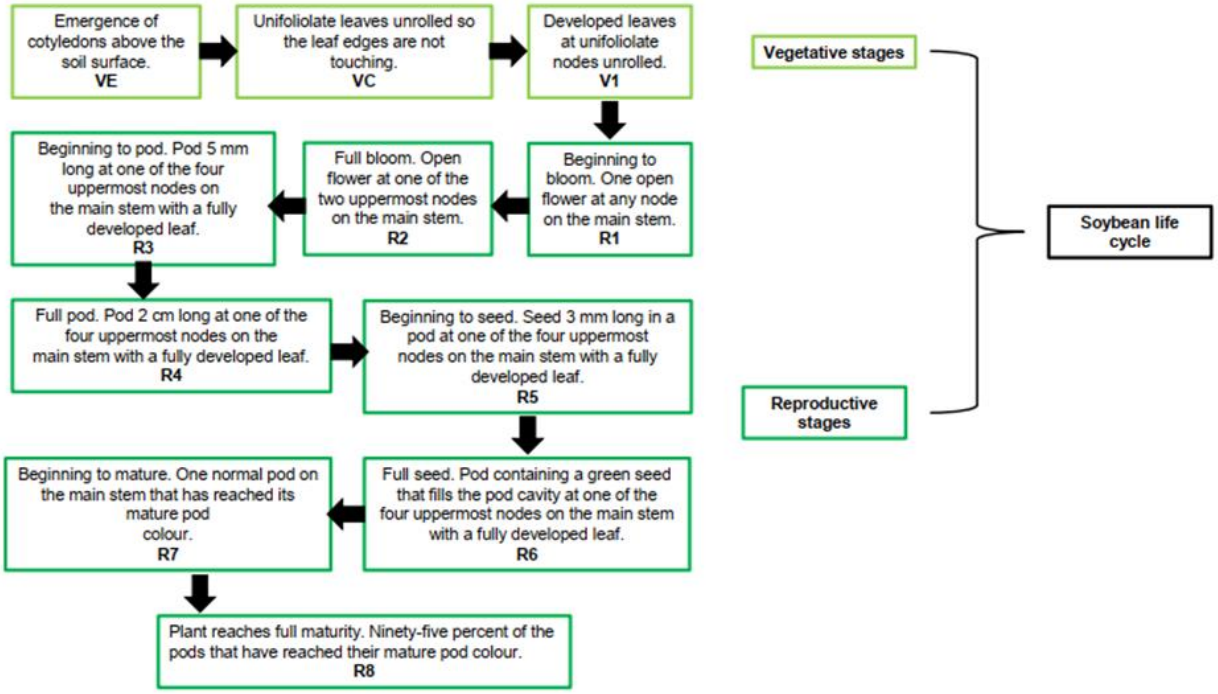

Figure 1.1: The life stages of a soybean plant. Adapted from Hodgson *et al*. (2012).

1.4 Soybean production

Countries such as the USA, Canada, Brazil, Paraguay, Argentina, China, and India (Yadava *et al*., 2011) are known to be some of the major soybean producing countries in the world. In a report released in 2022 by the United States Department of Agriculture (USDA), Brazil was identified as becoming the largest soybean producing country in the world with the estimated 2020/21 soybean production at a record 144 million MT. Second to Brazil, the USA produced an average of more than 114 million MT per year between the 2017/2018 and 2020/2021 seasons. The world's total soybean yield showed more than a 38 million MT increase from 2017/2018 to 2020/2021.

In SA the area dedicated to soybean production increased to 827 100 ha in 2020/2021, producing 1.79 million MT of seeds (Grain, 2021). This increase can be attributed towards the need for its oils and proteins (Hartman *et al*., 2011; Liebenberg, 2012). The production of soybean, just like any other crop, greatly depends on various abiotic and biotic factors. In SA soybean usually will be planted in November, after the first rains of the summer season in the warmer parts of the country (Kantolic & Slafer, 2007; De Beer & Prinsloo, 2013). Various production requirements of soybean include climatic requirements, selection of cultivars, planting and sowing, irrigation as well as weed and pest management (Zilli *et al*., 2009; Daff, 2010; Yao *et al*., 2010). Another factor

that needs to be considered is the usage of genetically modified, Roundup® Ready (RR) cultivars (cvs.) (Mc Donald *et al*., 2017).

In a report released by DAFF in 2010, the optimal temperature for soybean production is indicated as 25°C, but with a minimal soil temperature of 15°C. Although they are planted under rain-fed conditions (500-900 mm), irrigation (sprinkler or drip irrigation) can still be implemented. The planting and spacing of soybean in SA usually depend on the water availability and yield potential of the area, with 250 000-400 000 plants per ha being recommended (DAFF, 2010). However, recent droughts and increased pests and diseases of soybean have caused soybean cvs. selection to become a very important consideration that must be made by farmers. Therefore, traits such as grain yield, the improvement of the total pods per plant as well as seeds per pod and the seed mass are some of the most common traits for soybean cvs. selection (Cui & Yu, 2005; Li *et al*., 2017). Furthermore, the selection of cvs. that are RR, the treatment of seeds with fungicides and integrated practices, such as the use of registered pesticides and fungicides, also play an important role in the production practices of soybean (Zilli *et al*., 2009; DAFF, 2010).

1.5 Two important nematode pests of soybean in South Africa

The rapid expansion of local soybean production is a clear indication of the increase in the need for food security; however, it also results in an increased exposure to potential devastating diseases and pests (Liebenberg, 2012). In SA, these dangers include plant-parasitic nematodes (PPNs), specifically root-knot nematodes (RKN) and lesion nematodes (Table 1.1), which are of global economic importance due to their devastating effect on a variety of agricultural crops including soybean (Fourie *et al*., 2017). The continuous generation of knowledge of nematode pests associated with soybean, particularly due to the substantial expansion of the crop since the early 2000s, is hence crucial.

Table 1.2.1: General information regarding the two major nematode pests (root-knot nematodes and lesion nematodes) of soybean in SA.

	***Meloidogyne* spp.**
Classification	*Meloidogyne* spp. (RKN) belong to the class Chromadorea; the order Rhabditida; with suborder Thylenchina; the family Hoplolaimidae and subfamily Meloidogyninae. They are obligate biotrophic sedentary endoparasites, and when plant roots are infected by them, they usually cause the formation of characteristic galls (Figure 1.2) (Jones & Goto 2011; Decraemer & Hunt 2013).
Morphology	The second-stage juveniles (J2) have slender vermiform bodies (Figure 1.3). The labial region is weakly cuticularized. The stylet of these nematodes is relatively weak with smaller basal knobs compared to that of the cyst nematode J2. The pharynx consists of the usual parts, but it has a notably long pharyngeal gland lobe. When focussing on the posterior region of the J2, the tail is tapered and elongated while the tail has a rounded tip in most species (Kleynhans *et al*., 1996; Eisenback & Hunt, 2009; Hunt & Handoo, 2012).

Figure 1.2: Heavily infected and galled soybean root. Photo by G. Engelbrecht, North-West University, Potchefstroom.

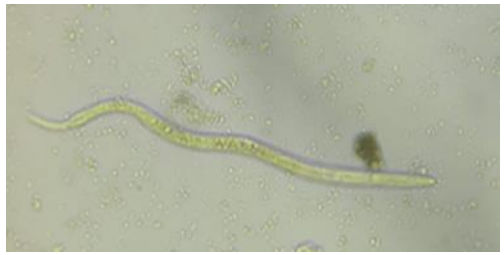

Figure 1.3: A *Meloidogyne* spp J2. Photo by G. Engelbrecht, North-West University, Potchefstroom**.**

However, for adult males the body shape is also vermiform, just like the J2, but it is longer (Figure 1.4a) compared to that of the J2. The stylet of the male is usually well developed (Figure 1.4b). The genital system consists of a single testis. However, there are exceptions in specimens where sex reversal has occurred. There will then be two testeis that represent the two female genital tracts. Males also have two spicules without any bursa. The tail of the males is short and the cloacal opening is found at the end of the body (Kleynhans *et al*., 1996; Eisenback & Hunt, 2009; Hunt & Handoo, 2012).

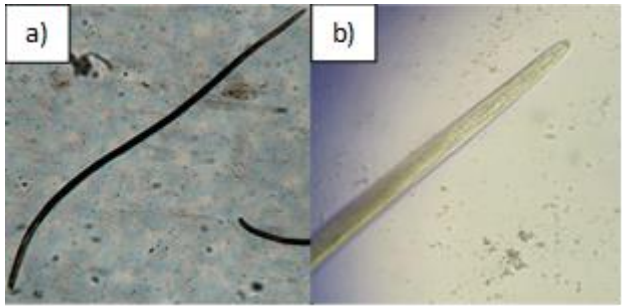

Figure 1.4: A full length *Meloidogyne* spp. male and b) the anterior part of *Meloidogyne* spp. male. Photos by a) R. Collet, North-West University, Potchefstroom and b) G. Engelbrecht, North-West University, Potchefstroom.

The mature female body (Figure 1.5) is pear-shaped and sedentary. Mature females have protruding neck regions, but it is still muscular to allow the head to move between feeding positions of several giant cells. The vulva opens in a terminal position and is surrounded by the perineal pattern. Close to the vulva are the phasmids, anal opening and tail tip. In the swollen part of the mature female body, two genital tracts are found. According to Hunt & Handoo (2012) the mature females are didelphic, prodelphic due to the location of the vulva which is located extremely posterior. The genital tracts are very long, complex structures that intertwine around the intestine and large ovaries. There are several well-developed rectal gland cells near the anus that produce the gelatinous matrix in which the eggs are laid (Kleynhans *et al*., 1996; Hunt & Handoo, 2012).

Figure 1.5: A stained mature *Meloidogyne* spp. female with eggs. Photo by R Collet, North-West University, Potchefstroom.

Reproduction

The life cycle (Figure 1.6) of RKN consists of an egg stage, four juvenile stages, and the adult male and females. The presence of males, however, is rare and in the case of some species absent due to parthenogenetic reproduction, yet some species are amphimictic. The life cycle from egg-to-egg stage can easily be completed within 3 weeks (Hunt & Handoo, 2012).

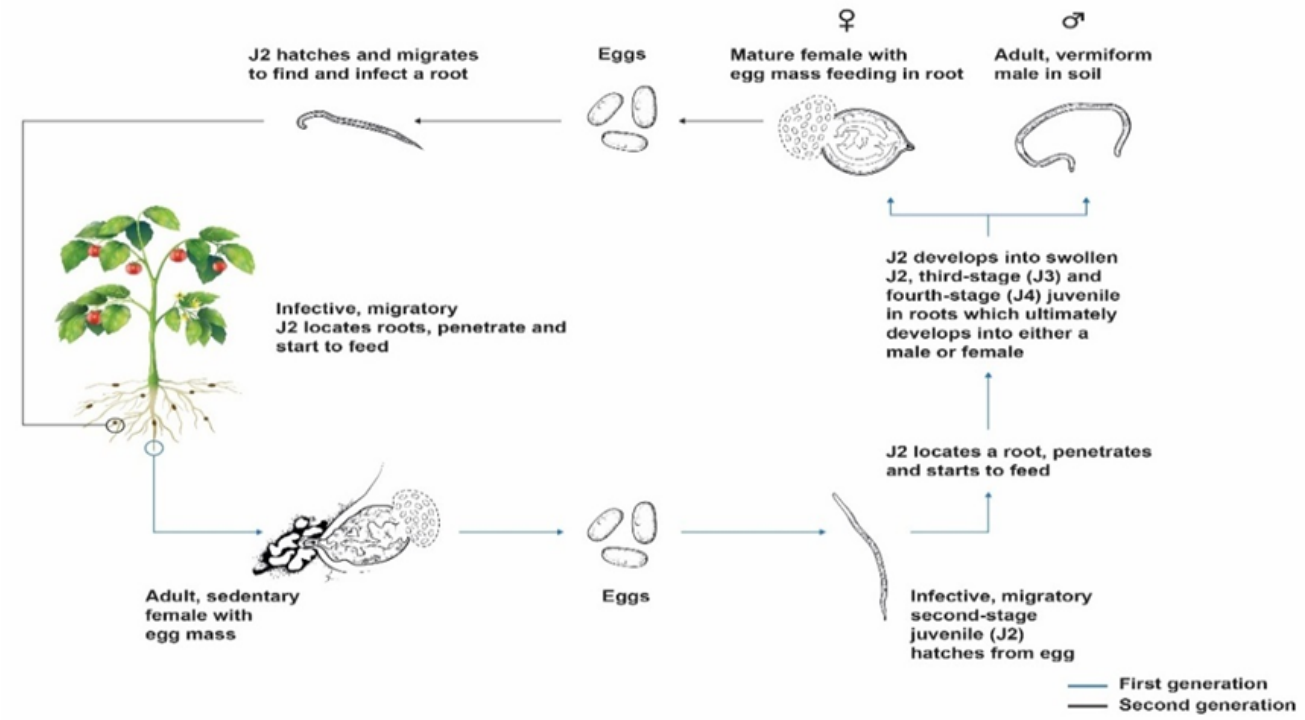

Figure 1.6: Life cycle of *Meloidogyne* spp. (Mashela *et al*., 2017).

Pratylenchus spp.

Classification

Pratylenchus (lesion nematodes) belong to the order Tylenchida, suborder Tylenchina the family Pratylenchidae and subfamily Pratylenchinae. They are obligate biotrophic, migratory endoparasites, but *Pratylenchus* can feed ectoparasitically. When plant roots are infected by them, they cause formation of necrotic root tissue (Figure 1.7) being visible as discoloured areas (Siddiqi, 2000; Castillo & Vovlas 2007; Fourie *et al*., 2017).

Figure 1.7: Soybean root infected by *Pratylenchus* spp. Photo by Suria Bekker.

Morphology

They have vermiform bodies with low labial regions which are flattened anteriorly, broadly rounded and annulated with strongly sclerotized framework. They have very strong stylets with large, rounded knobs (Figure 1.8). Another characteristic is the pharyngeal gland overlap of the intestine. Females are monoprodelphic with an anterior genital branch might contain a developed spermatheca. The tail is usually conoid and sub-cylindrical with a round to pointed tip depending on the species. The males also have bursa enveloping the tail. Even though there are clear identification features, the taxonomic separation of various species belonging to this genus is difficult. *Pratylenchus* is stenomorphic because of the small number of diagnostic features available and the intraspecific variability of some of these characteristics like the tail region (Kleynhans *et al*., 1996; Castillo & Vovlas, 2007; Castillo *et al*., 2012).

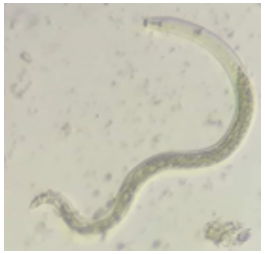

Figure 1.8: A *Pratylenchus* spp. Photo by G. Engelbrecht, North-West University, Potchefstroom.

Reproduction

The *Pratylenchus* life cycle, although simple and direct (Figure 1.9), remains poorly understood in field conditions. Under controlled conditions the complete life cycle of *P. vulnus* only took 3-4 weeks. After carrot discs were inoculated with *P. vulnus,* J2 started to appear 9 days after inoculation. The development of J3 started 14 days after inoculation and J4 where already present after 17 days. After 26 days mature females where already present and started to lay eggs. In comparison with *Meloidogyne* J2 which are known as the infective stage, none of the *Pratylenchus* stages are known as the "infective stage" as both adults and juveniles enter and leave the roots (Chitimbar & Raski, 1985; Castillo & Vovlas, 2007).

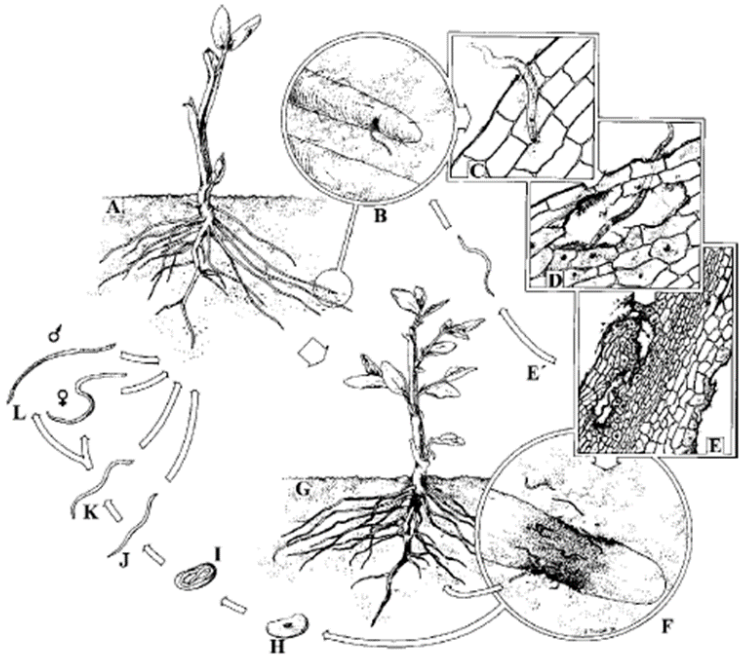

Figure 1.9: Representation of the disease cycle of *Pratylenchus penetrans* on faba bean. A= represents a healthy root system. B-D= describes the penetration of the roots by *Pratylenchus*, E and F= cortical damage caused, G= necrotic lesions caused, H-I= production of eggs, J-K= juveniles appear, L= adult appear (Vovlas & Troccoli, 1990).

1.6 Perspective, aim and chapter layout

1.6.1 Perspective of this study

To date two large-scale nematode-soybean surveys have been done in soybean producing fields across SA (Fourie *et al*., 2001; Mbatyoti, 2018). An upcoming threat, the root-knot nematode *Meloidogyne enterolobii* (Jones *et al*., 2013) has been identified since 2016 in grain production areas infecting maize (*Zea mays*) (Pretorius, 2018) and potato (*Solanum tuberosum*) (Visagie *et al*., 2018) in the Highveld region of Mpumalanga. This area is the largest soybean producing area after the Free State Province (Grain, 2021). Unfortunately, no extensive nematode survey of soybean which only focused on the Highveld area has been done to date. Due to *M. enterolobii* being listed as more pathogenic when compared to its counterpart species *Meloidogyne incognita* and *Meloidogyne javanica* (Jones *et al*., 2013), this scenario should be addressed.

Therefore, this project included an extensive survey of soybean in the Highveld region (the second biggest soybean producing area in SA) to determine the (a) abundance and diversity of *Meloidogyne* spp. that parasitise the crop and (b) whether *M. enterolobii* is more widespread than on the two farms where it has been found to date. Also, the past survey of Mbatyoti (2018) suggested that lesion nematodes (*Pratylenchus* spp.) became more abundant since the first survey (Fourie *et al*., 2001). Efforts must therefore also be directed towards determining the abundance and diversity of this genus as part of this survey.

The use of chemical nematicides to control *Meloidogyne* and *Pratylenchus*, as well as other less important but also problematic nematode pests on soybean, is seldom cost-effective (Fourie & McDonald, 2001). There are also no or limited chemical nematicides predicted to be registered on soybean for use in SA in the foreseeable future (Fourie *et al*., 2017). However, seed treatments such as AVICTA® Complete Beans, that contains abamectin as its active ingredient, is a registered nematicide in the USA and Brazil, but not yet in SA. Chemicals remain one of the most common methods for nematode management (Schneider *et al*., 2003), yet many have elevated levels of toxicity contributing to environmental and human safety concerns. This calls for the urgent development of more environmentally friendly management methods. These methods include crop rotation, use of genetic host plant resistance and biological control products.

Crop rotation as an approach to manage RKN and lesion nematode populations is not an effective management approach in soybean-based cropping schemes across SA. This is mainly because the crops that are frequently rotated with soybean locally, such as maize and sunflower (Mc Donald *et al*., 2017) are susceptible to RKN and lesion nematode infections. If crop rotation is used as a management approach, it is suggested that either poor host or RKN-resistant cvs. of

rotation crops should be used (Fourie *et al*., 2017). Genetic host plant resistance can be seen as the inhibition/limitation of the feeding sites and reproduction of various target PPN as a consequence of gene expression. Plants are usually seen as having either low, moderate or highly resistant traits (Starr *et al*., 2013). The use of genetic resistance against PPN, has been identified as one of the most cost-effective and environmentally friendly control strategy of RKN infection in soybean (Bridge & Starr, 2007). The use of biological control (bacterial and fungal strains) is an alternative method for the management of PPN infections (Ashoub & Amara, 2010; Dias-Arieira *et al*., 2018) and can lessen damage done to vital economical crops. Microorganisms that have been known to show nematicidal activity towards *Meloidogyne* and *Pratylenchus* populations include bacteria such as *Bacillus*, *Pasteuria* and *Pseudomonas* spp. as well as fungi which include *Polyphilus* and *Trichoderma* spp. (Kath *et al*., 2017; Ashrafi *et.al*., 2018; Confort & Inomoto, 2018; Engelbrecht *et al*., 2018; Watson *et al*., 2018).

1.6.2 Aims and objectives

This study aimed to 1) compile an up-to-date literature review about the importance of soybean and the threats posed by *Meloidogyne* and *Pratylenchus* on its production and 2) screen for the presence of *Meloidogyne* and *Pratylenchus* spp. and specifically the potentially destructive *M. enterolobii* and *P. brachyurus* in soybean crops of the Mpumalanga Highveld region, whilst determining the presence of nematicidal soil bacteria and fungi that can be used in biocontrol research.

Major objectives of the study included:

- Reviewing the current state of soybean in SA, with focus on the economically most important nematode pests and their control, against the background of the importance of the crop worldwide and particularly in sub-Saharan Africa.
- Determining soil microbial community structure using next generation sequencing for the identification of bacterial strains with biocontrol potential.
- Determining the presence, abundance and distribution of *M. enterolobii* and *P. brachyurus* on the highveld using molecular techniques.
- Comparing the impact of soybean dominated rotations vs soybean-maize rotations on PPN and soil bacterial communities.
- Assessing the ability of soil bacteria (especially *Bacillus* spp.) associated with soybean for their potential nematostatic activities against a mixed *Meloidogyn*e community.

This research is intended to assist producers of soybean and related industries in combatting nematode pests that adversely affect production of the crop. The study is also expected to create awareness surrounding RKNs (*Meloidogyne* spp.) and lesion nematodes (*Pratylenchus* spp.) that are present in soybean production areas, which might increase the risks of reduced yields and/or quality of soybean and rotation crops used.

1.6.3 Chapter layout

This thesis represents a compilation of published and unpublished manuscripts, where each chapter is an individual entity. Therefore, some repetition between chapters has been unavoidable.

Chapter 1 is the current chapter and introduces the study which describes the importance of soybean and the threats that certain plant-parasitic nematodes pose to this crop. The need for biocontrol research (as done in this thesis) is also explored. This chapter includes the perspective, aim, specific objectives and outline of the thesis chapters.

Chapter 2 reviews the literature on the state of soybean and its value in sub-Saharan Africa, especially SA. The review also focused on the threat posed by *Meloidogyne* and *Pratylenchus* spp. and how current management strategies are used against these nematode pests.

Chapter 3 discusses the screening of nematode population assemblages and endemic rhizosphere bacteria associated with soybean using Next Generation Sequencing (NGS). The abundance of the bacterial genera that were subsequently identified as being significantly more abundant using linear discriminant analysis (LDA) Effect Size (LEfSe), was compared to the abundance of the most prevalent plant-parasitic nematode genera found across all sampled sites, *viz*. *Meloidogyne* and *Pratylenchus*.

Chapter 4 discusses the identification of plant-parasitic nematode populations of 16 soybean-maize producing localities in the Mpumalanga Highveld region of SA. This was followed by the use of molecular techniques to characterise species of the two most predominant plant-parasitic nematodes, *viz*. *Meloidogyne* and *Pratylenchus* spp., associated with soybean-maize crops in the Highveld region of SA.

Chapter 5 assessed the potential impact of soybean-maize rotation schemes on both rhizosphere bacterial and PPN community composition of the plant roots.

Chapter 6 focused on the nematicidal activity of *Bacillus* spp. mixtures isolated from soybean producing localities on the motility of second-stage juveniles (J2) of a mixed *Meloidogyne* community that co-occurs in this region.

Chapter 7 concluded the thesis with focus on the major findings of this research and the way forward in terms of potential research to be done to further address the soybean-nematode problem in SA in a way that will be beneficial to stakeholders.

References and Supplementary information are provided at the end of each chapter.

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CHAPTER 2: LITERATURE REVIEW

“Scientific research is one of the most exciting and rewarding of occupations.”

Frederick Sanger

2.1 Abstract

With an increase in the global population, a protein-rich crop like soybean can help manage food insecurity in sub-Saharan Africa (SSA). The expansion of soybean production in recent years lead to increased land requirements for growing the crop and the increased risk of exposing this valuable crop to various pests and diseases. Of these pests, plant-parasitic nematodes (PPN), especially *Meloidogyne* and *Pratylenchus* spp., are of great concern. The increase in the population densities of these nematodes can cause significant damage to soybean. Furthermore, the use of crop rotation and cultivars (cvs.) with genetic resistance traits might not be effective for *Meloidogyne* and *Pratylenchus* control. This review builds on a previous study and focuses on the current nematode threat facing local soybean production, while probing into possible biological control options that still need to be studied in more detail. As soybean is produced on a global scale, the information generated by local and international researchers is needed. This will address the problem of the current global food demand, which is a matter of pressing importance for developing countries, such as those in sub-Saharan Africa.

Keywords: Africa; soybean; *Meloidogyne*; *Pratylenchus*; management

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2.2 The Potential of Soybean to Manage Food Insecurity

United Nations (UN) estimates indicated that the world population increased from 6 145 007 to 7 795 482 during 2000–2020, while the sub-Saharan Africa (SSA) population almost doubled (645 007 to 1 106 573) in the same period [1]. This increase in population density can result in severe food insecurity especially in SSA, where food demand can increase by more than 300% by 2050. Cereals, such as maize (*Zea mays*), millet (*Panicum* spp.), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), and wheat (*Triticum*) are the most important crops with regards to calorie intake in SSA, although large yield gaps still exist for crops like maize [2,3].

Recent estimates indicate that there are over 475 million farms worldwide that can be defined as smallholder entities, producing at least half of the world's food [4]. However, the situation is different in South Africa, where most farms are commercial and from which the bulk of food is produced while smallholder farms are more predominant in poor rural areas [5]. In SSA, smallholder farms face various challenges, including low productivity, and high levels of poverty and food insecurity, resulting in low agricultural growth that cannot match the rapid population increase [6]. To help manage the food demand in SSA, alternative crops, such as soybean (*Glycine max* (L.) Merr.), can be used. It is one of the most important summer legume crops worldwide that serves as an important dietary protein and oil source for animal and human consumption. Soybean seeds consist of about 18% oil and 38% protein while providing protein equal in quality to that of animal sources and has the possibility to nourish people in SSA. Global soybean production has already increased since the 1960s and future increases can also be expected due to larger areas being cultivated and higher yields obtained [7]. However, as with the other important food crops grown in SSA, soybean is also parasitised by a variety of pests and diseases, of which plant-parasitic nematodes (PPN) represent an important constraint [8,9]. Other than PPN, various insect pests damage soybean in SSA of which stem feeding pests are of great concern. Similarly, a range of pathogenic bacterial and fungal diseases of soybean have been listed in association with soybean crops in SSA countries of which the most important are most likely bacteria that cause bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*) and fungi (*Phakopsora pachyrhizi* and/or *P. meibomiae*) causing soybean rust.

2.3 Soybean Production in South Africa and Sub-Saharan Africa

Soybean production in countries, such as Argentina, Brazil, China, Paraguay, and the United States of America (USA), are significantly higher when compared to those of SSA. During the past few years, the USA and Brazil were the world's biggest soybean producing countries (Table 2.1) [10], producing over 120 and 117 million metric tons (MT), respectively, in 2018/19. However, projections for 2019/20 places Brazil′ s soybean production at 126 million MT [10]. Although SSA

has an estimated total land area of 21.2 million square kilometres with 600 million hectare (ha) of arable land, less than 10% is cultivated. Therefore, SSA can be considered as the most underutilized land reserve in the world [11]. The agro-ecological regions in SSA have a high potential for growing soybean [12]. The importance of soybean in SSA is evident in its increased production. In the early 1970s, SSA produced 13 000 MT of soybean, while 2019/20 estimates for soybean production are at 2.55 million MT when combining the top two soybean producing countries in this region, namely South Africa and Nigeria [10,11]. In 2018/19, South Africa was the largest soybean producer in SSA (1.17 million MT) followed by Nigeria and Zambia (Table 2.1). However, Zambia had very similar yield figures per ha compared to that of South Africa for 2018/19, indicating a steep increase in the production of soybean. Outside of these three countries, there is very little soybean production in the rest of SSA [10].

In South Africa, soybean, maize, and sunflower (*Helianthus annuus* L.) are the top grain crops produced in terms of area planted and production. The socioeconomic value of soybean in South Africa is of such importance that this crop is produced in each of the country's nine provinces. Numerous commercially available cultivars that are adapted to different climatic regions contribute to the crop being grown widely throughout the different climatic zones in South Africa [13]. The growing need for the proteins and oils provided by soybean is evident in the increased amount of land dedicated to its production during the past three decades [7,13]. In the early 1990s, fields in South Africa that were used for soybean cultivation made up as little as 87 000 ha. However, the area dedicated to soybean production increased to an astonishing 730 500 ha during 2018/19, which was slightly less than the record figure of 787 200 ha recorded during 2017/18 [14].

The Department of Agriculture, Forestry, and Fisheries (DAFF) estimated that in 2010, soybean production in South Africa ranged from 450 000 to 500 000 MT per annum [15]. However, in the 2016/2017 and 2017/2018 seasons, annual production was at a record high of 1.3 and 1.5 million MT per annum, respectively [14]. The Free State, Mpumalanga, and Kwa-Zulu Natal are the three provinces with the highest soybean production, with environmental conditions (particularly drought as experienced during 2018/19) being a main constraint that limit production under local conditions. In the past few decades, the average soybean yield in SSA was reported at 1.1 t ha^{-1}, while the world average was more than double (2.4 t ha^{-1}) [11]. However, sporadic reports exist of extraordinary high and healthy pod formation and yield of soybean in South Africa; for example, a farmer in the Highveld region of the Mpumalanga province recorded a record 1893 pods per plant cultivated under conservation agriculture tillage practices during the 2020 growing season [16]. This is due to the benefit of increased use of conservation agriculture practices in SSA countries, such as South Africa, Ghana, and Zambia. This type of production practice can

increase yields and reduce labour requirements while also improving soil fertility [17]. Yet, as with any crop, soybean production is threatened by abiotic (e.g., temperature) [7] and biotic (pests and diseases) [18,19] factors. The low soybean yields in SSA are most likely due to a combination of these factors since the cultivars used nowadays are genetically improved to deliver optimal growth and yield [20].

Table 2.1: Data regarding soybean production in the top sub-Saharan Africa countries and selected international countries from 2018/19 to projected data for 2019/20 [10].

Area	Country	2018/19			2019/20		
		Area Harvested (Million ha)	Yield (Metric Tons Per ha)	Production (Million MT)	Area Harvested (Million ha)	Yield (Metric Tons Per ha)	Production (Million Metric Tons)
International	Argentina	16.60	3.33	55.3	17.00	3.18	54.00
	Brazil	35.90	3.26	117.00	36.90	3.41	126.00
	Bolivia	1.40	1.93	2.70	1.40	2.00	2.80
	Canada	2.54	2.86	7.27	2.30	2.61	7.27
	India	11.33	0.96	10.93	11.25	0.83	9.30
	Indonesia	0.41	1.27	0.52	0.40	1.28	0.51
	Japan	0.15	1.45	0.21	0.15	1.69	0.25
	Paraguay	3.70	2.39	8.85	3.54	2.80	9.90
	Turkey	0.03	3.80	0.10	0.03	3.89	0.11
	USA	35.45	3.40	120.52	30.36	3.19	96.84
Sub-Saharan Africa	Nigeria	1.00	1.05	1.05	1.00	1.10	1.10
	South Africa	0.73	1.60	1.17	0.80	1.81	1.45
	Uganda	0.05	0.60	0.03	0.05	0.60	0.03
	Zambia	0.19	1.58	0.30	0.20	1.43	0.28

2.4 Value of Soybean in South Africa and Sub-Saharan Africa

The increase in demand of soybean and its products in SSA led to an annual soybean import of 6.8 million MT from 2013–2016 at a cost of 4.4 billion USD [14]. Botswana, Kenya, Nigeria, Seychelles, South Africa, Zambia, and Zimbabwe are among the top soybean importers in SSA [21]. Yet, it has been reported that SSA achieved 4.6% inflation-adjusted annual mean increases in agricultural growth from 2000–2016 [6]. More people in SSA countries realize the potential of this protein-rich crop, resulting in its use in several food products, such as dawadawa (fermented dried seeds of the African locust bean *Parkia biglobosa*), mahewu (non-alcoholic home-brewed drink made of thin slightly fermented maize-meal porridge), and nshima (a dish made from ground maize flour), consumed by local people in many SSA countries. Furthermore, some SSA countries use soymilk and soup as daily meals for malnourished children and patients infected with HIV/AIDS. Nigeria is also an SSA country that took advantage of soybean consumption and this resulted in the development of processing technologies for soy-based food [11]. In other countries, such as Rwanda, more than half of the smallholder farmers consume their entire soybean harvest as an unprocessed food source, while Zambia exports large amounts of their soybean to Botswana and Zimbabwe [21].

South Africa has seen a dramatic increase in the demand for protein sources, such as soybean, as feed for the growing livestock sectors. This led to significant investment in soybean production since the early 2000s [14,22]. In a 2016 report released by Meyer and co-authors [22], the gross value of agricultural output for field crops contributed 23% to the gross domestic product (GDP) of South Africa, compared to 29% from horticulture and 47% from livestock. Soybean contributed 8% to the total value of field crop output [22,23]. Moreover, the production of soybean increased to 1.55 million MT in 2017/2018, with a gross value of approximately ZAR7 139 million compared to approximately ZAR4 598 million for 2015/2016. The estimated gross value for soybean in 2018/2019 was approximately ZAR6 023 million, although the price per MT (ZAR/MT) decreased from 2015/2016 (ZAR6 197) to 2018/2019 (ZAR4 719) [24].

Besides socioeconomic benefits, soybean and associated *Rhizobium* and *Bradyrhizobium* microbes contribute to nitrogen fixation in soils. Nitrogen fertilization is tremendously expensive and poses ecological risks, such as water eutrophication and the emission of greenhouse gases, that contribute to global warming [24,25]. In South Africa, the expense of applying synthetically derived nitrogen to crop fields as part of fertilization programs is one of the biggest financial outputs that farmers undertake to obtain target or expected yields [26]. Therefore, biological nitrogen fixation is a valuable and ecologically safe alternative that warrants further utilization as an added value factor when growing a legume crop, such as soybean. The increasing development and release of *Rhizobium* and/or *Bradyrhizobium* products, such as RhizoFlo® and HiStick® (BASF South Africa (Pty) Ltd, Port Elizabeth, South Africa) [27], to optimize nitrogen fixation additionally contributes towards increasing biodiversity in agricultural soils [28].

2.5 Pests and Diseases of Soybean

Soybean crops are susceptible hosts to various pests and diseases. The threat of fungal diseases, such as frogeye leaf spot (caused by *Cerospora sojina*), red leaf blotch (caused by *Coniothyrium glycines*), rust (caused by *P. pachyrhizi* and/or *P. meibomiae*), and sudden death syndrome (caused by various pathogenic species of *Fusarium*), are common to soybean production in Africa [29]. Red leaf blotch appears to be endemic to SSA and is of major concern; there is little information about this disease with regards to its occurrence, as it has only been documented in Africa [30]. Soybean rust has, however, already been reported in Argentina, Brazil, and some of the top SSA soybean producing countries, such as South Africa, Nigeria, and Zambia [21].

Diseases caused by bacteria, such as bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*), bacterial pustule (*Xanthomonas axanapodis* pv. *glycines*), and wildfire (*Pseudomonas syringae*),

are also known to occur worldwide on soybean and limit production of the crop [21]. Insect and nematode pests of soybean are also of major concern [19]. Stem feeding pests of soybean in Africa are the soybean stem flies (*Melanagromyza sojae* and *Ophiomyia phaseoli*). Soybean foliar pests include the American serpentine leafminer (*Liriomyza trifolii*), the bean leafroller (*Omoides diemenalis*), and the soybean looper (*Thysanoplusia orichalcea*). Other insects, including the legume pod borer (*Maruca vitrata*) and the southern green stink bug (*Nezara viridula*), feed on soybean pods [19,31].

Although there are also various foliar pests on soybean, the damage caused by these organisms often results in little yield loss [31]. Along with the diseases and insect pests that were mentioned previously, PPN are also known to be major pests of soybean and various other crops [19,32,33]. Of the known PPN that cause damage to soybean globally [32], soybean cyst (*Heterodera glycines*), root-knot nematode (*Meloidogyne*), and lesion (*Pratylenchus*) nematode are the most important pests [18]. However, in South Africa, the soybean cyst nematode *H. glycines* has not yet been found [26]. The soybean cyst nematode causes severe infections and yield losses in various countries in Asia as well as North and South America [9].

Root-knot nematodes, together with *Heterodera* and *Globodera* (the cyst nematodes) are generally seen as economically important nematode pests of various crops worldwide [34,35]. Concerning root-knot nematodes, *Meloidogyne arenaria* was reported in soybean fields of South Africa as early as 1959 [36], with the list of root-knot nematode species associated with soybean expanding over the years (Table 2.2) [33]. Root-knot nematodes are obligate biotrophic sedentary endoparasites, have a global distribution, and can cause major damage to almost all vascular plants. Inevitably, their parasitism results in substantial damage to various economically important crops, such as fruit, grain, industrial, potato, and soybean crops [35–37]. Lesion nematodes are also considered as one of the most economically important nematode pests of several crops, including banana (*Musa* spp.), cereals, coffee (*Coffea arabica* L.), maize, and soybean (Table 2.2) [18,38]. The first lesion nematode found to be associated with soybean in South Africa was *P. brachyurus* in 1984 [39], with reports thereafter highlighting the presence of this nematode species in soybean fields [40,41].

As soybean production in South Africa and SSA increases, the risk of exposing this crop to various pests and diseases also increases. This is evident in the number of root-knot and lesion nematodes already reported to be associated with soybean in South Africa (Table 2.2). Although soybean production in SSA is on the rise, the extent of research on the root-knot and lesion nematodes associated with soybean in SSA remains insufficient. The research done in South Africa [33] with regards to predominant nematode pests associated with soybean could greatly benefit soybean production in the rest of SSA. Thus, there is a need to understand the potential

impact of the two predominant nematode pest genera, *Meloidogyn*e and *Pratylenchus*, can have on soybean production, as well as a discussion of the current and future management strategies to ensure sufficient yields.

Table 2.2: Countries where *Meloidogyne* and *Pratylenchus* spp. have been found to be associated with soybean.

Genus	Species	Countries
Meloidogyne	*M. arenaria*	South Africa [36], USA [42]
	M. ethiopica	South Africa [40]
	M. enterolobii	USA [43]
	M. hapla	China [44], South Africa [40]
	M. incognita	Brazil [45], China [44], South Africa [41,46], Pakistan [47], USA [42]
	M. javanica	Brazil [45], Greece [48], Nigeria [49], South Africa [41,46], Pakistan [47], USA [42]
	Meloidogyne spp.	Germany [18], South Africa [39]
Pratylenchus	*P. brachyurus*	Brazil [50], South Africa [39,41]
	P. crenatus	Germany [18], South Africa [40,41]
	P. flakkensis	South Africa [41]
	P. neglectus	Germany [18], South Africa [41]
	P. scribneri	South Africa [41]
	P. penetrans	Germany [18], South Africa [51]
	P. teres	South Africa [40,41]
	P. thornei	Australia [52], South Africa [40,41]
	P. zeae	South Africa [41]
	P. vulnus	South Africa [41]
	Pratylenchus spp.	South Africa [53], USA [54]

2.6 Impact of *Meloidogyne* and *Pratylenchus* on Soybean

2.6.1 Meloidogyne

When plant roots are infected by root-knot nematode second-stage juveniles (J2), this specific life stage as well as feeding females usually cause the formation of characteristic galls, making their presence in the roots of soybean and other crops easy to identify and study [33]. Symptoms caused by root-knot nematode infection can be divided into above- and belowground symptoms. The formation of galls on soybean roots interferes with the anatomical and physiological functioning of the roots, since infected roots cannot optimally take up and translocate water and nutrients to the aerial plant parts. Although gall formation caused by root-knot nematodes varies with the population density and or species [9], it is not advisable to attempt species identification of this genus using the extent of galling, or the size and shape of galls. However, a clear difference exists between galls caused by root-knot nematodes (Figure 2.1a), and the nodules formed by *Bradyrhizobium* or *Rhizobium* species (Figure 2.1b) [13].

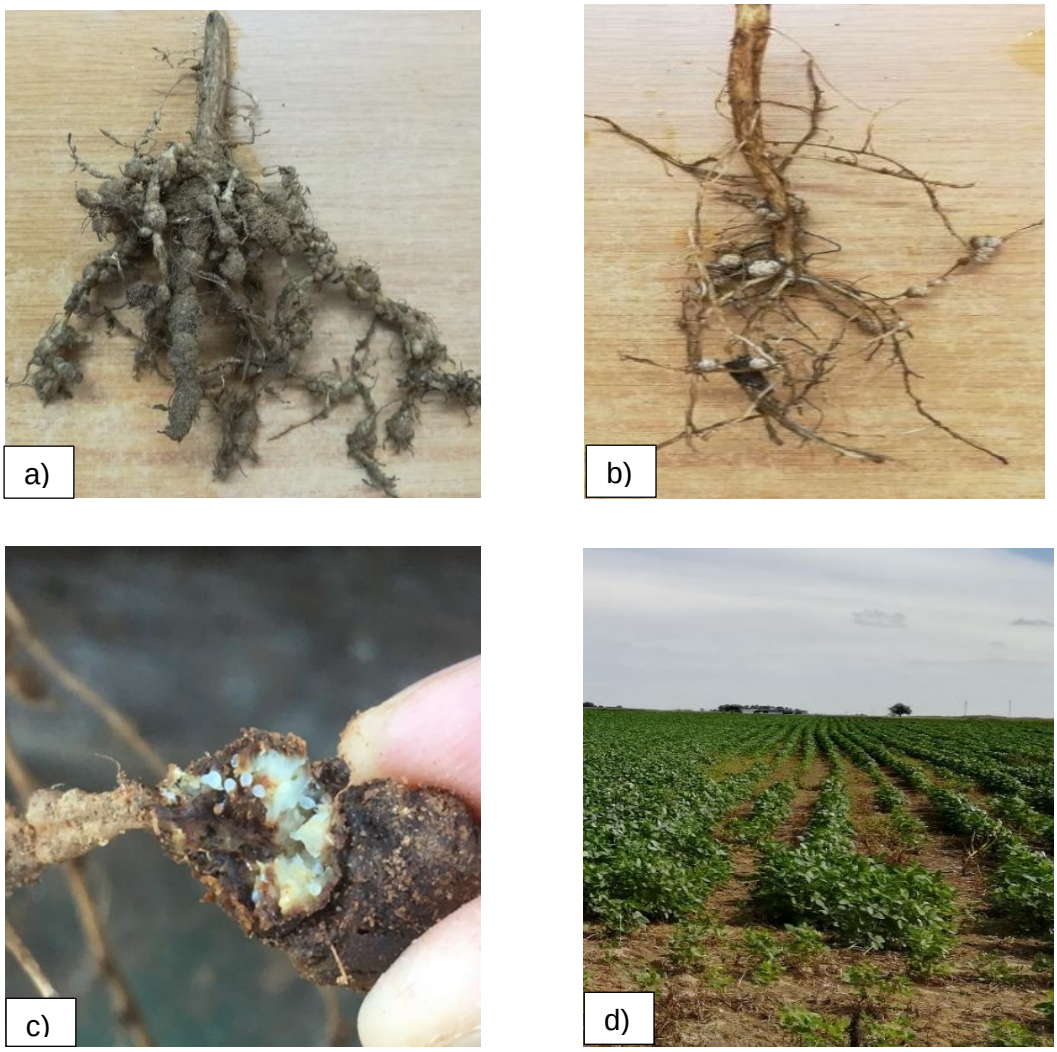

Figure 2.1: (a) Heavily infected and galled root, (b)healthy soybean root with no root-knot nematode galls visible and with nitrogen-fixing nodules, (c) *Meloidogyne* females found in nitrogen-fixing nodules, and (d) stunted growth of soybean plants infected with root-knot nematodes. Photos by: (a,b,d) G. Engelbrecht, North-West University, Potchefstroom and (c) by Suria Bekker, Econemaria, Potchefstroom.

These nitrogen-fixing nodules are easy to remove from the root system, as compared to the root-knot nematode galls, which form part of the root system. Although nodules can easily be removed, Bridge and Starr [55] indicated that these structures might also be infected with root-knot nematodes, with the same being found in South Africa (Figure 2.1c). Additionally, depending on the stage of development and infection, the color of the tissue inside the nodules will differ. In soybean, the nodules are usually pink when they are healthy as opposed to the greenish color when infected [36], but in cowpea, for example, nodules that have a pink color are healthy while infected nodules can be soft and dark brown-black in color [55]. Aboveground symptoms resulting from root-knot nematode infection generally represent stunting, wilting, yellowing, and, in severe cases, the death of plants (Figure 2.1d) [9,36]. However, these symptoms do not provide a conclusive diagnosis for root-knot nematode infection since it may be represented by other abiotic and/or biotic constraints, such as drought, lack of nitrogen-fixing ability of

Bradyrizobium/*Rhizobium*, water logging, and others. It is therefore recommended that plants are uprooted to see if the galling is present on the roots.

2.6.2 Pratylenchus

Individuals of this genus are obligate biotrophic migratory endoparasites. The belowground symptoms caused by lesion nematodes to soybean roots are characterised by the formation of necrotic tissue (inside the roots) being visible as discolored (greyish, brownish, or blackish) areas (as seen in Figure 2.2a) on the surface of infected roots [37]. Infections can occur along the entire length of the root, with damage done to the epidermis, cortex, and root endodermis [32]. Necrotic root tissue caused by feeding lesion nematodes can, however, also be confused with damage caused by other pest and/or diseases and therefore it is not an easy task to exclusively link it to the infection of lesion nematodes [37]. Aboveground symptoms of lesion nematode infection usually resemble that caused by plant-parasitic nematodes in general but can also include patches of stunted (Figure 2.2b) and chlorotic (yellowish) plants, and a reduction in leaf size and the number of leaves produced on heavily infected plants for which yields can be substantially reduced. It is also possible for symptoms of *Pratylenchus* infection to mimic those of soil-borne diseases and insect damage [37]. One of the most notable symptoms of *Pratylenchus* infection is the stubby and discolored roots (lesions caused by necrosis seen in Figure 2.2a).

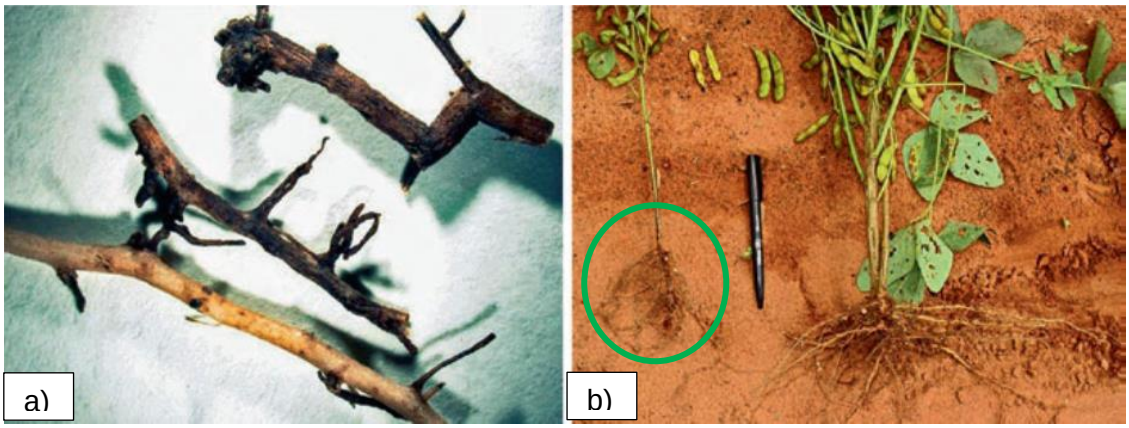

Figure 2.2: (a) Necrotic soybean roots infected with lesion nematodes, and (b) a stunted plant with a reduced and necrotic root system (green circle) compared to a noninfected plant (Photos: (a) Suria Bekker and (b) Driekie Fourie, North-West University, Potchefstroom, South Africa).

2.6.3 Interactions between *Meloidogyne* and/or *Pratylenchus* and Other Soilborne Pathogens

Root-knot and lesion nematodes can form associations with various pathogenic bacteria and fungi, leading to the development of disease complexes. This is a result of the wounds caused by the nematodes when parasitizing on the plant roots, which then act as an entry point for other

soilborne pathogens. The resulting symptoms of the host plant can then be different as compared to when the host is infected by only one of these pathogens [56]. For example, wounds made by *M. incognita* have been found to result in a disease complex with *Ralstonia solanacearum* (causing bacterial wilt) [57] and *M. hapla* to *Agrobacterium tumefaciens* [56]. These two nematode genera are amongst the most commonly reported to be involved in disease complexes with fungal pathogens [58]. *Meloidogyne incognita* has been known to form disease complexes with the fungal pathogen *Rhizoctonia solani* (seedling blight), which is of great concern for soybean production in Canada [58,59]. A synergistic co-infection of these pests can result in plant damage that exceeds the sum of individual damage caused by the pest and pathogen (1 + 1 > 2) [58]. It has also been reported that *M. incognita* can form synergistic disease complexes with *Fusarium graminearum* and *F. equiseti* (wilt fungus) in soybean [60]. High populations of *Pratylenchus* spp. were found in soybean plants infected with *R. solani* in the USA [61]. Although there is limited research regarding *Fusarium* pathogens of soybean in SSA, and the disease complex it can form with nematodes [62], Hartman and co-authors [62] found *F. incarnatum-equiseti* and *F. sambucinum* isolates from infected soybean roots in Ethiopia and Ghana. In South Africa, the increased root-knot and lesion nematode densities in soybean fields [40,41] can also lead to increased disease complexes with other soilborne pathogens, such as *Fusarium brasiliense* (soybean sudden death syndrome) [63].

2.6.4 Potential Yield Losses

Root-knot and lesion nematodes are known to cause substantial yield loss of soybean on a global scale. The damage caused to the host crop by these two nematode genera depends on the susceptibility of the infected crop genotype and the population density of the nematode [9]. However, other factors, such as the pathogenicity of the nematode population, environmental conditions, soil type, and others [7,18,19,52], can also contribute towards the damage caused to soybean crops by these genera. It is estimated that plant-parasitic nematodes, including root-knot and lesion nematodes, are responsible for yield losses of up to 80% in fields that are heavily infested [64]. Some reports suggest that the soybean yield reduction of 73 600 MT in Argentina, 313 600 MT in Brazil, and 16 600 MT in China in 1998 was caused by PPN parasitism [45].

Soybean losses in Florida, USA, due to *M. incognita* were estimated at 90%, while other root-knot species contributed to 93 000 MT of the annual yield losses in Canada and the USA from 1999–2002 [65,66]. With regards to lesion nematode infections, a soybean yield loss of 30% to 50% was reported in Brazil [67], while the lesion nematode, *P. brachyurus*, was able to result in a 31% yield loss of ‘Lee’ soybean [68]. For SSA and South Africa, yield loss figures for lesion nematodes infecting soybean crops are not available [26]. However, early reports found that the damage caused by PPN to soybean in South Africa contributed about 9% of the annual yield losses prior

to and/or in the 1980s [39]. In some cases, such as that reported by Smit and De Beer [69], severe infection of PPN assemblages, consisting predominantly of root-knot nematodes, caused a total loss of soybean in the Mpumalanga province of South Africa. More recent reports suggested that *M. incognita* caused up to a 41% yield loss on the soybean cultivar Prima2000 [56]. Along with the direct effect of root-knot and lesion nematodes on soybean yield, feeding of these genera also disturbs the nitrogen fixation process, which can lead to reduced N fixation, resulting in reduced yield [18].

The main reasons why root-knot and lesion nematodes continue to be problematic pests in soybean production are their wide host ranges, the fact that several species can be present in one field [70,71], and the lack of adequate nematicides, whether chemical or biological, being registered on soybean [72]. Yet, the main strategies used to manage root-knot nematode pests in soybean fields worldwide are nematicides (chemical and biological), crop rotation, and genetic host plant resistance [55]. Although chemical control is the preferred choice for nematode management, the application of a combination of management strategies, like those mentioned above, represents an integrated pest management (IPM) strategy. Such a strategy will be the best way to limit the impact of PPN on soybean.

2.7 Nematode Management Strategies

2.7.1 Chemical Control

Chemical control refers to the usage of products containing chemical compounds, either synthetically or naturally derived, that are either lethal to nematodes or cause disruption of their behavior, with the latter generally referred to as ‘nematistatic’ [73]. The effective use of chemical nematicides against PPN has been in practice since the 1950s [74,75]. Chemically derived nematicides, such as carbofuran, furfural, oxamyl, organophosphates, and halogenated compounds, are used on a large scale across the world to combat PPN [76,77]. However, the development of new synthetically derived nematicides has decreased over the past few decades mainly due to the withdrawal of many chemical products from international markets (as they have elevated levels of toxicity detrimental to environmental, animal, and human safety). As a result, there is an increase in the research and use of environmentally friendly nematicides and approaches. Specifically, seed-coat products are seen to have a more targeted effect of the chemical compounds applied, limiting the potential negative effects of these products [78,79].

Despite the downscaling in the development and registration of synthetically derived nematicides, research is still ongoing with regards to these chemicals. Field studies in the USA reported that the synthetically derived nematicides Bolster 15G (aldicarb) and Counter 20G (turbufos) were

able to significantly decrease nematode pests of soybean, which included *Pratylenchus* [80,81]. Although a pre-planting fumigant Telone® II is also registered for use on various crops (including soybean) against both root-knot and lesion nematodes [82], the active ingredient of this product (1,3-dichloropropene) is a methyl bromide alternative, which faces increased regulatory restraints [83]. A greenhouse study also identified methyl pelargonate as being effective in the management of M. incognita in soybean [84].

The use of seed treatments is, however, gaining more attention, especially in Brazil where products, such as Ecolife®, Cruiser®, Maxim Advanced®, Avicta®, and other abamectin-cointaining products, have been found to reduce population densities of target species, such as *M. javanica* and *P. brachyurus*, in glasshouse and in vitro studies [85–87]. Another seed treatment product ILeVO® (Bayer CropScience) that uses fluopyram as an active ingredient was registered in 2014 against sudden death syndrome and p nematode pests of soybean [88]. The use of chemical nematicides on soybean, especially in South Africa, has seldom been found to be cost-effective [89]. Of the granular and fumigant nematicides tested, only EDB® (AL; 1800 g^{-l}) was found to consistently reduce root-knot nematode populations. However, the use of aldicarb and terbufos treatments did not always differ significantly ($p \leq 0.05$) from the control [89].

There is currently only one nematicide, the seed treatment Avicta® (contains abamectin as its active ingredient), that is in the process of being registered for use on soybean in South Africa [26,90] with limited or no other chemically derived products predicted to be registered in the foreseeable future [37]. Avicta® Complete Beans is also a registered nematicide for soybean in the USA, Argentina, and Brazil [26,90]. Although products, such as ALVURAN® 100 G and CROP GUARD 80, are known nematicides, they remain unregistered for use on soybean in South Africa but can be used on maize and sunflower (usually used in rotation with soybean) [26]. Although chemicals remain one of the most common methods for root-knot nematode management [91], the safety concerns that these chemicals pose [79] calls for the urgent development of more environmentally friendly PPN control methods. Biochemicals or semiochemicals are natural compounds that can be used instead of synthetic nematicides [92] and are products derived from biological organisms, such as microorganisms, plants, and/or by-products of animals and plants. The pyrethroids produced by *Chrysanthemum cinerariaefolium*, citronella oil, and garlic extract are only a few examples of natural compounds that have nematicidal activity [93–95]. Semiochemicals, on the other hand, are compounds that can influence the behavior patterns of pests [93]. Sordidin, a male pheromone produced by *Cosmopolites sordidus*, has been used as a trapping mechanism of various pests [96].

2.7.2 Crop Rotation

The use of crop rotation as a management strategy for root-knot and lesion nematodes is difficult since species of both genera have wide host ranges [9,71]. When using crop rotation as a management strategy, it is important to use the rotation of susceptible crops with non-host or resistant crops [9]. Before any crop/cultivar is planted to form part of a rotation management strategy, it is also important to test the susceptibility of that host against the target nematode pest, as the population density [52] and species present [97] can impact that decision. In the USA, a maize genotype (Pioneer 3223), pearl millet (*Pennisetum glaucum* L.) cultivar (Tifgrain 102), and Bahia grass (*Paspalum notatum*) have been known to reduce *M. arenaria* densities [98,99]. In Australia, certain maize genotypes have also been found to cause reduced *M. hapla* densities [100], while growing the barley (*Hordeum vulgare*) genotype Clipper reduced *Pratylenchus* spp. densities [101]. In Brazil, the rotation of soybean with forage crops (*Andropogon gayanus* genotype Planaltina, *Cajanus cajan* genotype Caqui, and *Macrotyloma axillare* genotype Java), *Crotalaria* species, and oilseed radish reduced *P. brachyurus* densities [102,103]. Concerning the African continent, African marigold (*Tagetes erecta*) and sunn hemp (*Crotalaria juncea*) have been found to reduce *M. incognita* densities in soybean fields of Nigeria [104]. It is evident that crop rotation as a strategy to reduce root-knot and lesion nematode populations is not an effective control strategy in soybean-based cropping systems across South Africa [37,105]. A significant increase in RKN densities was detected when soybean was included once in four years in a maize-based cropping system [105]. Crops that are usually rotated with soybean in South Africa, such as dry bean (*Phaseolus vulgaris* L.), maize, potato (*Solanum tuberosum* L.), sunflower, and various vegetable crops, are susceptible to root-knot and lesion nematode infections [106]. If crop rotation is considered as a control strategy, it is recommended that poor host or root-knot nematode-resistant cultivars (cvs.) of rotation crops be used [37]. Yet, the use of such cvs. in rotation might cause an increase in the population density of other non-target PPN, such as a lesion nematode species for which no cultivar evaluation has been done in South Africa [26]. Coupled with this shortcoming is the lack of screening of newly released commercial cultivars of rotation crops on an annual or ongoing basis. The possibility also exists that the use of non-host or resistant crop cvs. have low or even no local interest or value to farmers [107].

2.7.3 Host Plant Resistance

Genetic host plant resistance translates to the inhibition/limitation of the penetration, feeding, development, and reproduction of target PPN species as a result of gene expression. Plants usually have none, low, moderate, or high levels of resistance to a target nematode species [37]. The use of genetic resistance against PPN, such as root-knot and lesion nematodes, have been identified as one of the most cost-effective and environmentally friendly management strategies

of these pests in soybean [55]. There are soybean cvs. that are either resistant or tolerant towards a few root-knot nematode species [9]. The USA soybean genotype PI 567516C was identified as being an excellent source of resistance as it displays resistance against not only *M incognita* but also soybean cyst nematode and reniform (*Rotylenchulus reniformis*) nematodes [108]. An early study also identified three soybean cultivars, Centennial, Forrest, and Hartwig, that were resistant against both the soybean cyst nematode (race 3) and *M. incognita* (race 3) [109]. Out of 29 soybean cultivars tested in Brazil for their resistance against *M. javanica*, none were found to be resistant, with only two (TMG 1288 RR and NS 8270) having moderate levels of resistance [110]. In Pakistan, local soybean varieties/lines viz. (Ajmeri and NARC-4) were found to be resistant to *M. incognita* in a greenhouse trial when they were inoculated with 1000 J2 per plant. However, the same cultivars were susceptible to *M. incognita* at *Pi* = 2000 J2 per plant [47], demonstrating the ability of resistance to be rendered ineffective at high population levels. In the USA, some of the Edamame soybean breeding lines have also been identified as having resistance towards *M. incognita* and these traits can be introduced into commercial cultivars [111]. Certain USA soybean genotypes, like Henderson, have moderate levels of resistance towards *M. incognita* but are again susceptible to *M. arenaria* and *H. glycines* races 2, 3, and 14 [78,112].

A substantial amount of research has been done in Africa assessing the host status of soybean genotypes to root-knot nematodes [33,113]. Fourie and Mbatyoti [113,114] found that a few soybean cvs. had different levels of resistance to different root-knot nematode species and races, while Mbatyoti [113] performed a similar work but only for *M. incognita*. Further research focused on introgression of *M. incognita* and *M. javanica* resistance into conventional high-yielding commercial cultivars [33]. However, such research is dated since new cvs. enter the market on an annual basis. In addition, introgression of resistance traits should be done for Roundup Ready® cvs. as they dominate the local market [41]. In South Africa, more than 90% of soybean cultivation used glyphosate-tolerant cultivars during the 2017 growing season [11]. Another major drawback in terms of the potential use of host plant resistance in SSA countries is the lack of information about the host status of soybean cultivars to infection by lesion nematode species. The same situation applies to Brazil, where soybean crops are adversely affected by *P. brachyurus* without any resistant sources being identified to date [113,115,116]. However, some soybean genotypes (BRSGO Chapadões, BRSGO Paraíso, M-Soy 7211 RR, M-Soy 8008 RR, Emgopa 313 RR, M-Soy 8411, BRSGO Juliana RR, Emgopa 316 RR, BRSGO Luziânia RR, and TMG 103 RR) were found to reduce *P. brachyurus* densities [117]. Ultimately, due to the impact that population density, species, and the race of nematode pests have on resistance levels, it is recommended that screening for resistance should be done with different populations and races [37,118].

With the impact of climate change also being evident on pest abundance, distribution, and diversity [119], evaluation of cultivars should also be done at different temperature regimes so that those with superior levels of resistance can be identified. Mbatyoti [113] demonstrated that the superior *M. incognita* resistance exhibited by 'LS5995' and some of its progeny (into which the trait has been introgressed) was not compromised by an increase in the temperature regime of between 3 and 4 °C. However, the same study also showed that the *M. incognita* densities in the roots of susceptible cvs. increased under the same increased temperature regimes at which soybean crops are likely to be exposed. Besides the valuable information to be generated at increased temperature regimes, there is a need to evaluate soybean cultivars for their reaction to drought. Most soybean production in South Africa occurs under rain-fed conditions, with severe drought conditions being experienced from time to time [11]. Although this may not be the case for some SSA countries that are situated in the tropics or subtropics, climate change is foreseen to be coupled with periods of severe droughts [11,120].

2.7.4 Biological Control

Biological control is a management strategy to combat PPN and refers to the use of live microbial agents to reduce population densities of target nematode pests [89]. The use of bacterial and fungal strains is an alternative management strategy for root-knot and lesion nematode infections [121,122] and can reduce the damage done to economically important crops like soybean (Table 3). Bacteria known to exhibit nematicidal activity against *Meloidogyne* and *Pratylenchus* populations include several species of *Bacillus* as well as *Pseudomonas* and *Pasteuria*, including: *B. megaterium*, *B. cereus*, *B. nematocida*, *B. subtilis*, *B. thuringiensis*, *B. pumilus*, *B. firmus*, *B. amyloliquefaciens*, *Pseudomonas fluorescens*, and *Pasteuria thornei* [115,123–126]. *Bacillus* spp. have various distinctive attributes that emphasize their potential use as bionematicides, including the production of biologically active molecules [127], such as antimicrobial compounds (antibiotics) [128], enzymes, exotoxins, and metabolites with reported nematicidal activity [129]. They are ubiquitous within the rhizosphere [128], can colonize plant roots [124], and are plant growth-promoting rhizobacteria [125,130]. Ultimately, *Bacillus* and *Pseudomonas* spp. used as biological control agents are safe to the environment and non-target organisms (animals, humans, and other soil-dwelling organisms) [131]. Some fungal species, such as the novel *Polyphilus sieberi* and *P. frankenii* [132], can penetrate and colonize nematode eggs. These two species can directly kill the eggs and thus reduce the parasite load on host plants. Kath [133] also found that some *Trichoderma* spp. can produce various enzymes, such as chitinase, lipase, and protease, which have nematicidal effects on *P. brachyurus* [133,134].

Table 2.3: Bacteria and fungi used in biocontrol of *Meloidogyne* and *Pratylenchus* spp. on soybean.

Microorganism	Species	Results	Country
Bacteria	*B. subtilis* [135]	Reduced egg and second-stage juvenile densities of *M. incognita* and *M. javanica* on susceptible genotypes by more than 70%	Brazil
	Pasteuria thornei [85]	Reduced *P. brachyurus* densities by up to 50%	Brazil
	Lactobacillus plantarum + *B. subtilis* + *Enterococcus faecium,* and *B. licheniformis* + *B. subtilis* + *Trchoderma longibrachiatum* [136]	Substantially reduced densities of *M. javanica* and *P. brachyurus*	Brazil
	T. harzianum T22 [137]	Reduced egg, second-stage juvenile and female densities of *M. incognita*	Nigeria
	Mixture of *Bacillus* isolates [138]	*In vitro* studies showed larval mortality of >80%. Greenhouse studies showed reduced gall formation and egg densities of *M. javanica*	South Africa
Fungi	*Pochonia chlamydosporia* var. *chlamydosporia* [139]	Reduced egg densities of *M. incognita*	Brazil
	Pleurotus ostreatus, P. tuberregium [140]	Reduced gall formation and second-stage juvenile densities	Nigeria

The use of bacteria and fungi as a biocontrol management strategy has led to the development of commercial products, such as Compost-Aid™, Nem-Out™ [136], and Rhizotec ® [139]. Although it is evident that research is now focused on the identification of microorganisms with anti-nematode properties, the use of such products is still limited [78]. Biocontrol products are not necessarily limited to the use of bacteria and fungi as some algae also have nematicidal characteristics [141]. *Botryocladia cabillaceae* have been reported to reduce *M. incognita* gall and egg mass numbers significantly on soybean roots in Egypt [141].

Of fundamental importance for effective biocontrol is that endemic microbial agents are exploited and that these are adapted to the environmental conditions of a country/climatic zone to enable optimal adaptation and proliferation in the rhizospheres of soybean crops [142]. Such an approach will also prevent a potential 'biological contamination' scenario resulting from the use of exotic microbes that are imported and released within soils, where they may outcompete their native microbe counterparts and negatively affect the preservation of endemic biodiversity and soil health [142,143].

2.8 Conclusions

This review emphasizes the important economic and socioeconomic impacts of soybean in South Africa and the wider SSA, while identifying the major risks posed by *Meloidogyne* and *Pratylenchus* infections. Soybean is a crop that is grown globally and is of great importance to

human and animals as a high-protein-content food source. Although, only as little as 2% of the yearly soybean production is used for direct human consumption [7], the high protein content of this crop has excellent potential for undernourished people worldwide, especially SSA. A recent study showed that 40–65% of people in SSA countries are employed in the agriculture sector, which contributes on average to 25% of the GDP [6]. With the steep increase in the SSA human population, the importance of food security also intensifies, thus contributing to the need for the production of food crops with high protein contents, such as soybean. However, it is evident that the PPN problem faced by soybean production in Brazil has caused major yield losses. It is also known that species of *Meloidogyne* and *Pratylenchus* are co-occurring in soybean fields in South Africa (and other SSA countries) and that such pests might lead to soybean yield losses similar to those experienced in Brazil. The current nematode management practices used in South Africa (highest soybean producer in SSA) might not be sufficient or sustainable enough to deal with the rising PPN threat. An IPM approach for the management of root-knot and lesion nematodes on soybean on a worldwide basis is suggested as a viable option to protect the crop and other crops grown in rotation. A major concern that still faces soybean producers on a local and global scale is the availability of registered environmentally friendly biocontrol products for soybean. It is therefore crucial that both governments and the private sectors of SSA countries invest adequate resources into the research, development, registration, and production of biocontrol products, thereby contributing to food security and preserving the environment. More research is also needed on a local and SSA scale to truly understand the interaction of *Meloidogyne* and *Pratylenchus* communities as well as their associated microbe populations play in soybean rhizospheres, and how the co-occurrence might impact soybean yield losses.

2.9 References

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CHAPTER 3: SCREENING OF RHIZOSPHERE BACTERIA AND NEMATODE POPULATIONS ASSOCIATED WITH SOYBEAN ROOTS IN THE MPUMALANGA HIGHVELD

"If agriculture goes wrong, nothing else will have a chance to go right."

M.S. Swaminathan

3.1 Abstract

Soybean is among South Africa's top crops in terms of production figures. Over the past few years there has been increasingly more damage caused to local soybean by plant-parasitic nematode infections. The presence of *Meloidogyne* (root-knot nematodes) and *Pratylenchus* spp. (root lesion nematodes) in soybean fields can cripple the country's production, however, little is known about the soil microbial communities associated with soybean in relation to different levels of *Meloidogyne* and *Pratylenchus* infestations, as well as the interaction(s) between them. Therefore, this study aimed to identify the nematode population assemblages and endemic rhizosphere bacteria associated with soybean using Next Generation Sequencing (NGS). The abundance of bacterial genera that were then identified as being significant using linear discriminant analysis (LDA) Effect Size (LEfSe) was compared to the abundance of the most prevalent plant-parasitic nematode genera found across all sampled sites, *viz*. *Meloidogyne* and *Pratylenchus*. While several bacterial genera were identified as significant using LEfSe, only two with increased abundance were associated with decreased abundance of *Meloidogyne* and *Pratylenchus*. However, six bacterial genera were associated with decreased *Pratylenchus* abundance. It is therefore possible that endemic bacterial strains can serve as an alternative method for reducing densities of plant-parasitic nematode genera and in this way reduce the damages caused to this economically important crop.

Keywords: bacteria; biological control; *Meloidogyne*; *Pratylenchus*; soybean

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3.2 Introduction

Plant parasitic nematodes (PPN) cause substantial yield losses to agricultural crops, with annual global crop losses estimated at $78 billion [1]. *Aphelenchoides bessseyi*, *Bursaphelenchus xylophilus*, *Ditylenchus dispaci*, *Globodera* spp., *Heterodera* spp., *Meloidogyne* spp., *Naccobus aberrans, Radopholus similis*, *Rotylenchulus reniformis* and *Xiphinema index* are considered the top 10 nematode pests worldwide [2]. Due to their global distribution and wide range of host plants, of all the PPN genera and species, root-knot nematodes (RKN; *Meloidogyne* spp.) and lesion nematodes (*Pratylenchus* spp.) are particularly harmful to crops in South Africa and can cause substantial damage and adversely affect production figures of a wide range of economically important crops, such as the potato, grain, oilseed, industrial and fruit crops produced in this country [3,4]. Of all *Meloidogyne* spp. documented to parasitise crops on a global scale, 22 are reported to occur in Africa [5], while 14 *Meloidogyne* spp. and 10 *Pratylenchus* spp., respectively, have been listed for South Africa [6–9].

In the Mpumalanga Highveld region of South Africa crops that are usually planted include maize (*Zea mays*), wheat (*Triticum* spp.), groundnut (*Arachis hypogaea*), soybean (*Glycine max* (L.) Merr.), sunflower (*Helianthus* spp.) and potato (*Solanum tuberosum*) [10] of which all are known hosts of both RKN and lesion nematodes. Of these crops, soybean is considered an important crop in South Africa, with the Mpumalanga Highveld region being one of the most important production regions [11]. Soybean is one of the most important summer legumes produced worldwide and serves as an important dietary protein and oil source for both animal and human consumption [12,13]. A major benefit of growing soybean is its ability to fix nitrogen, providing an environmentally friendly alternative to synthetic nitrogen application [13]. South African soybean production dates back to the 1960s when production was only 2631 metric tons (MT) [14]. Production of the crop drastically increased since then and during the 2019/2020 growing season the area planted to soybean were estimated at 705,000 hectares (ha) from which 1,245,500 MT seeds were produced. During the following season (2020/2021), South Africa experienced a record crop production, represented by 827,100 ha planted and 1,793,650 MT of seeds harvested [11]. With the local increase in, and expansion of soybean and maize production since the beginning of the century, the risk of the crop being infected by a wide range of diseases and pests was expected [15,16]. The continuous generation of knowledge regarding nematode pests associated with soybean and maize is hence crucial.

Since these crops are usually grown in warmer climates, PPN such as *Meloidogyne* and/or *Pratylenchus* are the genera that cause major damage to these crops [13]. Soybean roots infected by *Meloidogyne* are usually distinguished by the formation of galls which

interfere with several root functions, including water uptake, while roots infected by *Pratylenchus* can be characterised by the formation of necrotic root tissues. These nematode pests also cause various above ground symptoms like stunted growth and reduced leaf size [4,9]. Apart from nematodes, soybean production is also impacted by microorganisms such as bacteria that are present in the soil. Bacteria like *Bradyrhizobium* or *Rhizobium* are applied as a standard practice to increase the nitrogen fixation of soybean while other bacterial genera such as *Bacillus* have the potential to reduce nematode densities and resultant damage due to their nematicidal activities. Chemical nematicides remain one of the most used methods in nematode management, yet increasingly more research is being done to identify and develop eco-friendly products by using bacteria with nematicidal potential [9]. This study was performed in stages, by firstly stratifying the two top PPN in the localities into high, medium and low according to their abundance. Secondly, the bacterial community structure in these strata were determined. Therefore, the aims of this study were: (1) to determine the PPN communities and bacterial rhizosphere communities associated with soybean grown in the Highveld region of South Africa, which is the second biggest local production area for the crop [11] and (2) to determine whether a potential biocontrol link exists between these endemic rhizosphere bacteria and the prevailing PPN communities.

3.3 Materials and Methods

3.3.1 Site description

South Africa is situated between the 22 and 35° S latitudes in the southern hemisphere and is characterised by diverse climatic conditions when compared to most sub-Saharan African countries. Located in Mpumalanga, one of the nine provinces of the country, the Mpumalanga Highveld (where this study was conducted) has a mean annual rainfall of 800–900 mm and an annual temperature range of 6–30° C [10]. The grassland biome of this province, which contains rich and fertile upper layers, together with its annual rain and wide temperature ranges, makes it suitable for cultivation of crops such as soybean. In the 2019 summer growing season, rhizosphere samples (soil and roots) were taken from 15 fields where soybean was grown in the Mpumalanga Highveld of South Africa (Figure 3.1).

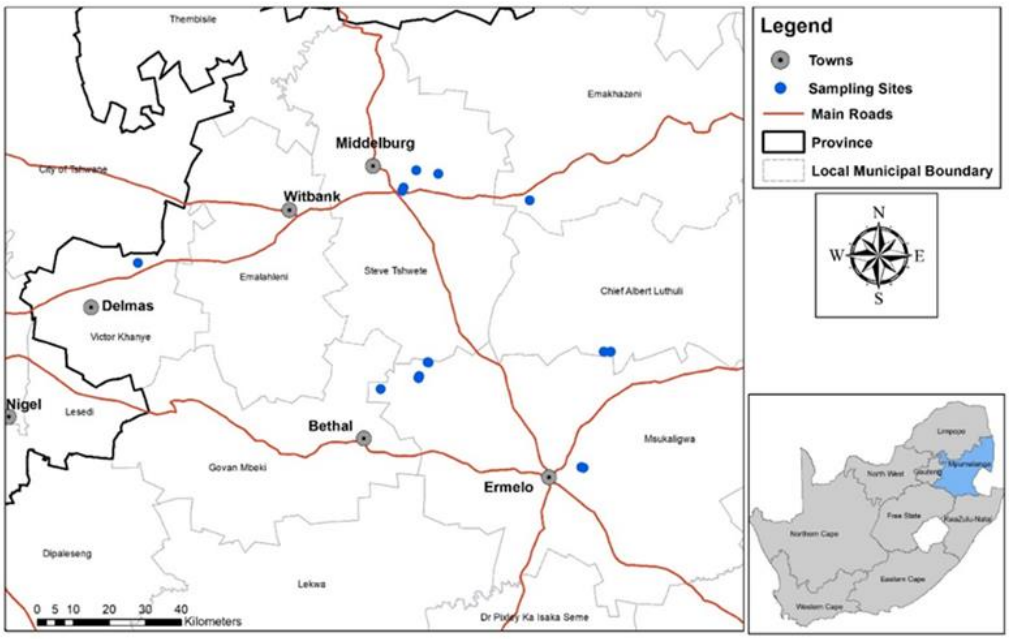

Figure 3.1: Soybean localities, situated in the Mpumalanga province of South Africa, where rhizosphere samples were obtained for nematode and microbe analyses during flowering of the crops in the 2019 summer growing season. (Illustration: Wiltrud Durand, BFAP, GIS & Crop Modelling).

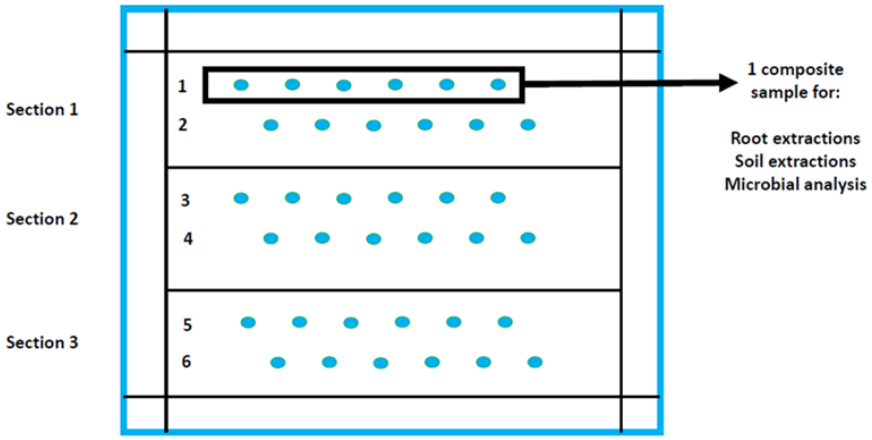

Figure 3.2: Sampling strategy at each of the 15 soybean localities sampled during the 2019 growing season for nematode and microbe analyses (Illustration: Gerhard Engelbrecht, North-West University).

The fields were spread across the province as seen in Supplementary Table (Table S1), from locations located between 1518–1747 m above sea level with maize-soybean rotations being practiced. Each field was divided into three sections depending on the size of the locality (Figure 3.2). Sampling of roots and soil was done in a W shape in each section and the distance between points differed in size according to the size of the locality. Therefore, in each section two rows were selected where the roots and soil (approximately 30 g of soil around the root per plant) of 6 soybean plants were sampled per row. The root samples of each row were cut up into 1 cm pieces, pooled and homogenised before being

used for nematode analyses. Six soil samples collected in each row were also pooled and homogenised, of which 50 g was taken for microbial analyses. This was done for 6 rows (2 rows per section) per field.

3.3.2 Extraction of PPN from Soybean Roots

Nematodes were extracted from 20 g of composite root samples, for each of the fields using the adapted centrifugal-flotation method [17] and transferred to a De Grisse counting dish [18]. The nematodes were counted and concurrently identified to the genus level using an ECLIPSE TS100 inverted microscope (Nikon Corporation, Tokyo, Japan) at 40× magnification.

3.3.3 DNA Extraction of Microbial Communities from the Soil

To extract the DNA of microbial communities from the composite soil samples, 0.25 g of each composite sample was used (Figure 4.2). This was done by using the NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) with the optimal lysis buffer system (a combination of SL 2 and Enhancer SX). The concentration of the extracted microbial DNA (absorbance at 260 nm) and its purity (absorbance ratio 260/230 and 260/280) were measured using a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To ensure the integrity of extracted DNA, it was analysed by means of 1.5% agarose gel electrophoresis in 1 x TAE buffer, containing ethidium bromide (Bio-Rad) was run at 100 V for 30 min [19].

3.3.4 Next Generation Sequencing of the Soil Bacterial Community 16s rRNA

The diversity of the total rhizosphere bacterial community was assessed by next generation sequencing (NGS) of 16S rRNA amplicons obtained from extracted DNA. The first step was to perform a polymerase chain reaction (PCR) with the bacterial primers (linked to the adapter sequences needed for Illumina MiSeq analysis) 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') to amplify the hypervariable regions V3 and V4 of the 16S gene [20]. The thermal conditions were: 95 ◦C for 3 min, 25 cycles of 95 ◦C for 30 s; 55 ◦C for 30 s and 72 ◦C for 30 s and finally followed by 75 ◦C for 5 min. All PCR reactions were done using the 1000 Cycler (BioRad, Hercules, CA, USA) thermal cycler.

All the samples consisted of a total volume of 25 µL. This volume consisted of 1 µL DNA (20–60 ng/µL), 12.5 µL KAPA Hifi Hotstart Ready Mix (2.5 mM $MgCl_2$, 0.3 mM of

each dNTP, KAPA HiFi HotStart DNA Polymerase at 0.5 U per 25 µL reaction) (Roche, Basel, Switzerland); 5 µL (1 µM) of the forward primer, 5 µL (1 µM) of the reverse primer and nuclease free water. To ensure the PCR was successful a 1.5% agarose gel electrophoresis in 1 x TAE buffer, containing ethidium bromide (Bio-Rad) was run at 100 V for 30 min.

This was followed by the first PCR clean-up with Agencourt AMPure XP beads (Beckman Coulter Genomics, Chaska, MIN, USA) to purify the amplicons and eliminate free primers and primer dimers. After the first product clean-up, a second PCR with limited cycles were performed to attach dual-index barcodes to the amplicons (Nextera XT Index Kit, Illumina, San Diego, CA, USA) as recommended by the library preparation protocol from Illumina [21]. A second PCR clean-up was performed to clean up the library before quantification. The libraries were quantified with a fluorescence-based method (Invitrogen) using a Qubit 3.0 (Life Technologies, Carlsbad, CA, USA) before normalization and pooling to 4 nM. The pooled library (5 pM) was denatured and 2 × 300 bp paired-end sequencing was conducted with a MiSeq V3 600 cycle reagent cartridge (Illumina) on an Illumina MiSeq according to the manufacturer's instructions.

3.3.5 NGS Data Bio-Informatics Analysis

Demultiplexing of reads was performed using the on-board MiSeq reporter software (Illumina). The Quantitative Insights into Microbial Ecology 2 (QIIME2) pipeline [22] was used for the processing of NGS data. The quality of reads was evaluated and filtered with demux for elimination of random sequencing errors, deletion of unreliable data from the libraries and removal of reads shorter than 200 bp. Based on the quality control parameters for DADA2, sequences were adjusted and forward and reverse reads assembled. The assembled reads were classified into amplicon sequence variants (ASV) using the feature classifier from QIIME2 software.

The processed sequences were aligned against the SILVANGS rRNA database (SILVA 132 release) [23] for taxonomic assignment. The generated ASV count table was summarized in QIIME2. Graphs of statistically significant bacteria were done using STAMP [24]. Metagenassist was used to do taxonomic to phenotype mapping [25]. MicrobiomeAnalyst was used to do abundance analysis between various stages [26,27]. Using this online tool, alpha diversity was produced using the Chao1 and Shannon diversity indices. With regards to beta-diversity, results were generated using the Bray-Curtis dissimilarity distance distribution. In order to detect the genera with significant differential abundance among the sample fields, linear discriminant analysis (LDA) Effect size (LEfSe) was used [28].

3.3.6 Statistical Analysis of Nematode and Microbial Data

Plant-parasitic nematode population assemblages extracted from the six 20 g composite root samples per field, were pooled and the frequency of occurrence, mean population density (MPD) and prominence value (PV) of each nematode genus calculated [15,29]. Frequency of occurrence was calculated as: (number of localities at which the genus occurred in the root and soil sub-samples of each cultivar/number of localities sampled) × 100. To determine the mean population density (MPD) at each field the total number of individuals of a genus present in root samples of each field was divided by the number of localities in which the genus occurred in root samples. Finally, to determine the prominence value (PV) the mean population density of each genus was multiplied by the frequency of occurrence and divided by 100. Density classification for *Meloidogyne* and *Pratylenchus* were done as follows: low $x \leq 600$; medium $601 \leq x \leq 2999$ and high $x \geq 3000$ (x = individuals per 20 g roots).

The alpha diversities of microbial communities, reflected by the bacterial abundance and diversity with regards to the population densities of *Meloidogyne* and *Pratylenchus* individuals in 20 g of soybean roots for each field, were demonstrated using Chao1 boxplots (abundance of bacterial ASV) and Shannon boxplots (community richness). A high Chao1 index indicates a high level of species richness, while a high Shannon index indicates a high level of diversity. Non-metric multidimensional scaling (NMDS) diagrams were used to show the differences between the various rhizosphere microbial communities, beta diversity, of the sample localities. The population densities of *Meloidogyne* and *Pratylenchus* were based on the number of individuals per 20 g of roots. Since the diversity of bacterial communities in each of the fields has its own unique taxonomic abundance profile, the fields with similar taxonomic profiles will group together. Similarities or differences in taxonomic profiles were determined by the Bray-Curtis dissimilarity distance distribution which uses read counts of the bacterial communities. Fields that are plotted close to zero indicate similar taxonomic abundance profiles, whereas sites that don't plot close to zero have different taxonomic profiles.

Differences in bacterial genus abundance with regards to the population densities of *Meloidogyne* and *Pratylenchus* were evaluated using the LEfSe algorithm [30]. The LEfSe was done using the following parameters: an LDA score of 1 and a cut off *p*-value of 0.05. To determine the link between the abundance of the rhizosphere bacteria that were identified using LEfSe and nematode densities, a functional response model described by Holling [31] was used. The abundances of *Meloidogyne* and *Pratylenchus*, respectively, were compared to the sequence read count (SRC) of each individual bacterial genus ASV to determine

whether an increase in the abundance of a certain bacterial genus might cause a decrease in the abundance of the respective said nematode genera. A Windows-based program (CANOCO version 5, Microcomputer Power, Ithaca, NY, USA) was used to generate the response graphs.

3.4 Results

3.4.1 PPN Associated with Soybean Roots

Eleven PPN genera were identified, while those individuals that could not be identified to genus level were listed as belonging to the Order Tylenchida and/or the families Aphelenchoididae and Criconematidae (Table 3.1). The highest number of nematode genera (9) was present at S9 and S15, while S2 had the lowest number of nematode genera (three) present (Table 3.1). Of the 12 genera present across the 15 fields, only *Meloidogyne* and *Pratylenchus* were present in each of these localities (Table 3.1). The highest number of *Meloidogyne* spp. (Table 3.1) was present at S7 (24,402 individuals/20 g of roots), with S14 having the lowest (183 individuals per 20 g of roots).

With regards to *Pratylenchus* spp., S18 (7851 individuals/20 g of root) and S11 (107 individuals/20 g of root) had the highest and lowest levels, respectively. The PV the nematode genera in all 15 fields ranged from 7 (*Ditylenchus*) to 5291 (*Meloidogyne*) (Table 4.2). The MPD of *Meloidogyne* were the highest (5291) with *Ditylenchus* and Tylenchida both having the lowest MPD of 28 (Table 3.2). Some of the other nematode genera present in root samples from the Highveld region were observed in only a few fields. These were *Tylenchorhynchus*, *Ditylenchus*, *Rotylenchus*, *Tylenchus* and nematodes belonging to the Order Tylenchida, and the individuals of the Aphelenchidae and Criconematidae families. Individuals belonging to the genera *Tylenchus* and *Tylenchorhynchus* as well as those identified as belonging to the Criconematidae family are usually ectoparasitic [32] and were potentially feeding actively on the roots when sampling and extractions were done.

Table 3.1: The community structure and abundance of plant parasitic nematodes in 20 g soybean root samples collected during the 2018/19 growing season from 15 fields of commercial producers in the Highveld region of the Mpumalanga province of South Africa.

Genus and/or family	**Field no.**														
	S1	**S2**	**S5**	**S6**	**S7**	**S8**	**S9**	**S11**	**S12**	**S13**	**S14**	**S15**	**S16**	**S17**	**S18**
Meloidogyne	344	2548	3625	1141	24402	4913	4980	7400	999	5097	183	21757	1059	518	394
Pratylenchus	518	9350	550	784	3584	655	1826	107	243	4331	270	1004	229	335	7851
Helicotylenchus	44	132	28	28	170	28	248	87	28	28	34	110	83	28	0
Scutelonema	87	0	66	66	101	34	83	38	110	41	77	41	41	50	28
Hoplolaimus	96	0	37	105	89	28	118	72	96	34	69	65	143	57	0
Rotylenchulus	28	0	28	34	60	46	1000	62	37	0	55	41	0	0	0
Tylenchorhynchus	0	0	0	0	0	0	37	14	0	0	0	193	0	7	69
Ditylenchus	0	0	0	0	28	0	0	0	0	0	0	0	0	0	0
Rotylenchus	0	0	0	0	0	0	39	0	0	94	0	0	0	0	37
Tylenchus	0	0	0	0	0	28	55	0	28	0	0	0	0	0	46
Tylenchida	0	0	0	0	0	0	0	0	28	0	0	28	0	0	0
Aphelenchidae	28	0	28	0	0	0	0	0	0	28	0	97	0	0	0
Criconematidae	0	0	0	28	0	0	0	14	0	0	0	0	28	0	55
Level of *Meloidogyne* infection	Low	Medium	High	Medium	High	High	High	High	Medium	High	Low	High	Medium	Low	Low
Level of *Pratylenchus* infection	Low	High	Low	Medium	High	Medium	Medium	Low	Low	High	Low	Medium	Low	Low	High

Table 3.2: Prominence values, frequencies of occurrence and mean population densities of plant parasitic nematode genera occurring in 20 g soybean root samples collected during the 2018/19 growing season from 15 fields of commercial producers in the Highveld region of the Mpumalanga province of South Africa.

Genus and/or family	[b]Mean population density (MPD)	[a]Frequency of occurrence (FO)	[c]Prominence value (PV)
Meloidogyne	5291	100	5291
Pratylenchus	2109	100	2109
Helicotylenchus	77	93	74
Scutelonema	62	93	60
Hoplolaimus	78	87	72
Rotylenchulus	139	67	113
Tylenchorhynchus	64	33	37
Ditylenchus	28	7	7
Rotylenchus	57	20	25
Tylenchus	39	27	20
Tylenchida	28	13	10
Aphelenchidae	45	27	23
Criconematidae	31	27	16

[a]FO = (Number of samples containing genus/number of samples collected) x100

[b]MPD= total number of individuals of a genus present in root samples of each site/number of localities in which the genus occurred in root samples of each site

[c]PV= MPD x √absolute frequency)/100

3.4.2 Rhizosphere Bacterial Community Associated with Soybean

3.4.2.1 Alpha Diversity

The boxplots in Figures 3.3 and 3.4 represent the alpha diversities which are reflective of the bacterial abundance and diversity of *Meloidogyne* and *Pratylenchus* individuals in 20 g of soybean roots for each field (Table 3.1). In Figure 3.3 the Chao1 index reveals that sites with high levels of *Meloidogyne* had higher levels of bacterial ASV abundance (±290–300), whereas sites with low levels of *Meloidogyne* had the lowest bacterial ASV abundance. Yet, when compared to the Chao1 index of Figure 3.4, it is evident that sites with lower *Pratylenchus* densities had higher levels of bacterial ASV abundance, while sites with higher *Pratylenchus* densities had the lowest bacterial ASV abundance. With regards to the species diversity (Shannon index), Figure 3.3 showed that sites with the highest *Meloidogyne* densities had less diverse bacterial communities (±4.56–4.69) as compared to those with low and medium densities of *Meloidogyne*. In the case of *Pratylenchus*, (Figure 3.4) it was evident that sites with medium densities of *Pratylenchus* showed higher bacterial diversity (±4.56–4.75) compared to those with high and low densities of *Pratylenchus.*

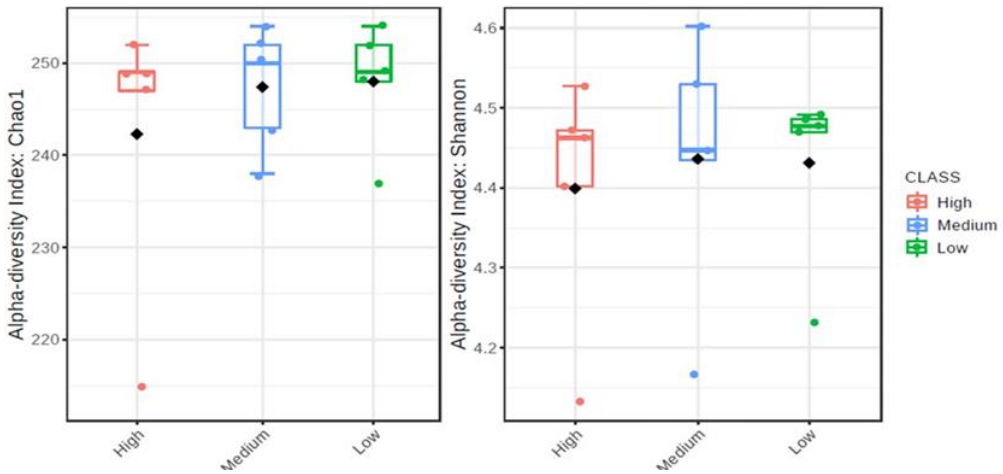

Figure 3.3: The alpha diversities with regards to *Meloidogyne* infection in soybean roots from the Highveld production area (Mpumalanga province) in South Africa presented as boxplots. The data was plotted with the Chao1 and Shannon diversity indices with $p<0.05$; the median as well as highest and lowest values are indicated on each boxplot.

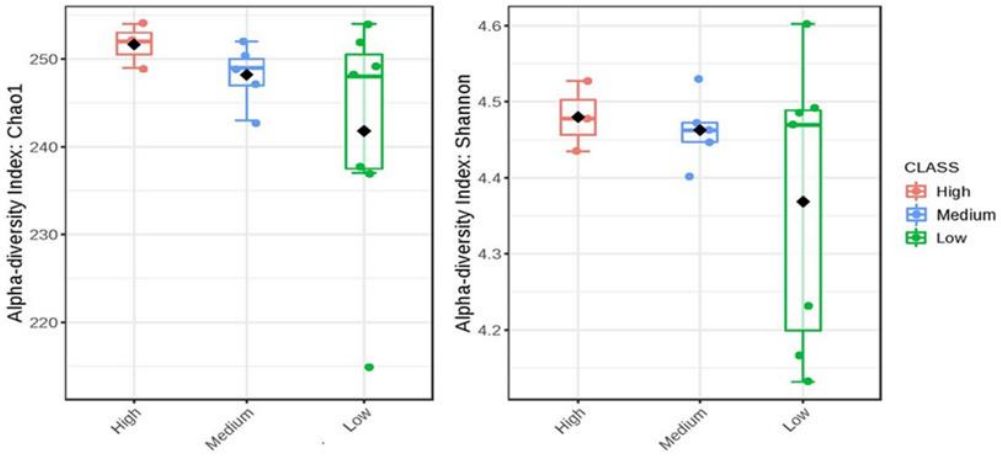

Figure 3.4: The alpha diversities with regards to *Pratylenchus* infection in soybean roots from the Highveld production area (Mpumalanga province) in South Africa presented as boxplots. The data was plotted with the Chao1 and Shannon diversity indices with p<0.05; the median as well as highest and lowest values are indicated on each boxplot.

3.4.2.2 Beta Diversity

The non-metric multidimensional scaling diagram (Figure 3.5) shows the differences between the various rhizosphere microbial communities of the fields sampled. From Figure 3.5 it is evident that the following fields, S17 and S18, did not group with the others resulting in each of these fields having a different taxonomic microbe profile when compared to those of the other fields. Most of the fields grouped relatively close to zero on both the *x* and *y*-axis, indicating that they share similar bacterial taxonomic profiles.

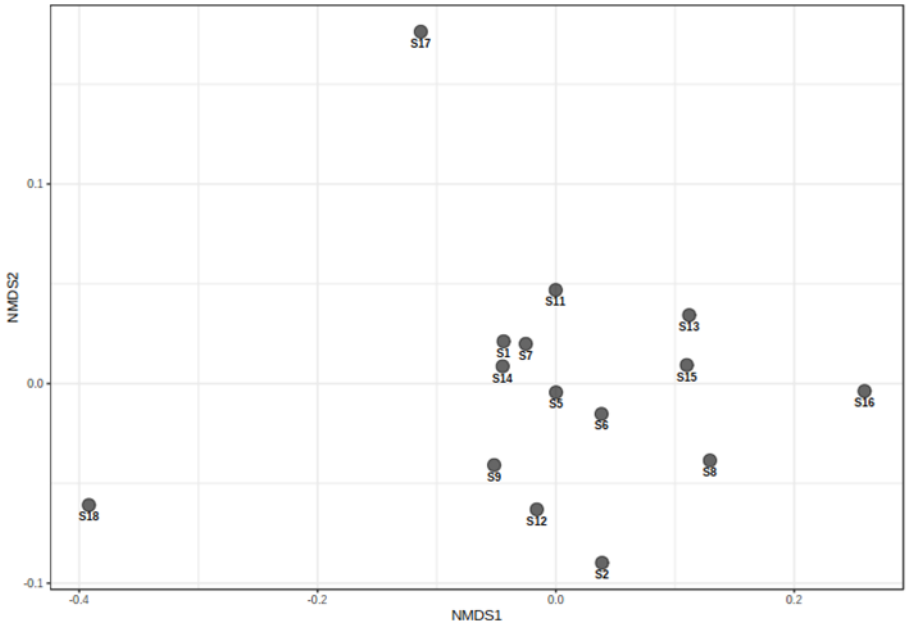

Figure 3.5: The NMDS diagram shows the beta-diversity of microbe communities among 15 soybean fields sampled from the Highveld region, Mpumalanga province, South Africa. The statistical method used to analyze group similarities was ANOSIM (p<0.85) and applied a Bray-Curtis dissimilarity distance distribution with the sample sites using a correction of R=-0.12262.

3.4.2.3 Bacterial Populations Associated with Soybean

All the NGS sequences obtained for bacterial populations from soil collected from the 15 fields sampled could be divided into 15 phyla (Figure 3.6), 47 classes, 55 orders, 91 families and 148 genera. Actinobacteria (33.88%) was the most abundant phyla across the 15 fields followed by Proteobacteria (25.14%). The least abundant phyla were Cyanobacteria (0.11%) Latescibacteria (0.09%) and Chlorobi (0.02%).

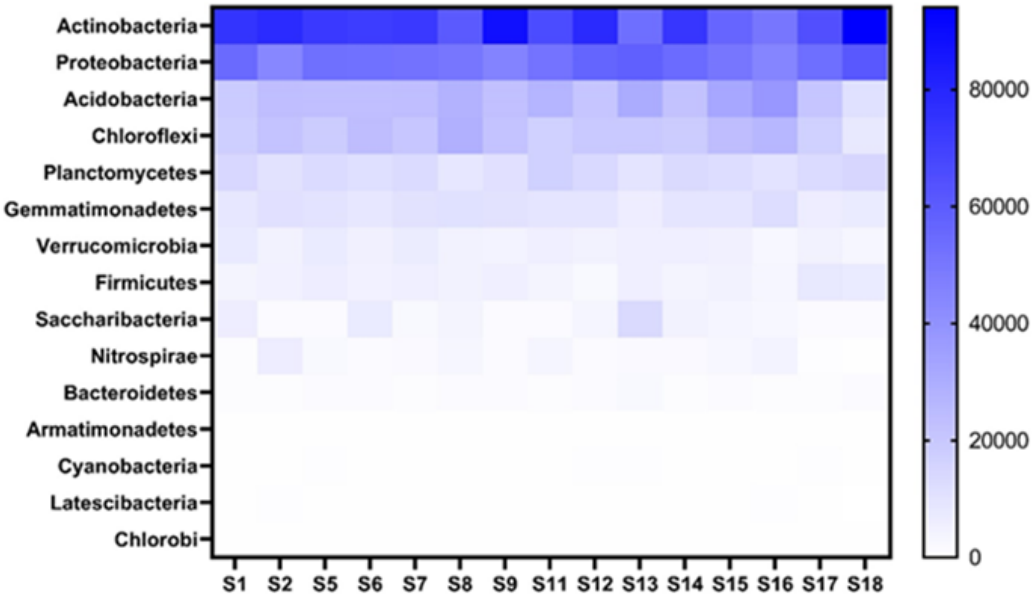

Figure 3.6: Heatmap indicating the top bacterial phyla and their abundance associated with the soybean rhizosphere of the 15 fields (S1-S18) sampled from the Highveld region, Mpumalanga province, South Africa. The intensity of the blue colour indicates the abundance, with darker colours being more abundant.

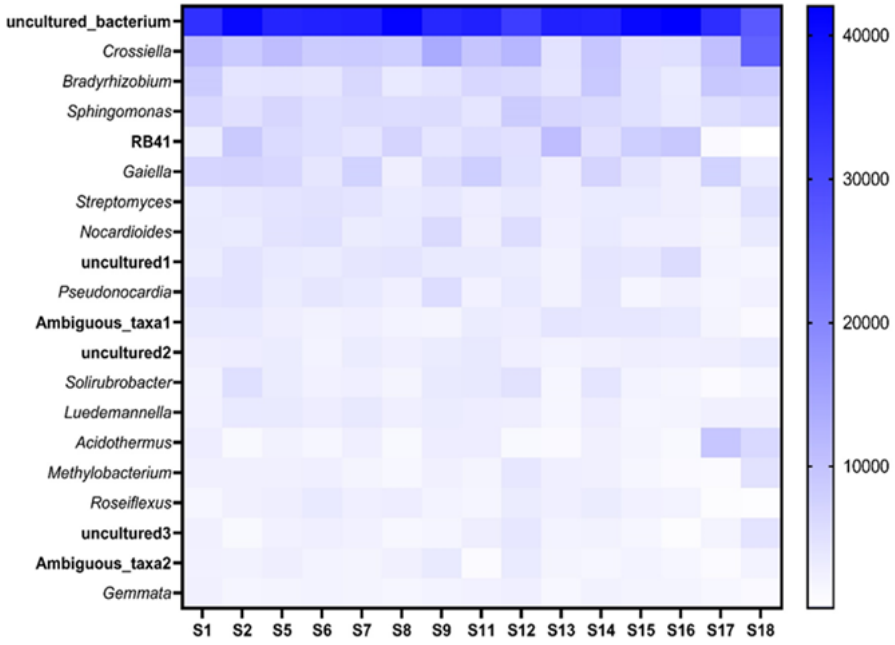

Figure 3.7: Heatmap indicating the top 20 bacterial genera and their abundance associated with the soybean rhizosphere of the 15 fields (S1-S18). The intensity of the blue colour indicates the abundance, with darker colours being more abundant.

Figure 3.7 indicates the top 20 genera present across all fields. Of these genera uncultured_bacteria and *Crossiella* had the highest abundance across all the fields. Some genera such as *Bradyrhizobium*, *Sphingomonas* and *Acidothermus* amongst others could be identified while there were many genera that could not be identified with the database used and were listed as uncultured. Of the 148 genera identified, all the similar genus names were assigned a number to identify which of these uncultured bacterial genera is being referred to in further analysis.

3.4.2.4 Linear Discriminant Analysis (LDA) Effect Size (LEfSe)

A total of 9 bacterial genera (Table 3.3) were found to be significantly more abundant in the soybean rhizospheres of plants sampled for this study. With regards to *Meloidogyne* densities, the genera *Bacillus*2 (p = 0.01) and uncultured15 (p = 0.03) had significantly higher abundances in fields with medium densities of *Meloidogyne* (Table 3.1). However, the genera *Gemmata*1 (p = 0.02), *Streptomyces*2 (p = 0.04), *Roseiflexus*2 (p = 0.034), *Pirellula*3 (p = 0.034) and Ambiguous_taxa10 (p = 0.007) had significantly higher abundances in fields with low densities of *Pratylenchus*. Moreover, the genera uncultured15 (p = 0.025) and uncultured30 (p = 0.0355) as well as Ambiguous_taxa16 (p = 0.026) were significantly more abundant in fields with medium densities of *Pratylenchus.*

Table 3.3: Classification of bacterial genera that were significantly more abundant in the rhizosphere of the 15 soybean fields (sampled from the Highveld region, Mpumalanga province, South Africa) used in this study according to LefSe.

Phylum	Class	Order	Family	Genus
Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Ambiguous_taxa10
Proteobacteria	Deltaproteobacteria	Myxococcales	Sandaracinaceae	uncultured15
Firmicutes	Bacilli	Bacillales	Bacillaceae	Ambiguous_taxa16
Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	*Gemmata*1
Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	*Pirellula*3
Chloroflexi	Chloroflexia	Chloroflexales	Roseiflexaceae	*Roseiflexus*2
Firmicutes	Bacilli	Bacillales	Planococcaceae	uncultured30
Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	*Streptomyces*2
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*2

3.4.3 Potential Link between Significantly Abundant Rhizosphere Bacteria and PPN Population Density

Of the 9 bacterial genera that were identified using LEfSe, only Ambiguous_taxa16 (Hyphomicrobiaceae family) was associated with high abundance of both *Meloidogyne*

and *Pratylenchus* (Figure 3.8). In fact, where a high abundance of the Ambiguous_taxa16 (±300 ASV) was evident, both *Meloidogyne* (±8000 individuals) and *Pratylenchus* (±4000 individuals) abundances were also high. Furthermore, from the 9 genera identified using LEfSe, high abundances (SRC) of 6 were associated with low *Pratylenchus* densities (Figure 3.9). However, of these 6 genera, a high abundance (SRC) of uncultured15 was associated with the lowest *Pratylenchus* abundance. Although the high abundance of these bacterial genera correlated with high *Meloidogyne* densities, high abundance of in *Gemmata*1 (Figure 3.9b) were inversely correlated with *Meloidogyne* densities. An SRC count of ±10,000 for *Gemmata*1 was associated with ±8000 *Meloidogyne* individuals (Figure 3.10b), compared to an SRC count of ±5000 for both *Bacillus*2 (Figure 3.9a) and *Streptomyces*2 (Figure 3.9d), associated with ±12,000 *Meloidogyne* individuals.

The two remaining genera Ambiguous_taxa10 (Figure 3.10a) and *Roseiflexus*2 (Figure 3.10b) were the only two of which high abundances (SRC) were associated with low densities of both *Meloidogyne* and *Pratylenchus*. Of these two genera, high SRC of *Roseiflexus*2 (Figure 3.10b) were associated with the lowest *Meloidogyne* densities. A *Roseiflexus*2 SRC count of ±600 was, for example, associated with *Meloidogyne* densities of ±2000 individuals. In comparison Ambiguous_taxa10 with an SRC count of ±800 was associated with *Meloidogyne* densities of ±3500 individuals. In both cases of Ambiguous_taxa10 (Figure 3.10a) and *Roseiflexus*2 (Figure 3.10b), low *Pratylenchus* densities were found to be associated with increased abundances of these genera.

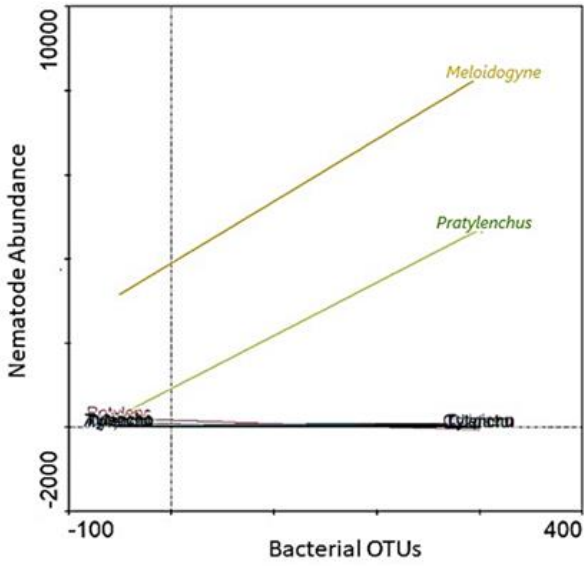

Figure 3.8: A functional response graph showing the correlation between the abundance (Bacterial OTUs) of the genus Ambiguous_taxa16 and the abundance of *Meloidogyne* (orange line) and *Pratylenchus* (green line) that were extracted from soybean roots sampled from the Highveld production area in the Mpumalanga province, South Africa.

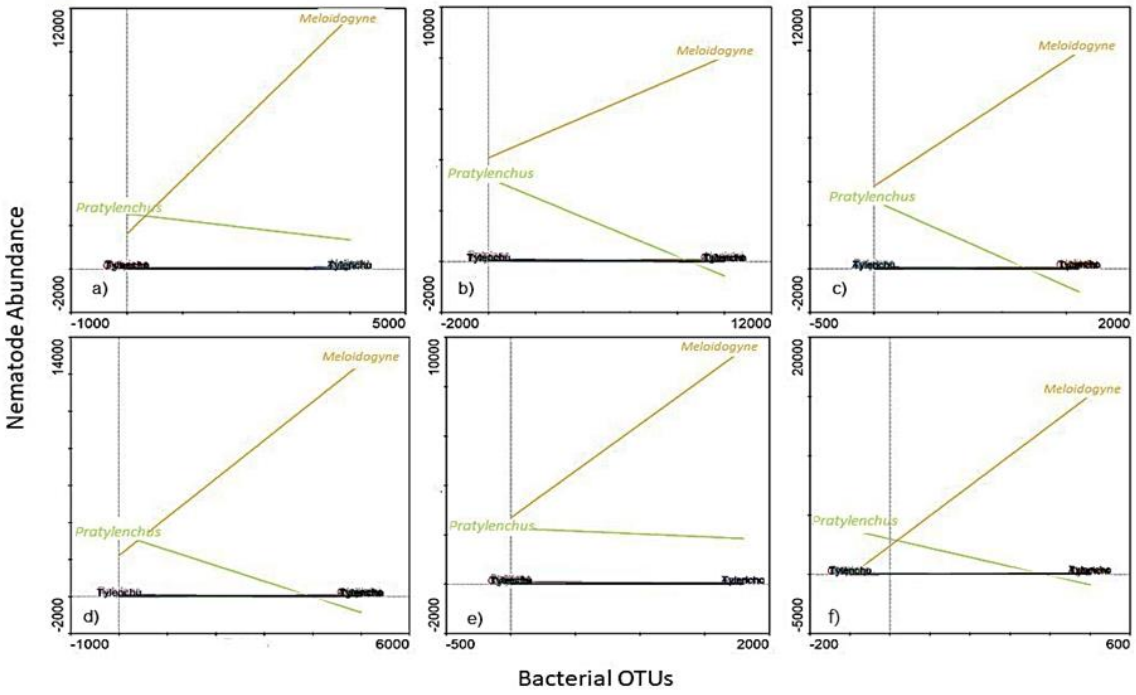

Figure 3.9: A functional response graph showing the correlation between the abundance (Bacterial OTUs) of the genera *Bacillus*2 (a), *Gemmata*1 (b), *Pirellula*3 (c), *Streptomyces*2 (d), uncultured15 (e), uncultured30 (f) and the abundance of *Meloidogyne* (orange line) and *Pratylenchus* (green line) that were extracted from soybean roots sampled from the Highveld production area in the Mpumalanga province, South Africa.

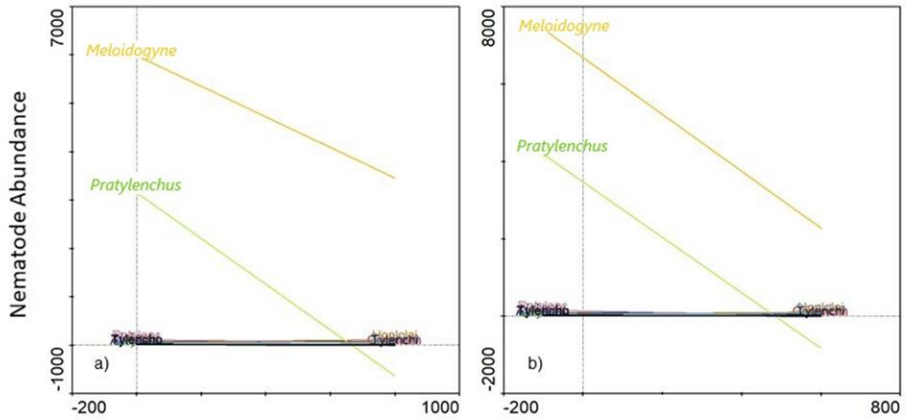

Figure 3.10: A functional response graph showing the correlation between the abundance (Bacterial OTUs) of the genera Ambiguous_taxa10 (a), *Roseiflexus*2 (b) and the abundance of *Meloidogyne* (orange line) and *Pratylenchus* (green line) that were extracted from soybean roots sampled from the Highveld production area in the Mpumalanga province, South Africa.

3.5 Discussion

The current study reports a similar number of PPN genera associated with soybean roots (11) than the 11 reported in 2020 [33] and more than the 7 genera previously reported in 2001 to be associated with soybean in South Africa [15]. This can likely be explained by the improved, adapted methods used for PPN extraction from soybean roots—during this study the protocol of Swart and Marais [17] was used. Also, the expansion of soybean production compared to the beginning of the century when a previous study [15] was done could add to the

explanation of this phenomenon. However, when results from this study were compared to the results from a previous study [15], the predominant endoparasites were still found to be *Meloidogyne* and *Pratylenchus* spp. on both occasions. One of the most important observations that was made is the high PV of both the *Meloidogyne* and *Pratylenchus* genera (Table 3.2). The PV of *Meloidogyne* (Table 3.2) was higher when compared to that reported by previous studies [15,34]. Crop rotation used in the Mpumalanga Highveld region, especially in the fields used in this study, usually include soybean rotated with grain crops such as maize (*Zeae mays* L.) [35], which is also susceptible RKN. This rotation practice therefore contributes to aggravated strain being placed on the sustainable crop production of grain and legumes in this region.

Moreover, other factors that are not known to the authors could have impacted on the higher PVs of these two genera in the Highveld region compared to those of the 2001 study. With regards to the PV of *Pratylenchus* (lesion nematode), this study reports similar findings to that of Mbatyoti [34]. Although *Pratylenchus* was not considered to be an important pest of soybean [36], recent studies have found that *Pratylenchus* spp. severely impact soybean, causing potential losses of up to 85% in some cases [34,37]. The high PV of *Pratylenchus* in this study might also be caused by the rotation practices used. It has been reported that rotation of soybean with maize in Brazilian production areas, favoured the reproduction of *P. brachyurus* [37] and this might have similar effects in South African production areas, such as the Mpumalanga Highveld. Moreover, the common practice of using maize and grain legumes in rotation in the Mpumalanga Highveld will therefore contribute to higher RKN and lesion nematode population densities since these crops have been found to all being susceptible to the two predominant nematode pest genera [10,35]. The impact of climate change is another factor that should be considered in terms of higher abundance of the two predominant endoparasitic nematode genera found in Mpumalanga Highveld study since combined changes in temperature and moisture is for example factors that will and is foreseen to impact on plant-parasitic nematode abundance [38]. Due to high population densities of both RKN and lesion nematodes in soybean roots, more studies are needed that are aimed at the impact of the co-occurrence of these harmful PPNs on soybean yields. The use of poor-host or resistant cultivars of soybean and rotation crops has until recently generally been the only way to reduce PPN numbers in local farmer's fields. Such a strategy requires that commercially available cultivars are annually screened for their host status to target nematode pest species and that resistance to such pests be introgressed into high yielding genetic material to develop high levels of resistance. This is, however, not receiving priority and therefore this approach cannot be used optimally. Furthermore, Velum 1GR (a.i. fluopyram) has recently been officially registered for use on soybean in

South Africa representing the only nematicide available for use by producers [39]. An alternative and/or supplementary approach to the non-optimal use of poor-host or resistant cultivars and limited chemical control options can be the use of endemic biological control agents such as bacteria or fungi. However, management of PPN remains difficult [40]. Although chemicals remain the most common method for RKN management [41], many have elevated levels of toxicity contributing to environmental and human safety concerns. Various chemical nematicides are also increasingly being removed from international markets [42]. This calls for the urgent development of more environmentally friendly PPN control methods.

Previous studies have shown that factors such as the crop that was planted, soil type and the root exudates of the cultivated plants can affect the bacterial community structure of the rhizosphere microbiome by changing the physical and chemical properties of soil [43–45]. Although the alpha diversity of rhizosphere bacterial communities with regards to either *Meloidogyne* or *Pratylenchus* were found to be different, the beta diversity (Figure 3.5) and therefore, the taxonomic profile of the fields was relatively similar. Only the taxonomic profiles of S18 and S17 were different compared to those of the other localities. The difference in the taxonomic profile of S18, when compared to other localities, might be caused by the monocropping of soybean at this locality. Yet, S17 also had a different taxonomic profile, although the reason for this might not be attributed to monocropping like that of S18, but to other complementary and unknown factors warranting further analysis. However, although the differences in the alpha diversity observed in this study were not significantly different, it might be caused by several factors including the root exudates of soybean plants. Root exudates are known to influence rhizosphere bacterial assemblages [43]. As the soybean plants respond to varying PPN densities, the root exudates can act as attractants/stimulants as well as inhibitors/repellents, which may have a profound effect on rhizosphere bacteria potentially causing the observed differences in bacterial diversity and richness [46].

The phyla that were identified in this study were very similar to those reported [47] when examining the soybean rhizosphere in Kyoto, Japan and that of soybean fields across China [48]. However, in these studies Proteobacteria was identified as the most abundant phylum. *Bradyrhizobium*, *Sphingomonas*, *Bryobacter* and *Streptomyces* were identified from the top 20 listed genera in the soybean rhizosphere in two similar studies [33,49]. There are microorganisms present in the soil that are not pathogenic towards plants and of the bacterial genera identified in Figure 3.8, *Bradyrhizobium* and *Sphingomonas* are examples of these. *Bradyrhizobium*, a nitrogen-fixing symbiont of legumes, would usually be abundant

in higher numbers when analyzing the rhizosphere of legumes [50] and explains the high abundance of this genera reported in this study. The plant growth-promoting endophytic bacteria (PGPEB) *Sphingomonas* can occur in diverse environments. Together with its plant growth promoting capabilities, this genus can also decompose various pesticides such as those that contain the active ingredient cypermethrin [51,52]. Other reports suggest that bacteria belonging to the genera *Methylobacterium* have been found in soils that are suppressive against the genus *Meloidogyne* in vegetable production sites in Grossbeeren, south of Berlin, Germany [53] and sites with a history of RKN infestation in Spain [54]. This corresponds with the abundance of *Methylobacterium* identified in the localities investigated in this study. The *Bacillus* genus has been associated with the soybean rhizosphere and promotes its plant growth [55] as well as being present in soils with low densities of *P. neglectus* and *M. chitwoodi* in potato farms of the San Luis Valley, Colorado, USA [56]. Other genera such as *Gemmata*, *Streptomyces* and *Roseiflexus* have also been reported from the rhizosphere of soybean fields in Kyoto, Japan and the Heilongjiang Province of China [48,57]. Furthermore, although a previous study found that bacteria belonging to genera such as *Lysobacter*, *Steroidobacter*, *Flavobacterium*, *Chryseobacterium* and *Flexibacter* were present in soils with low densities of *Meloidogyne*, none of these genera were identified as significant in this study [54].

Several studies have reported the presence of the *Gemmata* genus [58] in environments ranging from bogs in Russia [59], a compost heap in Northern Germany [60] as well as a water spring in South Africa [61]. Although these studies did not aim to study the nematicidal potential of this genus, a study done [62] found that *Gemmata obscuriglobus* is capable of polyketide and non-ribosomal peptide synthesis. These compounds can activate plant defences and contribute to a potential decrease in nematode infections [63,64]. In a study done in China [47], they compared the rhizosphere of soybean and another legume plant, alfalfa (*Medicago sativa*) and found that the genus of the Planctomycetes phylum, *Pirellula* to be more abundant in the rhizosphere of alfalfa than that of soybean. A strain of this genus, also known as *Rhodopirellula*, has been identified in the soybean root endosphere. This genus was found to be present in soybean monoculture systems in north-eastern China with suppressive effects against the soybean cyst nematode, *Heterodera glycines* [65]. It is possible that a higher abundance of several bacterial genera, such as those mentioned above, might cause reduced levels of parasitic nematodes like *Meloidogyne* and *Pratylenchus*. The identification of such bacterial genera and their abundance will therefore provide valuable information regarding bacteria that might be used as potential biocontrol agents in nematode management.

It has been reported that the *Streptomyces* genus has high abundances in the soybean rhizosphere [66]. This genus has been reported to be suppressive against *Fusarium* wilt disease [67] as well as the soybean cyst nematode, *Heterodera glycines* [68] and the RKN, *M. incognita* [69,70]. A novel strain belonging to the *Streptomyces* genus was also isolated from nematode-suppressive soil in Costa Rica [71]. Furthermore, *Streptomyces* spp. were found to have suppressive effects against the lesion nematode, *P. penetrans* that parasitises alfalfa in Minnesota and Wisconsin field soils [72]. In the case of *Bacillus*2 (Figure 4.9a), this genus belongs to the family Bacillaceae. Various species of the genus *Bacillus* has been known to have nematicidal activity against harmful nematode pests such as *Meloidogyne* spp., *Pratylenchus* spp. and *Heterodera* spp. Amongst these are *B. pumilus*, *B. megaterium*, *B. thuringiensis* and *B. soli* [69,73–76].

Ambiguous_taxa10 belongs to the Hyphomicrobiaceae family, which has been identified in soybean monoculture systems in Minnesota, USA [77], with the genus *Rhodoplanes* (Hyphomicrobiaceae family) identified in potato farms of the San Luis Valley (Colorado, USA). However, *Rhodoplanes* was found to be positively correlated with *M. chitwoodi* in a previous study. Yet, our results suggest that the abundance of the Ambiguous_taxa10 genus, belonging to the Hyphomicrobiaceae family, shows a negative correlation with relation to both *Meloidogyne* and *Pratylenchus* densities, contrasting results previously reported [56]. The relation of *Roseiflexus*2 (Figure 3.10b) abundance towards *Meloidogyne* and *Pratylenchus* densities proves quite interesting, as this genus has been found to be related to uncultivated filamentous phototrophic bacteria, predominately present in microbial mats of hot springs [78]. To our knowledge, there has not been any reports of the potential nematicidal activity of this genus and future studies will thus generate novel information in this regard.

3.6 Conclusions

Plant-parasitic nematodes cause extensive losses to various economically important crops in South Africa, including soybean. Notably, most research has been done on species of PPN genera such as *Heterodera*, *Meloidogyne* and *Pratylenchus* and their potential impact on soybean production. There are various control strategies such as nematicides, both chemical and biological, that can be used to manage the impact of the PPN. However, more research is being done on the use of microorganisms as potential biocontrol agents of nematodes to fill the gap left by the removal of various chemical nematicides from the international markets. Research relating to biocontrol remains challenging as the nematicidal effects observed for microbes in *in vitro* studies often fail to reproduce upon the reintroduction of these strains into field studies [79]. While the identification of bacterial

strains with nematicidal activity *in vitro* remains helpful, DNA based classification of the microbiomes associated with the natural rhizosphere of soybean plants with low PPN densities can provide a more comprehensive understanding of bacteria with nematicidal activity in such an environment. Since less than 1% of bacterial spp. can be cultivated in a laboratory [80,81], 16S rRNA gene amplification and more recently NGS have emerged as powerful tools that can be used to study microbial populations [81]. Even so, identification of genera still proves difficult, resulting in numerous genera not being identified. A possible explanation for the observations made in this study, with regards to bacterial SRC as well as *Meloidogyne* and *Pratylenchus* densities might be a result of competition between organisms (including both bacteria and nematodes). This is caused by the environmental conditions or mixtures of different bacteria having various nutritional and environmental requirements that influences certain metabolic capabilities of these bacteria [82,83], potentially causing changes in their nematicidal activity. In a similar study [57], the authors concluded that a consortium of bacteria with nematicidal properties can exist on a spatial scale within a field of soybean that is infected by RKN. There could then be a possibility of identifying several biological control agents that are potentially available *in situ* without introducing any “foreign” bacterial strain(s). Improving our understanding of the natural rhizosphere bacterial and fungal communities and their relationship with both the plant and nematodes will help unravel the natural microbiome structure needed for biocontrol of PPN.

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3.8 Supplementary data

Table S1: Details of 15 fields of commercial producers in the Highveld region of South Africa where soybean rhizosphere (root and soil) samples were collected during the 2018/2019 growing season for nematode and microbe analyses

Field	GPS Coordinates	Altitude (m)	Crop rotation history prior to 2018/2019
S1	25°50'04.4"S, 29°54'02.2"E	1747	Soybean-Maize-Soybean
S2	25°49'28.2"S, 29°32'42.6"E	1604	Maize-Maize-Maize
S5	26°19'08.7"S, 29°30'27.2"E	1625	Maize-Soybean-Soybean
S6	26°14'50.4"S, 29°38'09.7"E	1661	Maize-Soybean-Maize
S7	26°14'52.6"S, 29°38'18.8"E	1652	Maize-Soybean-Maize
S8	26°16'59.3"S, 29°36'48.4"E	1659	Soybean-Soybean-Maize
S9	26°17'10.2"S, 29°36'44.0"E	1661	Soybean-Soybean-Maize
S11	25°49'00.3"S, 29°32'56.9"E	1591	Maize-Maize-Maize
S12	25°46'43.6"S, 29°38'36.4"E	1631	Maize-Maize-Maize
S13	26°17'17.2"S, 29°36'44.8"E	1660	Maize-Soybean-Maize
S14	26°12'06.5"S, 30°08'43.6"E	1731	Maize-Soybean-Maize
S15	26°12'09.6"S, 30°07'31.2"E	1714	Maize-Maize-Soybean
S16	26°29'30.6"S 30°04'38.5"E	1687	Soybean-Maize-Maize
S17	26°29'35.7"S 30°05'00.5"E	1677	Soybean-Maize-Maize
S18	26°01'43.0"S, 28°48'57.9"E	1518	Soybean-Soybean-Soybean

CHAPTER 4: MOLECULAR CHARACTERISATION OF *MELOIDOGYNE* AND *PRATYLENCHUS* SPECIES ASSOCIATED WITH SOYBEAN AND MAIZE IN THE MPUMALANGA HIGHVELD

"Education is the passport to the future, for tomorrow belongs to those who prepare for it today."

Malcolm X

4.1 Abstract

Plant-parasitic nematodes (PPN) have global distribution and an extensive range of host plants, causing considerable yield losses to agricultural crops with annual worldwide crop losses projected at billions of dollars. Of all the PPN genera and species, root-knot nematodes (RKN; *Meloidogyne* spp.) and lesion nematodes (*Pratylenchus* spp.) are particularly harmful to crops with no exception for South Africa. A total of 14 *Meloidogyne* and 10 *Pratylenchus* spp. have been listed for South Africa causing substantial damage to various economically important crops, such as grain and oilseed. However, recent reports suggest that a more pathogenic species of RKN, *M. enterolobii*, and the lesion nematode *P. brachyurus* is becoming more prevalent in soybean-maize rotation schemes in South Africa. Therefore, this study aimed at identifying the PPN community composition associated with soybean-maize rotations in the Mpumalanga Highveld, while using species-specific PCR and sequencing to identify the presence and distribution of especially *M. enterolobii* and *P. brachyurus*. Although species-specific PCR results indicated that *M. enterolobii* and *P. brachyurus* were the most abundant species of those tested, results obtained from sequencing of individual specimens differed. This might be due to the use of the NADH5 and D2-D3 genes that have been reported to be less accurate when trying to discriminate between nematode species.

Keywords: *Meloidogyne enterolobii*, *Pratylenchus brachyurus*, SCAR-PCR, sequencing

4.2 Introduction

Plant-parasitic nematodes (PPN) are the source of extensive yield losses to agricultural crops with annual crop losses valued at approximately $78 billion (Lima *et al*., 2017). According to Jones *et al*. (2013) *Aphelenchoides bessseyi, Bursaphelenchus xylophilus, Ditylenchus dispaci*, *Globodera* spp., *Heterodera* spp., *Meloidogyne* spp., *Naccobus aberrans, Radopholus similis*, *Rotylenchulus reniformis* and *Xiphinema index* are seen as the top 10 most destructive nematode pests internationally. In South Africa, the PPN genera known as root-knot nematodes (RKN; *Meloidogyne* spp.) and lesion nematodes (*Pratylenchus* spp.) are particularly harmful to crops (Jones *et al*., 2017; Fourie *et al*., 2017a). Of the *Meloidogyne* and *Pratylenchus* spp. known to parasitise crops on an international scale, 14 *Meloidogyne* and 10 *Pratylenchus* spp. have been recorded for South Africa (SA) (Kleynhans *et al*., 1996; Marais, 2012; Marais *et al*., 2017; Van den Berg *et al*., 2017; Mbatyoti *et al*., 2020). Due to their global distribution and extensive host plant range, RKN and lesion nematodes can cause considerable damage to various economically important crops, such as grains, fruits, industrial crops, oilseed and potato (Jones *et al*., 2017).

In the Mpumalanga Highveld region of SA, crops that are typically planted include groundnut (*Arachis hypogaea*), maize (*Zea mays*), potato (*Solanum tuberosum*), soybean (*Glycine max*), sunflower (*Helianthus* spp.) and wheat (*Triticum* spp.) (Nel, 2005; Fourie *et al*., 2017b), of which all are identified as hosts for both RKN and lesion nematodes (Fourie *et al*., 2017a; Jones *et al*., 2017; McDonald *et al*., 2017). Of these crops, soybean and maize are considered important crops in SA (Acevedo-Siaca and Goldsmith, 2020). Soybean is one of the most vital summer legumes produced internationally and acts as a vital dietary protein and oil source for both animal and human consumption (Hartman *et al*., 2011; Elhady *et al*., 2018). South African soybean production dates to the 1960s with only 2,631 metric tons (MT) being produced (Shurtleff and Aoyagi, 2009). Production of the crop steeply increased since then and during the 2019/2020 growing season the area of soybean planted was estimated at 705,000 hectares (ha) from which 1,290,750 MT seeds were produced. However, during the 2017/2018 season, South Africa experienced its best ever production of the crop, represented by 787,200 ha planted with 1,540,000 MT seeds being harvested.

With regards to maize, the total area planted for both white and yellow maize during the 2019/2020 season stands at 2,610,800 ha with a combined production of 15,589,400 MT. Record maize production for SA was recorded during the 2016/2017 season at combined 16,744,000 MT with 2,628,600 ha dedicated to maize production (GrainSA, 2020). With the local increase in, and expansion of soybean and maize production since the beginning of the century, the risk of these crops being exposed to and infected by a wide range of diseases

and pests was expected (Fourie *et al*., 2001, Fourie *et al*., 2015). As these crops are usually grown in warmer climates of SA, RKN and lesion nematodes can cause major damage to these crops (McDonald *et al*., 2017; Elhady *et al*., 2018; Mbatyoti *et al*., 2021).

Although two nematode surveys in soybean have been done to date (Fourie *et al*., 2001; Mbatyoti, 2018), an upcoming threat RKN species *Meloidogyne enterolobii* (Jones *et al*., 2013; Collet *et al*., 2021), has been identified since 2016 infecting potato (Visagie *et al*., 2018) and maize (Pretorius, 2018) in the Highveld Region of Mpumalanga. This area is the second largest soybean and third largest maize producing area in SA (Grain, 2020). Unfortunately, no extensive nematode survey of soybean-maize rotations has been done in the Highveld area to date and since *M. enterolobii* is recorded as being more pathogenic than its counterpart species *M. incognita* and *M. javanica* (Jones *et al*., 2013), this scenario should be addressed. Furthermore, a past soybean survey (Mbatyoti, 2020) suggested that lesion nematodes became more abundant since the first survey (Fourie *et al*., 2001). This agrees with reports from Brazil that *P. brachyurus* is increasingly becoming a problem in soybean production areas, causing reductions of 30% to 50% in soybean yield (Rodrigues *et al*., 2014). Efforts therefore also must be directed towards determining the abundance of this genus as part of this study.

The application of molecular diagnostic tools can improve the rate and accuracy of PPN identification (Vallejo *et al*., 2021). Therefore, various molecular techniques can be used for the identification of *Meloidogyne* and *Pratylenchus* spp. that are present in soybean producing localities. This includes the use of Sequence Characterised Amplified Regions – Polymerase Chain Reaction (SCAR-PCR) and species-specific PCR for *Meloidogyne* and *Pratylenchus* spp. identification, respectively. Other molecular techniques that can be used for their identification include the sequencing of ribosomal DNA (rDNA) (e.g., 18S, the D2-D3 segment of 28S and ITS) and mitochondrial DNA (NADH5) (Zijlstra *et al*., 2000; Subbotin *et al*., 2006; Berry *et a*l., 2008; Jansen *et al*., 2016).

The continuous generation of knowledge of nematode pests associated with soybean and maize is hence crucial. Therefore, this study aimed at identifying the PPN community composition associated with soybean-maize rotations in the Mpumalanga Highveld. Because it is crucial to accurately identify the presence and distribution of species belonging to these two nematode genera in soybean-maize rotations, especially *M. enterolobii* and *P. brachyurus*, SCAR-PCR, species-specific PCR and sequencing was used for their identification.

4.3 Methodological approach

4.3.1 Site description

South Africa is placed between the 22 and 35 °S latitudes in the southern hemisphere and is characterised by diverse climatic conditions in comparison to other sub-Saharan African countries. The Mpumalanga Highveld (where this study was conducted) is situated in one of the country's nine provinces and has an average annual rainfall of 800-900 mm and an annual temperature range of 6-30 °C (Fourie *et al*., 2017b).

The grassland biome of this area contains soil that has a rich and fertile upper layer, and together with the annual rain and temperature ranges, makes it appropriate for cultivation of crops like soybean and maize. In the 2020/2021 summer growing season, root samples were taken from 16 fields (Engelbrecht *et al*., 2021) where soybean and maize are usually grown in rotation (Figure 4.1). The fields were located across the Mpumalanga province as seen in Table 4.1. Each field was divided into three sub-sections. Sampling of roots was done in a W shape in each section while the distance between sampling points differed according to the size of the fields (Engelbrecht *et al*., 2021). In each section two rows were selected where the roots of six soybean/maize plants were sampled per row. Root samples of each row were cut into 1 cm pieces, pooled and homogenised before being used for nematode analyses. This was done for all six rows (two rows per section) per field. Each field therefore had a total of six composite root samples that was analysed.

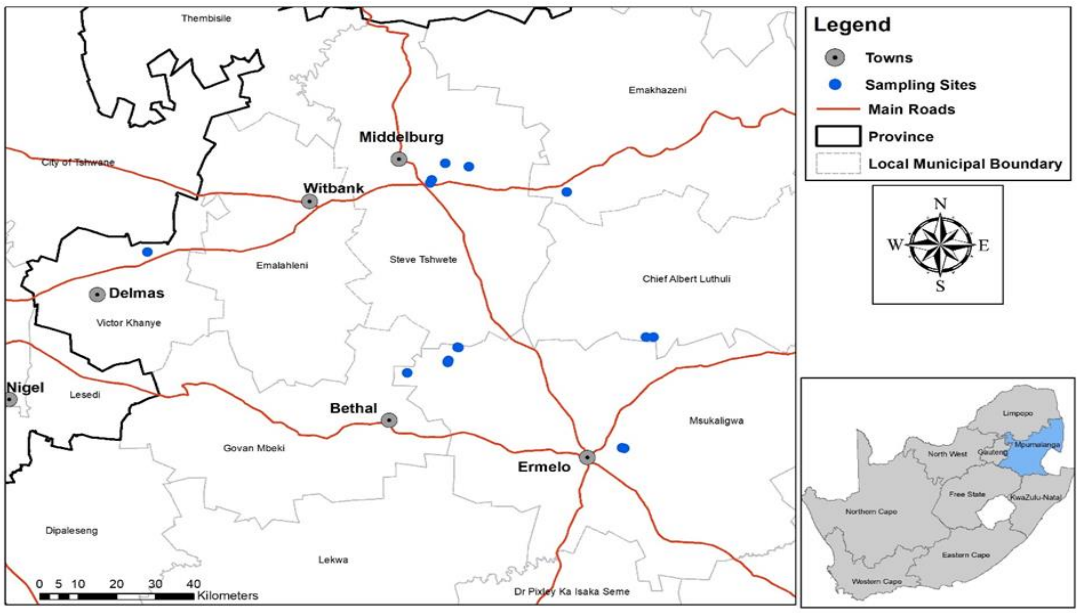

Figure 4.1: Soybean and maize localities, in the Mpumalanga province of South Africa, where root samples were obtained for nematode analyses during flowering of the crops in the 2020/2021 summer growing season. (Illustration: Wiltrud Durand, BFAP, GIS & Crop Modelling).

Table 4.1: Details of sample localities in the Highveld region of South Africa where soybean and maize root samples were collected during the 2020/2021 growing seasons for nematode analyses.

Field	Area	GPS Coordinates	Altitude (m)	2018/2019 crop	2020/2021 crop
S1	Belfast	25°50'04.4"S, 29°54'02.2"E	1747	Soybean	Maize
S2	Middelburg	25°49'28.2"S, 29°32'42.6"E	1604	Soybean	Soybean
S4	Middelburg	25°46'19.6"S 29°34'55.4"E	1600	Soybean	Maize
S5	Diepfontein	26°19'08.7"S, 29°30'27.2"E	1625	Soybean	Maize
S6	Hendrina	26°14'50.4"S, 29°38'09.7"E	1661	Soybean	Soybean
S7	Hendrina	26°14'52.6"S, 29°38'18.8"E	1652	Soybean	Soybean
S8	Ermelo	26°16'59.3"S, 29°36'48.4"E	1659	Soybean	Soybean
S9	Ermelo	26°17'10.2"S, 29°36'44.0"E	1661	Soybean	Soybean
S11	Middelburg	25°49'00.3"S, 29°32'56.9"E	1591	Soybean	Soybean
S12	Middelburg	25°46'43.6"S, 29°38'36.4"E	1631	Soybean	Maize
S13	Ermelo	26°17'17.2"S, 29°36'44.8"E	1660	Soybean	Soybean
S14	Carolina	26°12'06.5"S, 30°08'43.6"E	1731	Soybean	Soybean
S15	Carolina	26°12'09.6"S, 30°07'31.2"E	1714	Soybean	Soybean
S16	Ermelo	26°29'30.6"S 30°04'38.5"E	1687	Soybean	Maize
S17	Ermelo	26°29'35.7"S 30°05'00.5"E	1677	Soybean	Maize
S18	Delmas	26°01'43.0"S, 28°48'57.9"E	1518	Soybean	Soybean

4.3.2 Nematode extraction and morphological identification

Nematodes were extracted from 20 g of composite root samples, for each row of the fields using the centrifugal-flotation method described by Swart and Marais (2017) and transferred to a De Grisse counting dish (De Grisse, 1969). The nematodes were counted and simultaneously identified to genus level (Heyens, 1917) using a Nikon ECLIPSE TS100 (Nikon Corporation, Tokyo, Japan) inverted microscope (40× magnification) (Engelbrecht *et al*., 2021).

4.3.3 Molecular characterisation of *Meloidogyne* and *Pratylenchus* spp.

4.3.3.1 Species-specific characterisation of *Meloidogyne* and *Pratylenchus* spp.

As more than one *Meloidogyne* and *Pratylenchus* spp. can be identified in a population using SCAR-PCR and species-specific PCR, respectively, 15-20 randomly selected *Meloidogyne* (mature females) and *Pratylenchus* (adults) spp. from each field were placed in an Eppendorf tube that contained 15 µl double distilled (Milli-Q) water for DNA extraction. The tubes

containing individuals were stored at -10 °C until DNA extraction was done for molecular analyses. The DNA extraction of the *Meloidogyne* and *Pratylenchus* spp. from each field was done using the chelex-100 protocol (Musapa *et al*., 2013). Briefly, 20 µl chelex (5% w/v) and 5 µl proteinase K (20 mg/ml) was added to each tube containing the individuals from each field and incubated at 56-57 °C for 2 h followed by incubation at 95 °C for 10 min. Amplification of DNA was done by using a Alpha Cycler 1 PCRMax thermocycler (Vacutec, USA). The total PCR reaction volume of 25 µl constituted of 12.5 µl master mix (Promega Corporation, USA), 1 µl forward and reverse primers (10 µM), 2.5 µl DNA and 8 µl ddH_2O. Each of the fields were screened for the presence of *M. enterolobii*, *M. arenaria*, *M. incognita*, *M. javanica*, *P. brachyurus*, *P. zeae*, *P. penetrans* and *P. neglectus* using species specific primers (Table 4.2).

For the SCAR-PCR of *Meloidogyne* spp. conditions were set as follows: 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature and 1 min at 72 °C. Annealing temperatures depended on the use of species-specific primers, resulting in the use of 61 °C for Far/Rar primers, 64 °C for Fme/Rme and Fjav/Rjav primers, 54 °C for the Finc/Rinc primer (Zijlstra *et al*., 2000; Long *et al*., 2006). Species-specific PCR conditions for *Pratylenchus* spp. were set as: 95 °C for 3 min.; 35 cycles at 95 °C for 1 min.; 1 min. at 62 °C for PPEN/D3B and 63 °C for PNEG/D3B primers; 72 °C for 1 min. and a final extension step of 7 min. at 72 °C. For the 18sF/Praty-R primer set PCR conditions were 94 °C for 3 min., 94 °C for 1 min., annealing at 57 °C for 1 min.; 30 cycles of 72 °C for 1 min., followed by a final extension of 72 °C for 10 min. For the 18sF/ACM7R primer set conditions were 94 °C for 2 min. 45 s; followed by 40 cycles of the following: 94 °C for 1 min.; 57 °C for 45 s and 72 °C for 2 min., followed by the final extension cycle of 72 °C for 10 min. (Al-Banna *et al*., 2004; Machado *et al*., 2007; Berry *et al*., 2008).

The PCR product obtained from each sample (2 µl) (representing the *Meloidogyne* and *Pratylenchus* population of each locality) was electrophoresed on a 2% agarose gel to determine the presence/absence of *M. enterolobii*, *M. arenaria*, *M. incognita*, *M. javanica, P. brachyurus*, *P. zeae*, *P. penetrans* and *P. neglectus*. This was done by comparing the PCR fragment lengths of a known *Meloidogyne* and *Pratylenchus* spp. (positive controls) to that of the PCR products obtained in this study. The DNA bands were stained with GelRed (www.biotium.com) and visualized and photographed using a UV transilluminator.

Table 4.2: Name, sequence and amplification size of different SCAR and sequencing primers used for molecular identification of *Meloidogyne* and *Pratylenchus* populations obtained from 16 soybean-maize rotation localities.

Target spp.	Primer name	Primer sequence	Fragment size
M. arenaria	Far	TCG GCG ATA GAG GTA AAT GAC	420 bp
	Rar	TCG GCG ATA GAC ACT ACA ACT	
M. enterolobii	FMe	AAC TTT TGT GAA AGT GCC GCT	250 bp
	RMe	TCA GTT CAG GCA GGA TCA ACC	
M. incognita	Finc	CTC TGC CCA ATG AGC TGT CC	1200 bp
	Rinc	CTC TGC CCT CAC ATT AGG	
M. javanica	Fjav	GGT GCG CGA TTG AAC TGA GC	700 bp
	Rjav	CAG GCC CTT CAG TGG AAC TAT AC	
Meloidogyne spp.	NADH5-F	TAT TTT TTG TTT GAG ATA TAT TAG	560 bp
	NADH5-R	CGT GAA TCT TGA TTT TCC ATT TTT	
P. brachyurus	18sF	TTG ATT ACG TCC CTG CCC TTT	267 bp
	ACM7R	GCW CCA TCC AAA CAA YGA G	
P. zeae	18sF	TTG ATT ACG TCC CTG CCC TTT	250 bp
	Praty-R	CTG CAT TGG AAG CGC GCT TG	
P. penetrans	PPEN	TAA AGA ATC CGC AAG GAT AC	278 bp
	D3B	TCG GAA GGA ACC AGC TAC TA	
P. neglectus	PNEG	ATG AAA GTG AAC ATG TCC TC	290 bp
	D3B	TCG GAA GGA ACC AGC TAC TA	
Meloidogyne and Pratylenchus spp.	D2A	ACA AGT ACC GTG AGG GAA AGT TG	497 bp
	D3B	TCG GAA GGA ACC AGC TAC TA	
Pratylenchus spp.	18sF	TTG ATT ACG TCC CTG CCC TTT	800 bp
	ITS-R	GGA ATC ATT GCC GCT CAC TTT	

4.3.3.2 Sequencing for *Meloidogyne* and *Pratylenchus* spp. characterisation

For sequencing several individual *Meloidogyne* (mature females or juveniles) and *Pratylenchus* (adults*)* individuals was used per locality. Each individual *Meloidogyne* and *Pratylenchus* was surface sterilised and washed before being placed in an Eppendorf tube that contained 15 µl double distilled (Milli-Q) water for DNA extraction. The tubes containing

individuals were stored at -10 °C until DNA extraction was done for molecular analyses. The extraction of DNA was done with chelex-100 protocol (Musapa *et al*., 2013) mentioned previously. To amplify the DNA an Alpha Cycler 1 PCRMax thermocycler (Vacutec, USA) was used. Each PCR reaction (total volume of 25 µl) constituted of 12.5 µl master mix (Promega Corporation, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 5 µl DNA and 5.5 µl ddH_2O. Sequencing of the *Meloidogyne* spp. were done using the NADH5 and D2-D3 genes while for *Pratylenchus* spp. the D2-D3 and 18s-ITS genes were used (Table 4.2). Conditions for the amplification of the NADH5 gene were as follows: initial denaturation for 2 min at 94 °C, followed by 40 cycles of 60 s at 94 °C, 60 s at 45 °C, 90 s at 72 °C, and finally an extension for 10 min at 72 °C (Jansen *et al*., 2016). Sequencing of *Pratylenchus* spp. were done by the amplification of the D2-D3 expansion segments of 28S rRNA (Table 4.2) (Subbotin *et al*., 2006). For the amplification of the D2-D3 expansion segments of 28S rRNA, PCR conditions were set at 4 min for 94 °C followed by 35 cycles of 60 s at 94 °C, 90 s at 55 °C, 2 min at 72 °C and finally 10 min at 72 °C (Subbotin *et al,*. 2006). Amplification conditions for the 18S F-ITS primer set were: 95 °C for 3 min, followed by 35 cycles of 45 s at 95 °C, 45 s at 54 °C and 45 s at 72 °C followed by a final extension step of 5 min 72°C. The PCR products used for sequencing for each sample were then stored at -20 °C prior to sequencing done by a genomic company, Inqaba Biotec™, South Africa (www.inqaba-southafrica.co.za). Primers used for the sequencing reactions were the same as those used in the amplification step.

4.3.4 Statistical analysis of nematode data

Plant-parasitic nematode population assemblages extracted from the six 20 g composite root samples per field, were pooled and counted to determine the frequency of occurrence, mean population density (MPD) and prominence value (PV) of each nematode genus (Fourie *et al*., 2001; Karuri *et al*., 2017). Frequency of occurrence was calculated as: (number of localities at which the genus occurred in the root of each crop/ number of localities sampled) x 100. The mean population density (MPD) of each field was determined by dividing the total number of individuals of a genus present in root samples of each field by the number of fields in which the genus occurred in root samples. The prominence value (PV) was determined by multiplying the mean population density of each genus with the √frequency of occurrence and divided by 100. The DNA sequences obtained from Inqaba Biotec™ were edited using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and confirmed in a forward direction. All sequences from this study were uploaded to NCBI and available sequences for other *Meloidogyne* and *Pratylenchus* spp., as well as outgroups for each gene were downloaded from NCBI GenBank and aligned using the ClustalW alignment tool

implemented in Molecular Evolutionary Genetics Analysis (MEGA) version 11 (Tamura *et al*., 2021). To determine the appropriate nucleotide substitution a model test was run in MEGA 11 (Tamura *et al*., 2021). The best identified models were Tamura-Nei method for *Meloidogyne* NADH5 gene and Jukes-Cantor for the *Meloidogyne* D2-D3 gene.

With regards to *Pratylenchus,* the Kimura 2-parameter was used for both the18S-ITS and D2-D3 genes of *Histeromerus mystacinus,* Wesmael, 1838 (Accession number: Z83601) (Belshaw & Quicke, 1997) was used as outgroup for the *Meloidogyne* D2-D3 analyses. *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970 (Pereira *et al*., 2013) (Accession number: JQ514068) was the outgroup for the NADH5 gene. For the *Pratylenchus* D2-D3 gene *Mesocriconema ornatum* Siddiqi, 1980 (Accession number: AY780968) (Subbotin *et al*., 2005) and *Oncholaimellus* sp. NN022 (Accession number: LK054723) (Armenteros *et al*., 2014) for the 18S gene were used as the respective outgroups.

4.4 Results

4.4.1 Plant-parasitic nematode populations associated with soybean and maize roots

Only eight PPN genera, which were less than the 10 reported in the 2018/19 survey (Engelbrecht *et al*., 2021), were identified during 2020/2021, while those individuals that could not be identified were recorded as belonging to the family Aphelenchoididae. The highest number of genera (seven) during the 2020/2021 season were found at S1, S4, S5 and S7 with lowest number of genera (four) identified at S2, S12, S17 and S18 (Table 4.3). Of the genera present across the 16 fields in the 2018/2019 (Table 3.1). and 2020/2021 seasons, both *Meloidogyne* and *Pratylenchus* were present in each of these fields (Table 4.3). The highest *Meloidogyne* level in the 2020/2021 season (Table 4.3) was reported for S7 (6672 individuals/20 g of root) and S18 the lowest *Meloidogyne* level (87 individuals/20 g of root). *Pratylenchus* levels during this season were the highest at S6 (1253 individuals/20 g of root) with the lowest recorded at S16 (78 individuals/20 g of root). While the PV of nematode genera in all 16 fields, ranged from 4.2 (*Aphelenchus*) to 1598.6 (*Meloidogyne*) during 2020/2021 (Table 4.4). Some of the other nematode genera found in root samples from the Highveld region were detected in limited fields. These include: *Aphelenchus* and other individuals belonging to the Aphelenchidae family.

Table 4.3: The community structure and abundance of plant-parasitic nematodes in 20 g soybean/maize root samples collected during the 2020/21 growing season from 16 fields in the Highveld region of the Mpumalanga province of South Africa.

Genus and/or family	Field no.															
	S1	**S2**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S11**	**S12**	**S13**	**S14**	**S15**	**S16**	**S17**	**S18**
Meloidogyne	189	108	1390	432	2471	6672	4304	537	407	110	1175	3537	3922	128	107	87
Pratylenchus	85	311	1024	412	1253	824	290	340	118	168	433	325	385	78	132	267
Helicotylenchus	12	10	22	17	27	25	19	27	8	0	8	33	14	0	0	0
Scutelonema	17	39	29	18	35	29	19	30	44	21	25	28	37	24	23	8
Hoplolaimus	8	0	0	12	0	12	0	0	0	0	0	14	25	12	0	0
Rotylenchus	8	0	47	10	25	28	8	25	24	29	11	0	33	28	40	10
Aphelenchus	8	0	8	12	0	0	0	0	0	0	0	0	0	0	0	0
Aphelenchidae	0	0	25	0	0	17	0	0	0	0	0	0	0	0	0	0

Table 4.4: Prominence values, frequencies of occurrence and mean population densities of plant-parasitic nematode genera occurring in 20 g soybean/maize root samples collected during the 2020/21 growing season from 16 fields in the Highveld region of the Mpumalanga province of South Africa.

Genus and/or family	[b]Mean population density (MPD)	[a]Frequency of occurrence (FO)	[c]Prominence value (PV)
Meloidogyne	1598.6	100.0	1598.6
Pratylenchus	402.8	100.0	402.8
Helicotylenchus	18.6	75.0	16.1
Scutelonema	26.7	100.0	26.7
Hoplolaimus	14.1	37.5	8.6
Rotylenchus	23.4	87.5	21.9
Aphelenchus	9.6	18.8	4.2
Aphelenchidae	20.8	12.5	7.4

[a]FO = (Number of samples containing genus/number of samples collected) x100. [b]MPD= total number of individuals of a genus present in root samples of each site/number of localities in which the genus occurred in root samples of each site. [c]PV= $\left(MPD\ x\ \sqrt{absolute\ frequency}\right)/100$

4.4.2 Molecular characterisation of *Meloidogyne* and *Pratylenchus* spp.

4.4.2.1 Species-specific characterisation of *Meloidogyne* and *Pratylenchus* spp.

Use of specific SCAR primers established the occurrence of *viz*. *M. enterolobii*, *M. javanica*, *M. incognita, P. brachyurus* and *P. zeae* (Figure 4.2 to Figure 4.4). The following DNA fragments were amplified for the respective species, namely 250 bp for *M. enterolobii*, 700 bp for *M. javanica*, 1200 bp for *M. incognita,* 267 bp for *P. brachyurus* and 250 bp for *P. zeae*. These results were compatible with the lengths of DNA fragments obtained for the positive standards for each respective species. No amplification was evident for Far/Rar, which suggested that *M. arenaria* was not present among the populations studied. Of the three *Meloidogyne* spp. identified in this study *M. enterolobii* was the most predominant and was identified in 62.5% of the fields, followed by *M. incognita* (31.3%) and *M. javanica* (12.5%). Moreover, of the 16 localities 62.5% (10 localities) were found to contain single *Meloidogyne* spp., while 18.8% (3 localities) were found to have mixed populations of *Meloiodogyne* (Figure 4.2a-b and Figure 4.3).

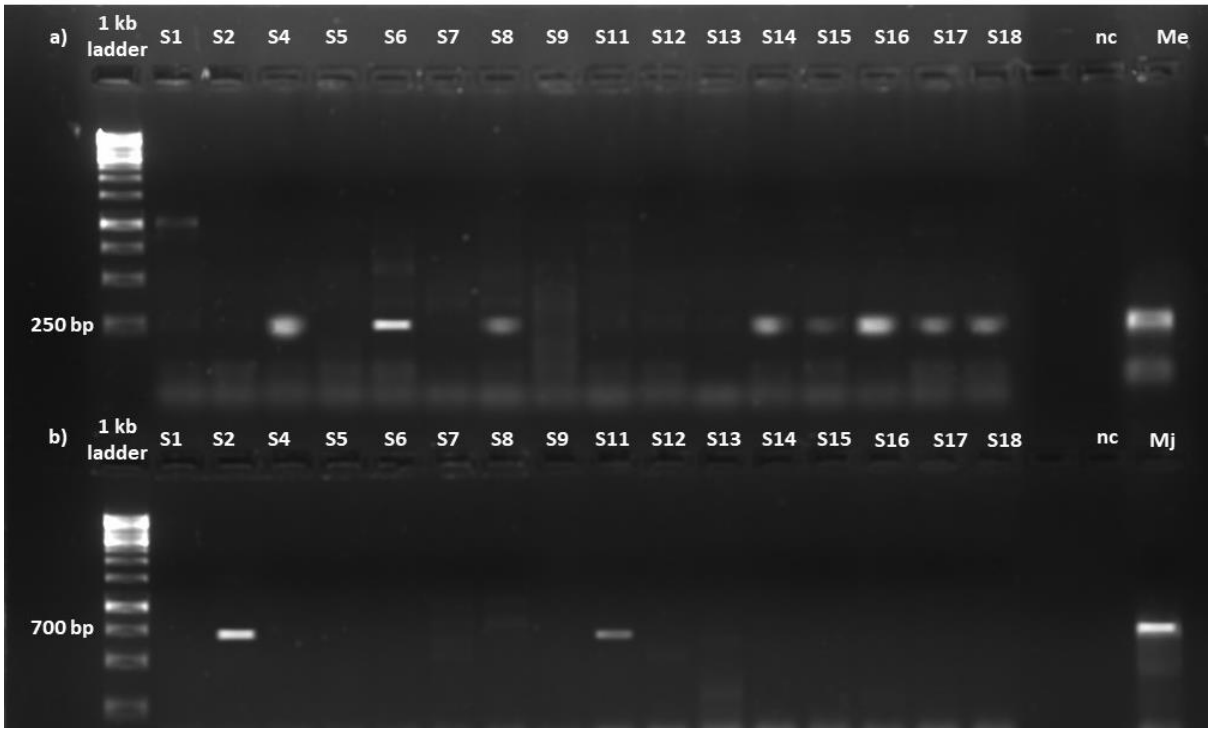

Figure 4.2a-b: Gel photo of DNA amplification products of *Meloidogyne* spp. females and second-stage juveniles obtained from 16 localities in the Highveld region of the Mpumalanga province sampled from soybean and maize, using SCAR-PCR. a) *M. enterolobii*, b) *M. javanica*; Me (*M. enterolobii*) and Mj (*M. javanica*) = DNA of standard (positive control) population used for each species, while nc = negative control.

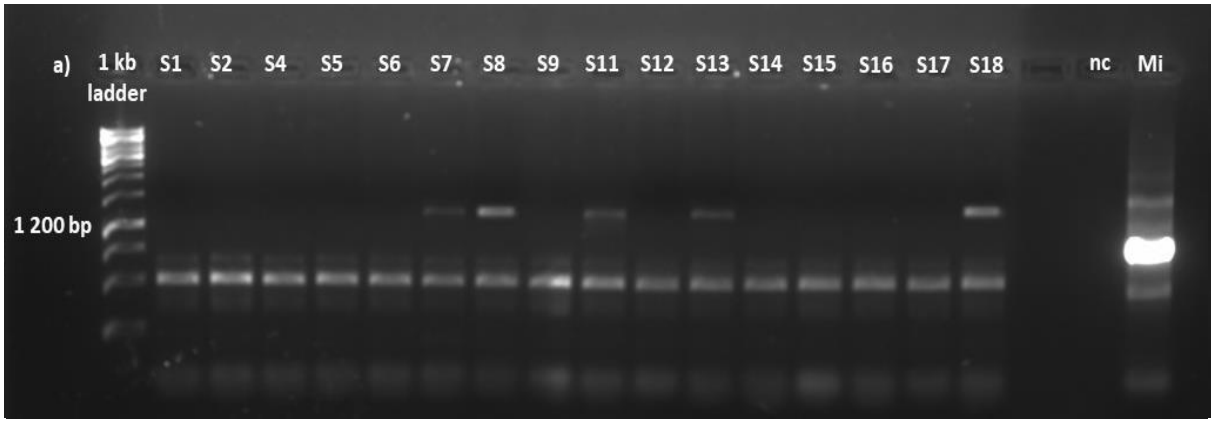

Figure 4.4: Gel photo of DNA amplification products of *Meloidogyne* spp. females and second-stage juveniles obtained from 16 localities in the Highveld region of the Mpumalanga province sampled from soybean and maize, using SCAR-PCR. a) *M. incognita*; Mi (*M. incognita*) = DNA of standard (positive control) population used for each species, while nc = negative control.

With regards to *Pratylenchus*, amplification was not evident for PPEN/D3B and PNEG/D3B, which suggested that *P. penetrans* and *P. neglectus* were not present among the populations studied. Of the two *Pratylenchus* spp. identified, *P. brachyurus* was the predominant species (75%) followed by *P. zeae* (18.75%). Of the 16 localities 56.3% (9 localities) contained single *Pratylenchus* spp., while 18.8% (2 localities) was found to have a mixed *Pratylenchus* population (Figure 4.4a-b).

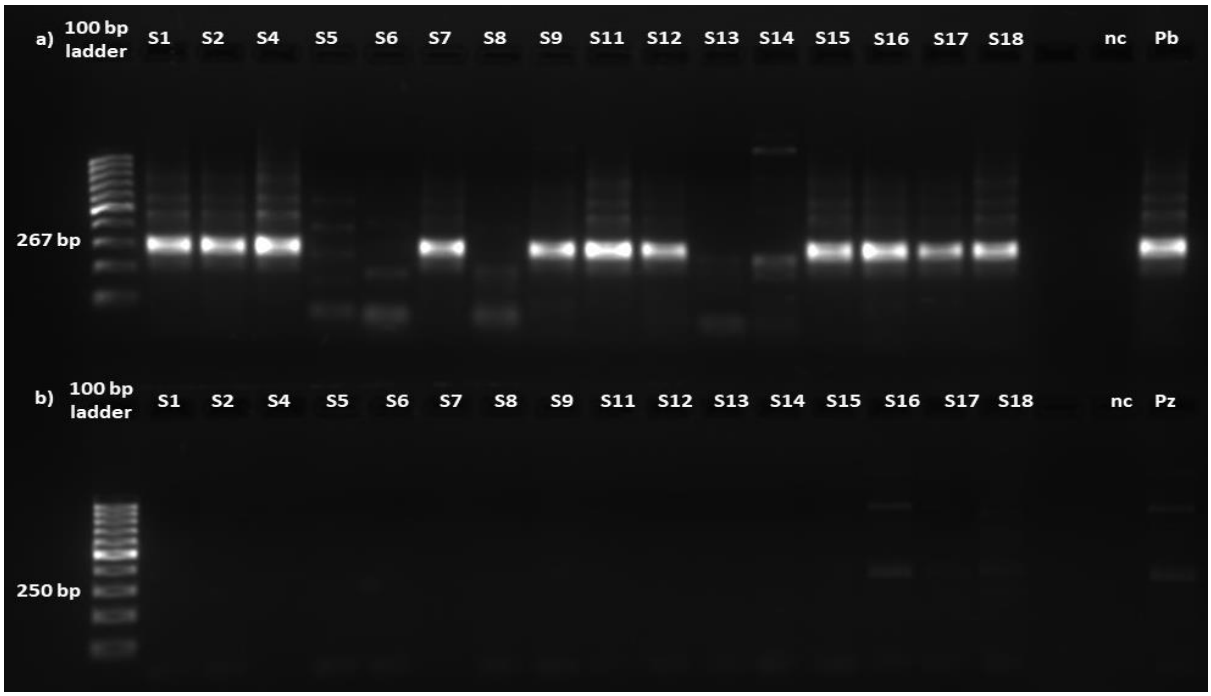

Figure 4.3a-b: Gel photo of DNA amplification products of *Pratylenchus* spp. obtained from 16 localities in the Highveld region of the Mpumalanga province sampled from soybean and maize, using species-specific PCR. a) *P. brachyurus*, b) *P. zeae*; Pb (*P. brachyurus*) and Pz (*P. zeae*) = DNA of standard (positive control) population used for each species, while nc = negative control.

4.4.2.2 Sequencing for *Meloidogyne* and *Pratylenchus* spp. characterisation

The sequences of the NADH5 gene, which were used to construct a Bayesian tree (Figure 4.5) revealed that 23 out of 28 sequences (identified using the before mentioned gene during this study) showed high similarity to *M. incognita* based on Blastn results (Table 4.5). They clustered with another sequence of this species selected from GenBank in a well-supported clade (Figure 4.5) with a 99 % posterior probability support. Furthermore, five out of 28 sequences identified using the NADH5 gene showed high similarity to *M. arenaria* according to Blastn results. Of these sequences only two *M. arenaria* sequences (OL469764 and OL469772) grouped close to the *M. arenaria* sequence extracted from GenBank (Figure 4.5). The remaining two sequences, although characterised as *M. arenaria* using Blastn, grouped together with the sequences of *M. javanica* obtained from GenBank (Figure 4.5).

With regards to the Bayesian tree constructed using the D2-D3 gene for *Meloidogyne* samples (Figure 4.6), 10 out of 14 sequences also showed high similarity to *M. incognita* based on Blastn results (Table 4.5). These *M. incognita* D2-D3 sequences grouped together with sequences selected from GenBank in a well-supported clade (Figure 4.6). Interestingly, two sequences *M. javanica* (OL505147) and *M. graminicola* (OL505148) were different from the other sequences and formed an individual group (Figure 4.6) based on D2-D3 sequences. One sequence was also identified as *M. haplanaria* (OL505152) and grouped together with the *M. incognita* sequences obtained in this study.

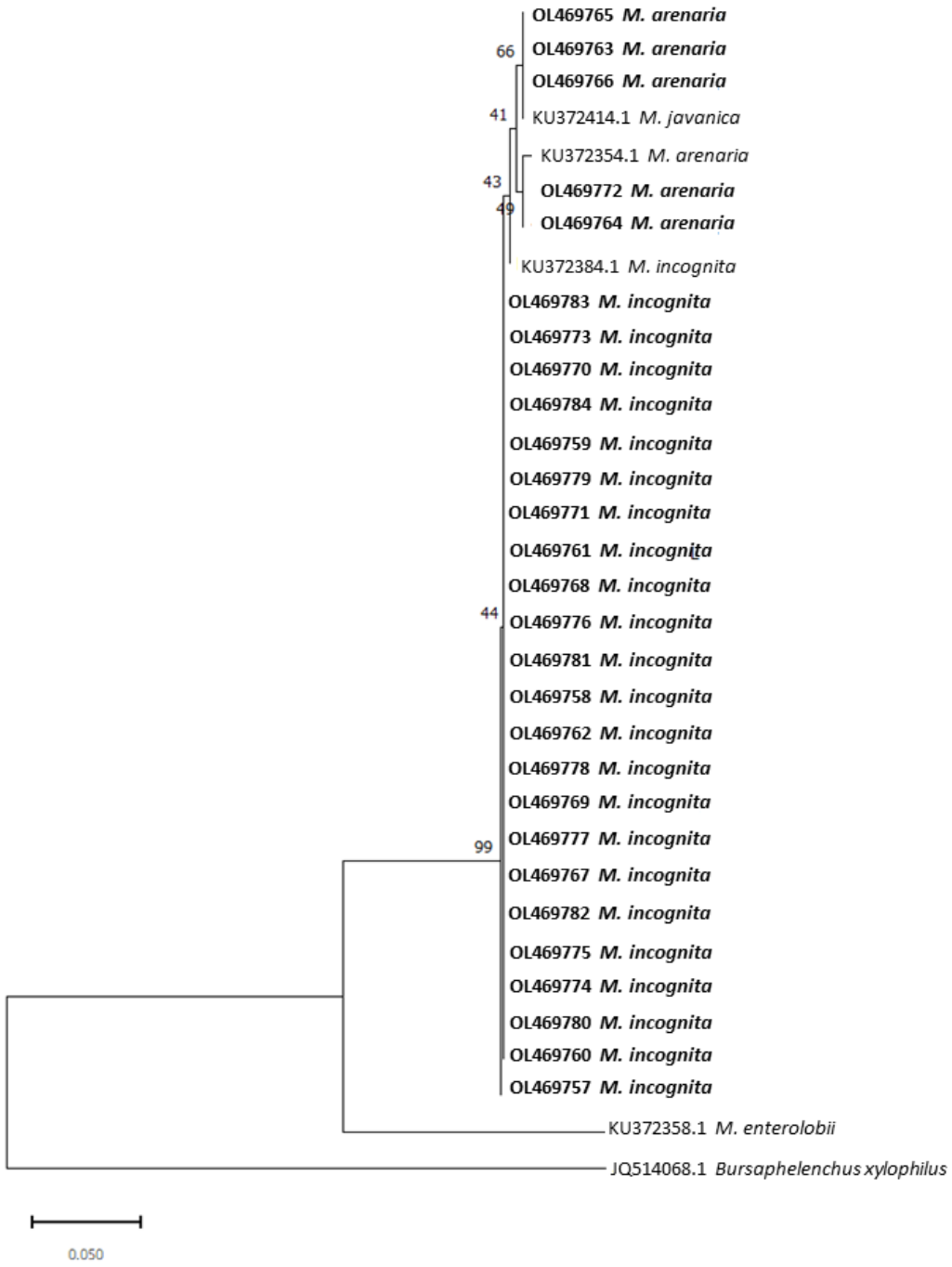

Figure 4.5: Bayesian inference (BI) of *Meloidogyne* spp. obtained from 16 soybean/maize producing localities in South Africa, using NADH5 mtDNA sequences were computed using the Tajima-Nei method (those populations which are from this study are shown in bold).

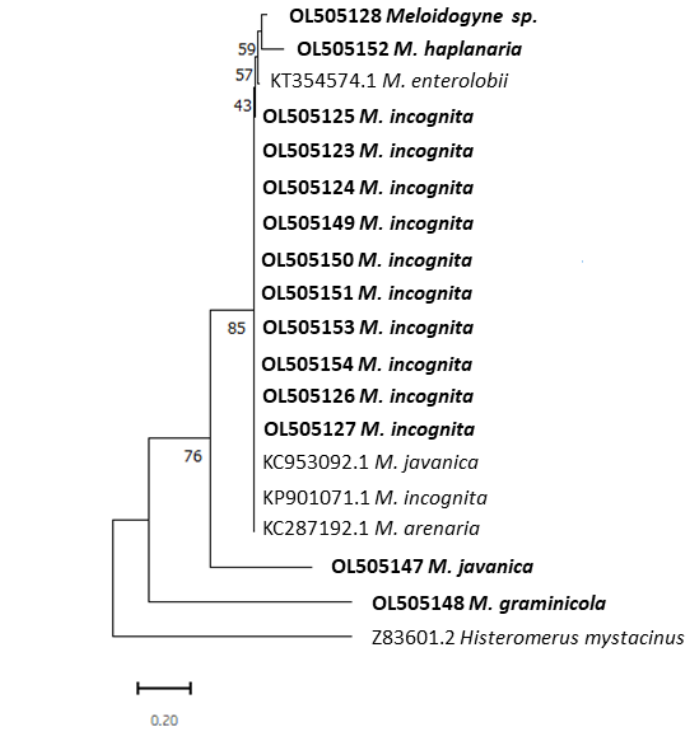

Figure 4.6: Bayesian inference (BI) of *Meloidogyne* spp. obtained from 16 soybean/maize producing localities in South Africa, based on partial D2-D3 28S rDNA region sequences, were computed using the Jukes-Cantor method (those populations which are from this study are shown in bold).

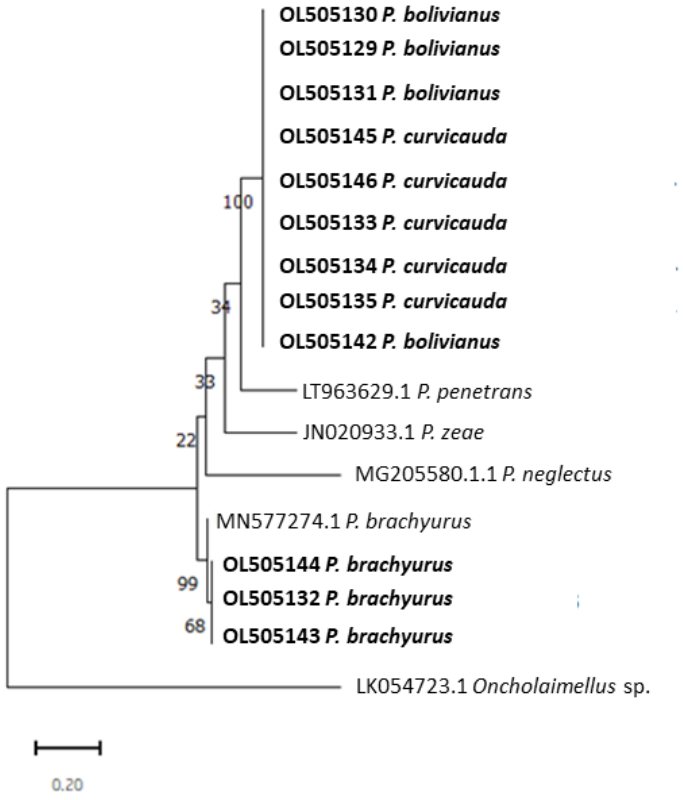

Figure 4.7: Bayesian inference (BI) of *Pratylenchus* spp. obtained from 16 soybean/maize producing localities in South Africa, based on partial 18s rDNA region sequences, were computed using the Kimura 2-parameter method (those populations which are from this study are shown in bold).

With regards to the Bayesian trees constructed using the 18S and D2-D3 sequences (Table 4.6) the following trees were constructed for *Pratylenchus* sp. from this study (Figure 4.7 and Figure 4.8). The Bayesian tree constructed using the 18S gene (Figure 4.7) revealed that only three (OL505144, OL505132 and OL505143) out of 12 sequences identified had high similarity to *P. brachyurus* after Blastn. These three sequences also had a well-supported grouping with a 99 % posterior probability support to *P. brachyurus* sequences retrieved from GenBank. Furthermore, the remaining nine sequences had high Blastn similarities to *P. bolivianus* (OL505130, OL505129, OL505131 and OL505142) and *P. curvicauda* (OL505145, OL505146, OL505133, OL505134 and OL505135) (Figure 4.7). Interestingly, all the *P. brachyurus* sequences (Figure 4.7) grouped together in a clade, with remaining *P. bolivianus* and *P. curvicauda* sequences forming part of clade with close association to the *P. penetrans* sequence obtained from GenBank (Figure 4.7). Moreover, Bayesian trees constructed from the D2-D3 genes of *Pratylenchus* sp. (Figure 4.8) formed two distinct clades. The *Pratylenchus* spp. from this study had high Blastn similarities to *P. brachyurus* (OL505139, OL505140, OL505141 and OL505162) formed one clade while the other *Pratylenchus* spp. that were found to be highly similar to *P. bolivianus* (OL505136, OL505137, OL505138, OL505155, OL505156, OL505157, OL505158, OL505159, OL505160 and OL505161) formed the other.

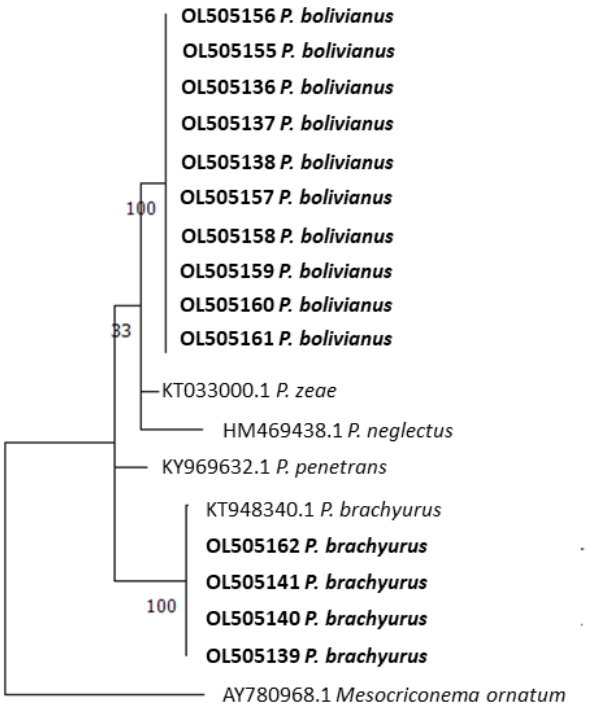

Figure 4.8: Bayesian inference (BI) of *Pratylenchus* spp. obtained from 16 soybean/maize producing localities in South Africa, based on partial D2-D3 28S rDNA region sequences, were computed using the Kimura 2-parameter method (those populations which are from this study are shown in bold).

Table 4.5: *Meloidogyne* spp., with their accession numbers deposited in NCBI Genbank, identified from 16 localities obtained from soybean/maize roots from the Highveld region of the Mpumalanga province.

Locality	SCAR-PCR	NADH5	D2-D3	Locality	SCAR-PCR	NADH5	D2-D3
1	*M. enterolobii*	*M. incognita* (OL469757); *M. incognita* (OL469758)	*M. incognita* (OL505123); *M. incognita* (OL505124)	2	*M. javanica*	*M. incognita* (OL469767); *M. incognita* (OL469773)	*M. incognita* (OL505150); *M. graminicola* (OL505148)
4	*M. enterolobii*	*M. incognita* (OL469762); *M. incognita* (OL469759)		5	-	*M. arenaria* (OL469764); *M. incognita* (OL469760)	M. incognita (OL505125)
6	*M. enterolobii*	*M. incognita* (OL469780); *M. incognita* (OL469774)	*M. javanica* (OL505147)	7	*M. incognita*	*M. incognita* (OL469775); *M. incognita* (OL469781)	*M. incognita* (OL505151); *M. haplanaria* (OL505152)
8	*M. enterolobii, M. incognita*	*M. incognita* (OL469782); *M. incognita* (OL469776)		9	*M. enterolobii*	*M. arenaria* (OL469772); *M. incognita* (OL469783)	
11	*M. javanica, M. incognita*	*M. incognita* (OL469768); *M. incognita* (OL469777)		12	-	*M. incognita* (OL469761)	
13	*M. incognita*	*M. incognita* (OL469769); *M. incognita* (OL469770)	*M. incognita* (OL505149); *M. incognita* (OL505153)	14	*M. enterolobii,*	*M. incognita* (OL469771); *M. incognita* (OL469778)	*M. incognita* (OL505154)
15	*M. enterolobii,*	*M. incognita* (OL469784); *M. incognita* (OL469779)		16	*M. enterolobii,*	*M. arenaria* (OL469765); *M. arenaria* (OL469766)	*M. incognita* (OL505126); *Meloidogyne spp.* (OL505128)
17	*M. enterolobii,*	*M. arenaria* (OL469763)	*M. incognita* (OL505127)	18	*M. enterolobii, M. incognita*		

Table 4.6: *Pratylenchus* spp., with their accession numbers deposited in NCBI Genbank, identified from 16 localities obtained from soybean/maize roots from in the Highveld region of the Mpumalanga province.

Locality	Specie specific-PCR	ITS-18s	D2-D3	Locality	Specie specific-PCR	ITS-18s	D2-D3
1	*P. brachyurus*	*P. curvicauda* (OL505145); *P. brachyurus* (OL505143)	*P. bolivianus* (OL505136); *P. brachyurus* (OL505140)	2	*P. brachyurus*	*P. brachyurus* (OL505132)	*P. brachyurus* (OL505162)
4	*P. brachyurus*	*P. curvicauda* (OL505146)	*P. bolivianus* (OL505137)	5	-	*P. bolivianus* (OL505142);	*P. bolivianus* (OL505138); *P. brachyurus* (OL505139)
6	-	*P. curvicauda* (OL505133); *P. curvicauda* (OL505134)	*P. bolivianus* (OL505157)	7	*P. brachyurus*		*P. bolivianus* (OL505158)
8	-	*P. curvicauda* (OL505135)	*P. bolivianus* (OL505159)	9	*P. brachyurus*	*P. bolivianus* (OL505129)	
11	*P. brachyurus*	*P. bolivianus* (OL505130); *P. bolivianus* (OL505131)	*P. bolivianus* (OL505160); *P. bolivianus* (OL505161)	12	*P. brachyurus*	*P. brachyurus* (OL505144)	*P. brachyurus* (OL505141)
13	-			14	-		*P. bolivianus* (OL505155); *P. bolivianus* (OL505156)
15	*P. brachyurus*			16	*P. brachyurus, P. zeae*		
17	*P. brachyurus*			18	*P. brachyurus*		

4.5 Discussion

The current study reports eight nematode genera associated with soybean roots similar to the 11 reported by Mbatyoti *et al*. (2020) and the seven genera reported earlier to be linked with soybean production in South Africa (Fourie *et al*., 2001). This can likely be explained by the diverse methods used for PPN extraction from soybean/maize roots, as during this study the Swart and Marais (2017) protocol was used. Furthermore, the expansion of soybean and maize production as compared to the start of the century, when the study by Fourie *et al.* (2001) was done, could aid in the clarification of this observed phenomenon. When results from this study were related to those from Fourie *et al*. (2001) and Engelbrecht *et al*. (2021), the major endoparasites were identified as being *Meloidogyne* and *Pratylenchus* spp. One significant observation that was made, was the high PV of both the *Meloidogyne* and *Pratylenchus* genera (Table 4.4), although it was lower than previously PV reported for these localities when they were all under soybean cultivation (Engelbrecht *et al*., 2021). Crop rotation practises in the Mpumalanga Highveld region, particularly in the fields used in this study, consist of soybean rotated with grain crops such as maize (Mc Donald *et al*., 2017), which is also vulnerable to RKN and lesion nematode infestations. This rotation practice therefore contributes to intensified strain being placed on the sustainable crop production of both grain and legumes in this region. Although both *M. incognita* and *M. javanica* have been reported to infect soybean in SA (Mbatyoti *et al*., 2020) the presence and distribution of *M. enterolobii* in both soybean and maize roots is of great concern. According to the knowledge of the authors this is the first report of *M. enterolobii* on soybean in SA. Moreover, recent studies in SA identified *M. enterolobii* in dry bean (*Phaseolus vulgaris*), eggplant (*Solanum melongena*), groundnut (*Arachis hypogaea*), guava (*Psidium guajava*), lettuce (*Lactuca sativa*), maize, potato and spinach (*Spinacia oleracea*) (Onkendi and Moleleki 2013; Pretorius, 2018; Visagie *et al*. 2018; Rashidifard *et al*., 2019).

The PV of *Pratylenchus* reported in this study, resemble that of Mbatyoti (2018). Although *Pratylenchus* was not considered to be an significant pest of soybean (Bridge and Starr, 2007), recent studies have found that *Pratylenchus* spp. severely impact soybean productions resulting in potential losses of up to 85% in some cases (Lima *et al*., 2015; Mbatyoti, 2018). The high PV of *Pratylenchus* observed in this study might also be caused by the rotation practices used. Lima *et al*. (2015) found that rotation of soybean with maize in Brazilian production areas, favoured *P. brachyurus* reproduction and such practises might have comparable effects in South African production areas, such as the Mpumalanga Highveld. Moreover, the common practice of using maize and grain legumes in rotation will consequently contribute to higher RKN and lesion nematode population densities since these crops have

been identified as being susceptible to these two predominant nematode genera (Fourie *et al*., 2017b; Mc Donald *et al*., 2017).

The value of the SCAR-PCR and species-specific PCR was demonstrated as it was able to positively identify and discriminate among three *Meloidogyne* spp. and two *Pratylenchus* spp. screened for in this study, respectively. However, the use of SCAR-PCR not only caused various non-specific bands to form but it was not able to identify *M. arenaria*. Such problematic results have also been reported in previous studies and it is therefore recommended that SCAR-PCR and species-specific PCR should be used in conjunction with other molecular and/or morphometrical techniques to ensure accurate identification/characterisation of both *Meloidogyne* and *Pratylenchus* spp. (Devran and Söğut, 2009; Rashidifard *et al*., 2019; Santos *et al*., 2019).

Although SCAR-PCR positively identified *M. enterolobii*, *M. javanica* and *M. incognita* as either mixed communities or as a single population, the use of the NADH5 and D2-D3 genes in this study mostly identified *M. incognita* except for a couple of samples that were found to be other *Meloidogyne* spp. (Table 4.7 and Figures 4.5-4.6). This was in contrast with SCAR-PCR results that indicated *M. enterolobii* was the predominant species and not *M. incognita*. This study reports similar *Meloidogyne* spp. to that of Visagie *et al*. (2018) and Mbatyoti *et al*. (2020). However, the use of the NADH5 gene for the molecular identification of *Meloidogyne* spp. might not be the most accurate as previous studies have reported similar problems with regards to discrimination of *Meloidogyne* spp. and conflicting results when compared to the use of other genes for example the D2-D3 and COI genes (Janssen *et al*., 2016; Rashidifard *et al*., 2019). Moreover, the use of the D2-D3 gene for discrimination between *Meloidogyne* spp. in this study has also proven to be unreliable, similar to findings of Rashidifard *et al*. (2019).

Likewise, to the discrepancies in the molecular characterisation of *Meloidogyne*, species-specific PCR of *Pratylenchus* samples found *P. brachyurus* to be the most abundant while sequencing results indicated otherwise (Table 4.8 and Figures 4.7-4.8). Sequencing results of *Pratylenchus* spp. in this study indicated that *P. bolivianus* together with *P. curvicauda* were the most abundant species. *Pratylenchus bolivianus* has previously been reported on crops such as rooibos tea (*Aspalathus linearis*) and potato (Troccoli *et al*., 2016; Daramola *et al*., 2021). As morphological identification and discrimination of *Pratylenchus* spp. are difficult, molecular identification and discrimination are becoming more important (Troccoli *et al*., 2016; Jansen *et al*., 2017a; Jansen *et al*., 2017b).

4.6 Conclusion

The impact of *Meloidogyne* and *Pratylenchus* spp. on various crops such as maize and soybean cannot be underestimated. The accurate identification of either a single population or a mixed community of RKN and lesion nematodes that parasitise a field is crucial as species such as *M. enterolobii* are considered highly pathogenic and can cause damage to the crop. Furthermore, the impact of *M. enterolobii* and *P. brachyurus* co-occurrence on any given crop should be investigated further. This study emphasises the fact that it is possible and common for mixed communities of both *Meloidogyne* and *Pratylenchus* spp. to parasitise a crop in a single field. The use of molecular techniques such as species-specific PCR can rapidly verify the presence and distribution of these nematode species. Although sequencing is an alternative molecular tool that can be used to identify nematode species, the sole reliance on DNA sequencing for identification might be flawed. The use of certain genes, like the NADH5 and D2-D3, might impact accurate species identification especially when investigating mixed communities. For example, NADH5 can provide reliable identification of only a few RKN species like *M. incognita*, *M. javanica* and *M. arenaria*. Moreover, the difficulty of morphological identification of *Meloidogyne* and *Pratylenchus* spp. has led to numerous misidentified species that are found on the GenBank database that was used to characterise the sequences obtained from *Meloidogyne* and *Pratylenchus* samples from this study, thus, impacting the accuracy of sequence identification when using this platform. The use of a single molecular technique as a sole method for nematode identification might not be accurate enough for the time being and must be combined with other identification techniques such as iso-enzyme analyses and/or morphological characterisation to aid in the accurate identification of nematodes.

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CHAPTER 5: SHIFTS IN RHIZOSPHERE BACTERIAL AND PLANT PARASITIC NEMATODE COMMUNITY STRUCTURES IN A SOYBEAN-MAIZE ROTATION SEQUENCE

"What you learn from a life in science is the vastness of our ignorance."

David Eagleman

5.1 Abstract

The main aim of agricultural intensification is to increase food/crop production, but these practices ultimately affect various soil ecosystem services negatively. Crop rotation sequences, for example, can have a major influence on soil health, by causing shifts in soil bacterial and plant- parasitic nematode (PPN) communities. Rotation of soybean and maize has economic and ecological benefits opposed to monoculture of either of these crops. However, both crop rotation and monoculture practices can result in important changes in bacterial and PPN communities that can be positive or detrimental to the sustainable crop production. Examining the soil bacterial community structure and PPN communities associated with different crop rotation schemes is thus important. Therefore, the focus of this study was to determine the impact of a soybean-maize rotation system on the diversity and/or changes on bacterial and PPN communities with samples being collected twice over three consecutive summer seasons in soybean and maize fields in the Mpumalanga Highveld region of South Africa. The roots of soybean and maize plants obtained were used to extract and determine the PPN community. Rhizosphere soil from the same plants was used to determine the bacterial community by means of Next Generation Sequencing (NGS). Results indicate that roots from soybean fields tend to have similar PPN communities in comparison to those from maize fields. A similar trend was observed for the bacterial diversity present in soybean and maize rhizospheres. Identifying crop rotation schemes that positively improve soil bacterial community structure, while reducing the damage caused by PPN, can be of value to improve soil health and enable sustainable crop production.

Keywords: crop rotation; maize; bacterial community structure; *Meloidogyne*; South Africa; soybean

5.2 Introduction

The increase in global population and need for food security calls for more sustainable agricultural practices, especially relating to crops such as soybean (*Glycine max*), maize (*Zea mays*), sorghum (*Sorghum bicolor*), and wheat (*Triticum aestivum*) (Engelbrecht *et al*., 2020). Soil quality is considered one of the most important factors that can contribute to the sustainable agricultural production of economically important crops such as soybean and maize. Since these crops have high economical value, the continuous practice of monoculture soybean or maize can have detrimental effects on soil quality while ultimately exacerbating the occurrence of various pests and diseases [including plant-parasitic nematodes (PPN)] (Bai *et al*., 2015; Zhang *et al*., 2019). Long-term economic and ecological benefits of soybean and maize production can be obtained by means of crop rotation with these Leguminosae and Gramineae crops (Mazzilli *et al*., 2019; Zhang *et al*., 2019). However, the global distribution and wide host range of PPN can negatively impact the sustainability of soybean-maize rotations (Fourie *et al*., 2017; Jones *et al*., 2017).

Globally PPN from the genera including *Criconemoides* (ring), *Helicotylenchus* (spiral), *Heterodera* (cyst), *Hoplolaimus* (spiral), *Meloidogyne* (root-knot), *Pratylenchus* (lesion) and *Tylenchorhynchus* (stunt) are known pests of soybean and maize, with *Meloidogyne* and *Pratylenchus* spp. being the predominant genera damaging both crops (Talwana *et al*., 2008; Fourie *et al*., 2017; Jones *et al*., 2017; Simon *et al*., 2018; Mbatyoti *et al*., 2019; Neher *et al*., 2019; Fourie & De Waele, 2019; Engelbrecht *et al*., 2021). In South Africa the predominant *Meloidogyne* species known to parasitise maize and soybean are *M. incognita, M. javanica, M. arenaria* and *M. enterolobii,* while for *Pratylenchus* spp. *P. zeae*, *P. brachyurus* and *P. crenatus* are dominant (Fourie *et al*., 2001; McDonald *et al*., 2017; Mbatyoti *et al*., 2020).

The use of soybean-maize crop rotations has economic value, but it can result in a shift in the PPN community composition (Neher *et al*., 2019) and lead to increased PPN densities within a field which is known to adversely impact on sustainable crop production (Simon *et al*., 2018; Mbatyoti *et al*., 2019). Likewise, the use of monoculture soybean or maize can also lead to increased PPN densities (Govaerts *et al*., 2006; Simon *et al*., 2018) threatening sustainable grain production. Both grain-based rotations and monoculture ultimately impacts other biotic soil quality factors, such as the rhizosphere microbial community (Zhou *et al*., 2018; Zhang *et al*., 2019; Liu *et al*., 2020).

Rhizosphere microbial communities play an important role in soil ecosystem functionality and sustainability (Waldrop *et al*., 2000; Liu *et al*., 2020), while greatly impacting important soil processes like biogeochemical cycles and metabolic processes (Cavigelli & Robertson, 2000).

Moreover, numerous bacterial and fungal genera have the potential to reduce PPN densities and mitigate their damage to crops (Poveda *et al*., 2020; Engelbrecht *et al*., 2020; Migunova & Sasanelli, 2021).

Although numerous studies have reported changes in microbial community compositions, these changes varied between studies. A study done by Tang *et al*. (2009) in the Heilongjiang Province of China, identified a significant increase in Actinobacteria abundance in soybean-maize rotation compared to that of soybean monoculture. In contrast to this, Proteobacteria, Actinobacteria and Firmicutes were reported to have significantly higher abundances in a soybean-maize rotation system compared to a soybean monoculture system in another Chinese study (Zhu *et al*., 2014). Furthermore, a study by Jangid *et al*. (2011) reported no significant changes in bacterial community composition between soybean-based rotations and soybean monoculture from fields in Michigan, USA. These inconsistent results can be attributed to various factors such as research methods applied (extraction methods, analyses, instrumentation etc.), rotation systems practiced (cultivars used, planting years and/or area studied) (Venter *et al*., 2016; Liu *et al*., 2020; Engelbrecht *et al*., 2021) and various other biotic and abiotic factors (e.g., climate, soil type and pH). Hence, the focus of this study was to investigate the impact of soybean-maize rotation systems on the diversity and/or changes on bacterial and PPN communities associated with the rhizospheres (roots and soil) of these grain crops over a three-year period in the second-largest soybean production area, the Mpumalanga Highveld of SA (Grain SA, 2021).

5.3 Materials and Methods

5.3.1 Site description

Located in one of the nine provinces of SA, the Mpumalanga Highveld (where this study was conducted) has a mean annual rainfall of about 900 mm and an annual temperature range of 6-30 °C (Botai *et al*., 2018). The grassland biome of this province (Nkuekam *et al*., 2018) which contains rich and fertile upper layers, together with its annual rain and wide temperature ranges, makes it suitable for cultivation of annual summer crops such as soybean and maize. In the summer growing season of 2018/2019, which represented the first sampling interval, composite root and soil samples were collected, as explained in Engelbrecht *et al*. (2021), from 15 fields where soybean was cultivated, while in the 2020/2021 summer growing (representative of the second sampling interval) samples were taken from the same fields where either soybean or maize was grown in rotation at the time. In total 15 fields were sampled during the two sampling intervals. The fields are located across the Mpumalanga province (see Figure 3.1 in Chapter 3).

5.3.2 Plant-parasitic nematode extraction and identification

Plant-parasitic nematodes were extracted from 20 g of composite root samples using the centrifugal-flotation method described by Swart and Marais (2017) and transferred to a De Grisse counting dish (De Grisse, 1969). The nematodes were counted and concurrently identified to genus level using a Nikon ECLIPSE TS100 (Nikon Corporation, Tokyo, Japan) inverted microscope (40× magnification) (Engelbrecht *et al*., 2021).

5.3.3 DNA extraction of microbial communities from the soil

The microbial community DNA extraction of the composite samples collected during the two sample intervals were done as explained in Engelbrecht *et al.* (2021). Briefly, 0.25 g of each composite sample was used for DNA extraction with the NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) with an optimal lysis buffer system (a combination of SL 2 and Enhancer SX). This was followed by a quality control check (absorbance ratio 260/230 and 260/280) using a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

5.3.4 High-throughput sequencing of rhizosphere bacteria and data processing

The diversity of the total rhizosphere bacterial community was assessed by next generation sequencing (NGS) of amplicons obtained from extracted DNA Sequencing of 16S rRNA (Engelbrecht *et al*., 2021). Initially the bacterial primers (linked to the adapter sequences needed for Illumina MiSeq analysis) 341F and 805R (Herlemann *et al*., 2011) was used to amplify hypervariable region V3-V4 of the 16S gene using the 1000 Cycler thermal cycler (BioRad, Hercules, CA, USA) (Kindelworth *et al*., 2013). All samples consisted of a final volume of 25 µL containing 1 µL DNA (20–60 ng/µL), 12.5 µL KAPA Hifi Hotstart Ready (Roche, Basel, Switzerland), 5 µL (1 µM) of the forward and reverse primers, respectively, and nuclease free water. After the initial polymerase chain reaction (PCR) samples (amplicons) were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). This was followed by library amplification with a limited-cycle PCR program to attach dual-index barcodes to the amplicons (Nextera XT Index Kit, Illumina, San Diego, CA, USA) as recommended by the library preparation protocol from Illumina (Gallego *et al*., 2019). The quality and sizes of the resulting DNA fragments were subsequently evaluated on a 2% (w/v) agarose gel. Obtained libraries were quantified with a fluorescence-based method (Invitrogen) using a Qubit 3.0 (Life Technologies, Carlsbad, CA, USA). Libraries were then pooled (4 pMol) and denatured with 0.1 N NaOH. This was finally followed by paired-end sequencing on an

Illumina MiSeq system (Illumina, San Diego, CA, USA) using a MiSeq Reagent Kit V3 600 cycles.

5.3.5 Bioinformatic analyses

Initially demultiplexed paired-end reads were checked for quality using MiSeq reporter software. This was followed by trimming the paired reads at both 5' and 3' ends to eliminate poor quality nucleotides. Paired reads were also denoised, merged, depleted of chimeric sequences, and clustered into amplicon sequence variants (ASV) (operational definition for a species) by means of the DADA2 denoiser (Divisive Amplicon Denoising Algorithm v. 2) and integrated into the Quantitative Insight into Microbial Ecology version 2 (QIIME2) software (Bolyen *et al*., 2019). However, quality of reverse reads resulted in a data loss of 80 %, especially for the 2021 sampling period. Due to this, focus was placed on the use of only the forward reads for further analyses. The alternative pipeline was as follows: Firstly, primers (17 bases) were removed from the forward reads using the following command: *vsearch\vsearch --fastx_filter forward_read_fastq --fastq_stripleft 17 --fastaout output_fastq* (Rognes *et al*., 2016). Next, Fastq files were converted to fasta files using the following command: *sed -n '1~4s/^@/>/p;2~4p' input.fastq > output.fasta*. The FASTA files obtained were then used for OTU picking by using the USEARCH SINTAX command with RDP training set v18 for taxonomy classification (Edgar, 2013; Edgar, 2018). The obtained SINTAX files were then processed to abundance tables using R scripts as in the publication of Mann *et al*. (2021).

5.3.6 Statistical analysis of nematode and microbial data

The 15 fields sampled during the two intervals, were divided for statistical analyses to represent the 10 fields where soybean was grown and sampled during both sampling intervals, and the five fields being under soybean cultivation during the first and under maize cultivation in the second sampling intervals, respectively. Nematode data obtained from the plant roots of the two sampling intervals, namely the first (Table 3.1) and second (Table 4.3) were used to construct correspondence analysis (CA) ordination biplots for the PPN community composition. This was done by using the Windows-based program CANOCO (CANOCO version 5, Microcomputer Power, Ithaca, NY, USA). The abundance data for the top six most abundant PPN genera (*viz*. *Meloidogyne*, *Pratylenchus*, *Helicotylenchus*, *Scutellonema, Hoplolaimus* and *Rotylenchulus*) were then log transformed [log(x+1)] and subjected to Factorial ANOVA, and the means separated by the Tukey's post-hoc test (HSD) where $P<0.05$ (Statistica 13.3; https://statistica.software.informer.com/13.3/) to determine whether there was significant changes in their mean abundance (Table S2a-c, for the 10 fields under soybean

cultivation in both intervals and Table S3a-c for the five fields under soybean cultivation during the first and under maize cultivation in the second sampling interval).

The online tool, MicrobiomeAnalyst, was used to do abundance and diversity analyses (Chong *et al*., 2020). Alpha diversities of microbial communities, reflected by the bacterial abundance and diversity with regards to the sampling interval for each field, were produced using the Chao1 (abundance of bacterial ASV) and Shannon (community richness) indices. A high Chao1 index represents a high level of bacterial species richness, while a high Shannon index is characteristic of high bacterial diversity levels. Principle component analyses (PCoA) diagrams were used to illustrate the differences between the various rhizosphere microbial communities, beta diversity, of the fields. Since the diversity of bacterial communities in each of the fields has its own unique taxonomic abundance profile, fields with similar taxonomic profiles will group together. Comparisons or variances in taxonomic profiles were analysed by using the Bray-Curtis dissimilarity distance distribution which uses bacterial community read counts. Fields that are plotted close to zero indicate similar taxonomic abundance profiles, while sites that do not plot close to zero have dissimilar taxonomic profiles. Significant differences in bacterial genus abundance with regards to the crop rotation scheme were evaluated using the LEfSe algorithm (Engelbrecht *et al*., 2021) with the following parameters: an LDA score of 3 and a cut off *p*-value of 0.05. Bacterial genera that were identified as having similar genus names, were assigned a number to identify which of these genera is being referred to in further analysis (Engelbrecht *et al*., 2021).

5.4 Results

5.4.1 Plant-parasitic nematodes associated with roots of soybean and maize

5.4.1.1 Plant-parasitic nematodes associated with 10 soybean fields sampled during the 2018/2019 (first) and 2020/2021 (second) summer growing seasons

The PPN communities extracted from the roots of 10 fields where soybean was cultivated during the two sampling intervals, consisted of *Meloidogyne, Pratylenchus, Helicotylenchus, Scutellonema, Hoplolaimus, Rotylenchulus, Tylenchorhynchus, Ditylenchus* and *Rotylenchus* with other species belonging to the families Tylenchida, Aphelenchidae and Criconematidae (genera and families listed in order of dominance). The CCA analysis (Figure 5.1), with axis 1 and 2 explaining 71.6% and 89.6% of the variation, respectively, suggested that fields that contained higher average densities of *Meloidogyne* spp. (n = 8055 individuals/20 g root) tended to have similar PPN community compositions. A similar trend, but to a lesser degree (regarding the number of fields identified), was evident for sites with high *Pratylenchus*

densities (n = 4159 individuals/20 g root) compared to those with high *Meloidogyne* densities. Most of the soybean fields sampled had similar PPN community compositions for the first sampling interval (Figure 5.1) and the 2020/2021 summer growing season/second sampling interval (Figure 5.1), except for S9 (S9_SY1) and S14 (S14_SY1) of which the PPN communities differed distinctly during the first sampling interval (Figure 5.1). These latter two fields had high densities of *Rotylenchulus* densities (n = 528 individuals/20 g root) (Figure 5.1). Furthermore, CA analysis indicated that a strong positive correlation exists between the spiral nematodes (*Helicotylenchus*, *Hoplolaimus* and *Scutelonema*) while *Meloidogyne* and *Pratylenchus* counts showing a negative correlation.

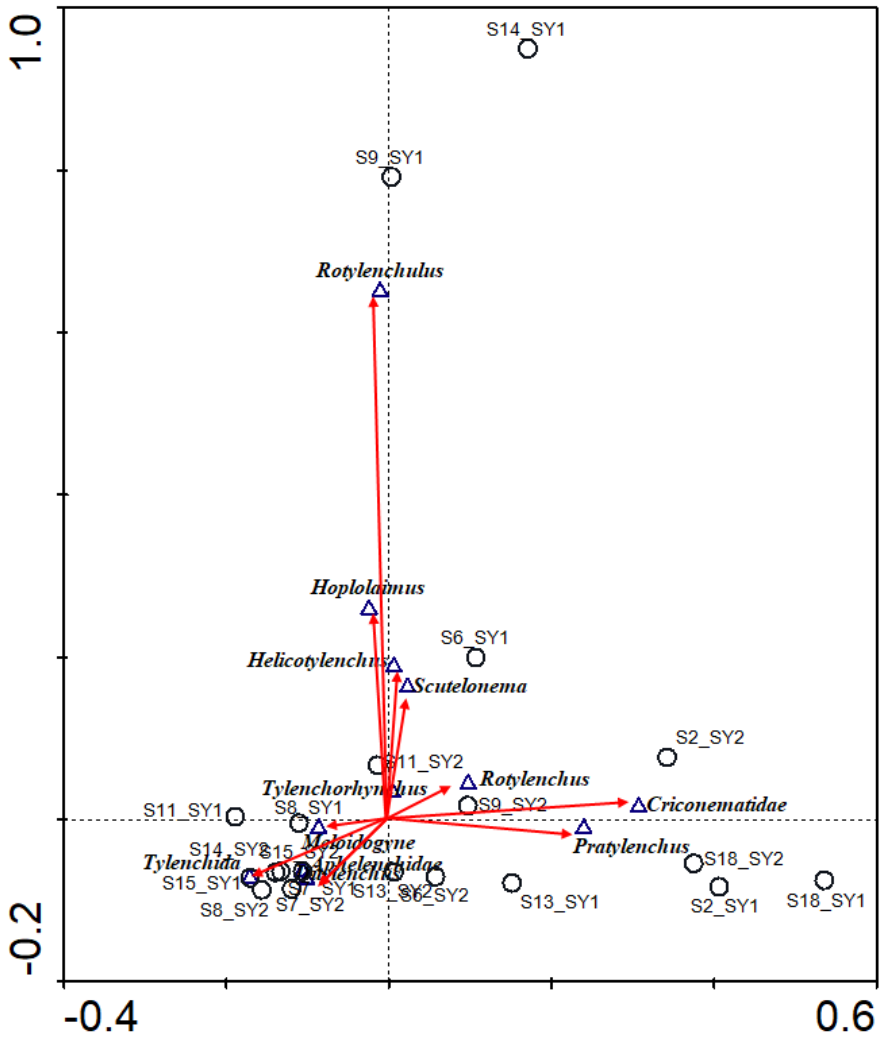

Figure 5.1: The correspondence analysis (CA) ordination biplot of the PPN community composition for 10 soybean fields in the Highveld production area (Mpumalanga province) of South Africa during the 2018/2019 (first sampling) and 2020/2021 (second sampling) summer growing seasons. Shorter distances between fields in the CA ordination indicate a greater degree of similarity between fields and their respective PPN community composition. Axes 1 and 2 represent 71.6 and 89.6% of the variation in the data, respectively. Data for the PPN community compositions are displayed in Chapter 3 and 4 (Tables 3.1 and 4.3). Fields are identified as S2_SY1 (S2=Second field, S=soybean cultivation and Y1=first sampling interval).

Analysis of variance indicated that the top six most abundant PPN genera (*viz*. *Meloidogyne*, *Pratylenchus*, *Helicotylenchus*, *Scutellonema, Hoplolaimus* and *Rotylenchulus*) differed significantly among the 10 fields and the two sampling intervals regarding their abundance per 20 g roots. This was indicated by significant interactions for Field x Sampling Interval (Table S2a-c). Abundance of *Meloidogyne* and *Pratylenchus* were high, generally <1 000 individuals /20 g roots (Table S2a) while that of *Helicotylenchus*, *Scutellonema, Hoplolaimus* and *Rotylenchulus* (Tables S2b & c) generally ranged from low (<100 individuals / 20 g roots) to intermediate (101 – 999 individuals /20 g roots).

With regards to *Meloidogyne* densities (Table S2a), S7 was found to have the highest across both the first (24402 ± 2680) and second (6672 ± 1449) sampling intervals. Furthermore, S2 was found to have significantly lower *Meloidogyne* densities in the second sampling interval (109 ± 31) when compared to the first (2548 ± 1177). *Pratylenchus* densities were significantly lower during the second sampling interval as compared to the first for fields S2, S7, S9 S13 and S18 (Table S2a). Of the 10 soybean fields S11 was found to have the lowest *Pratylenchus* densities at both sampling intervals (107 ± 34 and 118 ± 32, respectively). For *Helicotylenchus*, various fields (S2, S7, S9, S11 and S15) had significantly lower densities during the second compared to the first sampling interval, whereas *Scutellonema* densities were similar for the two sampling intervals, except for S2 in which it was higher during the second than the first sampling interval (Table S2b). *Hoplolaimus* densities (Table S2c) were generally significantly higher in the first sampling interval for all fields compared to those of the second interval. Furthermore, *Rotylenchulus* densities for S9 (1000 ± 494) was also significantly higher during the first than the second sampling interval (Table S2c).

5.4.1.2 Plant-parasitic nematodes associated with five fields under soybean cultivation during the 2018/2019 (first) and under maize cultivation in the 2020/2021 (second) summer growing seasons

Lower diversity in terms of PPN genera and/or families was recorded for fields on which soybean (first sampling interval) and maize (second sampling interval) were cultivated compared to that identified from soybean fields in both first and second sampling intervals with *Meloidogyne*, *Pratylenchus*, *Helicotylenchus*, *Scutellonema*, *Hoplolaimus*, *Rotylenchus*, *Aphelenchus* and specimens belonging to the family Aphelenchidae (listed in order of predominance) being recorded. Opposed to the PPN community composition of soybean fields (first and second sampling interval), those from five fields that was under soybean (first sampling interval) and maize (second sampling interval) cultivation, showed more diverse community compositions, e.g. S1 (S1_SMY1) and S5 (S5_SMY1) during 2018/2019 (Figure 5.2) and S12 (S12_SMY2), S16 (S16_SMY2) and S17 (S17_SMY2) during 2020/2021 (Figure

5.2) when compared to their respective corresponding samples of the alternate sampling time. Another difference is the positive correlation found between *Pratylenchus* and *Scutellonema* in soybean and maize fields during the second sampling interval, while a negative correlation between *Meloidogyne* and *Pratylenchus* was evident in soybean-maize rotations like it was recorded for the first sampling interval.

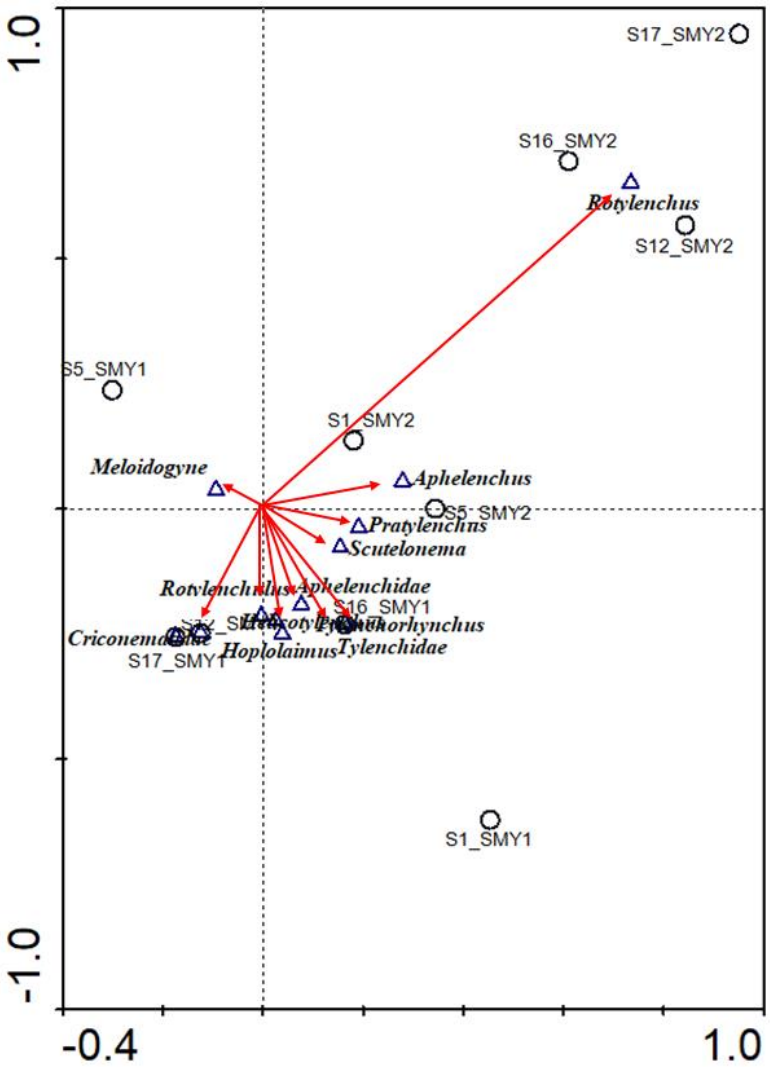

Figure 5.2: The correspondence analysis (CA) ordination biplot of the PPN community composition for five fields under soybean cultivation during the 2018/2019 and under maize cultivation in the 2020/2021 summer growing seasons in the Highveld production area (Mpumalanga province) of South Africa. Shorter distances between fields in the CA ordination indicate a greater degree of similarity between fields and their respective PPN community composition. Axes 1 and 2 represent 45.4 and 67.6 % of the variation in the data, respectively. Data for the PPN community compositions are displayed in Chapter 3 and 4 (Tables 3.1 and 4.3). Fields are identified as S1_SMY1 (S1=First field, SM=soybean and maize cultivated during first and second sampling intervals and Y1=first sampling interval).

The general trend for nematode abundance of the predominant plant-parasitic nematode genera was that substantial to significant reductions were evident for maize fields (second sampling interval) compared to their counterpart soybean fields (first sampling interval (Tables S3a-c). Abundance of *Meloidogyne* ranged from intermediate (101 individuals / 20 g roots) to high (>1 000 individuals / 20 g roots), while *Pratylenchus* abundance ranged from low to

intermediate (Table S3a). *Helicotylenchus* (Table S3b) and *Rotylenchulus* (Table S3c) abundance was low, while those for *Scutellonema* (Table S3b) and *Hoplolaimus* (Table S3c) ranged from low to intermediate. For the five fields that were under soybean (first sampling interval) and maize (second sampling interval) cultivation, lower densities of *Meloidogyne* were evident when compared to that of the 10 fields that were under soybean cultivation for both sampling intervals.

Analysis of variance indicated that abundance of *Meloidogyne* were significantly lower in three of the five maize fields (S12, S16 and S17) sampled during the second sampling interval when compared to those for the soybean fields (first sampling interval) (Table S3a). For *Pratylenchus* abundance one maize field (S1: second sampling interval) was significantly lower than that of the corresponding soybean field sampled in the first interval (Table S3a). *Helicotylenchus* abundance was significantly lower in three of the maize fields (S12, S16 & S17) compared to their counterpart soybean fields sampled in the first interval, while for *Scutellonema* two maize fields (S1 & S12) had significantly lower abundance than the corresponding soybean fields (first interval). Likewise, the *Rotylenchulus* abundance was significantly higher in two fields (S5 and S12) during the first sampling interval compared to their counterpart fields in the second interval (Table S3c). Concerning *Hoplolaimus*, all maize fields sampled in the second sampling interval had significantly higher abundance compared to the corresponding soybean fields sampled in the first interval (Table S3c).

5.4.2 Rhizosphere bacterial communities associated with 10 soybean fields during the 2018/2019 (first) and 2020/2021 (second) summer growing seasons

5.4.2.1 Alpha diversity

For the alpha diversity analysis, reflective of bacterial abundance and diversity, of the 10 soybean fields sampled during the first and second sampling intervals, the Chao1 index reveals that the soybean rhizospheres had similar ASV abundances; except for S6Y1 and S14Y1 with higher bacterial ASV abundances compared to the other fields sampled during the first sampling interval as well as those sampled during the second sampling interval (Figure 5.3a). Furthermore, S18 had lower levels of ASV abundance when compared to the other soybean fields sampled during both the first and second sampling interval (Figures 5.3a). With regards to the species diversity (Shannon index), Figure 5.3b showed that the 10 fields varied in their bacterial community diversity for the two sampling intervals, with distinctly higher diversity (±4.2–4.5) observed during the first as compared to the second sampling interval (±3.7–3.9).

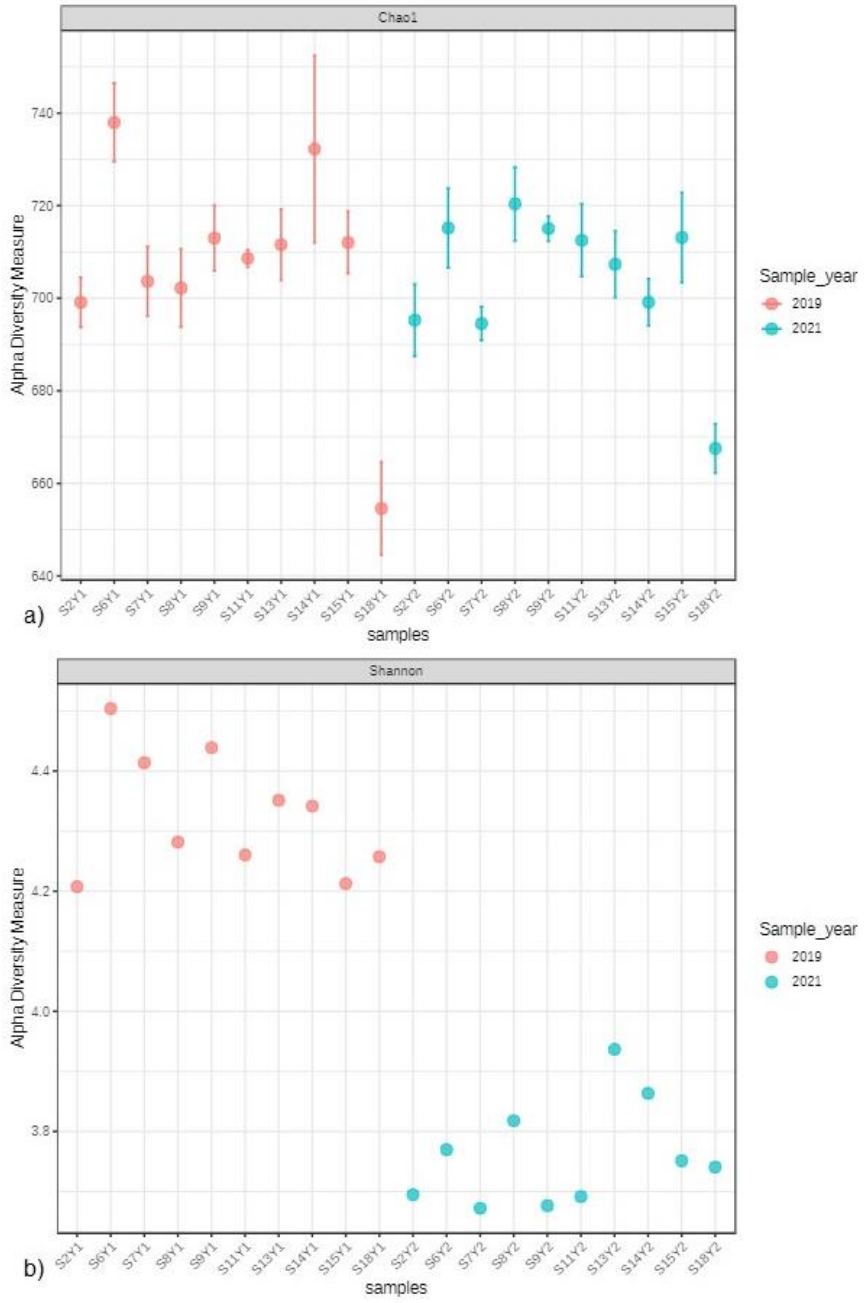

Figure 5.3: The alpha diversities of rhizosphere samples collected from 10 soybean fields in the Highveld production area (Mpumalanga province) of South Africa during the 2018/2019 (first sampling) and 2020/2021 (second sampling) summer growing seasons. The data was plotted with the a) Chao1 and b) Shannon diversity indices with $p<0.05$; the highest and lowest values are indicated for each field on Chao1. Fields are identified for example as S1Y1 (S1=First field and Y1=first year of sampling).

5.4.2.2 Beta diversity

Distinct differences in taxonomic profiles of the rhizosphere bacterial communities from soybean fields (first sampling interval) were evident compared to those from the same soybean fields during the second interval, since they grouped separately on the PCoA diagram (Figure 5.4). However, the rhizospheres from the 10 soybean fields (first sampling interval)

grouped relatively close to each other, except for one field that plotted to the bottom of the graph. With relatively close grouping being evident for the same soybean fields for the second sampling interval. The general similar grouping of the fields sampled during the two intervals relatively close to zero on both the *x* and *y*-axis indicate that they shared similar bacterial taxonomic profiles.

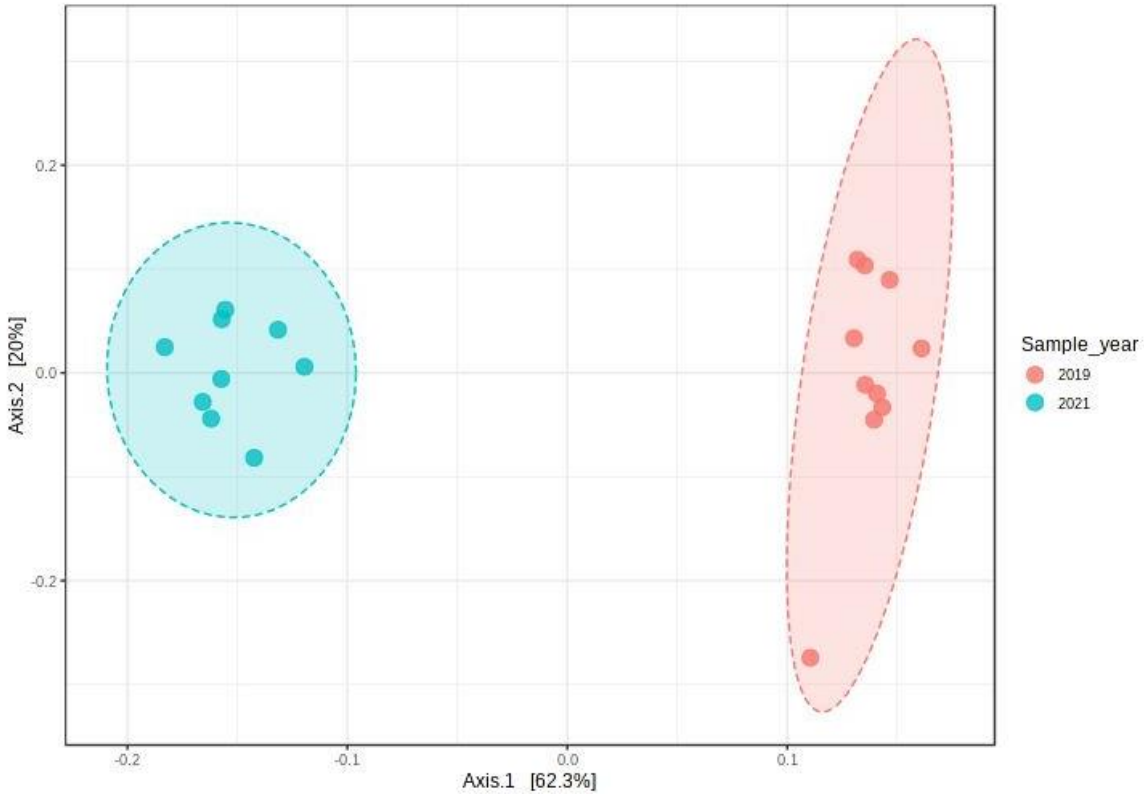

Figure 5.4: The 2D-PCoA diagram shows the beta-diversity of microbe communities among 10 fields sampled from the Highveld region, Mpumalanga province, South Africa during the 2018/2019 (first sampling) and 2020/2021 (second sampling) summer growing seasons. The statistical method used to analyse group similarities was PERMANOVA ($p<0.001$) and applied a Bray-Curtis dissimilarity distance distribution with the sample sites using a correction of R-squared=0.61622.

5.4.2.3 Relative abundance of bacterial populations associated with 10 soybean fields during the 2018/2019 (first) and 2020/2021 (second) summer growing seasons

From the NGS sequences obtained for bacterial populations from soil collected from 10 soybean fields sampled during the first and second intervals, Figure 5.5 lists the top 20 most abundant Phyla (Figure 5.5a), Class (Figure 5.5b), Family (Figure 5.5c) and Genera (Figure 5.5c) for each field. For nine of the 10 fields the phylum Acidobacteria had reduced abundance levels in the samples obtained during the second sampling interval as compared to the first sampling interval. Only S18 showed a small increase in Acidobacteria levels (3.82%) from the first to the second sampling interval. Furthermore, the abundance of Actinobacteria was also found to be lower in the second sampling interval when compared to the first. Moreover, of the 10 soybean fields sampled, six were found to have higher Proteobacteria abundance for the

second sampling interval than for the first sampling interval; by contrast four fields (S11, S13, S14 and S18) had lower Proteobacteria levels during the second compared to the first sampling interval. Samples obtained during the second sampling interval also showed more ASVs that were grouped under Uncultured phylum. Another notable change in phylum abundance was the increase in Firmicutes abundance from the first to the second sampling interval (Figure 5.5a). With regards to the top 20 Classes identified in the 10 soybean fields, a clear increase in the abundance of ASVs classified as an Uncultured was also evident from the first to the second sampling interval. Acidobacteria_Gp6 and Actinobacteria both showed lower abundance for the second compared to the first sampling interval. Small increases in abundance were also noted for Acidobacteria_Gp3, Chitinophagia and Bacilli from the first to the second sampling interval (Figure 5.5b).

Similar to the Uncultured phylum and class groups, the second sampling interval showed an increase in ASVs being classified as Uncultured family (Figure 5.5c). Furthermore, Bacillaceae_1 was the only other family showing increased abundance from the first to the second sampling interval (Figure 5.5c). Interestingly, reduced abundance for the Bradyrhizobiaceae family was observed across all 10 soybean fields from first to the second sampling interval (Figure 5.5c). Changes in the abundance of the top genera (Figure 5.5d) were also evident when comparing samples obtained during the first and the second sampling interval. Of the genera identified, all the similar genus names were assigned a number to identify which of these bacterial genera is being referred to for further analysis. Genera that were classified as ‘Other’, showed reduced abundance for the second sampling interval, with the genus named Uncultured87 also following a similar trend. Notable increases in the abundance of the genera listed as Uncultured586 and Uncultured172 were observed from the first to the second sampling interval.

A total of 178 bacterial genera were found to have significantly different abundances in the rhizospheres of the 10 soybean fields. Of the top 50 most significant bacterial genera, 35 had significantly higher abundances in the first sampling interval compared to the 15 genera that had higher significant abundances in the samples obtained during the second sampling interval (Figure 5.6). *Sphingomonas*, *Streptomyces*, *Nocardioides*, *Bradyrhizobium* and *Methylobacterium* are amongst those genera that had significantly higher abundances for the first compared to the second sampling interval with the genus *Bacillus* being amongst the 15 genera that were more abundant for the second sampling interval (Figure 5.6).

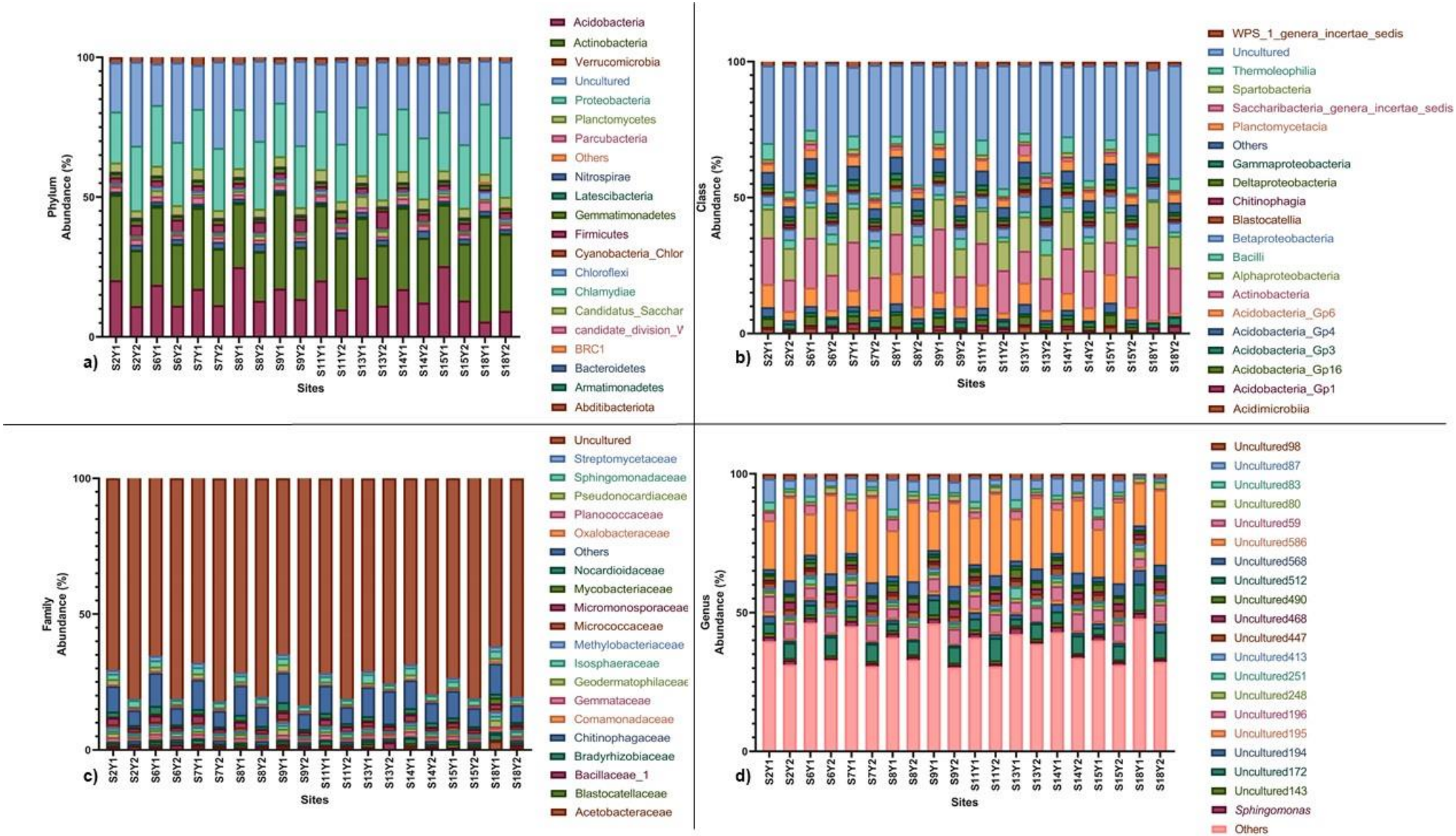

Figure 5.5: Stacked bar graphs indicating the top 20 most abundant bacterial a) Phyla, b) Class, c) Family and d) Genera associated with 10 soybean fields sampled from the Highveld region, Mpumalanga province, South Africa during the 2018/2019 (first sampling) and 2020/2021 (second sampling) summer growing seasons. Fields are identified for example as S1Y1 (S1=First field and Y1=first year of sampling).

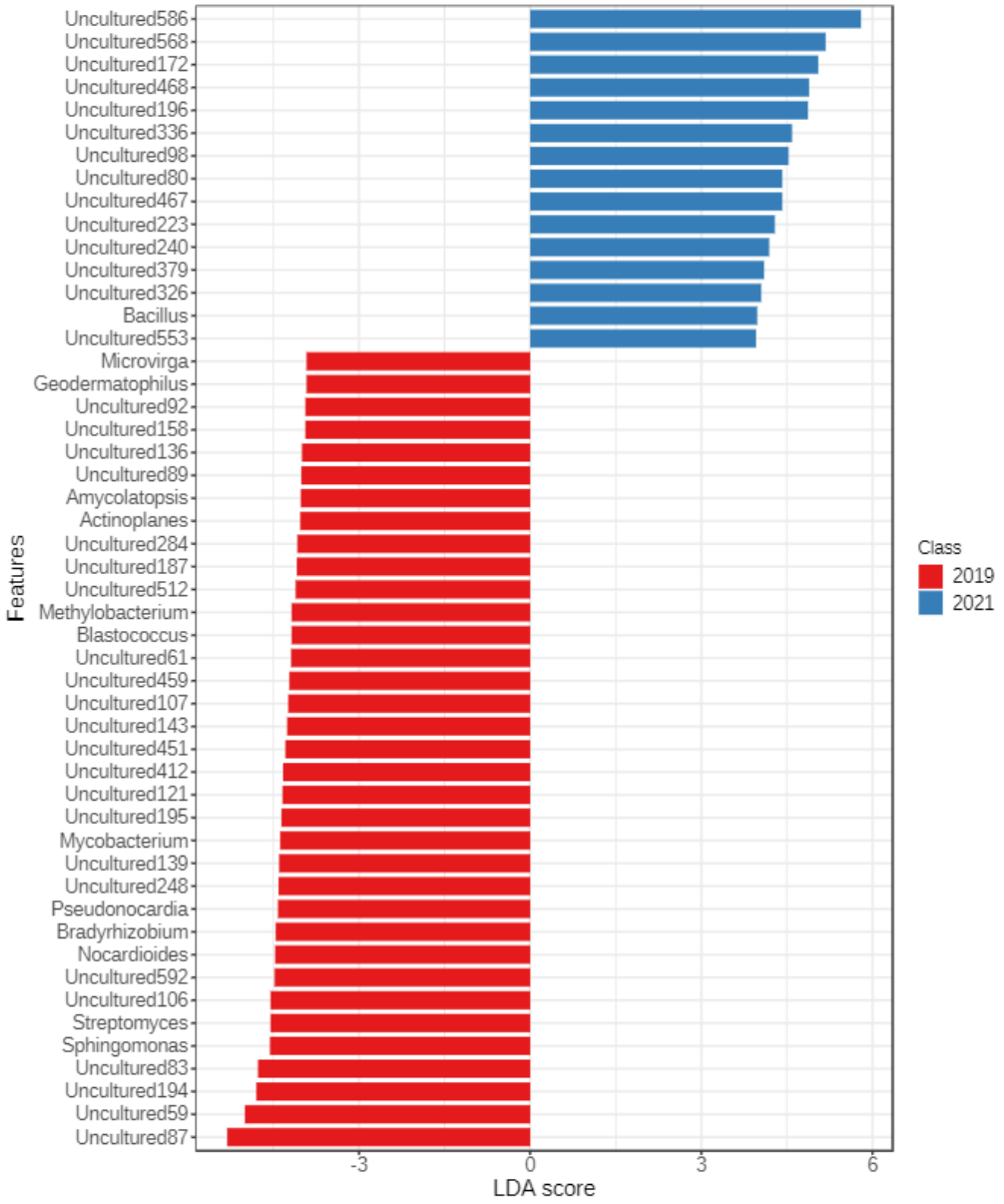

Figure 5.6: Graphical summary at the top 50 bacterial genera of 178 identified as having significantly different abundances using the Linear Discriminant Analysis (LDA) Effect Size (LEfSe) based on non-parametric factorial Kruskal-Wallis (KW) sum-rank test among the 10 soybean fields sampled from the Highveld region, Mpumalanga province, South Africa during the 2018/2019 (first sampling) and 2020/2021 (second sampling) summer growing seasons.

5.4.3 Rhizosphere bacterial communities associated with five fields under soybean cultivation during the 2018/2019 (first) and under maize cultivation in the 2020/2021 (second) summer growing seasons

5.4.3.1 Alpha diversity

The Chao1 index reveals that five soybean fields sampled during the first sampling interval had similar levels of bacterial ASV abundance, except for S17Y1 which was found to have the lowest level of bacterial ASV abundance during the first sampling interval (Figure 5.7a). Furthermore, during the second sampling interval, the same five fields sampled for the first

interval were under maize cultivation. Of these fields S1Y2, S12Y2 and S16Y2 (sampled during the second interval) had higher levels of bacterial ASV abundance when compared to the same fields sampled during the first sampling interval. With regards to the species diversity (Shannon index), Figure 5.7b showed that the five sampled fields had more diverse bacterial communities (±3.9–4.5) during the first interval (under soybean cultivation) when compared to the second sampling interval when they were under maize cultivation (±3.6–3.9).

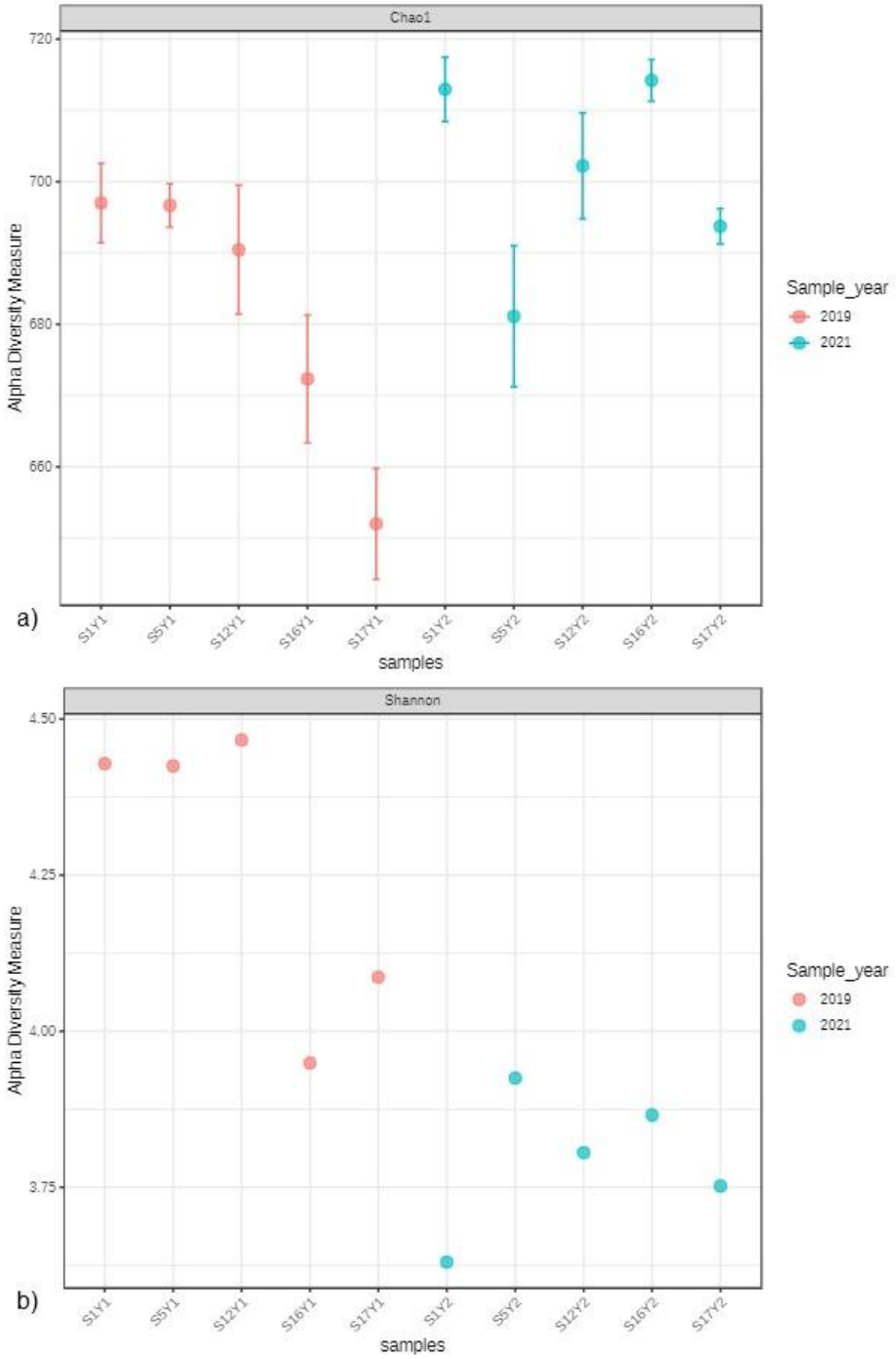

Figure 5.7: The alpha diversities of rhizosphere samples collected from five fields under soybean cultivation during the 2018/2019 and under maize cultivation in the 2020/2021 summer growing seasons in the Highveld production area (Mpumalanga province) of South Africa. The data was plotted with the a) Chao1 and b) Shannon diversity indices with $p<0.05$; the highest and lowest values are indicated for each field on Chao1. Fields are identified for example as S1Y1 (S1=First field and Y1=first year of sampling).

5.4.3.2 Beta diversity

The principal component analysis diagram (Figure 5.8) indicates the differences between the various rhizosphere microbial communities of the five soybean and maize fields sampled. The Beta diversity for the fields sampled during the first sampling interval when soybean was grown did not group with the samples obtained during the second sampling interval from the same fields when maize was grown. This indicates different bacterial taxonomic profiles for the five fields for both sampling intervals. However, during the second sampling interval, the five fields grouped closer together than when they were sampled during the first interval when soybean was grown, indicating similar bacterial taxonomic profiles. The five fields sampled during each respective interval grouped relatively close to zero on both the *x* and *y*-axis, indicating that they shared similar bacterial taxonomic profiles during each respective year.

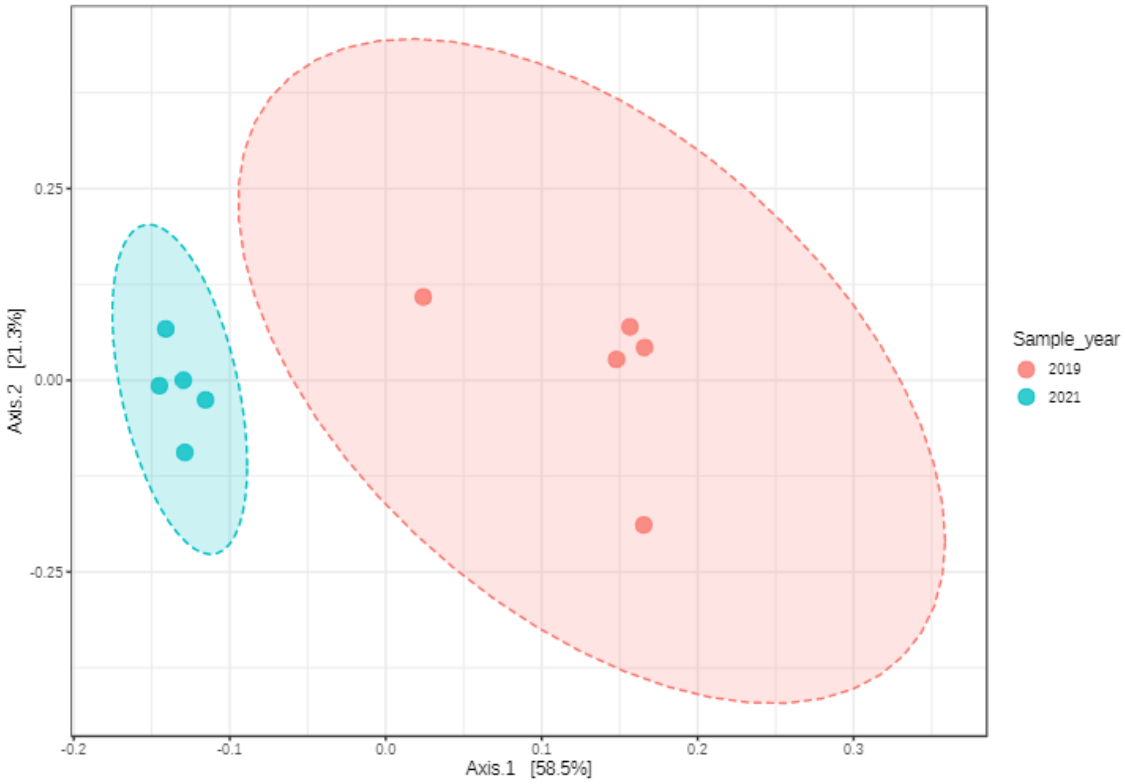

Figure 5.8: The 2D-PCoA diagram shows the beta-diversity of microbe communities from five fields under soybean cultivation during the 2018/2019 and under maize cultivation in the 2020/2021 summer growing seasons in the Highveld production area (Mpumalanga province) of South Africa. The statistical method used to analyse group similarities was PERMANOVA ($p<0.007$) and applied a Bray-Curtis dissimilarity distance distribution with the sample sites using a correction of R-squared=0.54771.

5.4.3.3 Relative abundance of bacterial populations associated with five fields under soybean cultivation during the 2018/2019 (first) and under maize cultivation in the 2020/2021 (second) summer growing seasons

From the NGS sequences obtained for bacterial populations from soil collected from five fields (under soybean cultivation in the first sampling interval and under maize cultivation in the second sampling interval) Figure 5.9 lists the top 20 most abundant bacterial Phyla (Figure 5.9a), Class (Figure 5.9b), Family (Figure 5.9c) and Genera (Figure 5.9c) for each field. Acidobacteria abundance was higher in all five sites during the first sampling interval when soybean was cultivated as compared to their abundance during the second sampling interval when maize was cultivated. The biggest reduction in Acidobacteria abundance was identified for S16 with a 21% difference from the first from the second sampling interval (Figure 5.9a). Furthermore, Actinobacteria abundance was lower during the second sampling interval compared to first sampling interval for four of the five fields, except for S16 that showed a 1.7% increase in Actinobacteria abundance for this period. The phyla Bacteroidetes, Gemmatimonadetes and those listed as Uncultured showed increased abundances for all five fields for the second compared to the first sampling interval (Figure 5.9a). With regards to the top 20 Classes identified in the five fields, increased abundance of ASVs classified as Uncultured was evident from the first to the second sampling interval. The ASVs belonging to class Actinobacteria showed slight increases in abundance from the first to the second sampling interval only for S5 (1.39%) and S16 (2.5%). Other classes such as Bacilli and Deltaproteobacteria showed lower abundance levels for the second sampling interval for maize fields (Figure 5.9b). The classes Alphaproteobacteria and Betaproteobacteria had reduced abundance levels for the second sampling interval compared to the first sampling interval, except for S16 that showed slight increases of 2.61% and 0.78% for Alphaproteobacteria and Betaproteobacteria, respectively, from the first to the second sampling interval (Figure 5.9b).

Similar to data obtained from the soybean fields (Figure 5.5a-5.5c), the Uncultured family had increased ASV abundance for the five maize fields for the second sampling interval. Furthermore, the families Bradyrhizobiaceae, Pseudonocardiaceae and Streptomycetaceae showed reduced abundance for the second sampling interval when compared to the first sampling interval. Notable reductions were also evident for the abundance of bacterial ASVs listed as Others from the first to the second sampling interval (Figure 5.9c). Changes in the abundance of the top genera (Figure 5.9d) were also evident when comparing samples obtained during the first sampling interval to those obtained during the second sampling interval. As explained before, genera identified with similar names were assigned a number to

identify which of these bacterial genera is being referred to in further analysis. Bacterial ASVs that were classified as Other, had reduced abundance during the second sampling interval. Notable increases in the abundance of the genera listed as Uncultured586 and Uncultured172 was observed from the first to the second sampling interval (Figure 5.9d) similar to results obtained from soybean fields (Figure 5.5d).

A total of 142 bacterial genera were found to have significantly different abundances in the rhizospheres of the five fields. Of the top 50 most significant bacterial genera, 29 had significantly higher abundances for the first sampling interval compared to the 21 genera that had higher significant abundances for the second sampling interval (Figure 5.10). *Bradyrhizobium, Streptomyces, Mycobacterium* and *Dictyobacter* are amongst those genera that had significantly higher abundances for the first sampling interval with several Uncultured genera amongst the more abundant genera during the second sampling interval (Figure 5.10)

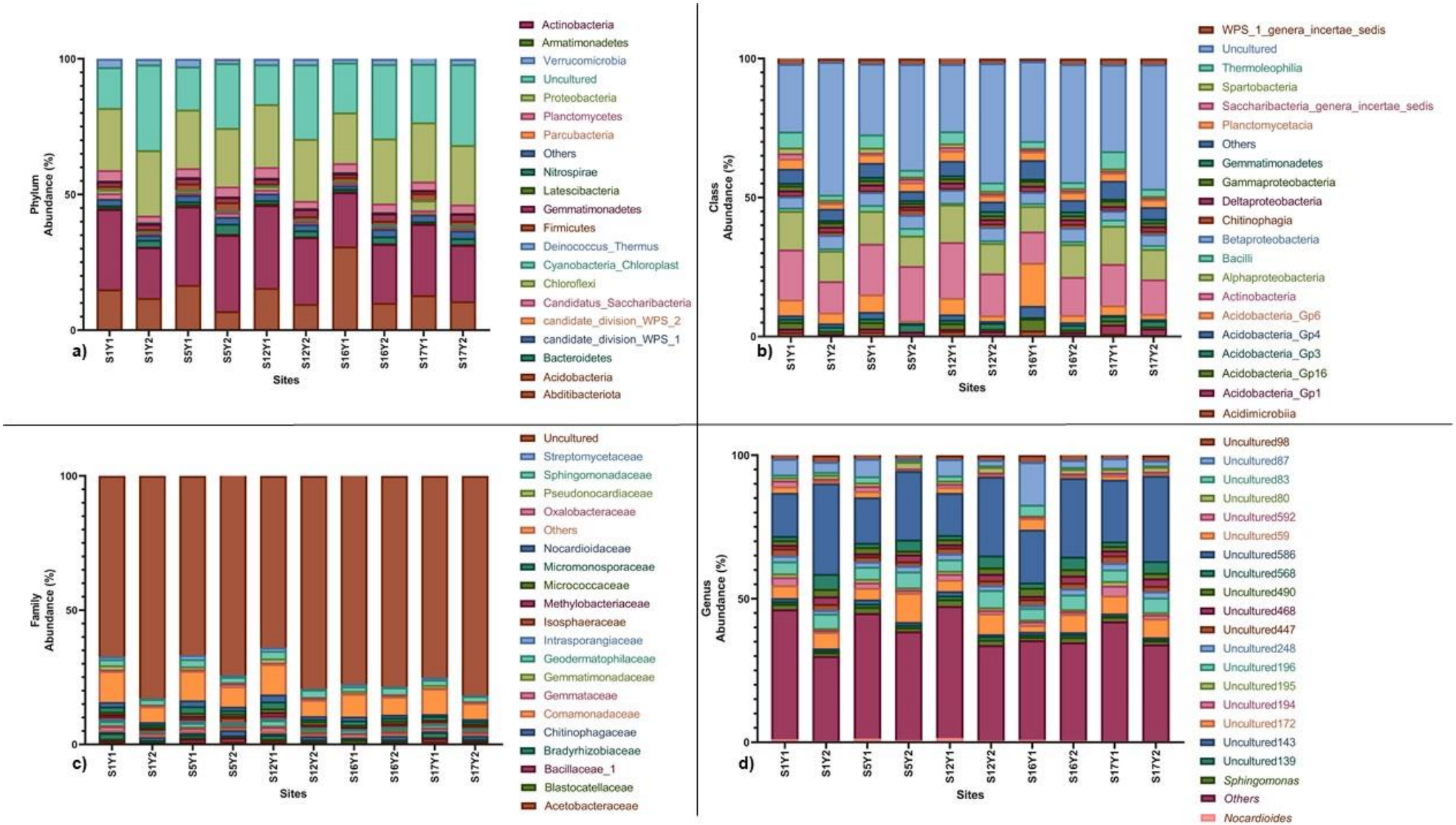

Figure 5.9: Stacked bar graphs indicating the top 20 most abundant bacterial a) Phyla, b) Class, c) Family and d) Genera associated with five fields under soybean cultivation during the 2018/2019 and under maize cultivation in the 2020/2021 summer growing seasons, in the Highveld production area (Mpumalanga province) of South Africa. Fields are identified for example as S1Y1 (S1=First location and Y1=first year of sampling).

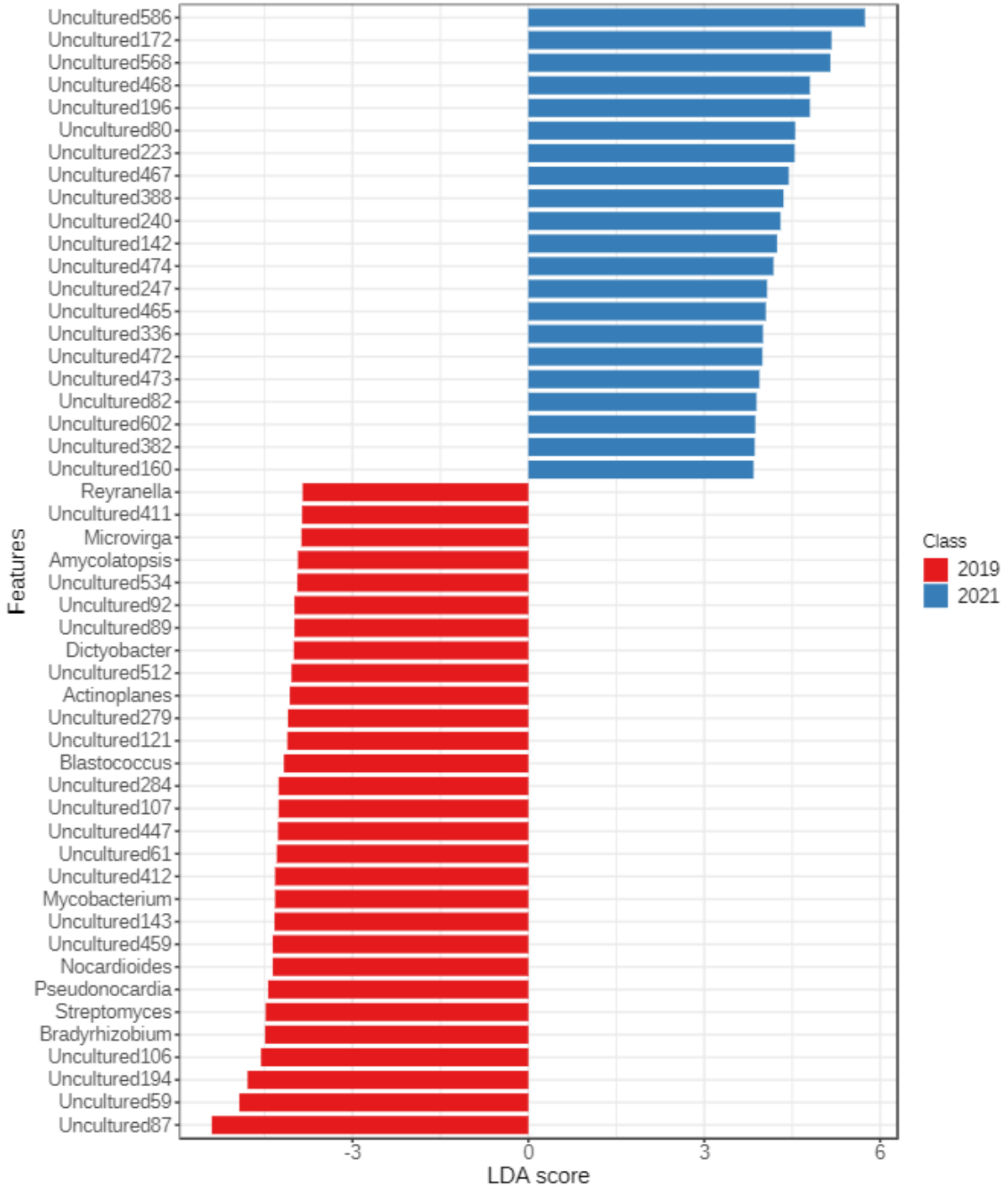

Figure 5.10: Graphical summary at the top 50 bacterial genera of 142 identified as having significantly different abundances using the Linear Discriminant Analysis (LDA) Effect Size (LEfSe) based on non-parametric factorial Kruskal-Wallis (KW) sum-rank test among the five fields under soybean cultivation during the 2018/2019 and under maize cultivation in the 2020/2021 summer growing seasons, in the Highveld production area (Mpumalanga province) of South Africa.

5.5 Discussion

The observation that genera such as *Meloidogyne, Pratylenchus*, and certain spiral genera such as *Helicotylenchus* and *Scutellonema* occur predominantly in soybean and maize

rhizospheres support the findings of other reports (Talwana *et al*., 2008; Simon *et al*., 2018; Mbatyoti *et al*., 2019). The association of these important nematode pests, particularly the presence of *Meloidogyne* and *Pratylenchus*, with soybean and maize accentuates that the potential damage to be caused by these pests should not be underestimated (Talwana *et al*., 2008). Results from this South African study showed that abundances of the top-six PPN genera were significantly lower (representing intermediate densities of 101-999 individuals / 20 g roots) in most maize fields (sampled during the second sampling interval) compared to those in the counterpart soybean fields, representing intermediate to high densities (>1 000 individuals / 20 g roots) that were sampled during the first sampling interval. This suggests that maize, although known as a susceptible host to particularly *Meloidogyne* and *Pratylenchus* species (Visagie *et al*., 2018; Mbatyoti *et al*., 2019) is not a superior host for these nematode genera in comparison to soybean. Furthermore, results suggested that the PPN communities associated with soybean rhizospheres had similar compositions during both sampling intervals and substantiate reports that monoculture of susceptible hosts can lead to a natural build-up of the nematode population originally present in the field (Neher *et al*., 2019). Moreover, resulting from our study, maize fields were found to have diffrent PPN community compositions compared to their soybean counterparts. In both rotation schemes, a negative correlation was observed between *Meloidogyne* (sessile endoparasite) and *Pratylenchus* (migratory endoparasite) which is most likely explained by which genus initially has a high population density. As these two genera occupy different feeding sites in root tissues (Volvas *et al*., 2005; Saikai & MacGuidwin, 2020), a high initial infestation of *Pratylenchus* will cause necrosis of the root tissue and *Meloidogyne* spp. will struggle to establish a functional feeding site. However, if *Meloidogyne* has a higher initial population density, the ability of females to produce more eggs, which are also more protected compared to *Pratylenchus* eggs, could prove to be advantages to *Meloidogyne* (Fontana *et al*., 2015). Similar observations were made by Ferraz (1995) and Fontana *et al*. (2018) that demonstrated the potential of *Meloidogyne* to substantially reduce the reproduction potential of *Pratylenchus* in soybean roots.

In the 10 fields that were under soybean cultivation at both sampling intervals (Figure 5.1), positive correlations were evident among the spiral nematodes *Scutellonema*, *Helicotylenchus* and *Hoplolaimus* as these genera are known to parasitise agricultural crops including soybean (Yan *et al*., 2017; Bandara *et al*., 2020). However, in a previous report, it was found that although spiral nematodes can parasitize soybean, their pathogenicity is low (Bao *et al*., 2013). Furthermore, a positive correlation was observed between *Pratylenchus* and *Scutellonema* in the five fields that were under soybean and maize cultivation at the time of sampling. This

corresponds to the findings of Talwana *et al*. (2008), where *Pratylenchus* and *Scutellonema* both had high abundances in fields where maize was planted.

Different cropping systems, such as the two systems used in this study, have been known to not only impact PPN communities that parasitise the crops, but they can also be a major factor in determining microbial community compositions (Zhou *et al*., 2018; Liu *et al*., 2020). When comparing the bacterial species diversity using the Shannon index, lower diversity was detected for samples obtained in the second sampling interval, while each sampling interval showed distinct beta-diversity profiles. Such trends were also observed by Fernandez-Gnecco *et al*. (2021), and it was attributed to potential water deficits. Furthermore, soybean dominated rotations in fields can also cause increased pH levels that might have negatively affected the microbial diversity and community structure (Berg & Smalla, 2009). Another factor that can contribute towards this scenario is that soybean is inoculated with *Bradyrhizobium* or *Rhizobium*, a practice that is not done for maize, that can also alter the microbiome dynamics in soils (Jabborova *et al*., 2020). These factors, amongst others, might also explain why more bacterial genera were found to have significantly higher abundances in 2019 as compared to 2021 for fields under a soybean cultivation (Figure 5.6).

When analysing the changes in bacterial abundance of rhizosphere samples from 10 fields under soybean cultivation, increases in certain phyla, classes, families and genera are reported in this study. This corresponds with similar findings of Liu *et al*. (2020) that indicated an increase in bacterial abundance in cropping systems under continuous soybean rotation. Corresponding with the findings of Liu *et al*. (2020), this study reported that both Actinobacteria and Alphaproteobacteria have high abundances in soybean fields. However, in contrast to this South African study, they reported that Betaproteobacteria had the highest abundance in soybean systems. The high abundance of Actinobacteria and Alphaproteobacteria in soybean fields reported in this South African study, might be due to relatively high nutrient availability (Li *et al*., 2014). Although the abundance of Betaproteobacteria reported in this study, is lower than that reported previously, several genera belonging to this class have a degree of plant pathogenicity, and reduced levels can aid in the crop's pathogen resistance capabilities (Liu *et al*., 2020).

Diversity analysis of five fields under soybean cultivation, were found to have generally higher bacterial species diversity when compared to the same fields under maize cultivation. However, beta-diversity analysis of these fields found that under maize cultivation in the second sampling interval, fields were found to have more similar bacterial profiles in comparison to when they were under soybean cultivation in the first sampling interval. With regards to the difference observed in beta-diversity in this study, Fernandez-Gnecco *et al*.

(2021) reported similar findings while highlighting the significant effect cropping regimes can have on bacterial community structure. Amongst the bacterial genera that were found to have significantly higher abundance under soybean cultivation (first sampling interval), when compared to the same fields under maize cultivation (second sampling interval) was *Bradyrhizobium* (Figure 5.10). This bacterial genus is known for its nitrogen fixation capabilities (Lopez *et al*., 2017), and the fact that it acts as crucial symbiotic bacteria genus for legumes (Venter *et al*., 2016). This corresponds with the significantly high abundance of a *Bradyrhizobium* spp. in the rhizosphere of fields under soybean cultivation (Liu *et al*., 2020).

Several phyla such as Actinobacteria, Acidobacteria, Bacteroidetes, Firmicutes and Proteobacteria have been reported to be amongst the most abundant phyla in the maize rhizosphere (Correa-Galeote *et al*., 2016). These phyla also dominated rhizosphere samples from this study taken form fields under maize cultivation. The current study identified phyla such as Gemmatimonadetes and Parcubacteria as also having increased abundances in fields when under maize cultivation. Akinola *et al*. (2021) reported that the bacterial community of maize rhizosphere samples were dominated by classes including Bacilli, Deltaproteobacteria, Gemmatimonadetes, Planctomycetacia, Spartobacteria and Thermoleophilia. Results from this South African study substantiate this, as increases in abundance of Bacilli, Deltaproteobacteria and Gemmatimonadetes were observed for samples taken from fields in the second sampling interval when under maize cultivation, relative to the samples obtained from the same fields under soybean cultivation (first sampling interval). Contrasting to findings of Akinola *et al*. (2021), Thermoleophilia was found to have higher abundances in fields when they were under soybean and not maize cultivation.

5.6 Conclusion

Cultivation practices including crop rotation sequences, result in physical disturbance of soil as well as in shifts in microbial and PPN community structures (Cheng *et al*., 2018; Simon *et al*., 2018). Seeing that the nematode populations vary not only among different cropping sequences, but between fields using the same rotation schemes, managing them might prove to be difficult. This is because different nematode genera, and even species of the same genus, respond differently to management strategies (Simon *et al*., 2018). Moreover, maize and soybean are hosts of the predominant nematode genera identified in this study which further allows increased abundance of such pests in grain fields. In addition, crop rotation greatly impacts the community composition of soil microbes. As numerous microbial genera present in soil can suppress PPN, changes in soil microbial community structure as a result of crop rotation can impact their metabolic activity and diversity; and potentially their nematicidal capabilities. By identifying the changes in microbial and PPN community

composition due to crop rotation sequences in a given field, it would be possible to determine which crop rotation sequence would be best suited for that field and can contribute to suppress nematode pest densities over time. Results from this study prove that incorporating soil microbes and PPN communities into the design and monitoring of agriculture practices can be valuable and should be attempted to investigate its long-term effect to contribute towards mitigating nematode damage in crops.

5.7 References

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5.8 SUPPLEMENTARY DATA

Table S2a: Log transformed data (logx+1) of the mean (+ SE) *Meloidogyne* and *Pratylenchus* individuals per 20 g of roots for 10 soybean fields sampled from the Highveld region, Mpumalanga province, South Africa during the 2018/2019 (first sampling) and 2020/2021 (second sampling) summer growing seasons

***Meloidogyne* spp. in first sampling interval**			***Meloidogyne* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**
S2	7.5 abcDEFG	2548 ± 1177	**S2**	4.5 eAB	109 ± 31
S6	6.7 abBCDEF	1141 ± 323	**S6**	7.7 acdDEFG	2471 ± 467
S7	10.1 dH	24402 ± 2680	**S7**	8.6 dFGH	6672 ± 1449
S8	7.3 abcCDEFG	4914 ± 2602	**S8**	7.9 adDEFGH	4354 ± 1386
S9	7.4 abcDEFG	4981 ± 3846	**S9**	6.2 bcABCDE	538 ± 69
S11	7.7 acdDEFG	7400 ± 3380	**S11**	5.8 beABCD	407 ± 142
S13	8.3 acdEFGH	5097 ± 1332	**S13**	6.9 abcCDEF	1175 ± 280
S14	5.1 bABC	184 ± 50	**S14**	6.9 abcCDEF	3538 ± 2022
S15	9.6 cdGH	21758 ± 7896	**S15**	8 adDEFGH	3922 ± 1129
S18	5.9 abABCD	395 ± 74	**S18**	4.2 eA	88 ± 26
F-value		8.33	F-value		18.30
P-value		0.001*	P-value		0.001*
Interaction data Field x sample interval					
F-value		4.71			
P-value		0.001*			
***Pratylenchus* spp. in first sampling interval**			***Pratylenchus* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**
S2	9. aG	9350 ± 1889	**S2**	5.7 abABC	311 ± 54
S6	6.6 bcdCDE	784 ± 111	**S6**	7.1 cDEF	1253 ± 114
S7	8.2 aeFG	3584 ± 234	**S7**	6.5 acCDE	824 ± 221
S8	5.9 bcCD	656 ± 215	**S8**	5.6 abABC	290 ± 35
S9	7.4 deEF	1826 ± 370	**S9**	5.8 aBC	340 ± 26
S11	4.5 fA	107 ± 34	**S11**	4.6 bAB	118 ± 32
S13	8.2 aeFG	4331 ± 1113	**S13**	5.8 aABC	433 ± 125
S14	5.4 bABC	270 ± 70	**S14**	5.7 abABC	325 ± 66
S15	6.7 cdCDE	1004 ± 353	**S15**	5.8 aBC	385 ± 80
S18	8.9 aG	7851 ± 351	**S18**	5.5 abABC	267 ± 40
F-value		13.52	F-value		8.012
P-value		0.001*	P-value		0.001*
Interaction data Field x sample interval					
F-value		20.8			
P-value		0.001*			

Note: Mean differences, standard error, p value and F ratio are indicated. Tukey's HSD (P<0.05) test indicates significant differences of each genus among fields per sampling interval with different small letters. Capital letters indicate significant differences of each genus per field between the two sampling intervals (thus comparing all the fields within one sampling interval to each other). Results obtained from sample replicates (n = 6).

Table S2b: Log transformed data of the mean (+ SE) *Helicotylenchus* and *Scutellonema* individuals per 20 g of root for 10 soybean fields sampled from the Highveld region, Mpumalanga province, South Africa during the 2018/2019 (first sampling) and 2020/2021 (second sampling) summer growing seasons

***Helicotylenchus* spp. in first sampling interval**			***Helicotylenchus* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**
S2	4.8 abEF	132 ± 27	**S2**	2.4 bcB	10 ± 1
S6	3.3 acBCD	28 ± 6	**S6**	3.1 acBC	27 ± 10
S7	5 bF	170 ± 32	**S7**	3.2 aBCD	25 ± 2
S8	2.5 cB	28 ± 10	**S8**	3 acB	20 ± 2
S9	4.9 bF	248 ± 97	**S9**	3.3 aBCD	27 ± 1
S11	4.4 abCDEF	87 ± 15	**S11**	2.2 bB	8 ± 1
S13	3.3 acBCD	28 ± 4	**S13**	2.2 bB	8 ± 1
S14	3.5 abcBCDE	34 ± 5	**S14**	3. aBCD	34 ± 9
S15	4.5 abDEF	114 ± 29	**S15**	2.7 abcB	14 ± 1
S18	0 dA	0	**S18**	0 dA	0
F-value		20.94	F-value		38.10
P-value		0.001*	P-value		0.001*
Interaction data Field x sample interval					
F-value		4.62			
P-value		0.001*			
***Scutellonema* spp. in first sampling interval**			***Scutellonema* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**
S2	0 cA	0	**S2**	3.6 aCD	39 ± 8
S6	4.1 abCD	66 ± 11	**S6**	3.5 aBCD	35 ± 7
S7	4.5 bD	101 ± 21	**S7**	3.2 aBCD	29 ± 8
S8	3.1 aBC	34 ± 8	**S8**	2.9 bBCa	20 ± 3
S9	4.1 abCD	83 ± 31	**S9**	3.3 aBCD	30 ± 5
S11	3.5 abBCD	39 ± 11	**S11**	3.4 aBCD	45 ± 18
S13	3.6 abCD	41 ± 7	**S13**	3.3 aBCD	25 ± 2
S14	4.1 abCD	78 ± 25	**S14**	3.2 aBCD	28 ± 7
S15	3.7 abCD	41 ± 4	**S15**	3.6 aCD	37 ± 2
S18	3.3 abBCD	28 ± 4	**S18**	2.2 bB	7 ± 1
F-value		18.42	F-value		3.950
P-value		0.001*	P-value		0.001*
Interaction data Field x sample interval					
F-value		3.38			
P-value		0.001*			

Note: Mean differences, standard error, p value and F ratio are indicated. Tukey's HSD ($P<0.05$) test indicates significant differences of each genus among fields per sampling interval with different small letters. Capital letters indicate significant differences of each genus per field between the two sampling intervals (thus comparing all the fields within one sampling interval to each other). Results obtained from sample replicates (n = 6).

Table S2c: Log transformed data of the mean (+ SE) *Hoplolaimus* and *Rotylenchulus* individuals per 20 g of root for 10 soybean fields sampled from the Highveld region, Mpumalanga province, South Africa during the 2018/2019 (first sampling) and 2020/2021 (second sampling) summer growing seasons

***Hoplolaimus* spp. in first sampling interval**			***Hoplolaimus* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**
S2	0 bA	0	**S2**	0 aA	0
S6	4.6 aDE	105 ± 25	**S6**	0 aA	0
S7	4.5 aDE	89 ± 9	**S7**	2.5 bB	13 ± 2
S8	2.5 cB	28 ± 10	**S8**	0 aA	0
S9	4.7 aE	118 ± 24	**S9**	0 aA	0
S11	4 aCDE	73 ± 25	**S11**	0 aA	0
S13	3.5 acBCD	34 ± 4	**S13**	0 aA	0
S14	4.2 aCDE	69 ± 8	**S14**	2.7 bB	14 ± 2
S15	4.1 aCDE	65 ± 16	**S15**	3.2 cBC	25 ± 2
S18	0 bA	0	**S18**	0 aA	0
F-value		35.50	F-value		484.3
P-value		0.001*	P-value		0.001*
Interaction data Field x sample interval					
F-value		4.62			
P-value		0.001*			
***Rotylenchulus* spp. in first sampling interval**			***Rotylenchulus* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**
S2	0 cA	0	**S2**	0 aA	0
S6	3.1 aB	34 ± 8	**S6**	0 aA	0
S7	4 abBC	60 ± 11	**S7**	3.2 bB	28 ± 6
S8	3.8 abB	46 ± 9	**S8**	0 aA	0
S9	5.4 bC	1000 ± 494	**S9**	0 aA	0
S11	3.9 abB	60 ± 14	**S11**	0 aA	0
S13	4.5 abBC	94 ± 19	**S13**	0 aA	0
S14	3.9 abB	55 ± 11	**S14**	0 aA	0
S15	3.7 abB	41 ± 3	**S15**	0 aA	0
S18	3.6 abB	37 ± 4	**S18**	0 aA	0
F-value		12.69	F-value		229.1
P-value		0.001*	P-value		0.001*
Interaction data Field x sample interval					
F-value		3.38			
P-value		0.001*			

Note: Mean differences, standard error, p value and F ratio are indicated. Tukey's HSD ($P<0.05$) test indicates significant differences of each genus among fields per sampling interval with different small letters. Capital letters indicate significant differences of each genus per field between the two sampling intervals (thus comparing all the fields within one sampling interval to each other). Results obtained from sample replicates (n = 6).

Table S3a: Log transformed data of the mean (+ SE) *Meloidogyne* and *Pratylenchus* individuals per 20 g of root for five fields under soybean cultivation during the 2018/2019 and under maize cultivation in the 2020/2021 summer growing seasons, in the Highveld production area (Mpumalanga province) of South Africa

***Meloidogyne* spp. in first sampling interval**			***Meloidogyne* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of maize root + Standard error**
S1	5.4 aABCD	344 ± 131	**S1**	5.2 aABD	189 ± 26
S5	6.9 aC	3627 ± 2501	**S5**	6 bABCD	432 ± 67
S12	6.7 aBC	999 ± 323	**S12**	4.6 aA	110 ± 19
S16	6.7 aBC	1059 ± 281	**S16**	4.8 aAD	128 ± 21
S17	6.2 aBCD	518 ± 77	**S17**	4.6 aA	107 ± 17
F-value		1.982	F-value		10.74
P-value		0.136	P-value		0.001*
Interaction data Field x sample interval					
F-value			2.84		
P-value			0.034*		
***Pratylenchus* spp. in first sampling interval**			***Pratylenchus* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of maize root + Standard error**
S1	6.1 aA	518 ± 113	**S1**	4.4 abBD	85 ± 10
S5	6.1 aA	550 ± 155	**S5**	6 cAC	413 ± 41
S12	5.3 aABC	243 ± 57	**S12**	5.1 bcABCD	168 ± 19
S16	5.1 aABCD	230 ± 65	**S16**	3.9 aD	78 ± 25
S17	5.5 aABC	335 ± 130	**S17**	4.8 abBCD	132 ± 28
F-value		2.184	F-value		10.41
P-value		0.108	P-value		0.001*
Interaction data Field x sample interval					
F-value			2.81		
P-value			0.035*		

Note: Mean differences, standard error, p value and F ratio are indicated. Tukey's HSD ($P<0.05$) test indicates significant differences of each genus among fields per sampling interval with different small letters. Capital letters indicate significant differences of each genus per field between the two sampling intervals (thus comparing all the fields within one sampling interval to each other). Results obtained from sample replicates (n = 6).

Table S3b: Log transformed data of the mean (+ SE) *Helicotylenchus* and *Scutellonema* individuals per 20 g of root for five fields under soybean cultivation during the 2018/2019 and under maize cultivation in the 2020/2021 summer growing seasons, in the Highveld production area (Mpumalanga province) of South Africa

***Helicotylenchus* spp. in first sampling interval**			***Helicotylenchus* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of maize root + Standard error**
S1	3.8 abAB	46 ± 9	**S1**	2.5 aA	13 ± 2
S5	2.5 aA	28 ± 10	**S5**	2.8 aA	17 ± 3
S12	3.3 abAB	28 ± 3	**S12**	0 bC	0
S16	4.4 bB	83 ± 13	**S16**	0 bC	0
S17	3.3 abAB	29 ± 6	**S17**	0 bC	0
F-value		3.082	F-value		319.4
P-value		0.040*	P-value		0.001*
Interaction data Field x sample interval					
F-value		23.5			
P-value		0.001*			
***Scutellonema* spp. in first sampling interval**			***Scutellonema* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of maize root + Standard error**
S1	4.4 aBC	87 ± 11	**S1**	2.8 aA	17 ± 2
S5	3.6 aABC	66 ± 19	**S5**	2.8 aA	18 ± 5
S12	4.7 aC	110 ± 14	**S12**	2.9 aA	21 ± 5
S16	3.7 aABC	41 ± 6	**S16**	3.1 aA	24 ± 4
S17	3.9 aABC	50 ± 4	**S17**	3 aA	23 ± 15
F-value		2.054	F-value		0.330
P-value		0.125	P-value		0.855
Interaction data Field x sample interval					
F-value		1.56			
P-value		0.199			

Note: Mean differences, standard error, p value and F ratio are indicated. Tukey's HSD ($P<0.05$) test indicates significant differences of each genus among fields per sampling interval with different small letters. Capital letters indicate significant differences of each genus per field between the two sampling intervals (thus comparing all the fields within one sampling interval to each other). Results obtained from sample replicates (n = 6).

Table S3c: Log transformed data of the mean (+ SE) *Hoplolaimus* and *Rotylenchulus* individuals per 20 g of root for five fields under soybean cultivation during the 2018/2019 and under maize cultivation in the 2020/2021 summer growing seasons, in the Highveld production area (Mpumalanga province) of South Africa

***Hoplolaimus* spp. in first sampling interval**			***Hoplolaimus* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of maize root + Standard error**
S1	4.5 bcBC	97 ± 16	**S1**	2.1 aA	8 ± 1
S5	3.6 cE	37 ± 6	**S5**	2.5 aA	12 ± 2
S12	4.5 bcBC	97 ± 15	**S12**	0 bD	0
S16	4.9 bC	143 ± 27	**S16**	2.5 aA	12 ± 2
S17	4 acBE	57 ± 7	**S17**	0 bD	0
F-value		6.908	F-value		232.6
P-value		0.001*	P-value		0.001*
Interaction data Field x sample interval					
F-value		48.0			
P-value		0.001*			
***Rotylenchulus* spp. in first sampling interval**			***Rotylenchulus* spp. in second sampling interval**		
Field	**Log transformed data**	**Mean individuals per 20 g of soybean root + Standard error**	**Field**	**Log transformed data**	**Mean individuals per 20 g of maize root + Standard error**
S1	3.3 aBC	28 ± 3	**S1**	2.2 aB	8 ± 1
S5	2.5 aBC	28 ± 10	**S5**	0 bA	0
S12	3.6 aC	37 ± 4	**S12**	0 bA	0
S16	0 bA	0	**S16**	0 bA	0
S17	0 bA	0	**S17**	0 bA	0
F-value		23.89	F-value		1283.0
P-value		0.001*	P-value		0.001*
Interaction data Field x sample interval					
F-value		19.67			
P-value		0.001*			

Note: Mean differences, standard error, p value and F ratio are indicated. Tukey's HSD ($P<0.05$) test indicates significant differences of each genus among fields per sampling interval with different small letters. Capital letters indicate significant differences of each genus per field between the two sampling intervals (thus comparing all the fields within one sampling interval to each other). Results obtained from sample replicates (n = 6).

CHAPTER 6: FILTRATES OF MIXED *BACILLUS* SPP INHIBIT SECOND-STAGE JUVENILE MOTILITY OF ROOT-KNOT NEMATODES

"The best insurance policy for the future of an industry is research, which will help it to foresee future lines of development, to solve its immediate problems, and to improve and cheapen its products."

Sir Harold Hartley

6.1 Abstract

The global expansion of soybean (*Glycine max* (L.) Merr.) exposes it to more diseases and pests such as nematodes. In South Africa particularly, *Meloidogyne incognita* and *M. javanica* are considered the predominant genera infecting soybean, but a more pathogenic root-knot species, *M. enterolobii*, was recently reported in the Mpumalanga Highveld of South Africa. The use of chemicals to manage these pests is usually preferred although various concerns exist regarding their potential impact on the environment. An increasing need for the development of less toxic alternatives for nematode management thus exists. This study determined the nematicidal activity of *Bacillus* spp. mixtures isolated from soybean rhizospheres on the motility of second-stage juveniles (J2) of mixed *Meloidogyne* communities co-occurring in these rhizospheres. Roots and soil from 10 soybean fields in the Mpumalanga Highveld were collected and *Bacillus* spp. isolated, while the population density and molecular identification of the co-existing *Meloidogyne* spp. were also done. The filtrates of the *Bacillus* spp. were then used in *in vitro* assays to determine their potential nematicidal activity. Results confirmed the presence of *M. enterolobii* (100%) *M. incognita* (50%) and *M. javanica* (40%) in the sampled fields, with single populations found in 20% and mixed communities in 80% of the fields. The filtrate mixture of *B. cereus*, *B. megaterium*, *B. subtilis* and *B. thuringiensis* caused approximately 85-90% immobility of *Meloidogyne* spp. J2 after 96 hours. The results show that the use of *Bacillus* spp. mixtures can aid in the development of biocontrol products to combat root-knot nematodes and might be more effective than products from a single species in limiting J2 motility

Keywords – Biological control; bioassay*, Glycine max, Meloidogyne,* root-knot.

6.2 Introduction

Soybean (*Glycine max* (L.) Merr.) is an important summer legume crop used particularly for its high protein content in food and fodder sources globally. Two countries, *viz*. Brazil and the United States of America (USA), are the largest soybean producers with both delivering >100 million metric tons of produce annually (USDA, 2020). This important crop has the potential to serve as an important dietary source of both protein and oil for animal and human consumption, as soybean seeds consist of approximately 18% oil and 38% protein (Hartman *et al*., 2011). The value and importance of this crop has also led to an increase in its production in lower-producing countries such as South Africa (SA). Soybean production in SA has substantially increased annually since the early 1960s with the area dedicated to its production estimated at a record 827 100 hectares during the 2020/2021 growing season (Grain SA, 2021). However, the increase in global production poses the risk of this important food and fodder crop being exposed to more pests and diseases, including plant-parasitic nematodes (PPN) (Sikora *et al.,* 2018).

Root-knot nematodes (*Meloidogyne* spp.) are important pests of various crops worldwide including soybean (Al-Banna *et al*., 2004; Xiong *et al*., 2015; Fourie *et al*., 2017). In SA, *Meloidogyne incognita* and *M. javanica* are the species being considered as the two economically most important root-knot nematode pests that parasitise soybean (Fourie *et al*., 2017; Mbatyoti *et al*., 2021). Recently an upcoming threat, *M. enterolobii* (Collet *et al*., 2021) has also been identified infecting maize (Pretorius, 2018) in the Mpumalanga Highveld area of SA where maize and soybean are rotated (Nel, 2005; Mc Donald *et al*., 2017). Compared to *M. incognita* and *M. javanica*, *M. enterolobii* caused greater galling on tomato (Cetintas *et al*., 2007). Adding to the potential devastating effect of *M. enterolobii* infection in crop roots, is its ability to reproduce on crop genotypes that contain root-knot resistance genes (*Mi-1*, *Mh*, *Mir1*, *N*, *Tabasco*, and *Rk*) (Ye *et al*., 2013). Therefore, *M. enterolobii* is listed as a more pathogenic species than its counterpart tropical/thermophilic species *M. incognita* and *M. javanica* (Jones *et al*., 2013). *Meloidogyne enterolobii* has already been reported from soybean roots in Brazil (Dias *et al*., 2010) and North Carolina, USA (Ye *et al*., 2013) where it causes substantial damage to the crop. Various studies have, however, reported that *M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica* can be found as either single populations or mixed communities in roots or other subterranean parts of agricultural crops (Karssen *et al*., 2013; Visagie *et al*., 2018). The latter scenario poses another challenge to producers in terms of protecting their soybean crops against a combination of these root-knot nematode species. Studies showed that the concomitant occurrence of more than one root-knot

nematode species in South African grain crop production areas where soybean is cultivated is a common phenomenon (Pretorius, 2018; Mbatyoti *et al*., 2021).

Although synthetic nematicides and the use of genetic host plant resistance remain common methods for PPN management (Schneider *et al*., 2003; Collet *et al*., 2021), many chemicals have elevated levels of toxicity with several being removed from the global market (Naz *et al*., 2015). Moreover, soybean genotypes with resistance to root-knot nematodes are commonly used in countries with extensive hectarage of the crop; however, resistant cultivars suitable for the SA growing conditions are limited and not widely available (Fourie, H. North-West University, Potchefstroom, North-West, South Africa. Personal communication, 2022). This scenario calls for the urgent development of additional alternative PPN management methods to protect local soybean crops rotated with other grain crops.

Since PPN inhabit soil, they are exposed to various indigenous soil microbiota which can possibly be used as biocontrol agents (Terefe *et al*., 2009). Biocontrol agents are not only environmentally friendly, but they also have different modes of action compared to chemical pesticides (Ongena & Jacques, 2008), making it possible for them to be applied when other management options are not feasible. Various bacterial species are known to exhibit nematicidal activity especially those belonging to the genus *Bacillus* which represents one of the most abundant genera of soil bacteria (Tian *et al*., 2007; Tiwari *et al*., 2017; Engelbrecht *et al*., 2018). Several *Bacillus* spp. have been tested internationally for their nematicidal activity against *Meloidogyne* spp. These include *B. amyloliquefaciens* (Jamal *et al*., 2017), *B. coagulans* (Askary, 2015), *B. cereus* (Gao *et al*., 2016; Engelbrecht *et al*., 2018), *B. firmus* (Geng *et al*., 2016; Jansen-Girgan *et al*., 2016; Engelbrecht *et al*., 2018), *B. licheniformis* (Miamoto *et al*., 2017; Lopes *et al*., 2020), *B. megaterium* (Huang *et al*., 2010), *B. nematocida* (Li *et al*., 2015) , *B. pumilus* (Lee & Kim, 2016; Engelbrecht *et al*., 2018), *B. soli* (Engelbrecht *et al*., 2018), *B. subtilis* (Higaki & Araujo, 2012; Zheng *et al*., 2016; Engelbrecht *et al*., 2018), *B. thuringiensis* (Li *et al*., 2015) and *Bacillus* spp. mixtures (Chinheya *et al*., 2017). Hence, several bionematicidal products containing *Bacillus* spp. as the active bacterial strain are already available in some countries (Askary 2015; Li *et al*., 2015; Rao *et al*., 2017). There are also several other potential sources for nematicidal biocontrol apart from *Bacillus*. These include products based on plant extracts and oils such as citronella oil, orange oil and garlic extract (Czaja *et al*., 2015) and several fungi that include *Purpureocillium lilacinum, Pochonia chlamydosporia, Arthrobotrys* spp., Myzocytiopsis spp., Nematoctonus spp. and Catenaria spp. (Timper, 2014; Yang & Zhang, 2014; Degenkolb & Vilcinskas, 2016).

However, many *in vitro* studies usually focus on the effect of single *Bacillus* spp. against PPN, while few studies determined the effect of *Bacillus* spp. mixtures that are increasingly

proposed to be a more sustainable and viable option to use (Jahagirdar *et al*., 2021). Therefore, this study focused on the nematicidal activity of *Bacillus* spp. mixtures (isolated from soybean rhizospheres in the Mpumalanga Highveld, SA) on the motility of second-stage juveniles (J2) of a mixed *Meloidogyne* community that co-occurred in such soil ecosystems. The hypothesis is that a combination of *Bacillus* species present in various fields will have an inhibiting effect on nematode motility.

6.3 Materials and methods

6.3.1 *Meloidogyne* spp. abundance

Soil and roots from 10 soybean fields (Table 6.1) in the Highveld region of Mpumalanga, SA was collected during the 2020/2021 growing season as explained by Engelbrecht *et al.* (2021). According to the Köppen-Geiger climate classification this region is classified as Cwb meaning that it is a temperate region with dry winters and warm summers (Beck *et al*., 2018).

Table 6.1: Details of the locations in the Highveld region of SA where soybean root and soil samples were collected during the 2020/2021 growing seasons for the isolation of microbes and root-knot nematodes.

Field	GPS Coordinates	Altitude (m)	2020/2021 crop	Average rain fall for January 2021 (mm)	Clay % of soil
S2	25°49’28.2”S, 29°32’42.6”E	1604	Soybean	184	20
S6	26°14’50.4”S, 29°38’09.7”E	1661	Soybean	239	15
S7	26°14’52.6”S, 29°38’18.8”E	1652	Soybean	239	10
S8	26°16’59.3”S, 29°36’48.4”E	1659	Soybean	239	10
S9	26°17’10.2”S, 29°36’44.0”E	1661	Soybean	239	10
S11	25°49’00.3”S, 29°32’56.9”E	1591	Soybean	184	20
S13	26°17'17.2"S, 29°36'44.8"E	1660	Soybean	239	15
S14	26°12'06.5"S, 30°08'43.6"E	1731	Soybean	210	20
S15	26°12'09.6"S, 30°07'31.2"E	1714	Soybean	210	15
S18	26°01'43.0"S, 28°48'57.9"E	1518	Soybean	200	6.9

Nematodes were extracted from 20 g of composite root samples from each field, using the adapted centrifugal-flotation method described by Swart and Marais (2017) and transferred to a De Grisse counting dish. *Meloidogyne* spp. life stages [eggs, J2, third- and fourth stage juveniles (J3 and J4), females and males] were counted and concurrently identified to genus level (Table 3) using a Nikon ECLIPSE TS100 (Nikon Corporation, Tokyo, Japan) inverted microscope (40× magnification).

6.3.2 *Meloidogyne* spp. identification and *in vivo* rearing

The soil from each field was transferred to individual 5-L capacity plastic pots and two tomato seedlings of 3-leaf stage cultivar Monica, which is susceptible to South African root-knot nematode species (Daneel *et al*., 2018), were planted in the pots and placed in a glasshouse (North-West University, Potchefstroom, SA). The pots were spaced 0.8 m apart to prevent cross contamination of the root-knot species and watered with borehole water (pH =7.75) three times per week. Glasshouse conditions were maintained between 20±1 °C and 26±1 °C with a 14D:10L photoperiod being using additional plant growth light sources.

The identity of the *Meloidogyne* spp., further referred to as populations when only one species is present and communities when mixed species are present (Bello *et al*., 2020), was verified for each of the fields with the use of the sequence characterised amplified region-polymerase chain reaction (SCAR-PCR). Using a scalpel, 15-20 randomly selected mature *Meloidogyne* females were removed from roots of infected tomato plants in which they were reared *in vivo.* The scalpel was sterilised in 70% alcohol between each female. Isolated females from each sampled field were placed in an Eppendorf tube that contained 15 µl deionised (Milli-Q; 18.2 MΩ.cm) water for DNA extraction. To extract the DNA from the *Meloidogyne* spp., the chelex-100 protocol (Musapa *et al*., 2013) was used. Samples were incubated at 56-57 °C for 2 h followed by incubation at 95 °C for 10 min. The concentration of the extracted microbial DNA (absorbance at 260 nm) and its purity (absorbance ratio 260/230 and 260/280) were measured using a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

To amplify the DNA an Alpha Cycler 1 PCRMax thermocycler (Vacutec, Sheridan, Wyoming, USA) was used. Each PCR reaction (total volume of 25 µl) constituted of 12.5 µl master mix (Promega Corporation, Madison, Wisconsin, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2.5 µl DNA and 8 µl ddH2O. Each of the fields were screened for the presence of *M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica*. Primers used for each of the species' SCAR-PCR has been known to be used for specific species relating to studies of population biology and dynamics (Qiu *et al*., 2006; Rashidifard *et al*., 2019). Conditions for

SCAR-PCR were programmed as follows: 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature and 1 min at 72 °C. Annealing temperatures depended on the use of species-specific primer (Zijlstra *et al*. 2000; Long *et al*., 2006; Rashidifard *et al*., 2019) resulting in annealing temperatures of 61 °C for *M. arenaria* primers, 64 °C for *M. enterolobii* and *M. javanica* primers and 54 °C for the *M. incognita* primers (Table 6.2). After the PCR of samples were completed, 2 µl of PCR product from each sample, representing the *Meloidogyne* spp. of each field, was loaded on agarose gel to determine the presence/absence of *M. arenaria*, *M. enterolobii*, *M. incognita*, *M. javanica.* This was done by comparing the PCR band lengths of a known and previously identified *Meloidogyne* spp. to that of the PCR products obtained from this study. The DNA bands were stained with GelRed (www.biotium.com) and visualized and photographed using a UV transilluminator.

Table 6.2: Name, sequence and amplification size of different SCAR primers used for molecular identification *Meloidogyne* spp. obtained from 10 soybean fields.

Target spp.	Primer name	Primer sequence	Fragment size
M. arenaria	Far	TCG GCG ATA GAG GTA AAT GAC	420 bp
	Rar	TCG GCG ATA GAC ACT ACA ACT	
M. enterolobii	FMe	AAC TTT TGT GAA AGT GCC GCT	250 bp
	RMe	TCA GTT CAG GCA GGA TCA ACC	
M. incognita	Finc	CTC TGC CCA ATG AGC TGT CC	1200 bp
	Rinc	CTC TGC CCT CAC ATT AGG	
M. javanica	Fjav	GGT GCG CGA TTG AAC TGA GC	700 bp
	Rjav	CAG GCC CTT CAG TGG AAC TAT AC	

6.3.3 Collection of *Meloidogyne* spp. egg masses and hatching of second-stage juveniles

After removing the females from infected roots for identification, all egg masses present on the roots of tomato plants per pot per field were collected directly from the galls that formed 30-40 days after the *in vivo* rearing process commenced. The collected egg masses from the 10 fields were pooled to ensure that all root-knot nematode species that occurred in the sampling fields were represented for the bioassay analysis. The pooled egg masses were placed on a 25 µm mesh sieve and submerged in a container filled with approximately 5 cm of borehole water. The container with eggs was then incubated at 26 °C for 48 - 72 h (Marais *et al.*, 2017). The water from the container was then passed through a 20 µm-mesh mesh

sieve to collect the hatched J2. Thereafter, the J2 were checked to ensure they were actively moving and then used for the bioassays.

6.3.4 Bacterial isolation and cultivation

Four fields, two with the lowest (S2 and S18), one with intermediate (S15) and one with the highest (S7) *Meloidogyne* spp. abundance per 20 g roots were selected to isolate the *Bacillus* spp. that co-inhabited the soybean rhizospheres (Table 6.3). To isolate and identify the *Bacillus* spp. present in the soil, 1 g of soil from the selected fields were suspended in 9 ml of sterile distilled water and subsequently diluted (up to10^{-8}) (Pugazhendhi *et al*., 2018). Of these, 0.1 ml of each field's diluted sample was spread on *Bacillus* ChromoSelect agar (BCS) containing Polymyxin B. The latter aids in the differentiation between *B. cereus* and *B. thuringiensis* and is also used for the inhibition of Gram-negative bacteria. Plates were incubated at 30 °C for 24 h – 48 h (Mortimer & McCann, 1974). *Bacillus* ChromoSelect agar acts as a selective and differential medium to distinguish between *B. subtilis*, *B. cereus*, *B. thuringiensis, B. coagulans* and *B. megaterium* based on colony morphology.

Individual *Bacillus* spp. isolated from the soil of each field were individually cultivated in Luria Bertani broth (LB broth) at 28 °C at 200 rpm for 48 h (Xiong *et al*., 2015; Engelbrecht *et al*., 2020). To ensure that an equal population ratio of the individual *Bacillus* spp. is present in the final filtrate mixture used in the bioassays, a 10% inoculum (Horak *et al*., 2021) of each individual species from the same field was added together and again cultivated in LB broth at the above-mentioned conditions. After incubation, the filtrates of the *Bacillus* spp. mixture were separated from the bacterial cells by centrifuging (HERMLE Z32HK centrifuge, Lasec South Africa (Pty) Ltd., Midrand, SA) 40 ml of each *Bacillus* spp. mixture at 1800 g for 30 min at 25 °C. The supernatant of each sample was filtered through a sterile 0.2 µm filter (Lasec South Africa (Pty) Ltd., Midrand, SA) to remove the remaining bacterial cells (Goodacre *et al*., 2017) and filtrates used for *in vitro* bioassays. The isolation and cultivation of *Bacillus* spp. were repeated for the second bioassay.

6.3.5 Nematicidal bioassay: J2 motility

The J2 that hatched from pooled egg masses from the 10 fields were used to determine the effect of the *Bacillus* filtrates on their motility by exposing 30 individuals to a control (LB broth without any *Bacillus* filtrates) and 25%, 50% and 100% concentrations of the *Bacillus* spp. filtrate mixture (Lee & Kim, 2016; Mendoza *et al*. 2008). Exposures were done using sterile 24-well, flat bottom, culture treated plates (Greiner Bio-one, Kremsmünster, Austria), with each well having a final volume of 1 ml. To avoid contamination, 100 µg/ml streptomycin was

added to each well of the assay (Gao *et al*., 2016). The plates were slightly closed and incubated at 25 °C in a temperature regulated growth cabinet. The J2 in each well were inspected and the number of motile and immotile (body completely straight) individuals were recorded after 48 h and 96 h. The percentage immotile J2 for each *Bacillus* spp. filtrate was determined based on the total number of J2, both motile and immotile at a given time (Xiang *et al*., 2016). In each bioassay, filtrate concentrations were replicated four times and the assay was repeated twice at different time intervals (7 days apart). Abbott's formula for corrected mortality, which in the case of this study refers to J2 immobility, was used (Abbott, 1925) to correct obtained assay data for a control response. This ensures that the nematicidal effects were caused by the filtrates of *Bacillus* spp. and not the culture media (LB broth).

$$Abbott's\ corrected\ immotility\ \% = \left(1 - \frac{immotile\ J2\ after\ treatment\ with\ Bacillus\ filtrates}{immotile\ J2\ after\ control\ treatment\ with\ LB\ broth}\right) \times 100$$

Corrected immotility data from the assays were then subjected to Levine's homogeneity test before performing Repeated Measures ANOVA and the means separated by the Tukey's post-hoc test (HSD) where P<0.05 (Statistica 13.3; https://statistica.software.informer.com/13.3/). This indicated whether the immotility of the mixed *Meloidogyne* J2 community was statistically significant (P<0.05) between cell-free filtrates at different time intervals for the different *Bacillus* spp. mixture concentrations.

6.4 Results

6.4.1 *Meloidogyne* spp. abundance

Of the 10 soybean fields sampled during the 2020/2021 season, *Meloidogyne* spp. life stages [eggs, J2, third- and fourth stage juveniles (J3 and J4), females and males] were present in each of them with abundance ranging from 87 individuals/20 g of root (S18) to 6672 individuals/20 g of roots (S7) (Table 6.3).

6.4.2 *Meloidogyne* spp. identification

The following DNA fragments were amplified with SCAR-PCR and confirmed the presence of three respective species: 250 bp for *M. enterolobii*, 700 bp for *M. javanica* and 1200 bp for *M. incognita* (Figure 6.1; Table 6.3). No amplification was evident for *M. arenaria*, indicating no presence among the populations and/or communities studied. *M. enterolobii* was the most dominant and found in 100% of the localities, followed by *M. incognita* (50%) and *M. javanica* (40%). These results also confirm that *Meloidogyne* spp. can occur either as single

populations (20% of localities) or mixed communities (80% of localities) (Figure 6.1). *Meloidogyne enterolobii* and *M. javanica* were found together in S2, S6, S7 and S9. *Meloidogyne enterolobii* and *M. incognita* were found together at S7, S8, S11, S13 and S18. Only locality S7 was found to contain *M. enterolobii*, *M. incognita* and *M. javanica*. Furthermore, *M. enterolobii* single populations were found at 20% of the localities with no *M. incognita* and *M. javanica* single populations present. *Meloidogyne javanica* and *M. incognita* only occurred together with *M. enterolobii* as a mixed community.

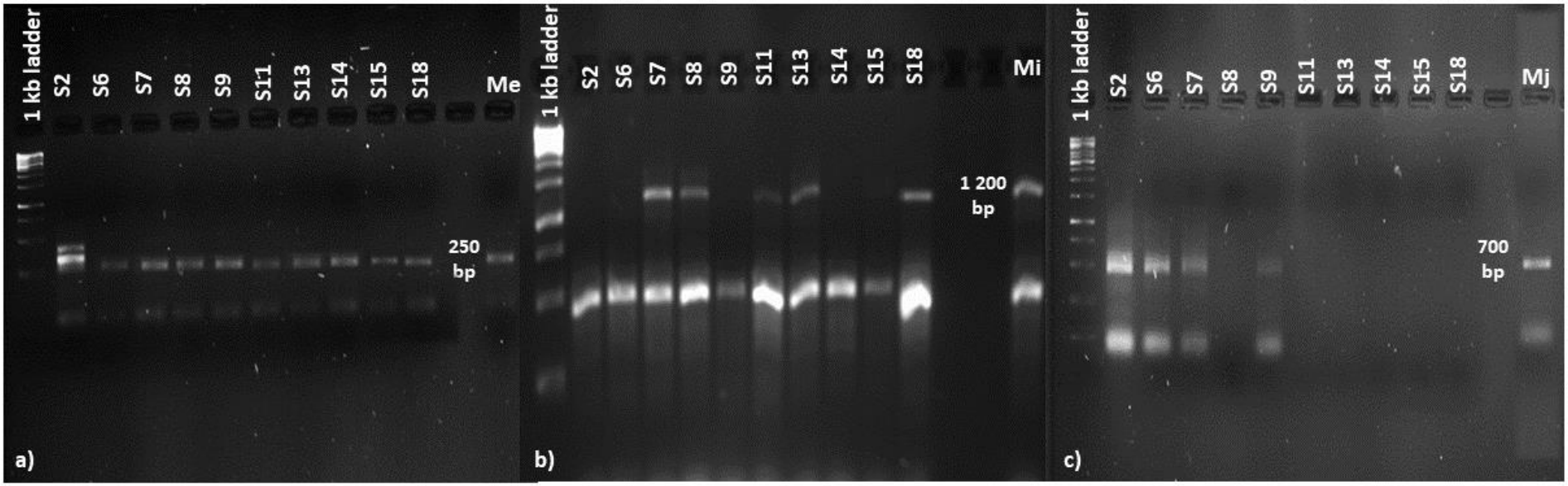

Figure 6.1: Photograph of agarose gel with DNA amplification products of *Meloidogyne* spp. second-stage juveniles obtained from 10 soybean localities in the Highveld region of the Mpumalanga province using SCAR-PCR. a) *M. enterolobii*, b) *M. incognita* and c) *M. javanica*; Me (*M. enterolobii*) Mi (*M. incognita*) and Mj (*M. javanica*) = DNA of standard (control) population used for each species.

Table 6.3: Population densities of *Meloidogyne* spp. [eggs, J2, third- and fourth stage juveniles (J3 and J4), females and males] present in 20 g soybean root from 10 soybean producing fields in the Mpumalanga Highveld of SA and the *Bacillus* spp. isolated from four selected localities (based on *Meloidogyne* abundance).

Field	*Meloidogyne* spp. densities	*Meloidogyne* spp. identified			*Bacillus* spp. identified				
		M. enterolobii	*M. incognita*	*M. javanica*	*B. cereus*	*B. coagulans*	*B. megaterium*	*B. subtilis*	*B. thuringiensis*
S2[1]	108	√		√	√	√	√		√
S6	2471	√		√					
S7[3]	6672	√	√	√	√		√	√	√
S8	4304	√	√						
S9	537	√		√					
S11	407	√	√						
S13	1175	√	√						
S14	3537	√							
S15[2]	3922	√			√	√		√	√
S18[1]	87	√	√		√		√	√	√

Fields selected for bacteria isolation: [1]S2 &S18 lowest *Meloidogyne* abundance, [2]S15 intermediate *Meloidogyne* abundance and [3]S7 highest *Meloidogyne* abund

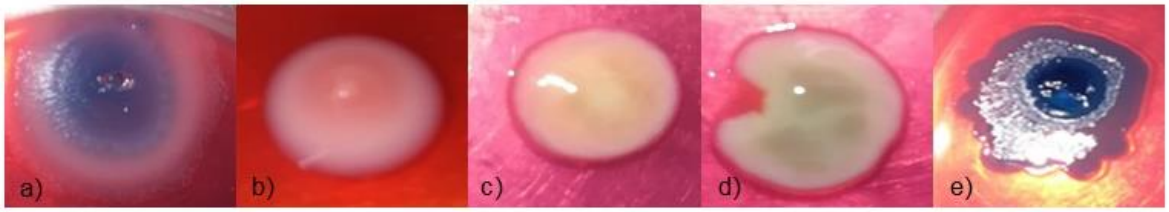

Figure 6.2: Morphology of *Bacillus* spp. grown on *Bacillus* ChromoSelect agar (BCS) representing a) *B. cereus*, b) *B. coagulans*, c) *B. megaterium*, d) *B. subtilis* and e) *B. thuringiensis* (Photo's: Gerhard Engelbrecht, NWU). [*B. cereus*: large light blue, flat colonies with blue centre; *B. coagulans*: small pink, raised colonies; *B. megaterium*: yellow, mucoid colonies*; B. subtilis*: light green to green colonies; *B. thuringiensis*: large light blue, flat colonies with irregular margins].

6.4.3 Bacterial isolation

Four fields, two with the lowest (S2 and S18), one with intermediate (S15) and one with the highest (S7) *Meloidogyne* spp. abundance per 20 g roots were selected to isolate the *Bacillus* spp. that co-inhabited the soybean rhizospheres (Table 6.3). The bacterial colonies isolated from the rhizosphere soil of the four selected fields (see section 6.3.4), grown on BCS, showed distinct morphological traits that corresponded to that of *Bacillus* spp. known for their nematicidal characteristics (Figure 6.2). Rhizosphere soil from two of the four fields (S7 and S18) were found to both contain *B. cereus*, *B. megaterium*, *B. subtilis* and *B. thuringiensis*. By contrast, soil from S2 was found to contain *B. cereus*, *B. coagulans*, *B. megaterium* and *B. thuringiensis*, with *B. cereus*, *B. coagulans*, *B. subtilis* and *B. thuringiensis* identified in soils from S15 (Table 6.3).

6.4.4 Nematicidal bioassay

Significant interactions were evident for treatment x time intervals (48 h and 96 h) for each of the two bioassays, showing that treatments differed significantly from each other for each of the time intervals (Table S4; Figure 6.3a-d). No significant interaction (p value of 0.143) was, however, observed when comparing the two assays (treatments x time intervals x bioassays). For both trials a linear increase in the immotility of the *Meloidogyne* spp. J2 occurred with an increase in the concentration of the *Bacillus* spp. mixtures selected from the four fields (Figure 6.3a-d). Furthermore, significant increases in immotility for J2 were also recorded for the individual *Bacillus* spp. treatment concentrations per bioassay over time (48 h – 96 h) (Table S4; Figure 6.3a-d).

For the first bioassay (Figure 6.3a-d), the 100% *Bacillus* spp. mixture filtrate concentration of field S18 caused the highest J2 immotility of 92.5%. after 96 h. However, for field S15 the *Bacillus* spp. mixture 100% filtrate concentration had the lowest J2 immotility when compared to the same concentration of the other *Bacillus* spp. mixtures in both bioassays for both 48 h and 96 h. Moreover, for field S2 the 25% filtrate concentration after 48 h in the first bioassay had the lowest J2 immotility (23.6%) followed by the 25% filtrate concentration of field S15 (30.7%). Still for the first bioassay, after 96 h J2 immotility was above 70% for all the *Bacillus* spp. mixture concentrations except for 25% filtrate concentration of field S15 (65.3%). After 48 h and 96 h in the first bioassay, J2 immotility for the 100% filtrate concentration of field S18 was significantly different compared to observed for the 50% and 25% concentrations (Table S4; Figure 6.3d).

In the second bioassay, after 48 h the 25% filtrate concentrations of fields S2 and S7 had the lowest J2 immotility of 20.5% and 40.2%, respectively. After 48 h, for fields S18 and S7 the 100% filtrate concentrations had the highest J2 immotility of 71.4% and 65.8%, respectively. Immotile J2 in 25% filtrate concentration of S2 was significantly lower compared to the that of all other

concentrations at 48 h. Although the 100% filtrate concentration for field S7 had 80.1% J2 immotility after 96 h, it was lower than that observed for the same concentrations of fields S2 and S18, namely 85%. Immotility of *Meloidogyne* J2 after 96 h of exposure was generally above 70% with the exception of the 25% filtrate concentrations of fields S2, S7 and S15. At 96 h J2 immotility for the 100% filtrate concentrations of fields S2 and S18 were significantly higher than that observed for the 25% filtrate concentrations.

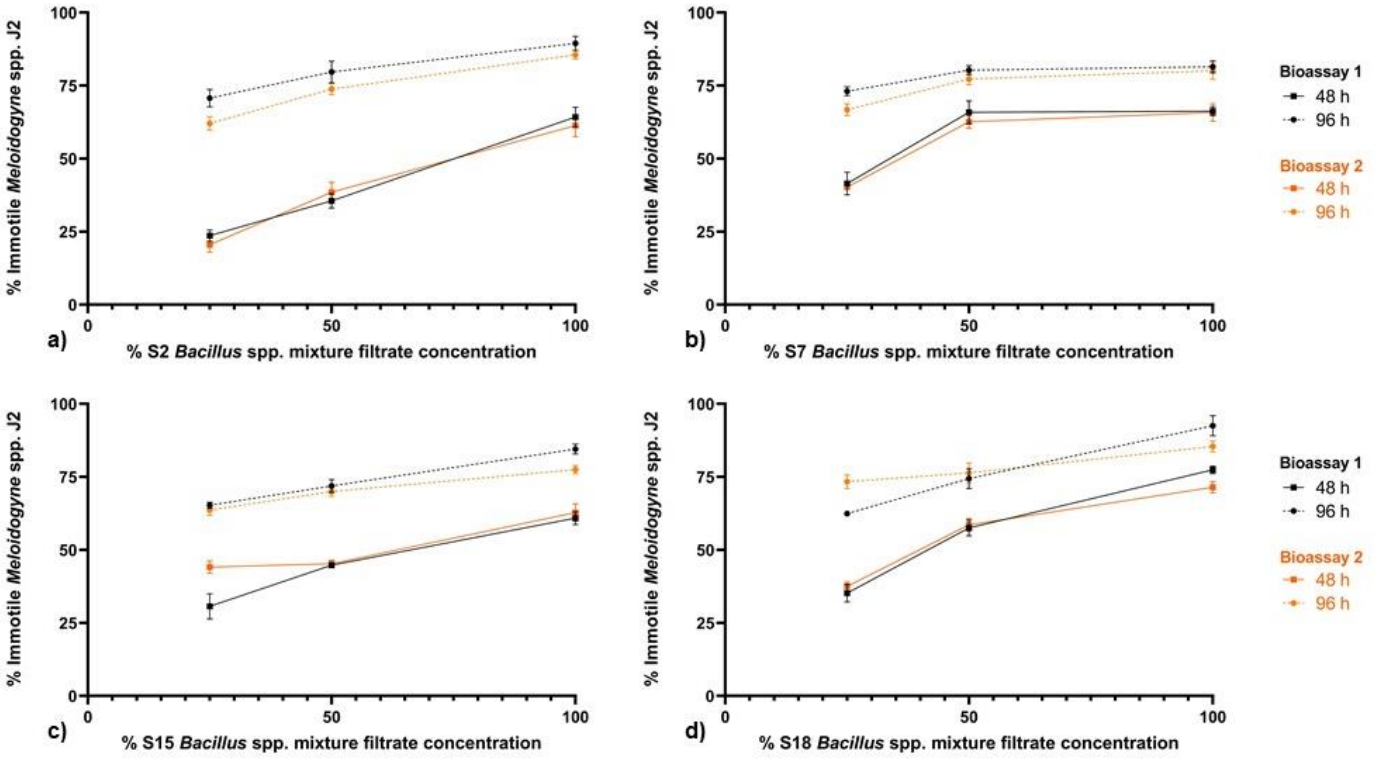

Figure 6.3: Results of nematode bioassays to determine the effect of filtrates of different *Bacillus* spp. mixture concentrations on the J2 of a mixed *Meloidogyne* community. For each bioassay immotile J2 were counted at 48 h and 96 h. Black and orange lines distinguish between the first and second bioassay respectively. Solid lines indicate results form 48 h while dotted lines indicate results of 96 h. a) filtrates of *Bacillus* spp. mixture isolated from S2, b) filtrates of *Bacillus* spp. mixture isolated from S7, c) filtrates of *Bacillus* spp. mixture isolated from S15 and c) filtrates of *Bacillus* spp. mixture isolated from S18. Results obtained from bioassay replicates (n = 4).

6.5 Discussion

Crop rotation used in the Mpumalanga Highveld region usually include soybean being rotated with grain crops such as maize, which is also susceptible to *Meloidogyne* spp. infection (Nel, 2005; Mc Donald *et al*., 2017). This rotation practice, therefore, is expected to contribute to greater nematode populations (Mbatyoti, 2019) and yield loss in soybean and other grain crops in this region. Moreover, other factors that are not known to or investigated by the authors for this specific study, e.g., climatic fluctuations, other biotic and/or abiotic factors, could have impacted the

increase in the population of this nematode genus in the Highveld region compared to those recorded for example for the baseline soybean-nematode audit done 20 years ago (Fourie *et al*., 2001). The identification of *M. incognita* and *M. javanica* in the Mpumalanga Highveld study site associated with local soybean crops is supported by their recent disclosure (Mbatyoti *et al*., 2020), while their impact on soybean yield is also known (Soares & Nascimento, 2021) and especially in countries that are known as the biggest producers such as Brazil (Silva *et al*., 2019) and the USA (Sikora *et al*., 2018). Although both *M. incognita* and *M. javanica* are known to predominantly infect soybean in SA (Fourie *et al*., 2001; Mbatyoti *et al*., 2021) the presence and distribution of *M. enterolobii* in soybean roots in the Highveld of the Mpumalanga during this study, is a novel finding for SA and of great concern. This species has, however, been reported from maize roots in this area by Pretorius (2018). *Meloidogyne enterolobii* is also known to infect soybean roots in Brazil (Dias *et al*., 2010) and North Carolina, USA (Ye *et al*., 2013) where it causes substantial damage to the crop.

Management strategies currently used to reduce PPN numbers in soybean fields in SA are the use of poor-host or resistant soybean cultivars (with only a few being identified) (Fourie, H. North-West University, Potchefstroom, North-West, South Africa. Personal communication, 2022), crop rotation (conducive for the build-up of predominant nematode pests infecting grains) and the application of a granular nematicide (Velum 1GR; a.i. fluopyram) (Bayer, 2021); only registered in 2021. Investigation into the use of alternative management strategies, e.g., biocontrol agents, is becoming increasingly important. The *Bacillus* spp. identified, isolated and reared from soybean rhizospheres in the Mpumalanga Highveld study, include *B. cereus*, *B. coagulans*, *B. megaterium*, *B. subtilis* and *B. thuringiensis* of which some have been listed for their nematicidal activity (Engelbrecht *et al*., 2018). These results are are similar to those from an Indian study in which 115 predominant bacilli were identified from soybean rhizospheres, with *B. cereus*, *B. anthracis*, *B. thuringiensis* and *B. subtilis* amongst those (Khande *et al*., 2017). Reports suggest that *B. cereus* can cause mortality of phytopathogens such as nematodes and as an added benefit result in increased soybean nodulation (Halverson & Handelsman 1991; Gao *et al*., 2016). Of the *Bacillus* spp. identified in our study, *B. megaterium* and *B. thuringiensis* are well known to adversely affect *Meloidogyne* J2 motility and inhibit their hatching (Engelbrecht *et al*., 2018*). Bacillus subtilis* is known for its plant growth promoting effect, increasing photosynthetic pigment levels while also resulting in *Meloidogyne* mortality (Xia *et al*., 2011; Bavaresco *et al*., 2020). The abundance of *Bacillus* spp. together with their distinctive properties, like plant growth promotion, emphasise their potential use and value in bionematicide research (Engelbrecht *et al*., 2018; Sahile *et al*., 2021).

From the four fields selected for this study, two were found to have similar *Bacillus* spp., while the other two contained different species. Interestingly, *B. cereus* and *B. thuringiensis* were isolated from soybean rhizospheres from all four fields, but *B. coagulans* was the least common and only present in two fields. Although the *Bacillus* communities from all fields showed nematicidal activity to *Meloidogyne* J2 (causing immotility), there were differences among the communities. Assay results showed that the combination of *B. cereus*, *B. megaterium*, *B. subtilis* and *B. thuringiensis* filtrates resulted in some of the highest incidences of *Meloidogyne* spp. J2 immotility (Table S1). Opposed to that, the filtrate combination of *B. cereus*, *B. coagulans*, *B. subtilis* and *B. thuringiensis* recorded some of the lowest observed immotile *Meloidogyne* J2. The combination and different strains of these *Bacillus* spp. might affect the nematicidal activity of the *Bacillus* spp. mixture. Furthermore, nematicidal activity of filtrates and combinations might differ over time as the production of potential nematicidal compounds are not conserved among *Bacillus* spp. or even strains (Horak *et al*., 2019). The differences in *Bacillus* spp. community composition can be influenced by sequence and rotation of crops and also organic matter inputs (Ashworth *et al*., 2017; Foo *et al*., 2017; Chamberlain *et al*., 2020).

The value of the current investigation lies into the evaluation of mixtures of *Bacillus* spp. for their nematicidal activity against *Meloidogyne* spp. communities which co-inhabit soybean rhizospheres. The majority of studies thus far focused on *in vitro* assays where only the effects of one of these microorganisms were tested. The latter is accepted as an approach to obtain valuable information about the nematicidal efficacy of microorganisms, e.g., *Bacillus* spp. against various *Meloidogyne* spp., but the adapted protocol followed during this study delivered results directly applicable to the field situation. This is substantiated by records showing that more than one *Bacillus* and *Meloidogyne* spp. can occur simultaneously in one field in local grain production areas (Pretorius, 2018; Visagie *et al*., 2018) and it is therefore proposed that combined opposed to single microorganism studies of this nature rather be done.

By conducting *in vitro* studies with various microorganisms including *Bacillus* spp. under controlled laboratory conditions, useful information regarding their nematicidal potential can be obtained. Although these conditions do not represent prevailing field conditions, *in vitro* studies can prove useful by determining the nematicidal mode of action used by the microorganisms to adversely affect either the biology (e.g., motility), physiology (e.g., oxygen consumption) and/or reproduction of the nematodes (Silva *et al*., 2017; Engelbrecht *et al*., 2018). A known nematicidal mode of action of several *Bacillus* spp. include the production of nematicidal compounds such as benzeneacetaldehyde and dimethyl disulphide produced by *B. megaterium* and the Cry proteins of *B. thuringiensis* (Huang *et al*., 2010; Li *et al*., 2015). *In vitro* nematicidal assays of *M. incognita* using *B. cereus* and *B. subtilis* cell-free filtrates reported 94% and 97% J2 mortality after 48 h of

exposure (Soliman *et al*., 2019) which is substantially higher than the immotile J2 observed at that time in the current study. Further reports suggest that *B. subtilis* Bs-1 showed 100% mortality of *M. incognita* J2 with ovicidal assays showing similar results (Cao *et al*., 2019). Biocontrol studies such as the one conducted by de Araujo *et al*. (2012) found that *B. subtilis* was able to cause more than 70% reduction in J2 and egg densities of *M. incognita* and *M. javanica* infecting roots of susceptible soybean genotypes. Under *in vitro* and *in vivo* conditions *B. thuringiensis* KYC was found to inhibit *M. incognita* J2 hatching and caused J2 mortality, with increased inhibitory effects as the incubation time increased (Choi *et al*., 2020). The latter is a similar trend to the increased J2 immotility reported in this study.

Although results from bioassays using single *Bacillus* and *Meloidogyne* spp. do show promising results (Engelbrecht *et al*., 2020), a study conducted by Khan & Siddiqui (2019) reported increased protection against *M. incognita* by using a mixture of *Pseudomonas putida* and *B. subtilis*. Therefore, assessing mixtures of *Bacillus* spp. and other naturally occurring microorganism communities should be the next approach to attempt elucidating their potential as biocontrol agents in crop rhizospheres. A multiple microorganism species approach to combat *Meloidogyne* spp. communities (containing up to three species in local grain crop production areas) (Pretorius, 2018) may be a more effective strategy to investigate for future application opposed to a single species biocontrol approach. This study generated valuable baseline knowledge about the natural occurrence of mixed *Bacillus* spp. in soybean rhizospheres co-inhabited by damaging *Meloidogyne* spp. communities. It also showed how the presence of these bacilli assemblages may be linked to reducing population densities of the number-one rated, root-knot nematode pest.

6.6 Conclusion

The high abundance of *M. enterolobii* together with *M. incognita* and *M. javanica* in soybean fields in the Mpumalanga Highveld of SA (second largest production area) is a concern and calls for innovative management strategies to protect the soybean industry. Research should focus on biological control organisms that are effective in reducing *Meloidogyne* community abundance in grain crop fields. Unravelling the biocontrol potential of mixed microbial communities co-occurring with *Meloidogyne* under natural field conditions, becomes more urgent to conserving natural resources (air, soil and water). An alternative and/or supplementary approach to use with these management strategies in an integrated pest management (IPM) system can be the use of endemic biological control agents such as bacteria or fungi. Although the management of PPN remains difficult (Xiong *et al*., 2015), the use of microbes with biocontrol characteristics represents a valuable tool to be used in IPM systems, which is the most sustainable way to produce and protect crops.

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6.8 Supplementary data

Table S4: Mean (+SE) of percentage immotile J2 of *Meloidogyne* spp. in bioassays from cell-free filtrates of *Bacillus* spp. mixtures isolated from different soybean fields in the Mpumalanga Highveld.

***Bacillus* spp. mixture concentration**	**1st Bioassay**		**2nd Bioassay**	
	48 h	**96 h**	**48 h**	**96 h**
	Corrected immotility (%)	**Corrected immotility (%)**	**Corrected immotility (%)**	**Corrected immotility (%)**
S2-25	23.6 ± 1.9 aA	70.7 ± 3.0 abEFGHIJ	20.5 ± 2.5 aA	62.1 ± 2.3 aCDE
S2-50	35.5 ± 2.5 abcABC	79.7 ± 3.7 bcdHIJKL	38.5 ± 3.4 bB	73.8 ± 1.9 bcdEFGH
S2-100	64.3 ± 3.4 efEFG	89.5 ± 2.4 deKL	61.3 ± 3.8 cdCD	85.5 ± 1.5 eH
S7-25	41.5 ± 3.9 bcBC	73.1 ± 1.5 abcFGHIJ	40.2 ± 0.7 bB	66.8 ± 2.0 abcCDEF
S7-50	65.9 ± 3.9 efEFGH	80.3 ± 1.6 bcdIJKL	62.6 ± 2.2 cdCDE	77.2 ± 2.0 cdeFGH
S7-100	66.3 ± 1.5 efEFGHI	81.4 ± 2.1 bcdeJKL	65.8 ± 3.0 cdCDEF	80.1 ± 2.9 deGH
S15-25	30.7 ± 4.3 abAB	65.3 ± 1.0 aEFG	44.1 ± 2.1 bB	63.6 ± 1.8 abCDE
S15-50	44.8 ± 0.6 cdCD	71.9 ± 2.2 abFGHIJ	45.3 ± 1.2 bB	70 ± 1.6 abcdCDEFG
S15-100	60.9 ± 2.2 eEF	84.5 ± 1.7 cdeJKL	62.8 ± 3.0 cdCDE	77.5 ± 1.4 cdeFGH
S18-25	35.2 ± 3.0 abcABC	62.4 ± 0.4 aEF	37.5 ± 1.5 bB	73.3 ± 2.3 bcdDEFGH
S18-50	57.5 ± 2.7 deDE	74.4 ± 3.4 abcFGHIJ	58.6 ± 2.2 cC	76.4 ± 3.4 cdeFGH
S18-100	77.4 ± 1.3 fGHIJK	92.5 ± 3.4 eL	71.4 ± 1.9 dDEFG	85.4 ± 1.8 eH
Interaction data per column				
p value	**0.001**	**0.001**	**0.001**	**0.001**
F ratio	41.72	13.91	36.65	11.9
Interaction data: treatments x time intervals per assay (48 h and 96 h)				
p value	**0.001**		**0.001**	
F ratio	8.8		8.7	
Interaction data: treatments x time intervals x assays (1 and 2)				
p value	0.143			
F ratio	2			

Note: Mean differences, standard error, p value and F ratio are indicated. Tukey's HSD (P<0.05) test indicates significant differences among treatments per bioassay per individual time interval with different small letters (thus comparing immobility results of all treatments within a time interval of a bioassay to each other). Capital letters following treatments indicate significant differences among respective time intervals (48 and 96 h) between the two assays (thus comparing all the treatments of the two respective time intervals within one bioassay to each other). Results obtained from sample replicates (n = 4). *Meloidogyne* spp. used were created from mixing populations from 10 soybean fields, while S2, S7, S15 and S18 refer to the fields used for the Bacillus spp. isolation. Furthermore, -25, -50 and -100 indicates the filtrate concentration that were used.

CHAPTER 7: CONCLUSION AND FUTURE PERSPECTIVES

“Knowing is not enough; we must apply. Willing is not enough; we must do.”

Johann Wolfgang von Goethe

7.1 Concluding observations

This study focused on determining the abundance and diversity of PPN communities associated with soybean (*Glycine max* (L.) Merr.) production in the second largest soybean production area in SA namely the Highveld region of Mpumalanga. This led to experimental work that focused on PPN communities associated with soybean. Additionally, attempts were made to identify (using advanced molecular methods) bacterial communities associated with soybean rhizospheres with potential as biocontrol agents to combat the predominant nematode pests identified. During this investigation quantitative data were generated with regards to both the predominant nematode pests of soybean roots in both a soybean dominated system and a soybean-maize rotation system and the soil bacterial communities of the same fields. To achieve the aim of this study, five specific objectives were put forward at the start of the investigation. The major findings of these objectives were as follows:

1. **Reviewing the current state of soybean in SA, with focus on the economically most important nematode pests and their control, against the background of the importance of the crop worldwide and particularly in sub-Saharan Africa**

This work started with a literature-based review (Chapter 2), that not only emphasised the importance of soybean internationally, but also on a more local scale, as the widespread use of soybean and related products play vital economic and socioeconomic roles. The increased production of this important crop also means that it will be exposed to more pests such as PPN that pose major risks to the sustainable production of the crop. Of these PPN, *Meloidogyne* and *Pratylenchus* species are of particular concern and known to parasitise soybean crops and cause major yield losses. Therefore, it is important to determine the composition of PPN communities associated with soybean roots. Furthermore, it is vital to ensure that sustainable nematode management practices be implemented. Unfortunately, current nematode management practices implemented in SA (chemical control, crop rotation and host plant resistance) might not be sufficient. Due to various economic and environmental factors, the need for more effective management strategies has developed over the years. One such management alternative is the use of biocontrol products. These products can be developed based on microbes that co-occur with the major PPN threats in the soil. Hence the importance of not only determining the composition of PPN communities, but studying the microbial populations associated with these pests in soybean rhizospheres, and how their co-occurrence might impact each other.

2. **Determining soil microbial community structure using next generation sequencing for the identification of bacterial strains with biocontrol potential.**

With this objective it was determined that a comprehensive understanding, of the bacteria with nematicidal potential present in the rhizosphere of crops that are parasitised on by PPN, can be obtained with 16S rRNA gene amplification and NGS. By implementing various downstream analyses of NGS data, it was possible to determine whether an increase in abundance of certain bacteria can be related to decreased nematode population densities as done in Chapter 3. It is also possible that a consortium of bacteria with nematicidal properties can exist on a spatial scale within a field. This observation can also potentially explain the low bacterial species diversities observed for sites with higher densities of *Meloidogyne* and *Pratylenchus* compared to those with lower *Meloidogyne* and *Pratylenchus* densities as seen in Chapter 3. Therefore, improving our understanding of the natural rhizosphere bacterial communities and their relationship with both the plant and nematodes will help unravel the natural microbiome structure needed for biocontrol of PPN.

3. **Determining the presence, abundance and distribution of *M. enterolobii* and *P. brachyurus* on the highveld using molecular techniques.**

Under this objective it was determined that it is possible for single populations or mixed communities of both *Meloidogyne* and *Pratylenchus* spp. to parasitise soybean and maize in a single field. These were also the only two genera that was found to be consistently present in the roots sampled during this study (Chapter 4). Of the species identified in this study, it is concerning *that M. enterolobii* and *P. brachyurus* occurred at the same time in a field. As *M. enterolobii* has been reported to be more pathogenic than its counterpart species and *P. brachyurus* is increasingly becoming a problem in soybean production areas, the impact of *M. enterolobii* and *P. brachyurus* co-occurrence on any given crop should be investigated further. Of the species screened for in this study belonging to *Meloidogyne* and *Pratylenchus*, *M. enterolobii* and *P. brachyurus* were identified as the most abundant when using SCAR-PCR and species-specific PCR, respectively. In contrast, sequencing results from Chapter 4 only identified a few samples as *M. enterolobii* with the majority being identified as *M. incognita*. Sequencing results of *Pratylenchus* samples showed a similar trend with a few being identified as *P. brachyurus* while most other samples were identified as *P. curvicauda* and *P. bolivianus*. Therefore, it is important that accurate identification of either a single population or a mixed community of RKN and lesion nematodes should be obtained to better understand the distribution of these species.

4. **Comparing the impact of soybean-maize rotations on PPN and soil bacterial communities.**

Chapter 5 investigated the intricate relationships between PPN, soil bacterial communities and crop management practices. The most important findings of this chapter indicated that nematode populations vary not only among different cropping sequences, but between fields using the same rotation schemes due the differences in how genera and species respond to management regimes, irrespective of the crop or cropping sequence. Another significant finding from this Chapter was that although maize and soybean are both hosts of the predominant nematode genera identified in this study, PPN were more abundant in fields under soybean cultivation (Chapter 4 and Chapter 5). Importantly, results from Chapter 5 also showed that crop rotation greatly impacts the community composition of soil microbes. As numerous microbial genera present in soil can suppress PPN, changes in soil microbial community structure because of crop rotation can impact their metabolic activity and diversity; and potentially their nematicidal activity. When comparing the bacterial species diversity using the Shannon index, lower diversity was detected for samples obtained in the second sampling interval while each sampling interval showed distinct beta-diversity profiles, irrespective of the cropping scheme of the fields used in this study. Furthermore, when cultivating soybean, *Bradyrhizobium* or *Rhizobium* are usually added to the soil, a practice that is not done for maize. Such practises can also alter the microbiome dynamics in soils while making certain genera more abundant in fields that are under soybean cultivation, as demonstrated in Chapter 5. Therefore, determining the presence of bacteria with potential nematicidal characteristics in a field using a certain cropping scheme, can provide insight into what cropping scheme can prove to be beneficial to these microbes and aid in the suppression of PPN.

5. **Assessing the ability of soil bacteria (especially *Bacillus* spp.) associated with soybean for their potential nematostatic activities against a mixed *Meloidogyne* community.**

With this objective it was determined that *M. enterolobii*, *M. incognita* and *M. javanica* presence in soybean production fields in the Mpumalanga Highveld of South Africa is of concern with single populations and mixed communities being identified for this region (Chapter 6). Current management strategies such as the use of poor-host or resistant soybean and crop rotation might not be efficient enough. Thus, requiring the use of alternative management strategies, e.g., biocontrol agents using bacteria with nematicidal properties. Soil bacteria such as *Bacillus* spp. are amongst the most dominant rhizosphere genera with nematicidal properties, and several species of this genus also occur in agricultural soil. The *Bacillus* spp. identified, isolated and reared from soybean rhizospheres used in Chapter 6, include *B. cereus*, *B. coagulans*, B.

megaterium, *B. subtilis* and *B. thuringiensis*. Although all of these species are known to have nematicidal properties (Engelbrecht *et al*., 2018), very few studies focused on the use of combinations of more than one *Bacillus* spp. in nematicidal research. Importantly, this objective verified that different nematicidal *Bacillus* spp. can occur in the same environment as *Meloidogyne*, while being associated with high levels of *Meloidogyne* spp. Immotility of *Meloidogyne* J2 of up to 93% reported from this study, was evident for a combination of *B. cereus*, *B. megaterium*, *B. subtilis* and *B. thuringiensis*.

7.2 Future perspectives

This study verified the potential threat that PPN, especially *Meloidogyne* and *Pratylenchus* spp., can have on soybean production and showed the need for more research on their presence and distribution with regards to the various crops that are rotated with soybean. The common occurrence of *M. enterolobii* and *P. brachyurus*, and other species of these two genera, in soybean producing fields is of great concern and hence it is crucial to understand the potential effect that the co-occurrence of these species can have on soybean yield as well as other crops that are commonly rotated with soybean.

The accurate identification of these *Meloidogyne* and *Pratylenchus* spp. using molecular techniques, is problematic due to the inter- and intraspecies variation between species. It is therefore recommended that when using molecular identification more than one technique/marker/gene should be used to aid in accurate identification/characterisation of *Meloidogyne* and *Pratylenchus* spp. One such technique that can be used is iso-enzyme identification. The use of several enzyme phenotypes like esterases, malate dehydrogenase and superoxide dismutase can possibly be implemented as a tool to characterise and distinguish between *Meloidogyne* spp. This tool is based on the use of electrophoresis to separate soluble proteins from macerated mature *Meloidogyne* females to distinguish between different phenotypes according to rate of migration of the proteins (Carneiro *et al*., 2000).

Meloidogyne and *Pratylenchus* spp. in the soil and roots of crops are also in close contact with and affected by the soil microbiome. The microorganisms in soil not only have plant growth promoting activities, but they can also lead to nematode suppression through various modes of action. It is therefore important to understand the potential interaction and potential suppressive effects of naturally occurring microbiomes on nematodes. The current study will provide a valuable baseline in this regard and future investigations could contribute knowledge by focusing on:

i) Expanding surveys that aim to identify the distribution and co-occurrence of both *M. enterolobii* and *P. brachyurus*, as well as other species of these two genera, in economically important crops.

ii) Determining the potential yield loss of a crop due to the co-occurrence of *Meloidogyne* and *Pratylenchus* spp.

iii) Improve the accurate identification of the soil microbes as new technologies are continuously developed.

iv) Improve and increase field-based studies that aim at identifying/validating the use of certain bacterial strains as possible biological control alternatives.

In the context of rotation practices and biological community structures, it is important to acknowledge the complexities of different rotation sequences and combinations of agricultural practices e.g., cropping systems × tillage practices (Habig, 2020). Any management regime will influence biological communities and not necessarily in the same way. When investigating such trends, allowances should be made for the frequency and sequence of a crop in a rotation as well as the intensity of disturbance associated with tillage regimes. Despite intensified research efforts into soil health, sustainable agriculture and biological control, there are still many unanswered questions and a clear need for a deeper understanding into the relationship between biological communities. Not only will such an understanding assist stakeholders in optimising management practices, but it will be valuable in ensuring long-term food security and agricultural sustainability.

7.3 References

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APPENDIX A

agriculture

MDPI

Review

South Africa: An Important Soybean Producer in Sub-Saharan Africa and the Quest for Managing Nematode Pests of the Crop

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Abstract: With an increase in the global population, a protein-rich crop like soybean can help manage food insecurity in sub-Saharan Africa (SSA). The expansion of soybean production in recent years lead to increased land requirements for growing the crop and the increased risk of exposing this valuable crop to various pests and diseases. Of these pests, plant-parasitic nematodes (PPN), especially *Meloidogyne* and *Pratylenchus* spp., are of great concern. The increase in the population densities of these nematodes can cause significant damage to soybean. Furthermore, the use of crop rotation and cultivars (cvs.) with genetic resistance traits might not be effective for *Meloidogyne* and *Pratylenchus* control. This review builds on a previous study and focuses on the current nematode threat facing local soybean production, while probing into possible biological control options that still need to be studied in more detail. As soybean is produced on a global scale, the information generated by local and international researchers is needed. This will address the problem of the current global food demand, which is a matter of pressing importance for developing countries, such as those in sub-Saharan Africa.

Keywords: Africa; soybean; *Meloidogyne*; *Pratylenchus*; management

1. The Potential of Soybean to Manage Food Insecurity

United Nations (UN) estimates indicated that the world population increased from 6,145,007 to 7,795,482 during 2000–2020, while the sub-Saharan Africa (SSA) population almost doubled (645,007 to 1,106,573) in the same period [1]. This increase in population density can result in severe food insecurity especially in SSA, where food demand can increase by more than 300% by 2050. Cereals, such as maize (*Zea mays*), millet (*Panicum* spp.), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), and wheat (*Triticum*) are the most important crops with regards to calorie intake in SSA, although large yield gaps still exist for crops like maize [2,3].

Recent estimates indicate that there are over 475 million farms worldwide that can be defined as smallholder entities, producing at least half of the world's food [4]. However, the situation is different in South Africa, where most farms are commercial and from which the bulk of food is produced while smallholder farms are more predominant in poor rural areas [5]. In SSA, smallholder farms face various challenges, including low productivity, and high levels of poverty and food insecurity, resulting in low agricultural growth that cannot match the rapid population increase [6]. To help manage the food demand in SSA, alternative crops, such as soybean (*Glycine max* (L.) Merr.), can be used. It is one of the most important summer legume crops worldwide that serves as an important dietary protein and oil source for animal and human consumption. Soybean seeds consist of about 18% oil and 38% protein

APPENDIX B

microorganisms

MDPI

Article

Screening of Rhizosphere Bacteria and Nematode Populations Associated with Soybean Roots in the Mpumalanga Highveld of South Africa

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Abstract: Soybean is among South Africa's top crops in terms of production figures. Over the past few years there has been increasingly more damage caused to local soybean by plant-parasitic nematode infections. The presence of *Meloidogyne* (root-knot nematodes) and *Pratylenchus* spp. (root lesion nematodes) in soybean fields can cripple the country's production, however, little is known about the soil microbial communities associated with soybean in relation to different levels of *Meloidogyne* and *Pratylenchus* infestations, as well as the interaction(s) between them. Therefore, this study aimed to identify the nematode population assemblages and endemic rhizosphere bacteria associated with soybean using Next Generation Sequencing (NGS). The abundance of bacterial genera that were then identified as being significant using linear discriminant analysis (LDA) Effect Size (LEfSe) was compared to the abundance of the most prevalent plant-parasitic nematode genera found across all sampled sites, *viz. Meloidogyne* and *Pratylenchus*. While several bacterial genera were identified as significant using LEfSe, only two with increased abundance were associated with decreased abundance of *Meloidogyne* and *Pratylenchus*. However, six bacterial genera were associated with decreased *Pratylenchus* abundance. It is therefore possible that endemic bacterial strains can serve as an alternative method for reducing densities of plant-parasitic nematode genera and in this way reduce the damages caused to this economically important crop.

Keywords: bacteria; biological control; *Meloidogyne*; *Pratylenchus*; soybean

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1. Introduction

Plant parasitic nematodes (PPN) cause substantial yield losses to agricultural crops, with annual global crop losses estimated at $78 billion [1]. *Aphelenchoides bessseyi*, *Bursaphelenchus xylophilus*, *Ditylenchus dispaci*, *Globodera* spp., *Heterodera* spp., *Meloidogyne* spp., *Naccobus aberrans*, *Radopholus similis*, *Rotylenchulus reniformis* and *Xiphinema index* are considered the top 10 nematode pests worldwide [2]. Due to their global distribution and wide range of host plants, of all the PPN genera and species, root-knot nematodes (RKN; *Meloidogyne* spp.) and lesion nematodes (*Pratylenchus* spp.) are particularly harmful to crops in South Africa and can cause substantial damage and adversely affect production figures of a wide range of economically important crops, such as the potato, grain, oilseed, industrial and fruit crops produced in this country [3,4]. Of all *Meloidogyne* spp. documented to parasitize crops on a global scale, 22 are reported to occur in Africa [5], while 14 *Meloidogyne* spp. and 10 *Pratylenchus* spp., respectively, have been listed for South Africa [6–9].

In the Mpumalanga Highveld region of South Africa crops that are usually planted include maize (*Zea mays*), wheat (*Triticum* spp.), groundnut (*Arachis hypogaea*), soybean (*Glycine max* (L.) Merr.), sunflower (*Helianthus* spp.) and potato (*Solanum tuberosum*) [10] of which all are known hosts of both RKN and lesion nematodes. Of these crops, soybean

APPENDIX C

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Filtrates of mixed *Bacillus* spp inhibit second-stage juvenile motility of root-knot nematodes

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ABSTRACT

The global expansion of soybean (*Glycine* max (L.) Merr.) exposes it to more diseases and pests such as nematodes. In South Africa particularly, *Meloidogyne incognita* and *M. javanica* are considered the predominant genera infecting soybean, but a more pathogenic root-knot species, *M. enterolobii*, was recently reported in the Mpumalanga Highveld of South Africa. The use of chemicals to manage these pests is usually preferred although various concerns exist regarding their potential impact on the environment. An increasing need for the development of less toxic alternatives for nematode management thus exists. This study determined the nematicidal activity of *Bacillus* spp. mixtures isolated from soybean rhizospheres on the motility of second-stage juveniles (J2) of mixed *Meloidogyne* communities co-occurring in these rhizospheres. Roots and soil from 10 soybean fields in the Mpumalanga Highveld were collected and *Bacillus* spp. isolated, while the population density and molecular identification of the co-existing *Meloidogyne* spp. were also done. The filtrates of the *Bacillus* spp. were then used in *in vitro* assays to determine their potential nematicidal activity. Results confirmed the presence of *M. enterolobii* (100%) *M. incognita* (50%) and *M. javanica* (40%) in the sampled fields, with single populations found in 20% and mixed communities in 80% of the fields. The filtrate mixture of *B. cereus*, *B. megaterium*, *B. subtilis* and *B. thuringiensis* caused approximately 85–90% immobility of *Meloidogyne* spp. J2 after 96 h. The results show that the use of *Bacillus* spp. mixtures can aid in the development of biocontrol products to combat root-knot nematodes and might be more effective than products from a single species in limiting J2 motility.

1. Introduction

Soybean (*Glycine* max (L.) Merr.) is an important summer legume crop used particularly for its high protein content in food and fodder sources globally. Two countries, *viz.* Brazil and the United States of America (USA), are the largest soybean producers with both delivering >100 million metric tons of produce annually (USDA, 2020). This important crop has the potential to serve as an important dietary source of both protein and oil for animal and human consumption, as soybean seeds consist of approximately 18% oil and 38% protein (Hartman et al., 2011). The value and importance of this crop has also led to an increase in its production in lower-producing countries such as South Africa (SA). Soybean production in SA has substantially increased annually since the early 1960s with the area dedicated to its production estimated at a record 827 100 ha during the 2020/2021 growing season (Grain SA, 2021). However, the increase in global production poses the risk of this important food and fodder crop being exposed to more pests and diseases, including plant-parasitic nematodes (PPN) (Sikora et al., 2018).

Root-knot nematodes (*Meloidogyne* spp.) are important pests of various crops worldwide including soybean (Al-Banna et al., 2004; Xiong et al., 2015; Fourie et al., 2017). In SA, *Meloidogyne incognita* and *M. javanica* are the species being considered as the two economically most important root-knot nematode pests that parasitise soybean (Fourie et al., 2017; Mbatyoti et al., 2021). Recently an upcoming threat, *M. enterolobii* (Collett et al., 2021) has also been identified infecting maize (Pretorius, 2018) in the Mpumalanga Highveld area of SA where maize and soybean are rotated (Nel, 2005; Mc Donald et al., 2017). Compared to *M. incognita* and *M. javanica*, *M. enterolobii* caused greater galling on tomato (Cetintas et al., 2007). Adding to the potential devastating effect of *M. enterolobii* infection in crop roots, is its ability to reproduce on crop genotypes that contain root-knot resistance genes (*Mi-1*, *Mh*, *Mir1*, *N*, *Tabasco*, and *Rk*) (Ye et al., 2013). Therefore, *M. enterolobii* is listed as a more pathogenic species than its counterpart tropical/thermophilic species *M. incognita* and *M. javanica* (Jones et al., 2013). *Meloidogyne enterolobii* has already been reported from soybean roots in Brazil (Dias et al., 2010) and North Carolina, USA (Ye et al.,

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