Population dynamics of *Meloidogyne* spp. and the effect of pre-plant practices on the pest in tomato net houses in South Africa

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DECLARATION BY THE CANDIDATE

I, Francinah L Matlala, declare that the work presented in this MSc thesis is my own work, that it is not been submitted for any degree or examination at any other University and that all the sources I have used or cited have been acknowledged by the complete reference.

Signature  Date 29 May 2020

DECLARATION AND APPROVAL BY SUPERVISORS

We declare that the work presented in this thesis was carried out by the candidate under our supervision and we approve this submission.

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Abstract

In South Africa, the largest tomato producer, ZZ2, practices extensive monocropping under net house structures. These net houses allow the farmer prolonged harvest and good quality yields, however, they also provide ideal conditions for the build-up of diseases and pest such as plant-parasitic nematodes (PPN). The aim of this study was to determine nematode population dynamics in net houses of ZZ2 over a period of three years and investigate the effect of pre-plant strategies on root-knot nematode population development under tomato production systems.

To achieve this, six net houses at ZZ2 farms, located in three different climatic regions in the Limpopo Province, were suveyed for three consecutive years to investigate the population dynamics of PPN. Nematode densities in soil fluctuated between the years, however, *Meloidogyne* spp. numbers significantly increased, irrespective of location or climatic conditons, from the first year to the third year for all net houses. Net houses also differed significantly among each other concerning *Meloidogyne* spp. densities recorded in tomato rhizospheres. Chemical soil analysis indicated that net houses with high potassium, calcium, pH and acid saturation levels had lower *Meloidogyne* spp. numbers, while those with higher Mg:K and Na:K ratios were associated with higher *Meloidogyne* spp. densities. *Meloidogyne* spp. numbers were higher in sandy soils. Planting time, however, had less influence on *Meloidogyne* spp. numbers, while cultivation of rootstocks RS2, RS3 and RS13 resulted in lower *Meloidogyne* spp. numbers compared to RS11 and scion ZZX132.

Root-knot nematode species associated with tomato crops in six net houses situated in different climatic areas were identified using morphological (perineal-pattern and oesophageal structure) and molecular (SCAR-PCR) approaches. The combination of both techniques (molecular and morphological) confirmed the presence of four *Meloidogyne* species namely *M. arenaria*, *M. enterolobii, M. incognita* and *M. javanica* and should be used to ensure that the correct information is gathered since both techniques did not present the same results for the different net houses.

Pre-plant strategies undertaken by ZZ2 producers to prepare net houses for a next tomato crop cycle were evaluated in the net house to determine their efficacy in delaying infection in tomato roots. The trial layouts were randomized, complete block

designs with five treatments representing i) the end of crop cycle, ii) fallow, iii) ridge disking, iv) addition of organic material (OM; compost, compost tea, fermented plant extracts and other organic products), and v) addition of OM combined with inoculation of the soil with ±2000 infective, second-stage juveniles (J2) of *M. javanica*. Soil from cold semi-arid (BSK) net houses contained both *Meloidogyne* spp. and *Pratylenchus* spp. individuals, and those from a warm subtropical (CWA) net houses only *Meloidogyn*e spp. individuals. Addition of OM to the potted BSK and CWA net house soils reduced *Meloidogyne* spp. densities in tomato roots and soil. Addition of OM to soil inoculated with ±2000 infective J2 of *M. javanica* and left fallow for both net houses also resulted in a reduction of *Meloidogyne* spp. denities in roots and soil of tomato plants. *Pratylenchus* spp. densities were not affected by the treatments.

Keywords: Physio-chemical, monocropping, protected structures, nematodes, tomato.

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Introduction and literature review

1.1 Introduction

The estimated global crop losses resulting from infection by plant-parasitic nematodes (PPN) are estimated to amount from $US 80 billion (Nicol et al., 2011) to $US 173 billion per year (Elling, 2013). In India, annual economic loss from 30 crops amounted to $US 1.58 billion (Kumar et al., 2020) while in South Africa losses per year in potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersium* L.) and maize (*Zea mays* L.) collectively are grossly estimated to amount to $US 216 million (Nemlab, 2012). Such damage is, however, likely higher as growers in developing areas are mostly unaware of the existence of PPN (Greco and Di Vito, 2009; Hassan et al., 2013; Jones et al., 2013b).

Root-knot nematodes, genus *Meloidogyne* Göldi, 1892, represents one of the most important nematode pest genera that damage vegetable crops. These nematode pests are microscopic, non-segmented roundworms found in soil and root-/other below-ground parts of host plants. Their short life cycle enables them to survive in their hosts causing measurable damage in a period of four to eight weeks after transplanting seedlings into infested soils (Briar et al., 2016). Controlling root-knot nematodes is hence crucial, but challenging.

An effective nematode management strategy requires the manipulation of nematode densities to non-injurious and sub-economical threshold levels (Njoroge, 2014, Sikora and Roberts, 2018), with the most effective generally being chemical control (Hussain et al., 2017). However, this method is expensive, not environmentally friendly and due to the withdrawal of many nematicides, it has become clear that other strategies have to be used as part of an integrated control strategy (Bernard et al., 2017). Under continuous monocropping, which is commonly practiced under intensive agricultural production, it is often not possible to apply crop rotation, intercropping, fallowing amongst other control strategies because of time and space constraints, and ultimately financial considerations. Additionally, continuous monocropping usually negatively affects soil fertility, physicochemical properties of soils and leads to a build-up of pests and diseases that result in lower crop productivity (Lovaisa et al., 2017). Also, soil

biodiversity is often unbalanced under monocropping and pests such as PPN are likely to become more abundant (Malherbe, 2016). Nevertheless, due to the human population increase that results in a higher demand for food, in combination with land shortage, monocropping seems to be the most popular solution (Ntalli et al., 2020).

As the largest tomato producer in South Africa, ZZ2's tomato production has been moving from open fields to net house production with the aim to improve quantity and quality of their products (Cadet et al., 2018). However, under net house production, fields cannot be left fallow as is the practice with open fields. This net house practice of continuous planting with crops presents an even higher risk of increased PPN population densities. To address this challenge, ZZ2, has adopted the principles of 'Natuurboedery/Nature Farming' which focus on addition of the following amendments to the soils in order to protect tomato plants from pests and diseases: inorganic fertilisers, organic soil amendments and plant extracts, effective micro-organisms (EM) products, compost tea and biological agents. This practice rejuvenates the soil after the intensive and continuous monocropping of tomato by ensuring a balanced soil biodiversity (Cadet et al., 2018; Taurayi, 2011).

1.2 Aim and objectives

In the farming system used by ZZ2 nematode analyses showed that regardless of high root-knot nematode densities recorded at the end of a tomato crop cycle, the first infected plants in a follow-up cycle are only observed two months after seedlings have been transplanted. This observation seems to indicate that pre-plant strategies followed when preparing the soils for planting a next crop have an impact on the development and reproduction of root-knot nematodes and other PPN.

Therefore, the broad aim of this study was to determine the root-knot nematode species and their population dynamics in net houses of ZZ2 over a period of three years, and investigate the effect of pre-plant strategies on root-knot nematode development under tomato production systems.

The specific objectives were to:

i) determine the root-knot nematode population development over a period of time in six net houses situated in different climatic areas by

identifying which parameters (climate, soil texture, planting periods, cultivated rootstocks) influence its spatial distribution,

ii) identify the root-knot nematode species associated with tomato crops in six net houses situated in different climatic areas using morphological (perineal-pattern and oesophageal structure morphology) and molecular (sequence characterised amplified region – polymerase chain reaction: SCAR-PCR) approaches and

iii) determine which pre-plant strategies impact on the delay of root-knot nematode development by identifying which pre-plant practice (fallow, ridge disking, addition of organic material, compost, EM, fermented plant extracts and compost tea) individually or in combination influence root-knot nematode population development.

1.3 Literature review

1.3.1 Tomato

Tomato (*Solanum lycopersium* L.), classified under the family Solanaceae (Darwin et al., 2003; OECD, 2017), is native to South and Central America and it is among the most important horticultural crops that are cultivated on over 4 million hectares of land worldwide (Nicola et al., 2009). More than 80% of global tomato crops are produced in Eastern Asia and North America (FAO, 2019). With respect to vegetables, tomato is ranked second in South Africa after potato (*Solanum tuberosum* L.) in terms of production; the crop is cultivated on approximately 8,006 ha with 610,237 metric tonnes (MT) produced in the 2017/18 production season, contributing 6.7% to the gross production figure in the same year (DAFF, 2018a; FAO, 2019). In South Africa, even though tomato is planted throughout the country, most of the production is done in the Limpopo Province, reported to have a production area of over 3 590 ha, followed by the Mpumalanga and Eastern Cape provinces (DAFF, 2018b). It is regarded as the most valuable cash crop grown in South Africa by both small and commercial growers (Malherbe, 2016).

1.3.1.1 Production

Tomato growth is influenced by climate, day length and soil characteristics (Shamshiri et al., 2018). The plant can be grown in all the provinces, however, it does well in the warmer area where it can be planted all year round. Planting periods in the lowveld of South Africa (frost free area) start from February to May, in the middleveld (moderate areas) from September to December and highveld (cold areas) from October to November (Starke Ayres, 2014). Tomato does well in well drained sandy loam soils, with pH range between 6 to 7. The production of tomato, however, is challenging due to its susceptibility to bacteria, fungi, virusses and also plant-parasitic nematodes (PPN) (Malherbe, 2016). Production requirements to optimise tomato yield are summarised in Table 1.1.

Table 1.1. A summary of production requirements to optimise tomato yield in South African climatic conditions.

1.	Climatic requirement	Optimal temperature for germination is between 15-30 °C, vegetative growth between 20-24 °C, fruit set night (14-20 °C) and day set (20-24 °C) red colour development (20-24 °C). Air relative humidity between 55-60% is important for pollination (OECD, 2017).
2.	Cultivar	The choice of cultivar is based on the agroecological area where a farmer is located. Fruit maturity, quality, reliability and susceptibility to disease and pest, plant growth habit, market and planting time are first taken into consideration (DAFF, 2010)
3.	Planting space	Planting distance is 25-50 cm between seedling and 1.5-1.8 m between rows, with 22 000-25 000 plants/ha, however, this depends on the production goal (OECD, 2017). Plant production under protection, however, is usually higher than open field production (Starke Ayres, 2014).
4.	Fertiliser application	The amount of fertiliser applied is determined by soil fertility status, season and cultivar (DAFF, 2010). Tomato requires both micro and macronutrients for growth and development. Nitrogen is more important in vegetative stage, potassium more important in flowering stage and during fruit set, calcium and magnesium are the most important elements (Starke Ayres, 2014).
5.	Irrigation	Frequent irrigation aids in delayed maturity and prolong plant productivity. Soil moisture varies with cultivation methods, variety and climate (OECD, 2017). Irrigation schedule is reliant on root system size (Starke Ayres, 2014). Drip irrigation is recommended as it uses water efficiently, and this type of irrigation is practiced more under protected structures (OECD, 2017).
6.	Weed control	Weed control can either be chemical, mechanical or both. Chemical control, *viz*. herbicides application is effective when done properly. Under protected structures, hand-hoeing is practiced (DAFF, 2010).
7.	Diseases and pest management	Integrated pest management practices such as chemical (*viz*. use of registered pesticides), biological, mechanical and other cultural practice (DAFF, 2010).

1.3.1.2 Anatomy and morphology

Tomato is a dicotyledonous perennial crop but is grown as an annual plant. It is a herbaceous diploid (2*n* = 24) plant (up to 3 m tall) with hairy, granular trichomes and weak trailing stems (Kimura and Sinha, 2008; OECD, 2017). The leaves alternate on the stems with shapes ranging from lobed to compound, with segments arranged pinnately. These compound leaves are made up of five to nine leaflets, which are petiolated and dentated. Furthermore, these leaves are covered by granular, hairy trichomes (OECD, 2017). The fruit shape can either be globular or ovoid, depending on the variety, being bilocular or multilocular. Within the locular cavities, there are about 50 to 200 lentil shaped seeds enclosed by a gelatinous membrane (Figure 1.1) (OECD, 2017).

According to Jones (2013a) and Garcia et al. (2011), there are five growth stages of tomato, and these include: germination and early growth (25-35 days), vegetative growth (20-25 days), flowering (20-30 days), early fruiting (20-30 days) and mature fruiting (15-20 days) (Figure 1.1). Each of these stages is dependent on the variety, environmental and other conditions (open field, greenhouse or shade nets, temperature, light, soil conditions and nutrients) (Shamshiri et al., 2018).

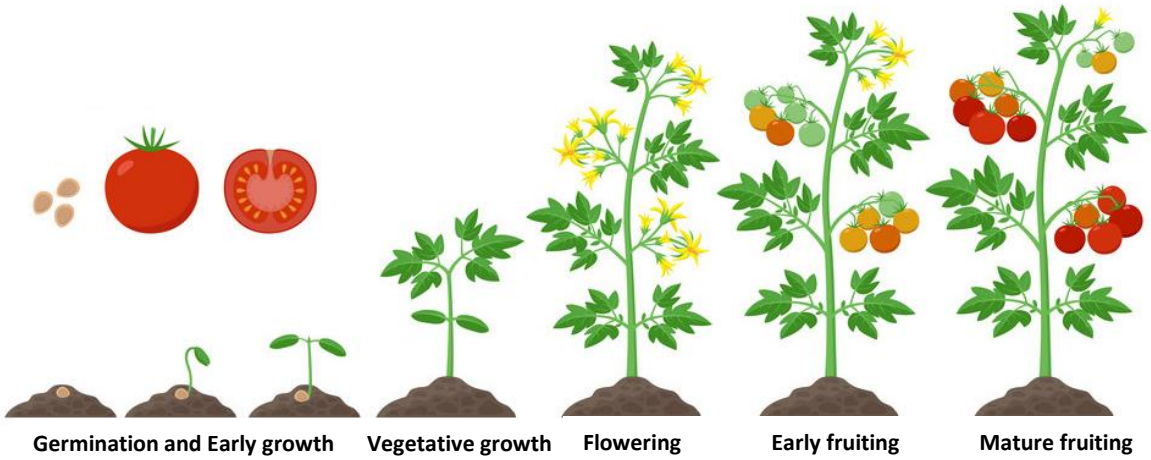

Figure 1.1: Demonstration of five growth stages of a tomato plant (adapted from www.vectorstock.com, assessed: 15/05/2020).

Vegetative growth and flowering occur when vegetative shoots terminates in a flower after development of leaves. This new shoot arises from an axillary bud just below the terminating inflorescence. The new shoot will terminate again and make new leaves.

The cycle will repeat continuously to form sympodial shoots. This action makes tomato to be determinate as shoots terminate in a flower. Fruit development has six stages that determines when they should be harvest: this starts from green, beaker, turning, pink, light red and red (Shamshiri et al., 2018).

1.3.1.3 Importance

Tomato is rich in micronutrients such as antioxidants, vitamins (A, C and B2) and minerals (K, Fe and P). The fruits are also good sources of lycopene (a pigment responsible for red color) which is an antioxidant that contributes to reduction of several cancer risks (Kimura and Sinha, 2008; Bhowmik et al., 2012). The fruits are used as salads, or cooked as vegetables, processed into tomato cans, juice, paste, puree, salsa and sauce (MOA, 2003). The plant is grown for both fresh market and processing industries as there is an increasing demand for processing (MOA, 2003).

1.3.2 Nematodes with focus on root-knot nematodes

Nematode pests adversely influence tomato production throughout the world, especially in the tropical and subtropical areas (Hussain et al., 2011; Lopes-Caitar, 2019). The rest of this chapter will therefore focus on nematodes, with special reference to root-knot nematodes (*Meloidogyne* Göldi, 1887) referring to their basic classification and identification, biology, survival, factors that influence their distribution and ultimately the management strategies that can be used (and are used to a certain extent by ZZ2) to protect tomato crops against these organisms.

1.3.2.1 Classification

Nematodes are unsegmented, bilaterally symmetrical roundworms, usually microscopic in size (Decraemer and Hunt, 2013). The taxonomic position of nematode taxa is nowadays determined using both the classical (morphology and morphometrics) and molecular analyses (phylogeny). For example, the systematic order classification of the target nematode genus focused on in this chapter, *viz*. root-knot nematodes (*Meloidogyne*), is as follows according to Decraemer and Hunt (2013):

Phylum Nematoda Potts, 1932

Class Chromadorea Inglis, 1983

Order Rhabditida Chitwood, 1933
Suborder Tylenchina Thorne, 1949
Superfamily Tylenchoidea Decraemer and Hunt, 2013
Family Hoplolaimidae Decraemer and Hunt, 2013
Subfamily Meloidogyninae Decraemer and Hunt, 2013
Genus *Meloidogyne* Göldi, 1887

These organisms are one of the most important and abundant metazoa groups on earth, found in almost all ecological systems (Ferris et al., 2001). The soil habitat, which is related to agriculture that is the focus of this study, is known to contain diverse trophic groups of nematodes, classified according to their feeding habits such as bacterivores, fungivores, omnivores, predators and plant-parasitic nematodes (PPN) also referred to as herbivores and nematode pests.

1.3.2.2 Identification of nematodes

Accurate identification of nematodes that are found in agricultural fields are of great importance to apply precise management strategies, *viz*. crop rotation, biological control, host plant resistance, plant quarantine and others (Adam et al., 2007; Blok and Powers, 2009). Application of these strategies is dependent on a good understanding of the taxonomy and biology of the targeted nematode species (Ahmed et al., 2016).

Since this dissertation focuses on root-knot nematodes (*Meloidogyne*) and its management, insight about the accurate identification of the specific species is crucial (Cunha et al., 2018). By 2020, 105 root-knot nematode species have been identified worldwide (Ghaderi and Karssen, 2020). Currently, in South Africa, 14 *Meloidogyne* spp. have been reported (Marais et al., 2017), while 23 records are known from the African continent (dos Santos et al., 2018).

Accurate identification of species is challenging mainly because they share similarities in terms of their morphological characteristics. For example, accurate identification of especially the tropical or thermophillic (preferring warmer climates; Karssen, 2002) species *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 and *Meloidogyne enterolobii* Yang and Eisenback, 1983 of South Africa have been

challenging and in some cases impossible using only morphology and morphometrics (Visagie et al., 2018; Rashidifard et al., 2019). Therefore, the latter authors showed that both morpholocial and molecular identification of these root-knot nematode species have to be done to ensure their correct identification. This is in agreement with Suresh et al. (2017) who stated that morphological characteristics and morphometrics, host preferences, biochemical and molecular techniques are essential for confirming the identify of root-knot nematode species with high precision.

1.3.2.2.1 Morphological and morphometrical (classical) identification

Characterisation of root-knot nematodes have been done previously mainly by studying the perineal patterns and eosophageal structures of females (Figures 1.2 and 1.3; Table 1.2) (Taylor and Sasser, 1978; Hunt and Handoo, 2009; Karssen et al., 2013). Also, characterisation is done by using numerous characteristics of J2 and males as described by Karssen (2002) and recently being compiled in an extensive compendium including 105 valid species (Ghaderi and Karssen, 2020). Characteristics used for such identification are listed in Table 1.2.

Table 1.2. Morphological and morphometrical characteristics used to describe life stages of root-knot nematodes and to discriminate among species.

Life stage	Morphological characteristic	Morphometrical characteristics	Figure
Female	Body form, head region, annulation, shape of stylet and basal knobs, oesophagus lumen lining (pro- and metacarpus), form of perineal pattern (overall shape, presence/absence of dorsal arch, presence of wings, development of lateral field, presence of tail-tip and anal punctuations and/or phasmids).	Length and width of body, stylet length, position of dorsal gland opening (DGO) and excretory pore, proportion of structures dorsal and ventrally positioned to vulva.	1.2 & 1.3
Male	Body shape, shape of head, form of annules, presence/absence of labial disc, shape of stylet and basal knobs, pharyngeal-gland overlap, form/shape of spicule, development of lateral field.	Position of DGO in relation to stylet knobs.	
Second stage juvenile (J2)	Body shape and length, head shape and form of annules, form/shape of stylet and stylet knobs, overlap of pharyngeal glands, form of rectum, shape of tail, form of tail tip and hyaline tail.	Length of body, DGO, stylet knob length, position of excretory pore, position of hemozonid in relation to excretory pore, length of hyaline region	

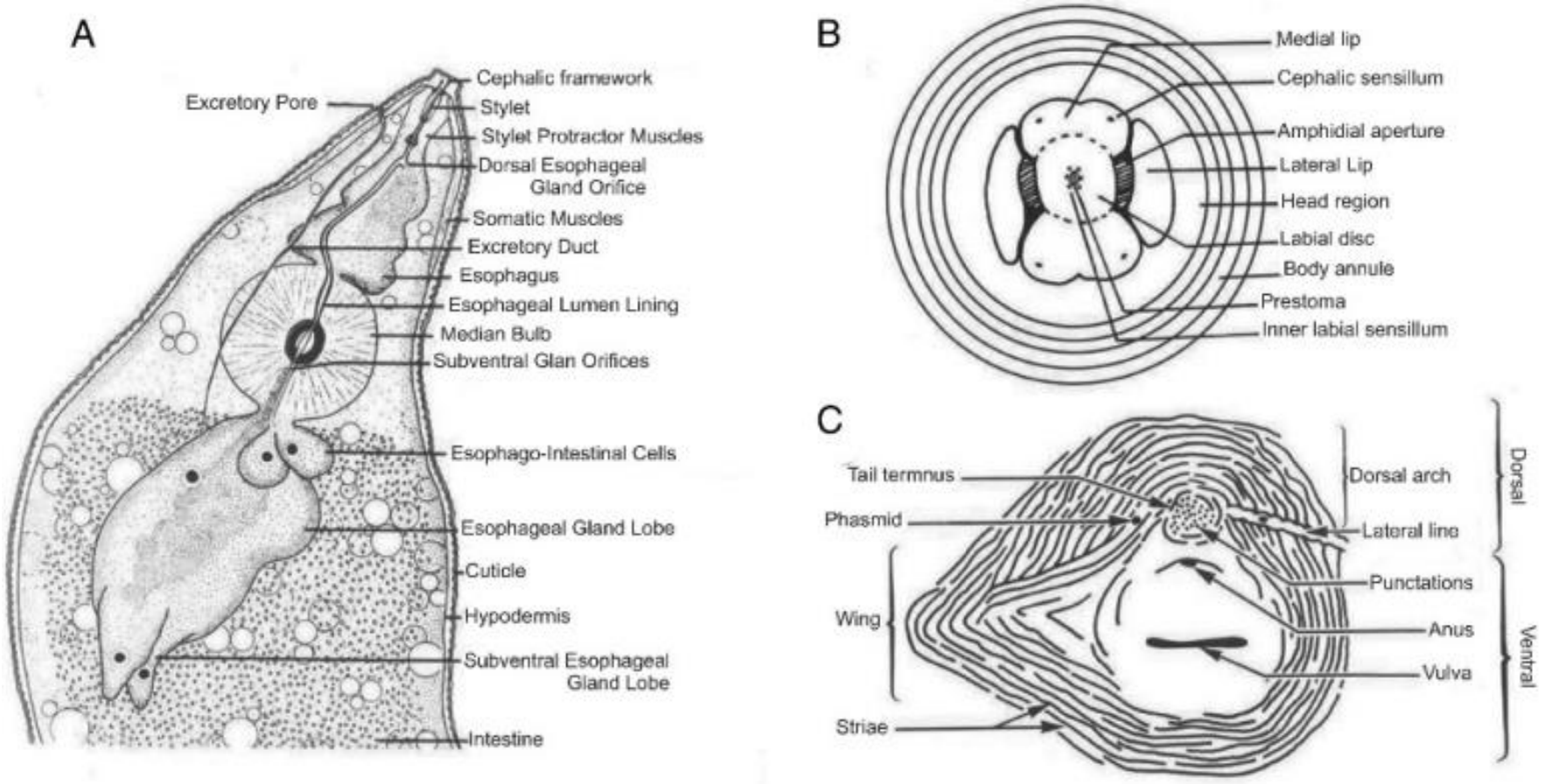

Figure 1.2: Female morphology of root-knot nematodes (*Meloidogyne* spp.). (A) Anterior region; (B) Head morphology as revealed by SEM, in face view; and (C) Perineal pattern in the posterior region of a female (Adapted from Eisenback and Triantaphyllou, 1991).

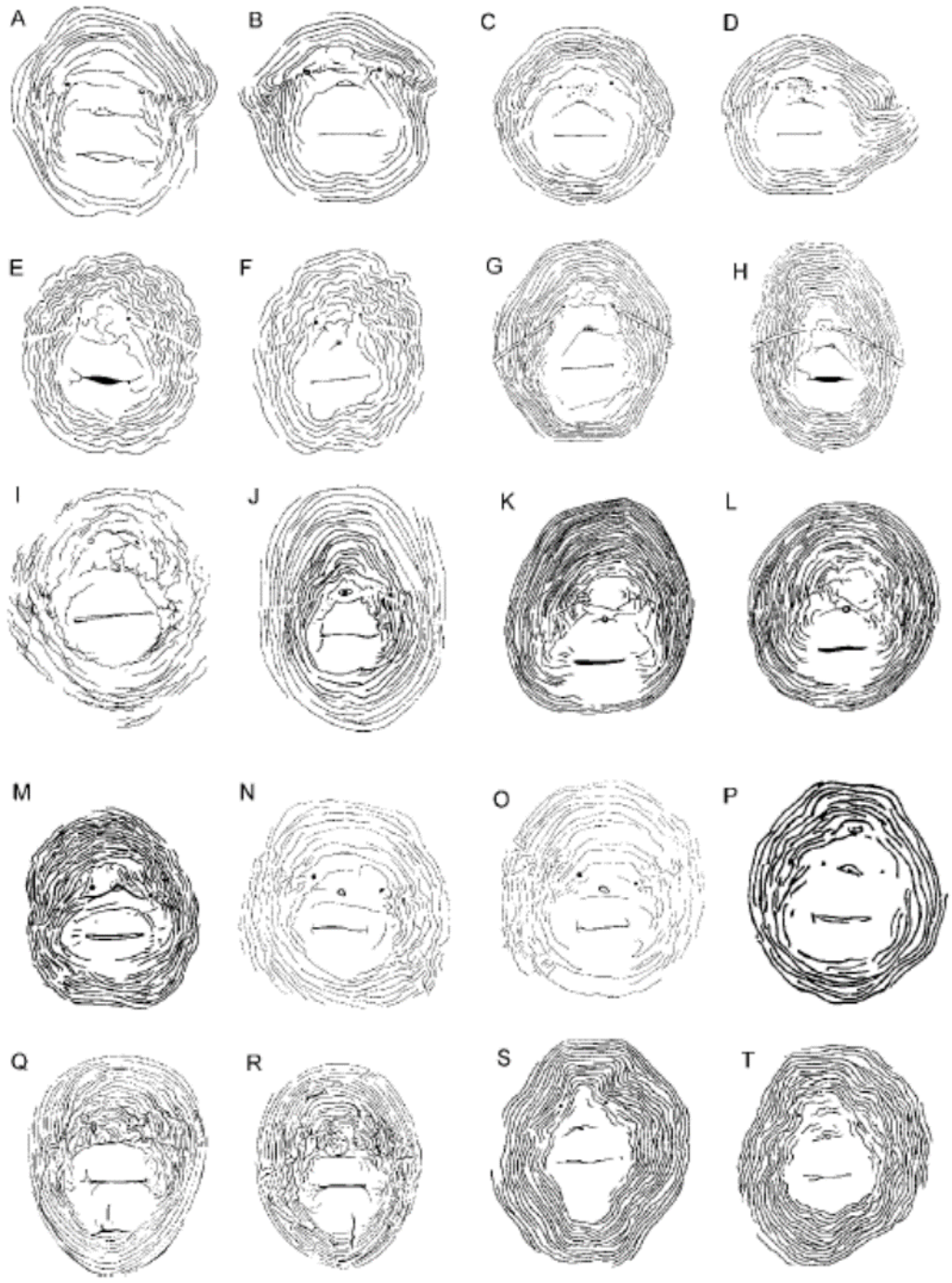

Figure 1.3: Variation of perineal patterns among some *Meloidogyne* spp. females. A, B: *M. arenaria*; C, D: *M. hapla*; E, F: *M. incognita*; G, H: *M. javanica*; I: *M. acronea*; J: *M. chitwoodi*; K, L: *M. enterolobii*; M: *M. ethiopica*; N, O: *M. exigua*; P: *M. fallax*; Q, R: *M. graminicola*; S, T: *M. paranaensis*. (Adapted from Hunt and Handoo, 2009).

These morphometric and morphological methods are not easy to do as they require a high level of expertise, are time consuming (Karssen et al., 2013) and there is a considerable overlapping of characteristics of root-knot nematode species (Brito et al., 2004; Hunt and Handoo, 2009). Challenges faced by reseachers in discriminating between *M. enterolobii* and *M. incognita*, for example, are demonstrated: the two species have slight differences regarding the female head and J2 morphology (Brito et al., 2004), while the perineal patterns of females of these two species are also similar. Ghaderi and Karssen (2020) grouped root-knot nematode species according to the tail length and the form of the tail tip of second-stage juveniles (J2) and certain characteristics of males. Radishifard et al. (2019) again listed perineal-pattern characteristics that differentiate South African females of *M. enterolobii* from other thermophilic species, *viz*. *Meloidogyne hapla* Chitwood, 1949, *M. incognita* and *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949. Although all these attempts generated insight in, and added to available knowledge on how to accurately distinguish *M. enterolobii* from other root-knot nematodes, more efficient techniques have been developed including biochemical and molecular methods (Adam et al., 2007).

1.3.2.2.2 Biochemical methods

1.3.2.2.2.1 Isozymes

Esterase patterns from 16 root-knot nematode species were first described by Esbenshade and Triantaphyllou (1985). These isozyme phenotypes have since been used to differentiate between root-knot nematode species with carboxylesterase/esterase being most effective (Blok and Powers 2009). Root-knot nematodes from sub-Saharan Africa were also successfully identified using esterase phenotypes, with the addition of the discovery of two new species, from vegetable fields in Benin, Kenya, Nigeria, Tanzania and Uganda (dos Santos et al., 2018). The cons of using this method are that only a specific gene may be used, which is expressed in mature females only (Carneiro et al., 2016). Interspecific variations that may occur in *M. javanica*, *Meloidogyne arenaria* Chitwood, 1949, *Meloidogyne exigua* Göldi, 1887 and *Meloidogyne paranaensis* Carneiro, Carneiro, Abrantes, Santos and Almeida, 1996 and that renders classical identification to be inconclusive (Carneiro et al., 2004) can hence be overcome by using the isozyme approach.

1.3.2.2.2.2 Antibodies

Adequate amounts of DNA from a nematode specimen, adults, juveniles or eggs (Blok and Powers 2009; Nega 2014), is required for a successful diagnosis of the species it represents. This method allows for the extraction of DNA from nematodes present in the soil. This is done by using the specific antibody that will recognise the surface of the targeted nematode by means of a magnetic bead coated with secondary antibodies (Chen et al., 2003; Blok and Powers 2009; Nega, 2014). The targeted nematodes can be extracted by using a immunomagnetic capturing system which has been effective for root-knot nematode species, *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 and *Xiphinema americanum* Cobb, 1913 (Chen et al., 2001; 2003).

1.3.2.2.2.3 DNA-based techniques

In 1985, Curran et al. reported the use of restriction fragment length polymorphisms (RFLPs) after DNA-based methods had been identified for root-knot nematode species earlier in the 1980s. This method has also been illustrated to, for example, give accurate results for species other than root-knot nematodes, e.g. *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936, being identified using DNA probes (Nega, 2014). Several DNA based methods, other than RFLPs, have been and are still being used to categorize root-knot nematode species. These include, satellite DNA probes, polymerase chain reaction (PCR), sequence characterised amplified regions (SCARs), random amplified polymorphic DNA (RAPDs), real-time PCR, single neocletide polymorphisms (SNPs), ribosomal DNA (rDNA), mitochondrial DNA (mtDNA) (Blok and Powers 2009) and more recently the genotyping by sequencing (GBS) approach (Rashidifard et al., 2018).

Restriction fragment length polymorphisms done through extraction and purification of genomic DNA, digestion and visualisation of banding patterns followed by gel electrophoresis enabled variation among root-knot nematode species (Blok and Powers, 2009). This method demands adequate amounts of DNA, but since the DNA bands are not always clear, PCR techniques were implemented and are used to replace this method (Blok and Powers, 2009). Satellite DNA probes and PCR are used by means of crushed nematode tissue placed in a membrane and hybridized. The

procedure is safe and stable and requires low levels of expertise (Blok and Powers, 2009).

Random amplified polymorphic DNA has been used to distinguish between intra- and interspecific relationships of root-knot nematode species. This is done by using characteristic amplified patterns from RAPD primers. The species-specific diagnostic primer is ideal for identification, however, requires high annealing temperatures (Blok and Powers, 2009). Real-time PCR make use of quantitative PCR assays, this technique is very effective as it can detect more than one species at a time and requires no post-PCR procedure (Block and Powers, 2009; Onkendi et al., 2014). Nucleic acids present can be quantified, and genotyping can be generated from high resolution curves which is only specific to certain root-knot nematode species (Blok and Power, 2009).

Previous reports have shown the accuracy of ribosomal and mitochondrial DNA PCR to distinguish between different *Meloidogyne* spp. (Onkendi and Moleleki, 2013, Onkendi et al., 2014). However, many of these primers could not discriminate among most *Meloidogyne* spp. Onkendi and Moleleki (2013) illustrated that South African *M. arenaria, M. incognita* and *M. javanica* were grouped in one clade. Furthermore, based on D2-D3 28S rDNA sequence *M. enterolobii* and *M. incognita* are grouped in the same clade, but *M. arenaria* and *M. javanica* in different clades. The COI, COII, COIII and 16S segments have been reported not to be ideal in differentiating between *M. arenaria*, *M. incognita* and *M. javanica* (Janssen et al., 2016; Rashidifard et al., 2018).

Genotyping by sequencing is another popular molecular method that has been used for genetic studies to identify and determine the genetic variation among organisms/populations of organisms and plants/plant genotypes (Elshire et al., 2011; Jarquin et al., 2014). Next generation sequencing of genomic fragments of organisms obtained from specific restriction enzymes are utilized using this approach (Jarquin et al., 2014). This technique is based on using single nucleotide polymorphisms (SNPs) situated at different loci on the genome (Mimee et al., 2015) and takes advantage of Pool-Seq (sequencing of composite samples), which removes the fastidious step of isolating and extracting DNA from single nematode juveniles (Rashidifard et al., 2018). Until 2018, when Rashidifard et al. used this method to characterize and discriminate

between South African root-knot nematode species *M. enterolobii*, *M. javanica* and *M. incognita*) only potato cyst nematode *Globodera rostochiensis* populations have been discriminated using this approach (Mimee et al., 2015).

A DNA-based method that needs to be accentuated is the sequence characterised amplified regions (SCAR) – polymerase chain reaction (PCR); another well-known and precise technique to distinguish among root-knot nematode species for which species-specific markers have been developed (Zijlstra, 2000; Blok and Powers, 2009; Long et al., 2006). This method is very useful in identifying species with low genetic differences according to DNA fragments length. Specific primers are deduced from sequences of specie-specific RAPD markers, for example RKN species *viz*. *M. arenaria*, *Meloidogyne chitwoodi* Golden, O'Bannon, Santo and Finley, 1980, *M. enterolobii*, *Meloidogyne fallax* Karssen 1996, *M. hapla*, *M. incognita* and *M. javanica* (Blok and Powers, 2009; Ahmed et al., 2016). This method's specificity and sensitivity enable identification of more than one *Meloidogyne* spp. by means of a single reaction, however, interference between primers can occur (Blok and Powers, 2009). Species-specific primers for *M. incognita*, *M. enterolobii* and *M. javanica* were effectively identified by multiplex PCR and DNA extract in South China (Hu et al., 2011). Furthermore, in South Africa, the same technique has been used with success to identify *M. arenaria*, *M. chitwoodi*, *M. enterolobii*, *M. fallax*, *M. hapla*, *M. incognita* and *M. javanica* occurring in agricultural fields (Fourie et al., 2001; Onkendi and Moleleki, 2013; Ntidi, 2016; Rashidifard et al., 2018; Visagie et al., 2018). This technique has been used for identification of root-knot nematode during this study and is elaborated on in Chapter 3.

1.3.2.3 Reproduction and life cycle

Root-knot nematode complete their life cycle through three different reproduction strategies. These includes:

i) obligatory cross-fertilization (amphimixis), this occurs in some diploid and polyploid nematodes; both male and female genetic material are fused to create a new genetic pool, e.g. *Meloidogyne kikuyensis* De Grisse, 1961 (Castagnone-Sereno, 2006; Moens et al., 2009; Karssen et al., 2013);

ii) facultative meiotic (automixis) parthenogenesis occurring in most polyploid nematodes. In the presence of males, mating amphimixis occurs, however,

in their absences female eggs undergo chromosome reduction through meiosis, e.g. *M. chitwoodi* and *M. hapla* (Castagnone-Sereno, 2006; Moens et al., 2009); and

iii) obligatory mitotic parthenogenesis (apomixis) occurring in most polyploid species. The egg develops directly from the embryo, with no amphimixis or automixis occurring, e.g. *M. incognita*, *M. enterolobii*, *M. javanica* (Castagnone-Sereno, 2006; Moens et al., 2009; Karssen et al., 2013).

The life cycle of root-knot nematode species is characterized by four developmental stages (Figure 1.4). The cycle begins with a zygote that is produced in an egg by a female. The egg then develops into a first-stage juvenile (J1) that moults within the egg. The vermiform J1 further develops into an infective J2, that will hatch in the soil and/or roots (or other below-ground parts) of the host. The J2 is mainly dependent on temperature and soil moisture to hatch, while root diffusates are known to also impact on J2 hatching of some species, to enable movement and host location (Moens et al., 2009; Jones et al., 2013b; Perry et al., 2013). This infective stage feeds on the tissues of the host plant by using its stylet and enzymes to break cell walls and enter host roots, rhizomes, pods and/or other below-ground parts (Bartlem et al., 2013; Karssen et al., 2013).

Under favourable conditions, an infective and motile J2 will establish a feeding site and will moult into immotile J2; then to a third-stage juvenile (J3) and subsequently to a fourth-stage juvenile (J4), which will eventually develop into the adult stages (either a swollen, immotile female or a vermiform male). The J3, J4 and males do not feed, with males only being present when conditions become unfavourable for female development (due to restricted food, space, unsuitable/resistant host plant genotypes (Moens et al., 2009; Karssen et al., 2013). A mature female can lay >1000 eggs into the gelatinous matrix made up of glycoprotein and produced by the rectal glands. The gelatin matrix keeps eggs together and shields them from harsh environmental conditions and has antimicrobial properties. The egg masses of root-knot nematodes are generally observed on the surface of galled, below-ground parts of host plants; however, they can also be embedded in the gall tissues (Moens et al., 2009; Jones et al., 2013b).

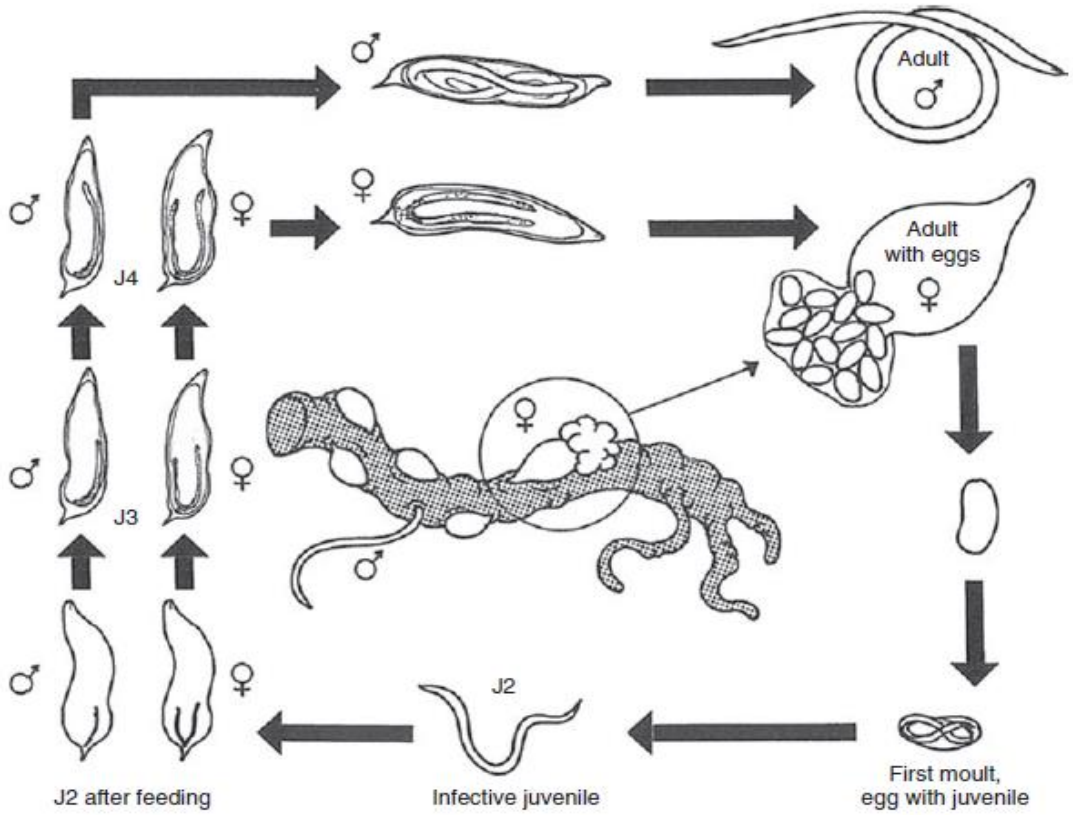

Figure 1.4: A schematic presentation of the life cycle of root-knot nematodes, *Meloidogyne* spp. (J2: second-stage juvenile; J3: third-stage juvenile; J4: fourth-stage juvenile) (Adapted from Karssen et al., 2013).

1.3.2.4 Spatial distribution and factors influencing nematode biology and survival

Physical and chemical soil characteristics are important determinates of soil microbial communities (Quist et al., 2019). Soil texture determines the type of microbes that can live in it, for example, fined-textures soils are known to contain more microbes and smaller nematode taxa than coarse textured soil (Sechi et al., 2018). Furthermore, species communities are influenced by habitat heterogeneity, food sources, nutritional inputs and the environment (eg. agricultural practices) (Villenave et al., 2013). These physio-chemical soil characteristics influence the spatial distribution of soil biota, thus, microbial patchiness is observed (Ettema and Wardle, 2002).

There are several factors that influence nematode patchiness in agricultural fields, for example, increased organic matter was reported to have correlated with higher diverse

group of energy rich, carbon-based compounds that stimulates primary decomposer nematode communities belonging to the coloniser persister (cp) groups 1-2. These are known to be opportunistic and have a short life cycle (Bongers, 1990; Quist et al., 2019). Soil preparation such as tillage, reported by Palomares-Rius et al. (2015) and Garcia et al. (2018) showed to result in increased *Pratylenchus* spp. numbers. Fungicides used by Garcia et al. (2018) densities showed to have a positive impact on *Pratylenchus* spp. and *Paratylecnhus* spp. These fungicides are known to sometimes decrease PPN densities (Van der Putten and Van der Stoel, 1998) or increase their population densities by stimulating hatching of juveniles from eggs (Rodriguez-Kabana and Curl, 1980) or by removing natural enemies of PPN, thus increasing their numbers (Garcia et al., 2018).

Stochastic processes driven by passive dispersal and ecological drift contribute to the spatial distribution of nematodes. Furthermore, functional equivalence between nematodes, from related species competing for space and food may lead species to sort themselves and promote patchiness (Zhou and Ning, 2017). Additionally, alternative hosts such as weeds can host root-knot nematodes during seasons when no crops are grown (Ntidi et al., 2012).

Taylor and Brown (1976) reported that climate, soil and plants are the three mechanisms of the environment that regulate the presence and dispersal of terrestrial nematodes. Environmental factors including extreme temperatures, relative humidity, soil moisture content, soil texture, and others impact on the distribution and survival of nematodes (McSorley, 2003). Temperature is an important environmental factor that influences the ability of nematode pests to penetrate and develop within a host (Jones et al., 2017b). Numerous studies recorded the influence of temperature on the biology, physiology and survival of root-knot nematodes associated with tomato crops (Khan et al., 2014; Espinoza-lozano, 2017). Plant growth, life cycle of root-knot nematode and the interaction among resistant plants and nematode populations are directly influenced by temperature. *Meloidogyne incognita*, for example, can complete its life cycle from 17 to 57 days depending on temperature, furthermore, it been observed to survive and reproduce in temperature ranging from 15.4 to 35 °C (Dropkin, 1963). However, depending on *Meloidogyne* spp. and plant host, these values may vary (Wong and Mai, 1973).

Tomato plants can be physiologically affected by high temperatures and heat stress. Camejo et al. (2005) illustrated that temperatures above 28 °C can affect normal development of plant caused by heat stress, thus interfering with physiological process such as photosynthesis, nutrient uptake and fruit setting. The stress created predisposed the plant to nematode attack (Haroon et al., 1993; Ornat and Sorribas, 2008).

An important initiative that is linked to the occurrence of higher temperatures is the use of protected cultivation of crops that has increased (Ramasamy and Ravishankar, 2018). The aim of protected cultivation is protecting the plants from pests, diseases (airborne), frost and hail while increasing harversting periods and obtaining improved quality and quantity fruit. These structures include net houses, shade nets, greenhouses and plastic tunnels (Sharma and Singh, 2009). However, the use of these structures brings forth increased pest and disease pressure, including infection by PPN (Sabir and Walia, 2017). The high temperatures prevailing in these structures favours the build-up of PPN once introduced into the fields especially after monocropping (Desaeger and Csinos, 2006). Sharma and Singh (2009) reported that the distribution of *M*. *incognita* can spread from 10 to 60% in vegetable production areas due to monoculture in polyhouse structures.

Tomato production is affected by relative humidity, especially under protected cultivation in hot and arid areas. Inadequate ventilation inside the structures during summer can lead to increase in air temperature and thus thus induces heat stress in plants (Harel et al., 2014). The more stressed the plant is, the more vulnerable it is to root-knot nematode damage (Ornat and Sorribas, 2008).

Soil moisture content directly influences the survival of root-knot nematodes, this was observed in Senegal where no root-knot nematodes were found in top 20 cm soil during dry seasons, with only 0.9% of the total population found in layers where moisture remained (Hallmann and Meressa, 2018). *Meloidogyne javanica* and *M. incognita* were observed by Asif et al. (2015) in tomato fields to be more abundant in layers where the soil moisture percentage ranged from 4.40 to 18.45%.

Soil texture, a mixture of solids *viz*. sand, silt and clay, provide information regarding aeration, porosity, soil moisture and soil compaction (Quist et al., 2019). These characteristics are also important in nematode survival and distribution (Moore and Lawrence, 2013). Sandy soils noted by Dropkin (1963) are favourable to larger populations of nematodes because they provide satisfactory aeration. *Meloidogyne incognita* J2 movement in sandy soil were found to be higher than in other soil textures where carrot was grown (Kim et al., 2017).

Soil tillage is practiced by many farmers as a means to prepare land for cultivation, however, in many cases it is said to have an influence on nematode populations. Fu et al. (2000) mentioned tillage to have a direct impact on nematode trophic levels due to the disturbance. Reported by Palomares-Rius et al. (2015) and Garcia et al. (2018) tillage increased *Pratylenchus* spp. numbers.

1.3.2.5 Survival strategies

Nematodes have superior survival strategies to overcome adverse conditions or factors impacting negatively on their biology and physiology. According to Wharton (2004) the ability of nematodes to survive in adverse conditions is a behavioural trait, inherent to an organism, that is activated when it encounters biological and physical constraints. Throughout the life cycle of root-knot nematodes, for example, there are various strategies they use when exposed to adverse environmental conditions and/or encountering adverse host responses. To prolong the survival of unhatched juveniles in the soil, root-knot nematodes use dormancy, quiescence and diapause. During quiescence, they use one of the following mechanisms to survive, namely cryptobiosis, thermobioisis, anoxybiosis, osmobiosis or desiccation (Perry, 2011; Perry et al., 2013).

In terms of root-knot nematodes in particular each of the life stages (eggs, four juvenile stages and adults) has an adaptive mechanism. The eggs have a mucoid protein mass that shields them from water loss and predators. In addition, utmost temperatures harden the glycoprotein that aids in mechanical pressure and prevent J2 from hatching in drought conditions (Hussain et al., 2015). The females are embedded in the tissues of below-ground plant parts and in this way are protected against predators, other pathogenic organisms and adverse climatic conditions (Karssen et al., 2013; Miyashita et al., 2014). Orion and Kritzman (2001) furthermore discovered that the gelatinous

matrix surrounding the eggs of *M. javanica*, protects it from being attacked by soil micro-organisms.

1.3.2.6 Root-knot nematode species: distribution, those associated with tomato, damage potential and pathogenicity

Root-knot nematodes are found in most temperate and tropical areas, but are also prevalent in cold areas, and are amongst the most destructive plant pathogens worldwide (Onkendi et al., 2014; Fourie et al., 2017; Ghaderi and Karssen, 2020). Root-knot nematodes have a wide host range, which includes most of the agronomic crops and weeds belonging to many plant families (Ntidi, 2016; Visagie et al., 2018).

Root-knot nematodes are generally the economically most important genus that parasitize tomato on a worldwide basis, with *M. incognita*, *M. javanica, M. arenaria* and *M. hapla* being known as the most common and pathogenic species (Villar-Luna et al., 2016). According to Jones et al. (2017b), root-knot nematode species found to cause damage to tomato in South Africa are *M. arenaria*, *M. chitwoodi*, *M. fallax*, *M. incognita*, *M. javanica, M. hapla*, *M. enterolobii* and *Meloidogyne hispanica* Hirschmann, 1986.

Another economically important nematode genus that causes damage to tomato is *Pratylenchus* Filipjev, 1936 or lesion nematodes, with *Pratylenchus penetrans* (Cobb, 1917) Filipjev and Schuurmans Stekhoven, 1941, *Pratylenchus brachyrurus* (Godfrey, 1929), Filipjev and Schuurmans Stekhoven, 1941, and *Pratylenchus coffeae* (Zimmermann 1898) Filipjev and Schuurmans Stekhoven, 1941 being the most damaging species worldwide (Blancard, 2012). In South Africa, *P. brachyrurus* has been identified as most damaging and predominant in potato (Jones et al., 2017b).

A wide range of nematode genera, other than root-knot and lesion nematodes, are also known to parasitize tomato globally (Bernard et al., 2017) and in South Africa, but their damage is usually confined to specific areas, and specific crops.

1.3.2.6.1 Above-ground symptoms

Aerial symptoms of root-knot nematode parasitism to crops, such as tomato (the target crop of this study), are often mistaken with those caused by biotic and abiotic stress factors such as drought and nutrient deficiency (Mashela et al., 2017). However, patches within a crop field showing poor plant growth due to wilting, stunting or yellowing of leaves usually is an indication of damage caused by nematode pests (Jones et al., 2017b). Small sized fruits, chlorosis or other abnormal coloration of foliage, failure to respond normally to fertilizers, small or sparse foliage, a tendency to wilt more readily than healthy plants (even in the presence of enough soil moisture) are frequently observed in affected tomato crops, especially when grown in root-knot nematode infested fields season after season as is the case with ZZ2 farmers, Limpopo Province, South Africa (Figure 1.5) (Jones et al., 2017b; Hallmann and Meressa, 2018).

Figure 1.5: Above-ground damage symptoms observed from root-knot nematode damage to tomato showing poor growth in a net house in the Mooketsi area, Limpopo Province, South Africa (Photo: Lesego Matlala, ZZ2-boedery).

1.3.2.6.2 Below-ground symptoms

Root-knot nematode damage symptoms are very distinctive because of the galls or swellings produced on roots and underground portions of stems on the lateral feeding

roots of tomato plants (Figure 1.6). The degree of root galling generally depends on the nematode population density and host plant species (Hallmann and Meressa, 2018).

Figure 1.6: Below-ground damage symptoms observed on tomato roots infected with root-knot nematodes from a tomato net house in the Mooketsi area, Limpopo Province, South Africa (Photo: Wiam Haddad, ZZ2-boerdery).

1.3.2.7 Interaction with other soil-borne organisms

Disease complexes that are formed due to root-knot nematode interaction with various pathogens are mainly evident in the tropical and subtropical areas (Manzanilla-Lopez and Starr, 2009; Karssen et al., 2013; Kumar et al., 2017). Diverse interactions occur between bacterial, fungal and viral organisms that occur or share habitats with nematodes in soil substrates (Manzanilla-Lopez and Starr, 2009). Rate of development, incidence and severity of wilt on *Fusarium oxysporum* susceptible and tolerant crop cultivars are, for example, increased in the presence of root-knot nematodes (Agrios, 2005; Kumar et al., 2017). Saikia and Bhagawati (2014) reported severe yield loss in tomato and okra (*Abelmoschus esculentus* (L.) Moench) that was caused by an *M. incognita* and *Rhizoctonia solani* disease complex. Another disease

complex caused by *Meloidogyne* spp. in tomato include *Ralstonia solanacearum* causing bacterial wilt (Hallmann and Meressa, 2018).

1.3.3 Management

The main objective of managing root-knot nematodes through plant protection initiatives is to protect the plant from secondary infections (Onkendi et al., 2014). Thus, a nematode management strategy is important to keep population densities of the target nematode pest below non-injurious and sub-economic threshold levels (Njoroge, 2014). Under intensive and continuous monocropping systems, management practices are highly dependent on nematode pest population densities and cost-effective strategies (Jones, 2017a). There are two general approaches used to control nematode pests, including chemical and non-chemical control. The restriction of nematicides and fumigants has given rise to alternative nematode control with increased focus on biological-based techniques (Hallmann et al., 2009), soil amendments, crop rotation and intercropping, cover crops as biofumigants, and local botanical nematicides as control agents of root-knot nematodes (Khosa, 2013; Fourie et al., 2016; Daneel et al., 2017; Mashela et al., 2017).

1.3.3.1 Chemical control

Chemical control is still the main and most effective control strategy to protect crops against root-knot nematode infection and it is practiced by most vegetable producers (Nyczepir and Thomas, 2009). The use of nematicides for this purpose constitutes 48% of the global nematicide market (Hallmann and Meressa, 2018) representing either fumigants (in liquid form) or non-fumigants (liquid or granular composites). Frequently used fumigants include 1,3-dichloropropene, while non-fumigants are represented by products containing carbamates and organophosphates as the active substances (Van Zyl, 2016). When applied according to the prescribed dosages and time of application set out in the labels, these products are generally highly effective in reducing nematode pest densities (Karssen et al., 2013). However, many of these chemicals (e.g. methyl bromide and aldicarb) have been withdrawn from the market due to detrimental effects on the environment and toxic effects on humans and animals (Jones, 2017a; Hallmann and Meressa, 2018).

1.3.3.2 Non-chemical control

1.3.3.2.1 Prevention

Prevention is better than cure and is a vital procedure to be implemented by ensuring that planting materials such as roots, tubers and bulbs, and even aerial parts of plants (applicable to nematodes infecting leaves, stems, seeds) are free of nematode pests. Human activities should be monitored to avoid dissemination of nematode inoculum by transportation of infested soil and plant debris to prevent that nematodes are transferred from infested to non-infested sites, with irrigation water, for example (from rivers and/or dams) also being a potential source of such contamination (Collange et al., 2011; Seid et al., 2015; Knoetze et al., 2017). Nematode-infected planting material is often cleaned by dipping it into hot water (temperature depends on the crop) or chemicals as a precaution to kill/inactivate any inoculum to prevent re-infestation into sterile or non-infested soil. In various parts of the world dormant grapevine rootstocks, for example, were immersed in water at 50 ˚C for 15 min to be free of *M. javanica* (Oka, 2010; Karssen et al., 2013; Storey et al., 2017). Cultivation of certified seed or planting material from trusted nurseries is essential since the growth media is expected to be free of nematode pests and other diseases (Seid et al., 2015, Pretorius and Le Roux, 2017; Storey et al., 2017). Irrigation water might be cleaned with chlorination, filtration and/or ozonation to purify it and farm equipment should be cleaned when moving it from one site to another to avoid contaminating of non-infested soil with nematodes from an infested field site (Hugo and Malan, 2010; Seid et al., 2015).

1.3.3.2.2 Biological control

A broad spectrum of biological control agents have been widely tested for nematode control including arbuscular mycorrhizae, rhizobacteria, fungal agents (including endophytes) that target nematode eggs and motile stages (Hallmann et al., 2009; Stirling, 2014).

In South Africa, biological control agents, for example, bacteria belonging to *Bacillus* and *Burkholderia*, and fungi such as *Trichoderma* and *Purpureocillium lilacinum* have been introduced for use on several crops including sugarcane (*Saccharum officinarum* L., banana (*Musa* L.), grapevine (*Vitis vinifera* L.) and tobacco (*Nicotiana tabacum* L.)

with varying degrees of success (Berry et al., 2017, Daneel and De Waele, 2017; Storey et al., 2017; Van Biljon, 2017). Plant growth-promoting rhizobacteria (PGPR) strains work by colonizing roots and rhizosphere of plants by producing metabolic by-products that adversely affect nematode motility and penetration. These strains i.e. *Bacillus aryabhattai* (A08) and *Paenibacillus alvei* (T30) were the most effective strains used to reduce gall numbers and eggs in tomato and carrot roots to control *M. incognita* (Viljoen et al., 2019).

1.3.2.2.3 Cultural and physical methods

These methods include, amongst others, cover crops, crop rotation (coupled with the use of poor- or non-host genotypes), intercropping, application of organic amendments and solarisation (Collange et al., 2011) and are commonly used worldwide by both commercial and small-scale farmers to reduce population densities of PPN.

Crop rotation is amongst the oldest technique used for environmental benefits, such as i) increasing biodiversity which will alter the microbial, soil community structure and activity, ii) reduce greenhouse gas emissions with the inclusion of legume-based sequences that will reduce nitrogen fertilisers and thus emissions of nitrous oxide, iii) reduce water pollution due to diversifying crop rotation which will limit the use of fertilisers and pesticides (Reddy, 2017) and iv) ultimately to increase crop yield. Effective use of this method has had positive results in reducing nematode populations when correctly applied with the use of resistant, poor- or non-host cultivars being non-debatable (Fourie and De Waele, 2020). For example, crops that are host/susceptible should be used in rotation with non-host/resistant to reduce the build-up of soil population densitites of the target nematode pest and allow the host crop to grow optimally and give satisfactory/expected yields (Talavera et al., 2009; Hallmann and Meressa, 2018). In South Africa, tomato farmers applying monoculture face various challenges of which nematode pests is a major constraint; especially in net houses where fields are not allowed to ‘rest’. However, rotation of susceptible and resistant cultivars could be implemented as shown from greenhouse and microplot trials done by Fourie et al. (2012). These results indicated resistant tomato ‘Rhapsody’ significantly reduced population densities of *M. incognita* race 2 when compared to susceptible ‘Moneymaker’. Results from Talavera et al. (2009) in Spain also showed

rotation of *Mi* resistant tomato ‘Monika’ with susceptible ‘Durinta’ over three years under unheated plastic house conditions reduced field populations of *M. javanica* and *M. arenaria* and *M. incognita* by 90%.

Alternating between crops was also found by Dhillon et al. (2019) to supress root-knot nematode population densities in vegetable crops. In okra, tomato and cucumber (*Cucumis sativus* L.) plantings, population densities of root-knot nematode were significantly reduced by rotating them with garlic (*Allium sativum* L.) (Dhillon et al., 2019). Hallmann and Meressa (2018) also noted rotation of tomato with sesame (*Sesamum indicum* L.) reduced root-knot nematode densities up to 75% as compared to when rotation was done with sweet potato (*Ipomoea batatas* (L.) Lam.). However, it is worth noting that some crops may be effective in reducing the population densities of the target nematode in one area/net house and not in another due to the occurrence of the aggressiveness of different populations of the target nematode species; thus looking into other alternative control is important (Hallman and Meressa, 2018).

Since the management of root-knot nematodes by ZZ2 farmers is based on the use of organic amendments (compost), botanical nematicides and mulching, focus will mainly be on these tools that are used worldwide to protect infected crops.

Soil organic amendments, another common traditional practices used to improve soil fertility and structure, has over the years proven to be successful in controlling several soilborne diseases, including PPN. Frequently used amendments include oil cakes, animal and urban wastes, agro-industrial wastes and plant residues (Oka, 2010; Sikora and Roberts, 2018). Compost has also been used in various studies across the globe as a measure for reducing root-knot nematode population densites. *Meloidogyne javanica* numbers in tomato roots were, for example, suppressed by using cattle manure and grape marc compost in Israel, with both compost and compost water extracts showing nematicidal effects (Oka and Yemiyahu, 2003). Furthermore, Raviv et al. (2005) illustrated a reduction in *M. javanica* population densities in tomato by using orange peel-separated cow manure (OP-SCM) and wheat straw-separated cow manure (WS-SCM) in Israel. Other composts prepared from poultry, sheep, cattle and horse manure were reported by Kerkeni et al. (2011) to suppress populations of *M. incognita* on tomato by increasing the population densities of predatory nematodes.

In South Africa, animal manures have also been used to reduce population densities of root-knot nematodes in tomato fields of developing farmers. In tomato, rhizospheres population densities of root-knot nematodes were reduced by 52% in small plots ammended with chicken manure (Fourie et al., 2012).

The effect of soil amendments derived from plants is another strategy exploited to reduce nematode pest numbers and is used by numerous farmers worldwide (Ntalli et al., 2020). The efficacy of this approach has been shown by various authors to be effective in reducing nematode population densities (Oka, 2010; Renco, 2013; Daneel et al., 2018; Dutta et al., 2019). Mashela et al. (2010) applied plant-based amendments in tomato sites in South Africa. Incorporated dried leaves of *Lippia javanica* L. into the soil before planting suppressed *M. incognita* densities over four seasons in two years. Khosa (2013) showed effective results from the use of *Cissus cactiformis* Gilg, *Tabernaemontana elegans* Stapf and *Maerua angolensis* DC. to suppress *M. incognita* race 2 numbers in tomato rhizospheres.

Other plant-based products used to combat nematode problems include the use of plant extracts. A range of extracts dervied from various plant parts such as *Myrtus communis* L. *Capsicum frutescens* L., *Hyoscyamus niger*, *Melia azedarach* L., *Xanthium strumarium* L. and *Achillea wilhelmsii* Koch have been reported with varying levels of effectivity in reducing root-knot nematode population densities (Oka et al., 2012; Kepenekci et al., 2016). Related to South Africa, Daneel et al. (2014) have used the fermented plant extract, Nemalan, from *Lantana camara* L. shoots and wild garlic (*Allium ursinum* L.) on tomato to reduce population densities of root-knot nematodes. In another study, *M. javanica* population densities were also reduced by using fermented extracts of different *L. camara* plant parts in tomato under net house conditions (Malahlela et al., 2019).

Effective micro-organisms (EM), isolated from soil are a mixture of micro-organisms, which include photosynthetic bacteria, lactic acid bacteria, yeasts, actinomycetes and fermenting fungi *viz*. *Aspergillus* and *Penicillium* (Higa and Parr, 1994). They are known to increase plant growth, yields and the photosynthesis rate (Olle and Williams, 2013). It is commonly used in aerobic and anaerobic respiration to produce disease-inducing, disease-suppressing, zymogenic and synthetic soils. Sangakara (2012)

demonstrated the benficial use of EM when it was added to compost made from rice and sawdust waste material “Bokashi”, and results were attributed to increased release of nutrients. The densities of PPN and non-plant parasitic nematodes decreased in a maize field by 43.21% and 29.32%, respectively, after application of EM (Hu and Qi, 2010).

Another technique includes the use of cover crops which involves transplanting plants, or seeds that are poor- or non-hosts to target nematodes. These plants are used, except for their cover-crop qualities, as a natural biofumigant to control soilborne pests and diseases (Fourie et al., 2016). One of the commonly used cover-crop and biofumigant plants are *Brassica* spp. that contain compounds like glucosinolates; the active substances that enable biofumigation (Youssef, 2015). In fields experiments, *M. javanica* and *M. incognita* densities in tomato rhizospheres were reduced after biofumigation with Brassicaceae including Indian mustard (*Brassica juncea* (L.) Czern.), rocket (*Eruca sativa* (Miller) Tell.), radish (*Raphanus sativus* L.) in tomato (Daneel et al., 2018). In Morocco under protected cultivation, aerial parts of mature marigold (*Tagetes patula* L.) plants were incoporated into soil beds to successfully control *M. incognita* prior the transplant of tomato seedlings (Hallmann and Meressa, 2018).

Another cultural method frequently practiced is bare fallowing, this method is effective to reduce population densities of root-knot nematodes as they require a host plant to survive (Netscher and Sikora, 1990; Hallmann and Meressa, 2018). Fallow has been used with success by farmers to reduce the numbers of root-knot nematodes in various parts of the world (Jones, 2017a). However, fallowing is not practical in most farms due to the availability of restricted agricultural fields, needed to be cultivated with crops to meet the demand to produce food and ensure an income. The need of frequent herbicide application to kill any weeds (that could act as hosts for nematode pests; Ntidi et al., 2012) re-emerging is another negative aspect associated with fallowing.

Soil solarisation, flooding, soil tillage, intercropping, quarantine and various others, are also representative of physical methods used for nematode management, but will not

be discussed in this chapter. Extensive information on the use, effectivity and benefits of these methods have been summarized by Hallmann and Meressa (2018).

1.4 References

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Chapter 2

Nematode population dynamics in tomato net houses over a three-year period, with focus on root-knot nematodes

Abstract

Continued monocropping practiced by commercial farmers in protected, net house structures provides ideal environmental conditions for crop growth, resulting in prolonged harvesting periods and good quality fruits. However, pests and diseases become difficult to manage under such optimal microclimatic conditions that make tomato crops vulnerable to attack by, for example, plant-parasitic nematodes. Tomato grown in six net houses at ZZ2 farms located in the Limpopo Province, located in three different climatic regions, were suveyed for three consecutive years to investigate the population dynamics of plant-parasitic nematodes. Data were subjected to ANOVA using Statistica 10 and Multivariate analyses (ADE-4). Nematode densities in soil fluctuated between the years, however, *Meloidogyne* spp. numbers significantly increased, irrespective of location or climatic conditons, from the first year to the third year for all net houses. Net houses also differed significantly among each other concerning *Meloidogyne* spp. densities recorded in tomato rhizospheres. Chemical soil analysis indicated that net houses with high potassium, calcium, pH and acid saturation levels had lower *Meloidogyne* spp. numbers, while those with higher Mg:K and Na:K ratios were associated with higher *Meloidogyne* spp. densities. *Meloidogyne* spp. numbers were higher in sandy soils. Planting time, however, had less influence on *Meloidogyne* spp. numbers, while cultivation of rootstocks RS2, RS3 and RS13 resulted in lower *Meloidogyne* spp. numbers compared to RS11 and scion ZZX132. Another observation was that when *Helicotylenchus* spp. was present, *Meloidogyne* spp. numbers were lower in tomato rhizospheres.

Keywords: Climate, monocropping, protected structures, root-knot nematodes, spiral nematodes.

2.1 Introduction

Crop health is influenced by a range of abiotic and biotic factors, including the composition of soil organisms, such as plant-parasitic nematode (PPN) populations and the interactions between genera and/or species (Brinkman et al., 2005; Vandegehuchte et al., 2010). The composition of PPN communities, presence of a plant host and the biophysical factors of environments mainly dictate the type and nature of the interactions between PPN (Brinkman et al., 2005). By manipulating one or several factors, this could provide a way of changing the balance amongst PPN genera/species constituting a community. For instance, Cadet et al. (2002) and Chavez et al. (2014) have seen that *Meloidogyne* Göldi, 1889 (root-knot nematode) and *Pratylenchus* Filipjev, 1936 (lesion nematode) are mutually exclusive in a host plant within the same rhizosphere.

The life cycle of PPN is amongst others dependent on climate, soil type and texture and the host plant it infects; hence PPN communities are influenced by habitat heterogeneity and changes in their food sources which are effected by agricultural practices such as crop rotation (Adediran et al., 2005; Colagiero and Ciancio, 2011) According to Palomares-Rius et al. (2015), PPN community structures in an olive orchard in Spain were predominantly defined by olive tree varieties and soil texture.

In South Africa, as in other parts of the world, farmers are opting to use net house structures for vegetable production as they can optimise their resources and increase crop productivity (De Visser and Dijkxhoorn, 2012). Tomato (*Solanum lycopersium* L.) is one of the popular vegetables grown in net houses. Prolonged harvesting periods with increased fruit quantity and improved fruit quality have been experienced by farmers (Sharma et al., 2009). However, the net house structures also provide a microclimate that promotes nematode reproduction providing optimal and favourable temperature and relative humidity ranges (Ramasamy and Ravishankar, 2018). Drip irrigation in net houses also provides the plant root system with adequate amounts of water on a continuous basis, which favours nematode infection (Sabir and Walia, 2017).

Adding to the favourable environment that nematode pests experienced in net houses is continuous monocropping of a susceptible host crop that provides the uninterrupted

availability of food for these organisms. Net house cultivation of tomato provides a longer growth period relative to open field cultivation which can lead to nematode population build-ups (Sabir and Walia, 2017). Additionally, net house production of tomato crops includes a resting period of only three to four months between growing seasons which is very short to reduce infestation (Cadet et al., 2018). Therefore, PPN are especially problematic under protected cultivation compared to open cultivation (Anupam, 2017).

Thus, the objectives of this study were to determine the effect of climate and soil physical and chemical characteristics over a three-year period on i) the development of root-knot nematode population (RKN) densities in ZZ2 tomato net houses as well as ii) the effect that these factors had on plant growth and yield.

2.2 Material and Methods

2.2.1 Characteristics of study areas

A net house structure covers an area of 5 ha, is sub-divided into 8 blocks of which each one consists of an area of 0.625 ha which is divided into 42 rows (ie. one net house will thus have 336 rows). Each row consists of 12 chambers, 5 m long with 18 to 23 plants (depending on the tomato variety used). Twenty-six net houses were constructed in 3 climatic zones in the Limpopo Province of which six, located in three districts Vhembe (WP: Moloi NH 1 and NH 3), Mopani (JP: Vlakfontein NH 1 and VS: Mellet NH 3 and Denneland NH 2) and Capricorn (DK: Makeppies NH 2), were selected for further studies (Figure 2.1).

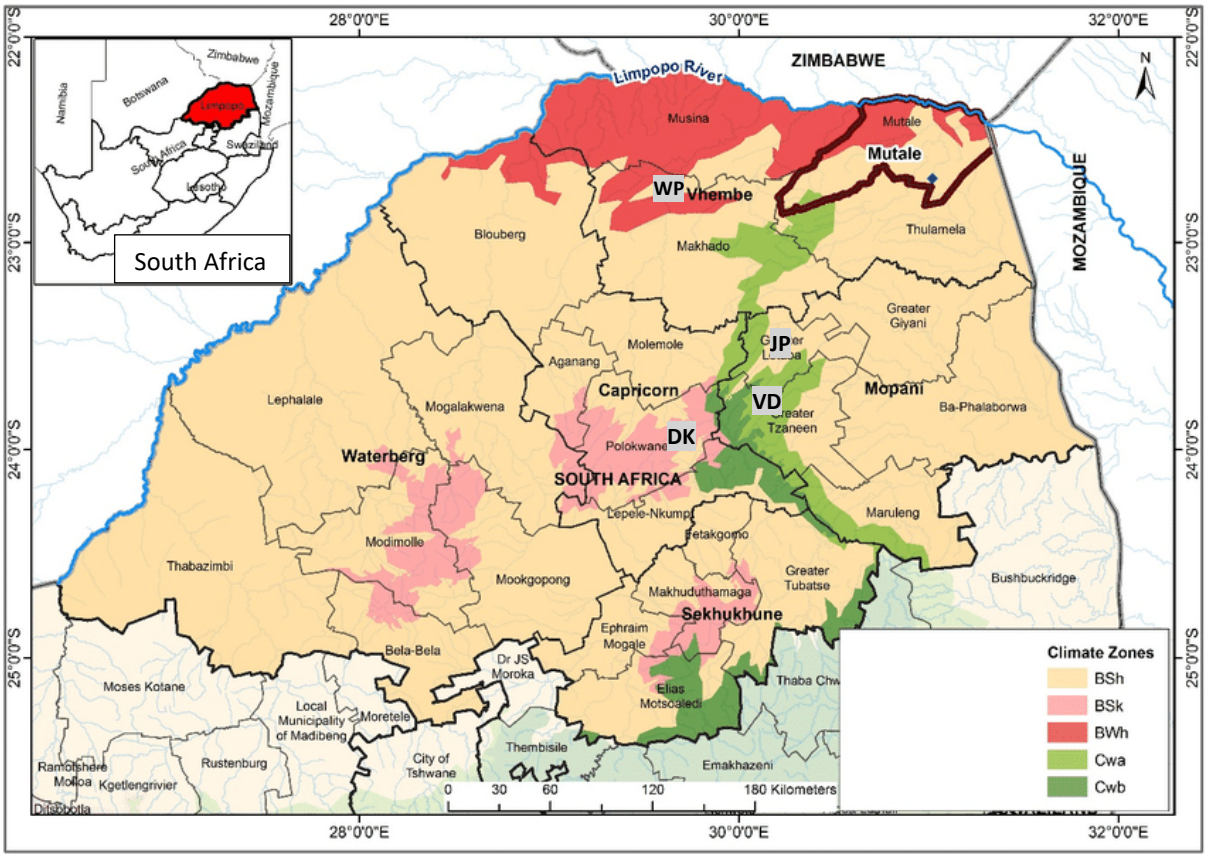

Figure 2.1: Map of the Limpopo Province (South Africa) depicting the climatic zones, i.e. 'Hot semi-arid climate (BSh)', 'Cold semi-arid climate (BSk)', 'Hot desert climate (BWh)', 'Humid subtropical climate (Cwa)' and 'Subtropical highland oceanic climate (Cwb)' where nethouses are located. (Adapted from Adeola et al., 2017).

2.2.2 Collection of pre-plant soil samples

Soil samples (250 cm^3 of each) were collected between 2015 and 2017 (depending on the development of each net house) from each of the 6 net houses selected for this study, before the tomato crop was planted. These samples were taken in a zig-zag pattern from 0-15 cm depth with an auger consisting of 10 subsamples each (Marais et al., 2017). The subsamples were combined in one sample (±5 kg) per block resulting in 8 samples per net house being analyzed.

The soil samples were sent to the nematode taxonomists of the Agricultural Research Council-Plant Protection Research, Queenswood, South Africa, for extraction, fixing and mounting of the nematodes to enable identification of specimens to genus level of PPN and non-parasitic nematodes (NPN). These nematode analyses served as the baseline data for each net house.

2.2.3 Collection of soil samples 8 weeks after tomato transplanting

Six net houses (out of the 26 existing ZZ2 net houses) located in Vhembe (Waterpoort), Mopani (Vreedsaam and Jachtpad) and Capricorn (Dikgale) districts were selected from the three climatic regions (Figure 2.1) for further studies and data were collected from 2016/17 to 2018/19 seasons. Nematode samples were collected 8 weeks after planting from 2016 until 2018 using the same sampling approach as described in Paragraph 2.2.2: Data collection for pre-plant soil samples. Similarly, the 10 subsamples obtained per net house were composited into one sample (±5 kg) resulting in 8 samples per net house to be analysed. Half of the samples were used for nematode analyses (processed at the ZZ2 laboratory), while the other half was analysed for the physico-chemical properties of the soil (see Paragraph 2.2.7).

2.2.4 Extraction of nematodes from soil samples

Nematodes were extracted from the 8 composite, 250-cm^3 soil samples per net house collected at 8 weeks after planting using the sieve sugar flotation technique described by Swart and Marais (2017). Each soil sample was washed through an 840-µm-aperture mesh sieve into a 5-liter bucket. The suspension was stirred, and then allowed to settle for 30 s. The suspension was then poured through 250-, 38- and 25-µm-aperture mesh sieves, respectively. This procedure was repeated twice, but the settling times were shortened to 20 and 10 s, respectively. The residue was then transferred from the 25-µm-aperture sieve to two 50-ml centrifuge tubes to which 5 cm^3 kaolin was added and which was then centrifuged for 5 min at 1 750 revolutions per min (rpm). The supernatant was decanted from the tubes and discarded. A sugar solution (484.5 g sugar/1 L water) was then added to the tubes, carefully mixed and centrifuged for 1 min at 1 750 rpm. The suspension was then poured through a 25-µm-aperture mesh sieve and collected into a beaker for examination and counting in a Peter's counting slide (Marais et al., 2017).

2.2.5 Counting of nematodes

The nematode-water suspension, representing each individual composite sample, in the beaker was adjusted to 100 ml by adding (depending on the initial volume of the suspension) tap water. The suspension was homogenized by blowing air into the solution. One millilitre (1 ml) aliquot of the suspension was collected using a pipette and placed onto a Peter's counting slide and counted using a light microscope (40x

magnification). For each sample, two aliquots of 1 ml were counted, and the means were multiplied by 100 to obtain the number of nematodes per 250 cm^3 soil for each sample. The PPN were determined to genus level, while free-living nematodes were classified only as NPN, with no distinction made between trophic levels or genera.

2.2.6 Plant data

2.2.6.1 Root gall ratings (*Meloidogyne* spp.)

Root gall ratings were determined annually for root-knot nematode infected tomato plants at the end of the crop cycle in each net house. A gall rating was given for each plant (512 per block) at the end of each crop cycle over a three-year period. Rating of root galls was done according to the index: 1 = 0 % galls, 2 = 1 – 40% galls, 3 = >40% galls (adapted from Coyne et al., 2014) (Figure 2.2).

Figure 2.2: Gall rating index used to quantify damage inflicted by *Meloidogyne* in roots of tomato plants grown in net houses in the Limpopo Province (Photo: Lesego Matlala, ZZ2-boerdery, Mooketsi).

2.2.6.2 Other plant parameters

Additional information collected per net house included rootstocks/scions used, planting season, and percentage RKN infected plants, where percentage RKN infection is calculated as follows:

$$\frac{\text{Total number of plants with index values 2 + 3}}{\text{Total number of plants (index values 1 + 2 + 3) at the end of crop cycle}} \times 100$$

2.2.7 Climatic and soil data

The parameters measured were temperature (˚C), heat (˚C), solar radiation (W/m^2), humidity (%), evapotranspiration (mm) and rainfall (mm). Weather data for each location was collected using the Davis Vantage Pro 2 (Hayward, California) weather station that reflected the average daily values per year. Rainfall data were, however, recorded as the total rain in mm per year.

Soil samples were sent to a commercial soil testing laboratory (Agri-technovation, Klapmuts, SA) for analysis of pH (KCl), calcium (mg/kg), magnesium (mg/kg), phosphorous (Bray l), potassium (mg/kg), sodium (mg/kg), sulphur (mg/kg), density (g/cm^3), acid saturation (cmol(+)/ kg), %Ca, %Mg, %K, %Na, Ca:Mg, (Ca + Mg)/K, Mg:K and Na:K. Furthermore, soil texture was classified as either sand, sandy loam or loamy sand for each net house.

2.2.8 Statistical analyses

The soil nematode data and percentage RKN infected plants for each net house were analysed with ANOVA using Statisica, version 10 (www.statsoft.com). Prior to statistical analysis, nematode data were transformed to $\log_{10}(x+1)$. Subsequently, proportions of PPN genus and NPN feeding group (bacterivore, fungivore, omnivore and predator) were determined as follows:

Proportions of each PPN =

$$\frac{\text{Total number of each PPN genus}}{\text{Total number of PPN}} \times 100$$

Proportions of each NPN group =

$$\frac{\text{Total number of NPN group}}{\text{Total number of PPN + NPN}} \times 100$$

For the coinertia analysis, proportions of RKN were further grouped according to three categories, being 1 = proportion of RKN being <20%, 2 = proportion of RKN between 21-50%, 3 = proportion of RKN >50%.

Soil, climate and plant data, as well as nematode data were analysed with principal component analysis (PCA) using ADE4 software (Thioulouse et al., 1997) to determine whether associations existed amongst these factors. Soil factors and weather data related to the nematode species distribution were identified using co-inertia analysis. Furthermore, T-test analyses were done on soil factors using excel Microsoft 2017.

2.3 Results

2.3.1 Nematode data: pre-plant soil samples

Fifty-six nematode genera (of which 45 were NPN), two families and two sub-families were identified (Figure 2.3). The 11 PPN genera identified included *Helicotylenchus, Hoplolaimus, Longidorus, Meloidogyne, Nanidorus, Pratylenchus, Rotylenchus, Scutellonema, Tylenchorhynchus, Xiphinema* and *Xiphinemella*. Specimens belonging to the PPN families Criconematidae and Dolichodoridae were also found but could not be identified to genus level (Addendum 1).

Meloidogyne was the most abundant genus, ranging from 0-7 670 individuals per 250 cm^3 soil (data not shown) for the 2016/17 to 2018/19 growing seasons (Figure 2.3). The second, third and fourth most abundant plant-parasitic nematodes were from the family Criconematidae (0-770 individuals per 250 cm^3 soil; data not shown), and the genera *Helicotylenchus* (0-150 individuals per 250 cm^3 soil; data not shown) and *Tylenchorhynchus* (0-135 individuals per 250 cm^3 soil; data not shown). *Meloidogyne* and Criconematidae each was recovered from 28% of the samples, respectively, and *Helicotylenchus* and *Tylenchorhynchus* each in 20% of the samples. The genus with the lowest abundance and frequency was *Hoplolaimus* occurring only in one sample (Addendum 1: Table 1).

In terms of the NPN, *Mesorhabditis* was most abundant with numbers ranging from 0-1 095 individuals per 250 cm^3 soil (data not shown), followed by *Acrobeles* (0-130 individuals per 250 cm^3 soil; data not shown) and *Acrobeloides* (0-120 individuals per 250 cm^3 soil; data not shown) which were recovered from 17, 30 and 31% samples, respectively (Figure 2.2; Addendum 1:Table 1). The genera with the lowest abundance were *Anaplectus, Monhystera, Prodorylaimus* and *Pungentus* with each occurring in 1 sample only.

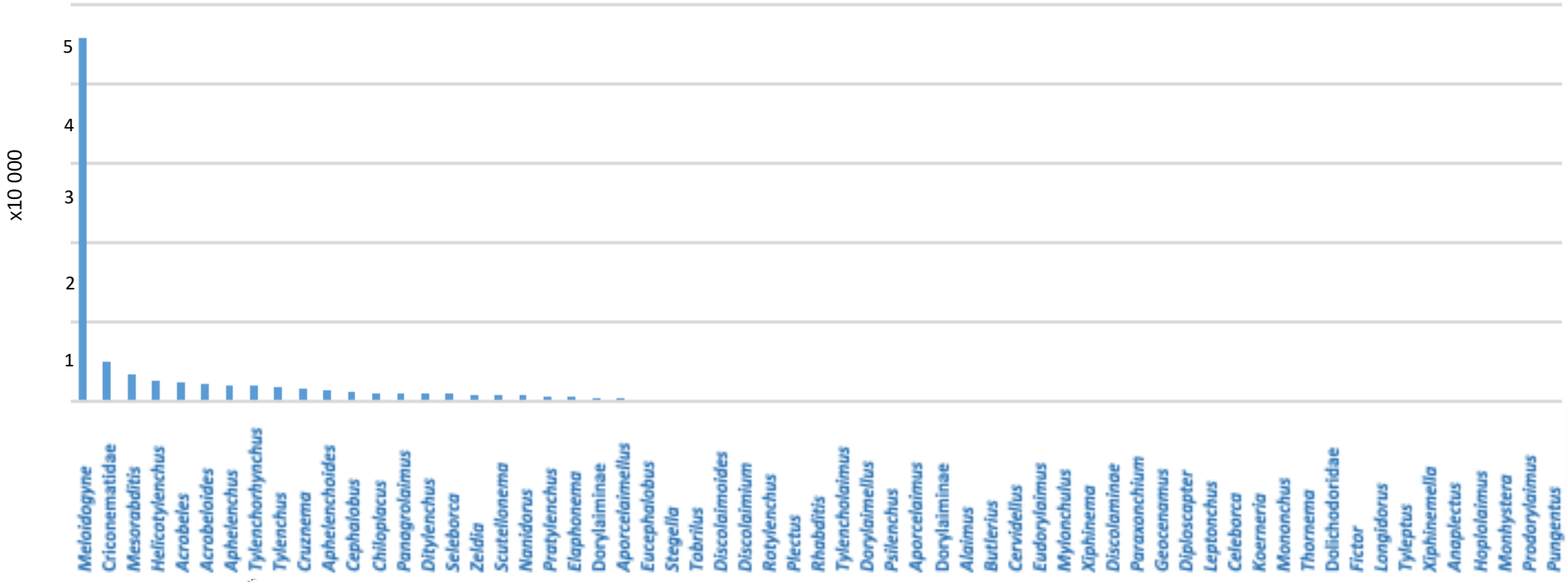

Figure 2.3: Abundance of plant-parasitic and non-parasitic nematode families, genera and sub-families in 250 cm^3 soil samples from 26 net houses located in the Limpopo Province (South Africa) over a 3-year period.

2.3.2 Nematode data: 8 weeks after transplanting for a 3-year cropping cycle

Significant differences ($P \leq 0.05$) were observed among the growing seasons for *Meloidogyne*, *Pratylenchus* and *Tylenchorynchus* (with their densities increasing from 2016/17 to 2018/19), while no significant difference in numbers were evident for the other PPN genera and the family Criconematidae (Table 2.1).

The densities of NPN differed significantly ($P \leq 0.05$) over time with densities increasing from 2016/17 to 2018/19 (Table 2.1).

Table 2.1. The average abundance of plant-parasitic (PPN) and non-parasitic nematodes (NPN) in rhizosphere soils (250 cm^3) of tomato plants sampled from six net houses located in the Limpopo Province of South Africa over a 3-year period from 2016/17-2018/19.

Growing season	*Meloidogyne*	*Pratylenchus*	*Scutellonema*	*Helicotylenchus*	*Tylenchorhynchus*	Criconematidae	*Nanidorus*	*Xiphinema*	NPN
2016/17	0.63* b (95** ± 292***)	0.26 b (3 ± 8)	0.03 a (0.4 ± 2)	0.53 a (8 ± 16)	0.09 b (4 ± 17)	0.59 a (12 ± 23)	0.00 a (1 ± 8)	0.00 a (1 ± 8)	1.47 c (183 ± 225)
2017/18	1.06 b (484 ± 1 357)	0.15 b (5 ± 19)	0.04 a (1 ± 7)	0.38 a (58 ± 113)	0.23 b (3 ± 10)	0.65 a (53 ± 68)	0.07 a (1 ± 7)	0.04 a	2.24 b (184 ± 195)
2018/19	1.88 a (1 057 ± 2 492)	0.85 a (45 ± 65)	0.07 a (2 ± 10)	0.33 a (3 ± 16)	0.73 a (51 ± 88)	0.67 a (19 ± 48)	0.15 a (6 ± 22)	0.00 a	2.61 a (587 ± 442)
P - value	**0.001**	**0.001**	0.736	0.410	**0.001**	0.908	0.151	0.403	**0.001**
F - value	11.57	13.33	0.31	0.76	9.83	0.10	1.92	0.92	47.03

*Log_{10} (x+1) transformed nematode mean data; **Real means in parenthesis followed by the ***Standard Deviation; Letters that differ indicate a significant difference at $P \leq 0.05$ between the treatments according to Tuckey HSD-test according to One-Way ANOVA.

Significant differences (*P*≤0.05) were observed among the net houses for the abundance of the PPN (Table 2.2). Makeppies NH 2 had significantly (*P*≤0.05) higher *Meloidogyne* numbers than the other net houses, while Moloi NH 1 and Moloi NH 3 had significantly (*P*≤0.05) lower *Pratylenchus* numbers than Makeppies NH2, compared to the other net houses.

Helicotylenchus population densities were significantly (*P*≤0.05) higher in Moloi NH1 compared to the other net houses (Table 2.2). Individuals of Criconematidae did not occur in Denneland NH2 but were comparable to the net houses in Vlakfontein NH 1, Mellet NH3 and Makeppies NH 2, whereas, they were significantly (*P*≤0.05) different from net houses in Moloi NH 1 and Moloi NH 3. *Tylenchorynchus* individuals did not occur in Moloi NH 3 but were comparable to the net houses in Vlakfontein NH 1, Moloi NH1 and Makeppies NH 2, whereas, they were significantly (P≤0.05) different from net houses in Mellet NH 3 and Denneland NH 2. *Nanidorus* individuals did not occur in Moloi NH 1, Moloi NH 3 and Denneland NH 2, but were comparable to the net houses in Vlakfontein NH 1, Makeppies NH 2 whereas, they were significantly (*P*≤0.05) different from Mellet NH 3. *Scutellonema* and *Xiphinema* nematode densities did not differ significantly among among the 6 net houses (Table 2.2).

The percentage of tomato plants that were infected (calculated from root gall indices as seen in Figure 2.2) showed a significantly (*P*≤0.05) higher infection in Makeppies NH2 than the other net houses, which confirms the high RKN numbers in the same net house (Table 2.2).

Table 2.2. Mean nematode densities per genus in rhizosphere soils (250 cm^3) of tomato plants, and the percentage *Meloidogyne* spp. infected tomato plants sampled from each of six selected nethouses over a 3-year period from 2016/17-2018/19.

Nethouse	*Meloidogyne*	*Pratylenchus*	*Scutellonema*	*Helicotylenchus*	*Tylenchorynchus*	*Criconematidae*	*Nanidorus*	*Xiphinema*	NPN	% plants infected with *Meloidogyne*
Vlakfontein NH 1	0.45* c (34** ± 122***)	0.48 ab (13 ± 25)	0.07 a (2 ± 10)	0.56 b (13 ± 19)	0.15 ab (6 ± 22)	0.16 c (5 ± 20)	0.07 ab (2 ± 10)	0.07 a (2 ± 10)	2.14 bc (278 ± 247)	0.96 b (20 ± 22)
Moloi NH1	0.52 bc (269 ± 822)	0.23 b (0.2 ± 1)	0.07 a (2 ± 10)	1.58 a (115 ± 138)	0.31 ab (5 ± 9)	1.42 a (84 ± 77)	0.00 b	0.00 a	2.01 bc (100 ± 112)	0.83 b (2 ± 2)
Moloi NH3	0.76 bc (19 ± 45)	0.03 b (8 ± 24)	0.00 a	0.00 c	0.00 b	0.93 ab (26 ± 32)	0.00 b	0.00 a	1.66 c (159 ± 129)	0.31 c (16 ± 21)
Mellet NH3	1.18 b (167 ± 280)	0.44 ab (15 ± 28)	0.05 a (0.6 ± 3)	0.00 c	0.61 a (46 ± 95)	0.50 bc (17 ± 32)	0.31 a (13 ± 30)	0.00 a	2.15 ab (404 ± 407)	1.42 b (32 ± 15)
Makeppies NH 2	2.77 a (2 078 ± 2 381)	0.89 a (43 ± 63)	0.00 a	0.00 c	0.41 ab (25 ± 66)	0.62 bc (25 ± 66)	0.07 ab (31 ± 64)	0.00 a	2.28 ab (418 ± 397)	1.63 a (50 ± 25)
Denneland NH2	1.87 b (1 009 ± 1 993)	0.53 ab (41 ± 82)	0.11 a (3 ± 13)	0.23 bc (9 ± 27)	0.87 a (50 ± 73)	0.00 c	0.00 b	0.00 a	2.77 a (731 ± 451)	0.88 bc (19 ± 22)
P - value	**0.001**	**0.001**	0.692	**0.001**	**0.011**	**0.001**	**0.040**	0.445	**0.001**	**0.001**
F - value	22.22	4.74	0.61	32.22	4.94	9.72	2.46	0.96	13.07	20.98

*Log_{10} (x+1) transformed nematode mean data; **Real means in parenthesis followed by the ***Standard Deviation; Letters that differ indicate a significant difference at P ≤ 0.05 between the treatments according to Tuckey HSD-test according to One-Way ANOVA

2.3.3 Effects of selected biotic and abiotic factors on nematode population densities during a 3-year crop cycle

Multivariate analysis showed that PPN, more specifically *Meloidogyne*, increased from the 2016/17 to the 2018/19 season (data not shown) agreeing with nematode abundance data (Table 2.1). To determine which factors influenced the increase in nematode numbers between the consecutive years, the effects of soil physical (texture) and chemical parameters, planting season and rootstocks/scions were evaluated as well as relationships between nematode numbers and climatic parameters.

2.3.3.1 Soil texture

The first two factors of the PCA (Figure 2.4A) described only 37.2% of the variability between the nematode data indicating a weak structure. *Meloidogyne* and *Pratylenchus* had the highest correlation on the positive side of F1, while *Helicotylenchus* and Criconematidae had the highest correlation on the positive side of F2. They were also correlated with the negative side of F1, although, not very strong. When the samples were projected on the F1XF2 factorial plan (Figure 2.4B) according to soil texture, the sandy loam soil (1) was situated in the negative part of F1 while the loamy sand (2) and sand (3) were positioned in the positive part of F1. The centre indicating sandy soil was more positively correlated on F1 than loamy sand soil (Figure 2.4B). Sandy soils seemed to contain more *Meloidogyne* and *Pratylenchus* followed by loamy sand and sandy loam, which was more prone in hosting higher *Helicotylenchus* numbers (Figure 2.4B).

When nematode numbers for each genus and percentage (%) RKN infestation were divided between the three soil textures, significant (P<0.05) differences were observed for the 6 genera and % infestation (Table 2.3). *Meloidogyne* and % infestation was significantly (P<0.05) higher in sand, followed by loamy sand and sandy loam. *Pratylenchus* had significantly (P<0.05) higher numbers in sand compared to sandy loam soil. *Helicotylenchus* had significantly (P<0.05) higher numbers in sandy loam soils, compared to loamy sand and sand.

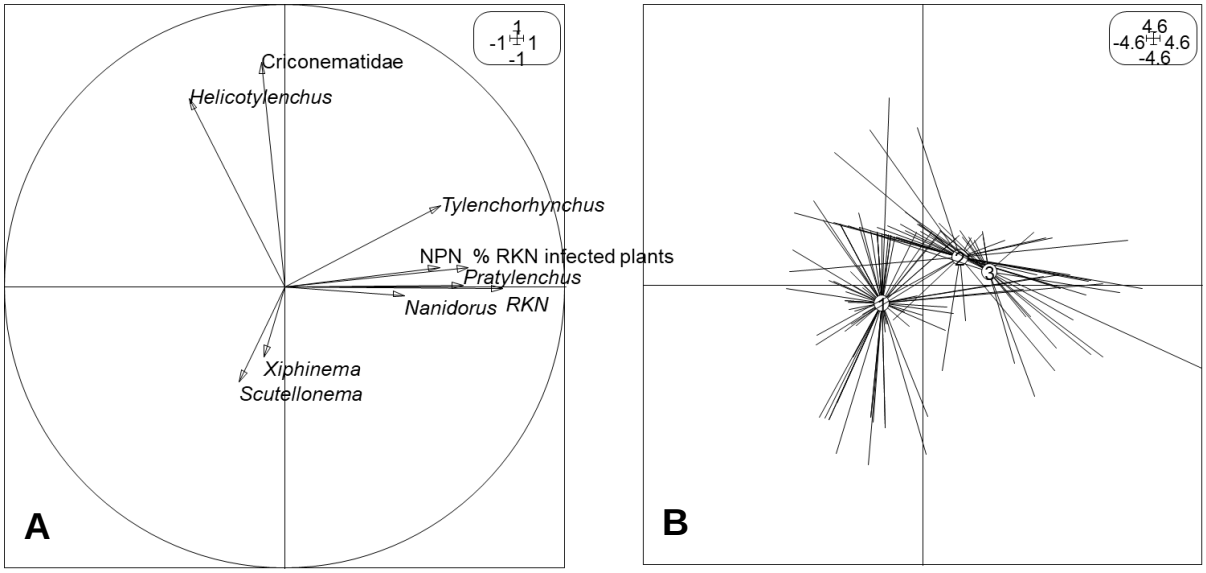

Figure 2.4: (A) Factorial plan of nematode variables (log transformed) and the percentage tomato plants infected with root-knot nematodes (RKN), and (B) factorial plan of samples grouped per soil texture with 1=sandy loam; 2=loamy sand and 3=sand.

Table 2.3. Nematode genera, identified in soils sampled from six net houses in the Limpopo Province (South Africa) from 2015-2018, divided per soil texture as compared with the Tukey HSD test (P<0.05).

Nematode genera	*Sandy loam*	*Loamy sand*	*Sand*	*F value*	*P value*
Meloidogyne	0.65 c	1.36 b	2.77 a	34.30	**0.001**
Pratylenchus	0.23 b	0.52 ab	0.89 a	7.73	**0.001**
Scutellonema	0.02 a	0.12 a	0.00 a	0.07	1.000
Helicotylenchus	0.72 a	0.04 b	0.00 b	16.16	**0.001**
Tylenchorynchus	0.23 b	0.59 a	0.41 ab	2.94	0.060
Criconematidae	0.81 a	0.34 b	0.62 ab	3.70	**0.030**
Nandorus	0.00 b	0.24 a	0.07ab	5.55	**0.001**
Xiphinema	0.02 a	0.00 a	0.00 a	0.42	0.660
NPN	2.00 b	2.34 a	2.29 ab	3.42	**0.040**
% RKN infected plants	0.66 c	1.30 b	1.63 a	37.37	**0.001**

Log_{10} (x+1) transformed nematode mean data

2.3.3.2. Root stocks and scions

Over the three growing seasons, different rootstocks and scions were used in the different net houses. When the samples were projected on the F1XF2 factorial plan and grouped according to rootstock/scion, variation in positions for the different rootstocks/scions was observed. Root stocks RS2, RS3 and RS13 (coloured in red) seemed to maintain lower PPN numbers, while RS11 and scion ZZX132 (also coloured in red) were positively correlated with higher PPN numbers (Figure 2.5B). Among the rootstocks/scions most commonly used, RS11 and ZZX132 had a tendency of supporting higher *Meloidogyne, Pratylenchus* and *Nanidorus* numbers (Table 2.4). It is also worth mentioning that scion ZZX64 and ZZX125 had a tendency of maintaining higher nematode numbers since *Meloidogyne*, *Pratylenchus*, *Tylenchorhynchus* densities were high in both scions; the same applied for NPN densities. However, most scions and combinations of rootstocks and scions were only planted in one season in one net house and therefore do not warrant further discussion.

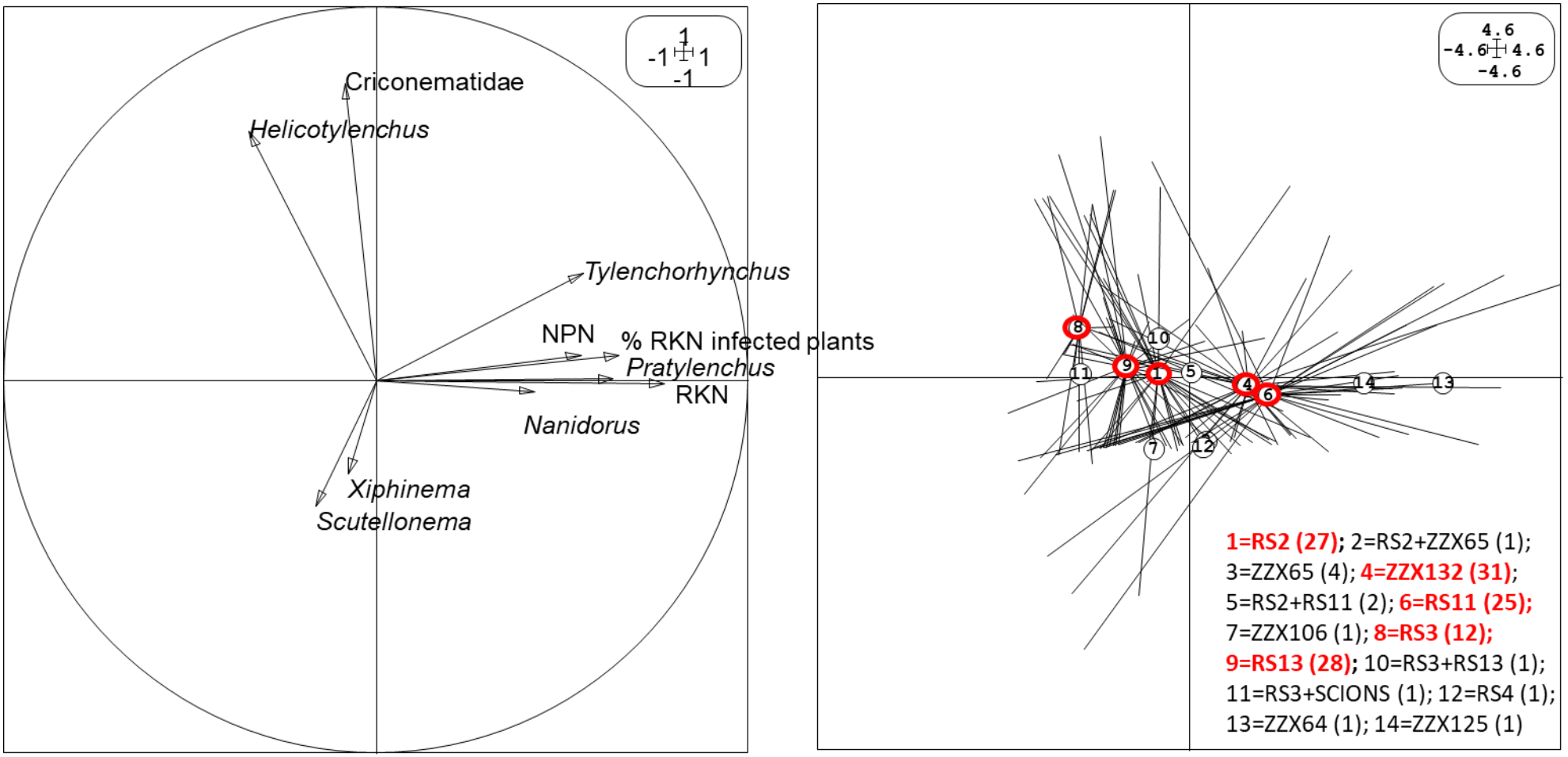

Figure 2.5: (A) Factorial plan of nematode variables (log transformed) and the percentage infected root-knot nematode (RKN) tomato plants, and (B) factorial plan of samples grouped according to rootstocks and scions where the rootstocks/scions are shown as well as the number of blocks planted with this rootstock/scion in brackets for the 3-year growing seasons in the six nethouses investigated.

Because it was previously established that soil texture played a significant role in RKN numbers, it was important to determine whether these root stocks/scions were possibly planted in areas with a specific soil texture for instance sandy soil. Significant differences (P<0.05) were observed between rootstocks/scion and *Meloidogyne*, *Pratylenchus*, *Helicotylenchus*, *Tylenchorynchus* and NPN, while no significant differences were evident for other PPN genera. Root stocks RS2, RS11 and ZZX132 was associated with significantly (P<0.05) higher *Meloidogyne* densities and were planted in the three different soil types (Table 2.4). However, RS3 was only planted in sandy loam soil, while RS13 was not planted in sandy soil (Table 2.4). This could influence the results observed in Figure 2.5B, where, these specific rootstocks/scions (RS3 and RS13) appeared to be more tolerant to RKN than others.

Table 2.4. Rootstocks and scions planted in different soil types in six nethouses (located in the Limpopo province, South Africa) compared by the Tukey HDS test *(P*<0.05) with regards to their preference to maintain plant-parasitic nematodes and to be associated with non-parasitic nematodes (NPN).

Rootstock/Scion		*Meloidogyne*	*Pratylenchus*	*Scutellonema*	*Helicotylenchus*	*Tylenchorynchus*	*Criconema*	*Nanidorus*	*Xiphinema*	NPN	Soil texture*
1	**RS2**	1.09 a	0.38 ab	0.06 a	0.62 ab	0.09 c	0.59 a	0.00 a	0.00 a	1.79 b	1, 2 & 3
3	**ZZX65**	0.00 b	0.30 ab	0.00 a	1.03 a	0.00 c	0.19 a	0.00 a	0.00 a	1.84 ab	1 & 2
4	**ZZX132**	1.52 a	0.50 ab	0.06 a	0.28 b	0.54 b	0.49 a	0.24 a	0.00 a	2.47 a	1, 2 & 3
6	**RS11**	1.82 a	0.83 a	0.05 a	0.07 b	0.79 b	0.54 a	0.06 a	0.07 a	2.47 a	1, 2 & 3
8	**RS3**	0.53 ab	0.00 b	0.00 a	0.65 ab	0.30 c	1.23 a	0.00 a	0.00 a	1.55 b	1
9	**RS13**	0.95 ab	0.18 b	0.06 a	0.57 ab	0.05 c	0.79 a	0.00 a	0.00 a	2.10 ab	1 & 2
P-value		**0.013**	**0.014**	0.988	**0.036**	**0.001**	0.156	0.082	0.545	**0.001**	
F-value		3.03	2.99	0.12	2.48	4.46	1.70	2.01	0.81	6.16	

Log_{10} (x+1) transformed nematode mean data; #Note: Only rootstocks and scions that had more than one replication were compared; *soil texture: 1 = sandy loam; 2 = loamy sand and 3 = sand

2.3.3.3 Climatic variables

Coinertia analysis was used to determine relationship between climatic parameters and nematode distribution. Positive relationships between temperature and heat, and *Helicotylenchus* and Criconematidae were observed, whereas, *Meloidogyne* and % RKN infected tomato plants were positively correlated with humidity evapotranspiration and solar radiation (Figure 2.6).

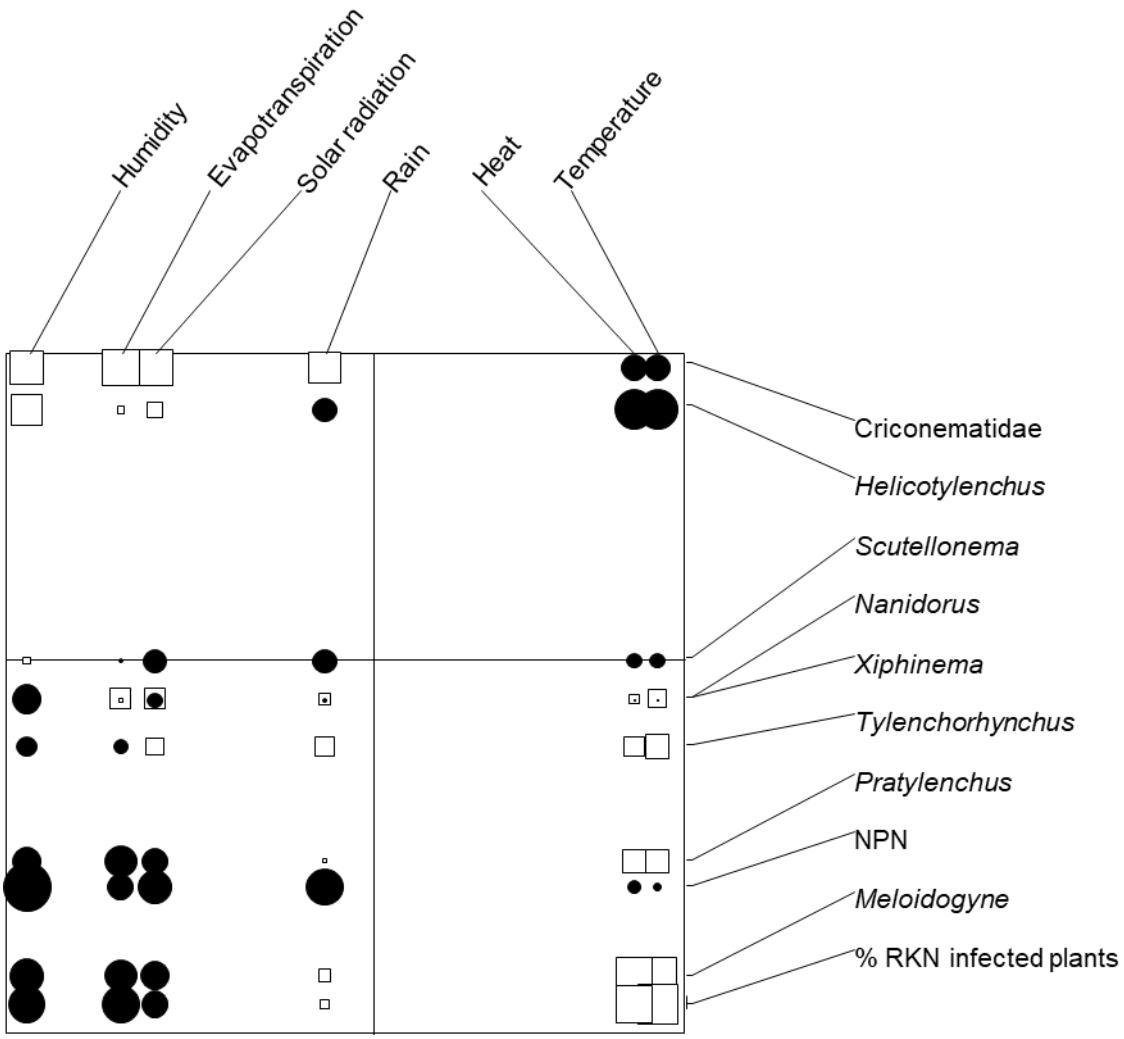

Figure 2.6: Summary of the correlations identified by the coinertia analysis between nematodes and the % *Meloidogyne* (root-knot nematode:RKN) infected tomato plants, and climatic parameters; with circles representing a positive correlation and squares a negative correlation, while the size of the symbol is proportional to the strength of the correlation.

2.3.3.4 Planting season

Nematode distribution was evaluated by grouping samples according to planting season, and no significant differences were observed between autumn, winter and spring plantings as the centres of each season on the F1XF2 factorial plan were grouped very close together (data not shown) indicating that time of planting did not play a significant role in *Meloidogyne* incidence and % RKN infected tomato plants.

2.3.3.5 Relationships between soil chemical variables and nematode population densities

The nematode data set was recalculated as proportions (as opposed to transformed data in previous analyses) and coinertia analysis were conducted to study the relationship between proportions of nematodes and soil chemical variables. The coinertia test showed that the calculated relationship between nematode communities and soil chemical data was never as good as the observed one. The relationships between nematode community and soil chemical data were therefore statistically significant according to the 5% limit.

On the factorial map for the nematode community, *Meloidogyne* and *Pratylenchus* were strongly correlated to the positive values of F1, while Criconematidae and *Helicotylenchus* were correlated to the negative values of F1 (Figure 2.7A). On the factorial map of the soil chemical parameters, Mg:K, Na:K, %Mg were strongly positively correlated to the positive value of F1 while pH, K, Ca and acid saturation were strongly correlated with the negative values of F1 (Figure 2.7B). Therefore higher levels of Ca, acid saturation, K and pH were associated with lower densities of *Meloidogyne* and *Pratylenchus*, while higher levels of Mg:K, Na:K, %Mg were associated with higher densities of *Meloidogyne* and *Pratylenchus* (Figure 2.7 A and B).

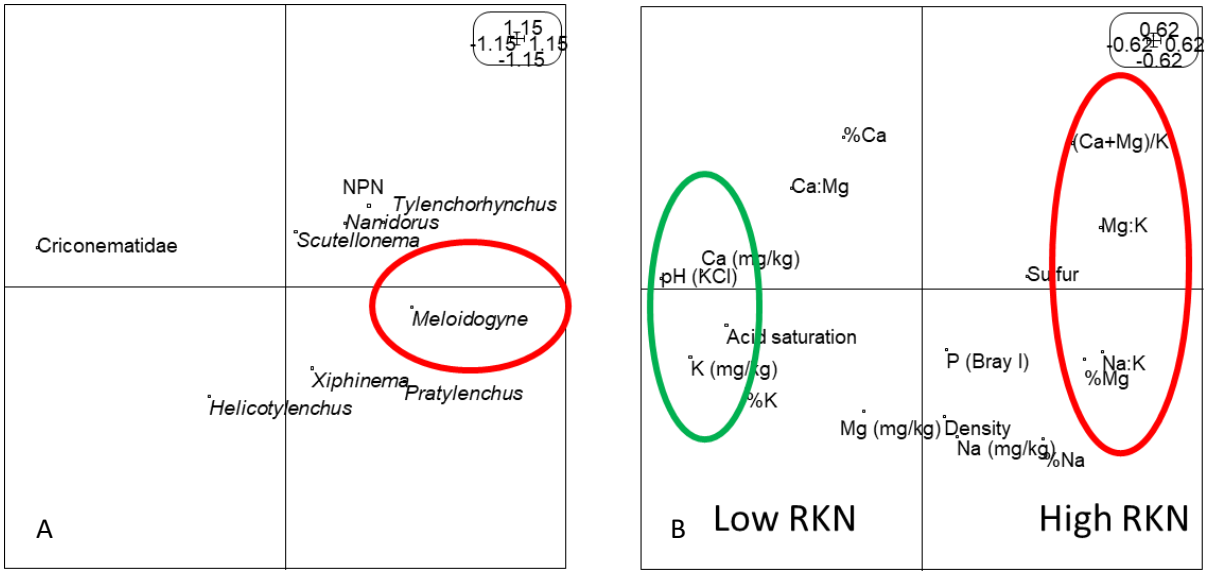

Figure 2.7: First factorial maps of the nematode variables (A) and soil variables (B) recalculated by the coinertia analysis.

The elements found to have the highest positive/negative correlation with *Meloidogyne* as seen in Figure 2.7, were each sorted from high to low levels after which the average of the 20 highest and 20 lowest values were determined and compared with averages of *Meloidogyne, Pratylenchus, Helicotylenchus* and Criconematidae proportions for these samples. Significant differences between *Meloidogyne* and the different parameters according to T-test analysis were observed for these parameters, except for Mg:K (Table 2.5). For *Helicotylenchus* and Criconematidae, the correlations were even more pronounced (Table 2.5).

Table 2.5. Comparison of *Meloidogyne, Pratylenchus, Helicotylenchus* and Criconematidae proportions in the 20 samples with the highest and lowest content of Ca, K, Acid saturation, Mg:K, Na:K and %Mg.

	High	SE	Low	SE	T - test
Acid saturation (%)	20.6	0.38	7.3	0.71	<0.001
***Meloidogyne* %**	9	3.6	32	5.3	0.011
***Helicotylenchus* %**	18	5.2	0	5.4	0.0016
K (mg/kg)	1046	15	220	19	<0.001
***Meloidogyne* %**	17	5.9	32	4.6	0.033
***Helicotylenchus* %**	24	4.6	5	5.1	0.002
Criconematidae *%*	39	5.9	17	5.3	0.015
Ca (mg/kg)	3074	77	876	48	<0.001
***Meloidogyne* %**	13	6.1	37	5.7	0.03
***Helicotylenchus* %**	12	4.2	0	5.0	0.005
Criconematidae *%*	36	8.2	16	8.8	0.044
Na:K	0.89	0.04	0.06	0.01	<0.001
***Meloidogyne* %**	35	9.5	18	9.1	0.047
***Pratylenchus* %**	17	5.9	5	5.4	0.025
Criconematidae %	6	3.4	35	3.6	0.0003
Mg:K	3.6	0.27	0.95	0.04	<0.001
Criconematidae %	5	3.2	34	4.9	0.0004
%Mg	25.4	0.4	11.1	0.3	<0.001
***Meloidogyne* %**	30	5.4	6	5.6	0.045
***Criconematidae* %**	8	3.7	34	4.0	0.006

#SE: standard error, T-test (Excel in Microsoft 2017) at *P*>0.005.

When the samples were grouped according to the three categories for *Meloidogyne* proportions (as explained in Paragraph 2.2.4), averages calculated for each *Meloidogyne* category differed significantly between the different categories for Ca, K and acid saturation (Table 2.6).

Table 2.6. Average values for the soil chemical parameters identified as strongly correlated with *Meloidogyne* population densities in soil samples obtained from six net houses in the Limpopo Province (South Africa) from 2015-2018 divided according to the three categories of proportion of *Meloidogyne*.

Category *Meloidogyne*	*Meloidogyne* %	Acid saturation	Ca (mg/kg)	K (mg/kg)	Mg:K	Na:K	%Mg
1	0.0 c	14.67 a	2003.50 a	631.07 a	1.85 a	0.46 a	17.22 a
2	37.32 b	13.02 ab	1703.50 ab	572.76 ab	1.96 a	0.43 a	18.07 a
3	83.13 a	11.85 b	1467.60 b	485.85 b	2.00 a	0.49 a	18.78 a
P-value	**0.001**	**0.006**	**0.001**	**0.038**	0.716	0.563	0.216
F-value	676.11	5.34	7.09	3.35	0.33	0.58	1.55

2.4 Discussion

Previous reports have indicated the abundance, diversity and distribution of RKN species in South Africa and their importance to the agricultural crops grown in the country (Ntidi et al., 2015; Agenbag, 2016; Rashidifard et al., 2019). Of the 11 PPN genera identified in pre-plant samples, it is not surprising that RKN were the most prevalent species infecting tomato in the net houses investigated at ZZ2 in Limpopo Province in this study. One of the reasons for high RKN population densities existing in such net houses is continuous tomato cropping cycle (coupled with the management strategies) used whereby very little or no time is allowed for a fallow or resting period, therefore enhancing population build up (Cadet et al., 2018). Moreover, prior construction of these net houses and before planting of the tomato crop, these fields were covered with weeds, which may have hosted RKN. This could also partly explain why higher population densities of this genus were found, relative to other genera recorded in this study. Ntidi et al. (2015) reported in a previous study that weeds such as *Chenopodium carinatum* (Brown) and *Datura ferox* L. were hosts for *Meloidogyne incognita* (Kofoid and White, 1919), Chitwood, 1949 and *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949. These are among many weeds that are abundant in and around the net houses investigated during this study.

Although biodiversity of nematodes (11 PPN and 45 NPN genera, 2 PPN families and 2 NPN sub-families) was evident from this study, RKN were by far the most abundant

genus and their abundance did not seem to be negatively influenced by competition from other nematode genera. On the contrary, RKN numbers increased throughout the three years of the study from an average number of 95 in the first year to 1 057 in the third year. These results are in agreement with those by Singh (2017) who illustrated an increase in numbers of *M. incognita* in soil from one year to the next for cucumber cucumber (*Cucumis sativus* L.); with a 68.8% frequency of occurrence in net houses and 16.6% in open-field cultivation in Punjab, India. Anupam (2017) also demonstrated similar trends for *M. incognita* in tomato roots and soil, with reproductive factors of 2.6-2.7 in poly houses and 1.7-1.9 in open fields in Punjab, India.

The present study revealed high RKN occurrences in net houses at ZZ2 in the Limpopo Province where sandy soils occur. The differences in occurrence of this nematode genus among the six net houses investigated might be related to soil types as the soil textures varied among these net houses. Kim et al. (2017) showed an increase of *M. incognita* numbers in carrot (*Daucus carota* L.) in Korea, when planted in soil with a higher sand percentage compared with those with a low sand content. Results of a study by Ferji et al. (2015) agreed to those of the latter authors and the present study since they found *Meloidogyne* spp. in higher numbers in sandy soils in banana (*Musa*, L.) fields in Morocco.

Hallmann and Meressa (2018) demonstrated that infection levels of RKN in rootstocks of tomato and cucumber were dependent on various factors, *viz*. nematode-rootstock variety combination, soil-nematode densities in the beginning of crop cycle, reproductive potential of the nematode, plant tolerance and duration of the cropping cycle. The current study suggested that rootstocks RS2, RS3 and RS13 were more tolerant to RKN species present in the net houses compared to the other scions and rootstocks, namely ZZX125, RS11, ZZX132 and ZZX64. Stevens (2018) also observed that RS13 showed tolerance to *M. javanica* which is in accordance to the results of this Limpopo-net house study. However, Stevens (2018) also reported ZZX132 as tolerant to *M. javanica,* which is in contrasts with results of the present net house study. This contrasting result obtained may be due to a mixture of RKN species present in the net houses, while micro-organisms found in the soil rhizosphere of the respective net houses, higher temperature regimes as well as soil chemical characteristics may also have an impact on the tolerance potential of the plants.

The six net houses investigated were situated in three different climatic areas and climate seemed to play a role in the soil infestation of RKN and their ability to infect tomato plants. However, when soil type was added to this equation, it was discovered that the net house selected in the cooler area (Makeppies NH 2), had a sandy soil, which has been demonstrated had a tendency to support higher RKN densities compared to the other net houses where a combination of different soil types were present. This might have compromised the effect of climate and therefore it might be interesting to investigate other net houses in the cooler area with a range of soil types with regard to the abundance and occurrence of particularly RKN. Planting date, also considered as a factor that might impact on nematode abundance, however, did not seem to influence nematode densities in the present study.

Except for plant host (variety), soil type and climate, plant-pathogenic microbes are reliant on mineral composition of the soil, which is dependent on soil type but can be manipulated by fertilisation, addition of compost and other organic amendments. A study by Ngeno et al. (2019) illustrated that high levels of phosphorus (as potassium phosphate), and Mg (magnesium) were associated with high population densities of *Meloidogyne exigua* Göldi, 1887. Their results concur with those from the present study, since the net house at Makeppies NH 2 that had higher of RKN densities also had higher P levels in the soils. Calcium components in the soil have also been associated with lower nematode damage to crop roots (Ngeno et al., 2019), thus enabling tolerance to RKN damage in susceptible plants. These results also agree with those from the present study since higher calcium availability in soils in net houses of Moloi NH 1 and Vlakfontein NH2 resulted in less nematode damage done to tomato roots due to a lower abundance of RKN and higher numbers of *Helicotylenchus* compared to other net houses. A similar phenomenon was reported from the KwaZulu-Natal Province of South Africa for sugarcane of which the yield was positively correlated with high densities of *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961 opposed to low densities of RKN species (Cadet et al., 2002).

Furthermore, a negative correlation was also observed in the present net house study between potassium levels and RKN population densities, similar to reports by Jothi and Poornima (2017). The latter authors showed that addition of K (potassium humate) reduced population densities of *M. incognita*. *Helicotylenchus* populations were

however positively correlated to K conforming with studies by Cadet et al. (2002) in sugarcane. Low cation contents and pH levels are associated with increased RKN population densities as was evident in the current study and conforms with reports by Ndava et al. (2018) for tomato grown in various soil pH levels and soil textures in Zimbabwe. These authors showed that soils with higher acidity levels had higher *Meloidogyne* and *Pratylenchus* infestation densities.

2.5 Conclusion

Insight was gained into how net house structures located in various climatic regions in the Limpopo Province of South Africa, where different soil types occurs influenced nematode population densities in tomato production over three years of continued cultivation. The differences in soil texture and soil chemical analyses seemed to influence the abundance and incidence of PPN together with the choice of rootstocks or scions used. The %RKN infected tomato plants increased from one year to the next, indicating that RKN abundance increased, which is a rising concern for the next cropping cycle as populations build up. More research is thus required to provide management strategies in these net houses, given the short fallow periods. The next part of this study, Chapter 3, focus on *Meloidogyne* spp. species found in the different net houses; this information will assist producers in choosing the correct resistant/tolerant tomato rootstock to produce the crop sustainably in the presence of these nematode pests.

2.6 References

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Chapter 3

Morphological and molecular identification of *Meloidogyne* spp.

Abstract

Identification of *Meloidogyne* spp. (root-knot nematodes: RKN) poses a challenge, thus the use of both morphological and molecular techniques has become important. Molecular methods are known for their accuracy, however, when in combination with morphological approach, higher precision is observed. During the current study that was conducted in tomato net houses at ZZ2 farms, located in three different climatic regions, 21 and 30 females, were collected from infected tomato roots from each site for morphological identification (oesophageal areas and perineal-patterns) and molecular identifcation (the sequence characterized amplified region – polymerase chain reaction; SCAR-PCR) respectively. Morphological identification confirmed that mixed communities of *M. arenaria*, *M. incognita* and *M. javanica* occurred in all the net houses, while *M. enterolobii* was seemingly present in 67% of the net houses. It was, however, a challenge to attempt identifying *M. enterolobii* females using morphology due to its resemblance with especially *M. incognita*. Molecular data showed that the six net houses had mixed communities comprising of two to four RKN species: X-land containing *M. enterolobii* and *M. javanica*; Vlakfontein, Mellet and Makeppies disclosing populations of *M. arenaria*, *M. enterolobii* and *M. javanica*; Denneland and Ferreira containing *M. arenaria*, *M. enterolobii, M. javanica* and *M. incognita*. The approach of using both techniques (molecular and morphological) to identify *Meloidogyne* spp. has confirmed the presence of four species and should be used to ensure that the correct information is gathered.

Keywords: *Meloidogyne enterolobii*, Perineal pattern, root-knot nematodes, SCAR-PCR, tomato.

3.1 Introduction

The significance of accurate identification of *Meloidogyne* spp., is highly important since effective management strategies such as crop rotation coupled with the use of non-, poor- or resistant genotypes, cover crops and others are dependent upon it (Adam et al., 2007; Bello et al., 2020). Common procedures used to identify these species include morphological taxonomy (Eisenback and Hunt, 2009), isozyme analyses (Esbenshade and Triantaphyllou, 1985; dos Santos et al., 2018), host-plant response (Hartman and Sasser, 1985), molecular (Zijlstra et al., 2000; Adam et al. 2007; Janssen et al. 2016) and genetic (Rashidifard et al., 2018) techniques.

Morphological characters have been used to determine the physiological and morphological functions of different life stages of *Meloidogyne* spp. (Dropkin and Bird, 1978; Karssen et al., 2013). These are helpful in clarifying interactions between nematodes and the environment and nematode and its hosts (Bird and McClure, 1976; Papadopoulou and Triantaphyllou, 1982). Female perineal-pattern morphology is among the most frequently used methods to differentiate between species (Eisenback and Hunt, 2009). Furthermore, different life stages of *Meloidogyne* spp. are used to emphasize the constraints between intraspecies variation through morphological and morphometrical characteristics (Hunt and Handoo, 2009; Ghaderi and Karssen, 2020).

Mature female characteristics such as vulval slit length, body shape, stylet length, shape of stylet knobs and shape of the lumen of the oesophagus (Kleynhans 1991; Brito et al. 2004; Karssen et al. 2013; Rashidifard et al., 2019a), and for males and second-stage juvenile (J2) characters such as the body and stylet length, shape of labial region and stylet knobs, dorsal gland orifice from stylet base and tail shape, hemozonid location (Kleynhans 1991; Karssen 2002; Rashidifard et al., 2019a; Ghaderi and Karssen, 2020) can be used for identification. This method, however, is not always reliable for certain species since many diagnostic characters overlap across species (Moens et al., 2009; Karssen et al., 2013). Due to intraspecific variation, interspecific similarities and indistinct species boundaries and complexities, relying only on these techniques for identification hinders verification, thus the use of molecular techniques to validate results is required (Pagan et al., 2015). The use of deoxyribonuclease- (DNA) based identification has become evident over the years providing more reliable results and not requiring a specific nematode life stage

(Powers, 2004; Powers et al., 2005; Qiu et al., 2006; Adam et al., 2007). Sequence Characterised Amplified Regions Polymerase Chain Reaction (SCAR-PCR), microarrays, DNA-based techniques and genotyping by sequencing (GBS) (Blok and Powers, 2009; Kiewnick et al., 2013; Rashidifard et al., 2018; Bello et al., 2020) are among molecular techniques being used. Thus, the use of morphology or molecular methods and its combination is essential and will provide reliable accurate identification of *Meloidogyne* spp. (Hunt and Handoo, 2009; Rashidifard et al., 2019a, b).

The aim of this study was to use morphology and morphometrics to identify *Meloidogyne* spp. found in the six-tomato grown net houses at ZZ2, Limpopo Province in South Africa and to validate the results with the commonly- and popularly used SCAR-PCR technique.

3. 2 Material and Methods

3.2.1 Characteristics of study areas

The study area where the net houses are located are the same as described in the study of nematode population dynamics (Chapter 2, paragraph 2.2.1).

3.2.2 Collection of *Meloidogyne* spp.

Infected root samples were collected in six net houses representing those used for the studies of Chapter 2 and 4. Mature RKN were isolated and removed for morphological and molecular identification. Root-knot nematode infected root samples were collected 10 and 20 weeks after planting of tomato crops from the six net houses during 2019/2020 season. The root samples were collected from one block (0.625 ha) in each net house using a zigzag pattern. Samples from each net house were kept in a labelled plastic bag and placed in a cooler bag that was taken to the ZZ2 Nematology laboratory for isolation of the mature females.

3.2.3 Isolation of mature *Meloidogyne* spp. females

Roots from each net house were washed separately with tap water to remove soil debris, placed in a container and labelled. Mature female specimens were removed from 20 random individual root pieces with a tweezer and scalpel that were dipped in 96% ethanol after removal of each female to prevent cross contamination among net

houses. Fifteen females isolated from each net house were placed in a 1.5 ml Eppendorf tube that contained 20 µl distilled water. This procedure was repeated once allowing 30 mature females isolated per net house (and contained in two tubes) to be stored for DNA extraction. The tubes were stored at -10 °C until molecular analyses were performed.

Another 21 mature females from each net house were concurrently, with isolation of females for the molecular study, transferred to the lid of a petri dish for preparation of morphological identification (see Paragraph 3.2.4).

3.2.4 Morphological and morphometrical identification of females

3.2.4.1 Oesophageal and perineal-pattern characteristics

For morphological identification, 21 randomly selected mature female specimens fixed in each of the individual tomato root systems sampled per net house, were carefully removed using a scalpel and tweezer (Figure 3.1A). The females were transferred to drops of lactic acid placed on the surface of the lid of a Petri dish and the cuticle of each female body was punctured with a scalpel blade behind the neck area to release pressure excess body tissue. A transverse cut midway through the female body was then made to separate the anterior/tail and posterior/head parts of the female. The tail area, containing the vulva and anus (perineum), tail terminus, phasmids, lateral lines and surrounding cuticular striae (perineal pattern) (Marais et al., 2017a) and its corresponding female's head area (Figure 3.1B). Both were transferred to a drop of glycerol on a glass microscope slide and a cover slide was placed over these structures. The cover slide was sealed with colourless nail polish (Cutex) and the microscope slide carefully labelled (Figure 3.1C and 3.1D), to enable examination and identification using a Nikon Eclipse 50*i* light microscope. From each of the six *Meloidogyne* spp. populations that were present in the six net houses, perineal patterns and oesophageal areas of 21 mature females were examined for identification purposes.

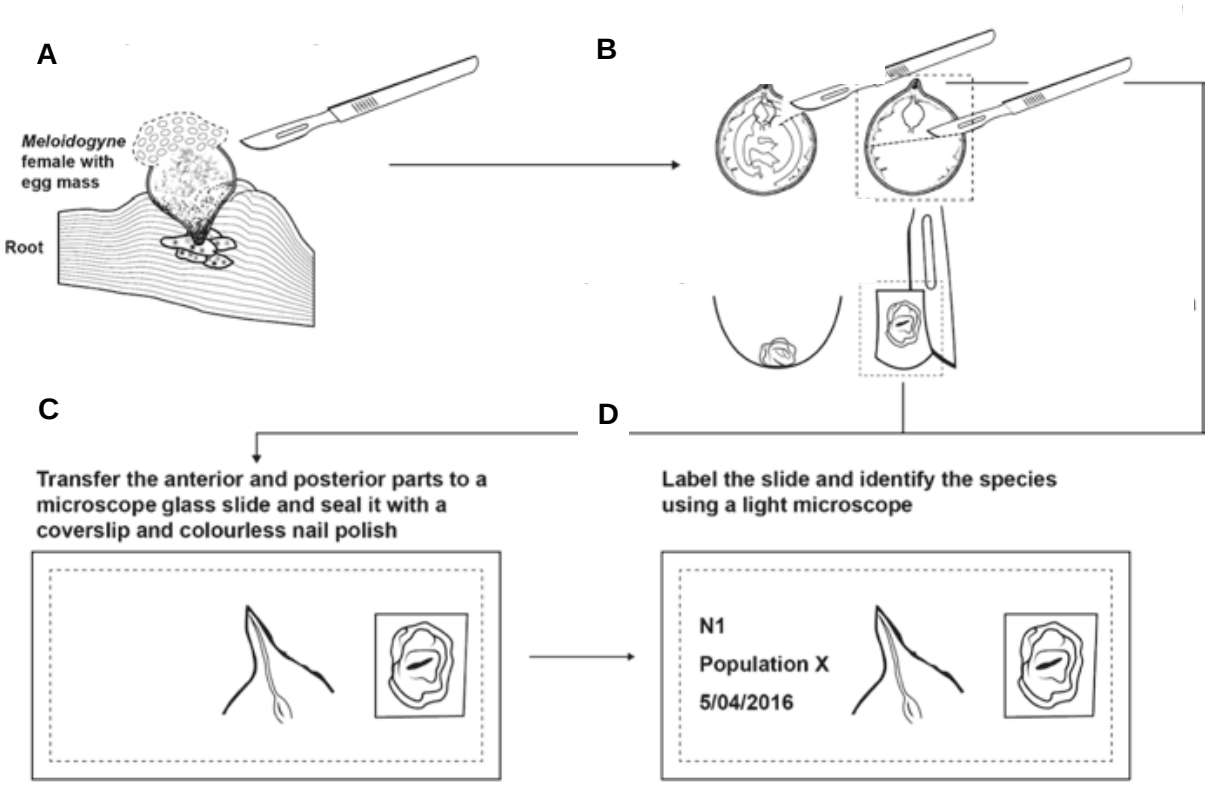

Figure 3.1: Illustrations of a technique used for mounting posterior and anterior ends of a mature *Meloidogyne* spp. female: (A) the female is removed from an infected root fragment, (B) the body is punctured, cut in half and the body content gently removed, and the cuticle around the perineal pattern is trimmed and (C) the oesophageal and perineal pattern of the females are mounted in glycerol on glass microscope slides and (D) clearly labelled. (Photo: adapted from Marais et al., 2017b).

3.2.4.2 Characteristics used to identify *Meloidogyne* spp.

Meloidogyne spp. were identified according to protocols of Yang and Eisenback (1983), Kleynhans (1991), Brito et al. (2004), Karssen et al. (2013) and Rashidifard et al. (2019a) and characteristics are summarised in Table 3.1.

Table 3.1. Morphological characteristics used to identify *Meloidogyne* spp. females isolated from infected tomato roots from six net houses obtained during the 2019/2020 season.

***Meloidogyne* spp.**	**Perineal-pattern morphology[1,2,3,4,5,6]**	**Shape of the lumen of the esophagus[1,3]**
M. arenaria (Neal, 1889) Cobb, 1890	Circular to oval, dorsal arch low to medium high, apex broadly rounded/squarish, short vertical lines may form along one/both lateral fields, around and outside phasmids.	Cylindrical or expands, narrows usually ovoid, occasionally spheroid metacorpus lining.
M. enterolobii, Yang and Eisenback, 1983	Round to ovoid; dorsal arch moderately high to high; apex usually rounded but square-like in some specimens; distinct phasmids in the tail-terminus, with striae found around phasmids in South African populations; lateral lines are usually lacking and/or not distinct with the exception that distinct lateral lines (similar to *M. javanica*) were observed occasionally in *M. enterolobii* specimens of a Florida and Mexico population, respectively; perivulval area is generally free of striae; striae on ventral area of pattern generally finer and smoother; tail tip is visible.	No information recorded for the lumen of oesophagus form/shape in pro- and metacorpus to be used as a discriminatory characteristic.
M. incognita (Kofoid and White, 1919) Chitwood, 1949	Circular to oval; dorsal arch medium high to high; apex broadly rounded or squarish; tail terminus clear/with some disordered phasmids.	Lumen lining expands, then narrows towards spheroid; occasionally visible as an ovoid metacorpus lining.
M. javanica (Treub, 1885) Chitwood, 1949	Circular to oval; dorsal arch low to medium high, apex squarish to broadly round; areas above lateral lines not bulged outwards; lateral lines usually visible as distinct double lines; distinct rectal punctuations also visible.	Procorpus lining usually cylindrical but may expand/narrow immediately in front of usually ovoid, occasionally spheroid metacorpus lining.

[1]Kleynhans (1991); [2]Yang and Eisenback (1983); [3]Brito et al. (2004); [4]Karssen et al. (2013); [5]Carillo-Fasio et al. (2018), [6]Rashidifard et al. (2019a)

3.2.5 Molecular identification

3.2.5.1 Extraction of DNA from females and the SCAR-PCR

The extraction of DNA from 15 *Meloidogyne* spp. females, per net house, that were contained in a single Eppendorf tube (see Paragraph 3.2.3) was done according to the

protocol of Musapa et al. (2013) using chelex-100. This included adding 30 µl chelex (5% w/v) and 5 µl proteinase K (20 mg ml-1) to each tube containing 15 females. The tubes were then placed on a vortexer for 1 min to mix and then centrifuged (Labnet Prism^{TM}) for 30 s at 13 500 rpm to settle the product at the bottom of each tube. The individual tubes, containing the RKN female homogenates, were then incubated at 56 °C for 2 h and then at 95 °C for 10 min before they were stored at -20 °C.

A Vacutec thermocycler (www.vacutec.co.za) was used to amplify the female DNA contained in each Eppendorf tube after they were removed from the -20 °C refrigerator. This was done by adding the following reagents/products to a new set of individual Eppendorf tubes: 25 µl of a PCR mix that was made up by adding 12.5 µl ready to use master mix (Promega Corporation, USA), 1 µl of the respective forward RKN species primers (10 µM), 1 µl of the respective reverse RKN species primer (10 µM), 5 µl of the female DNA of the respective net houses and 5.5 µl ddH_2O. Each of the *Meloidogyne* spp. forward and reverse SCAR primers followed the preceding programme throughout the PCR process, namely programmed for 2 min at 94 °C followed by 45 cycles of 30 s at 94 °C, 30 s at the following annealing temperatures: 1) 61 °C for Far/Rar primers, 2) 54 °C for the Finc/Rinc primers, 3) 64 °C for Fjav/Rjav and extension by 1 min at 72 °C for each species. *Meloidogyne enterolobii* primers followed the same procedure, only it had 35 cycles and its annealing temperature was 64 °C for the Fme/Rme primers (Zijlstra et al., 2000; Long et al., 2006). The primers codes are listed in Table 3.2.

The DNA of females of *M. enterolobii, M. incognita* and *M. javanica* (Rashidifard et al., 2019b), and *M. arenaria* (Visagie et al., 2018) were used as references during this study to establish whether such species were present in the six net houses studied (Table 3.3). The females from which such DNA were extracted by the latter, respective author groups were isolated from infected roots of various crops grown in South Africa as indicated in Table 3.3.

Table 3.2. Primer codes used for the identification of *Meloidogyne* spp. with their sequences, specificity, amplification size and reference sources.

Primer code	Primer sequence 5'-3'	Primer specificity	Reference
[1]**Far**	TCG GCG ATA GAG GTA AAT GAC	*M. arenaria*	Zijlstra et al. (2000)
[2]**Rar**	TCG GCG ATA GAC ACT ACA ACT		
Me-F	AAC TTT TGT GAA AGT GCC GCT G	*M. enterolobii*	Long et al. (2006)
Me-R	TCA GTT CAG GCA GGA TCA ACC		
Finc	CTC TGC CCA ATG AGC TGT CC	*M. incognita*	Zijlstra et al. (2000)
Rinc	CTC TGC CCT CAC ATT AGG		
Fjav	GGT GCG CGA TTG AAC TGA GC	*M. javanica*	Zijlstra et al. (2000)
Rjav	CAG GCC CTT CAG TGG AAC TAT AC		

[1]F=Forward primer; [2]R=Reverse primer

Table 3.3. DNA primers reference populations used.

Species	Life stage	Host crop	Origin (Country; Province; Population code)	DNA Fragment size (bp)
M. arenaria	Mature female	Tomato	South Africa; Limpopo, M36[1]	420
M. enterolobii	Mature female	Guava	South Africa; Mpumalanga, P8[2]	250
M. incognita	Mature female	Tomato	South Africa; Limpopo, P[2]	1200
M. javanica	Mature female	Tomato	South Africa; Limpopo, P[2]	700

[1]Population identified by Visagie et al. (2018); [2]Populations identified by Rashidifard et al. (2019b)

3.2.5.2 Gel electrophoresis

Subsequent to DNA amplification, 1% (m/v) agarose gel with TAE buffer was used to determine the DNA quality of the female products. This was achieved by adding 4 µl of PCR product from each sample represented by the six net houses into the individual wells of 1% agar gel. An O'GeneRuler™ 1kb DNA Ladder was loaded into the first well, on the left side, of the gel to show the size of the DNA bands for each of *Meloidogyne* spp. that were representative of the six net houses along with the reference populations used for this study. GelRed (www.biotium.com) was used to stain the DNA bands of the *Meloidogyne* spp. females and photographed by UV transilluminator to get visualised patterns. The gels containing the female DNA products were electrophoresed for 50 min at 80 V. Primers used for the sequencing reactions were the same as those used in the amplification step (Table 3.2).

3.3 Results

3.3.1 Morphological and morphometrical identification

Four *Meloidogyne* spp. *viz*. *M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica* were identified from the six net houses by means of morphological and morphometrical identification (Figure 3.2 - 3.5). Although it was relatively unchallenging to identify *M. arenaria*, *M. incognita* and *M. javanica* based on their respective perineal-pattern morphology mainly, it was difficult to be sure about the *M. enterolobii* female identification using this approach. However, the presence of the latter species was identified by RKN taxonomist Dr Mariette Marais (Agricultural Research Council–Plant Protection Research, Queenswood, South Africa).

Meloidogyne arenaria, *M. incognita* and *M. javanica* populations were detected in six net houses (Vlakfontein 2, Mellet 6, Makeppies 2, X-land, Denneland 2 and Ferreira 2), while *M. enterolobii* was identified in four net houses (Mellet 6, X-land, Denneland 2 and Ferreira 2) (Table 3.4).

Of the 126 females inspected across the six net houses for their oesophageal and perineal-pattern characteristics, *M. javanica* dominated (53), followed by *M. arenaria* (32), *M. incognita* (26) and *M*. *enterolobii* (13). For two of the females, the species could not be identified due to the non-optimal fixing and/or mounting of the anterior and posterior parts of the females on the slides.

According to the perineal-pattern morphology (Figure 3.2A), females belonging to *M. arenaria* had a circular to oval shape with apex broadly squarish and both lateral fields were present around and outside phasmids (Table 3.1). The general oesophageal identification (Figure 3.2B) was made upon the presence of an ovoid, metacorpus lining.

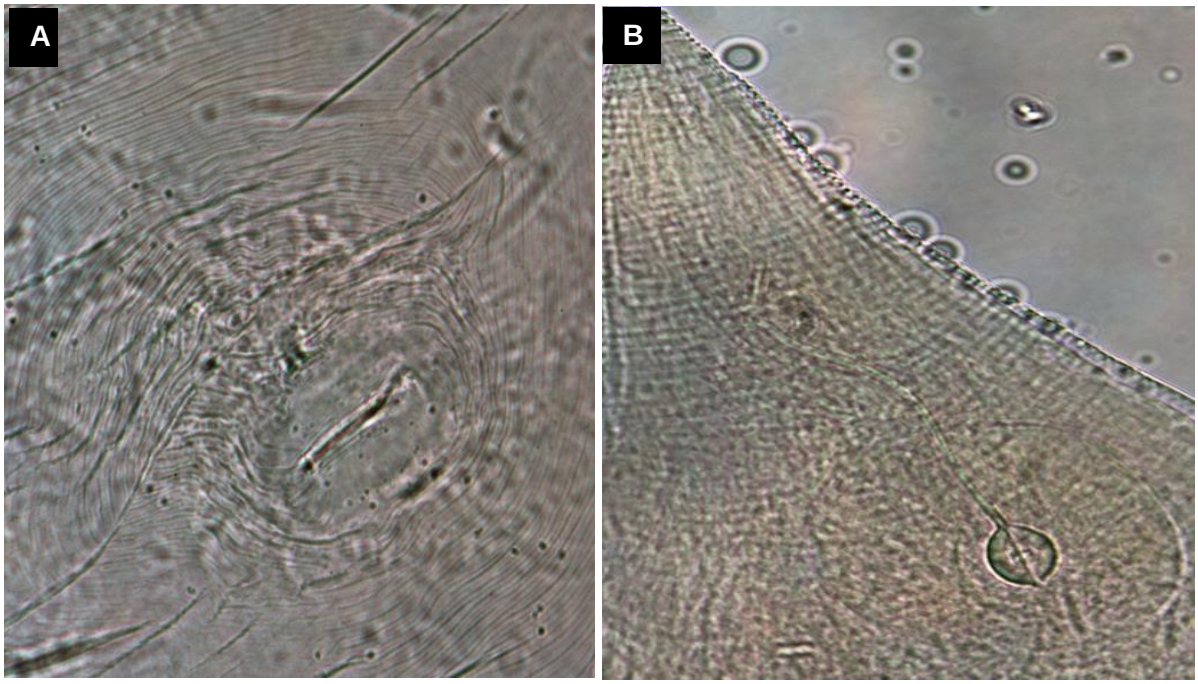

Figure 3.2: A perineal pattern (A) (from Vlakfontein net house) that resembles that of a *Meloidogyne arenaria* female (50x magnification using a stereomicroscope) and the oesophageal region (B) of the same specimen (50x magnification using a stereomicroscope) showing the lumen of the oesophagus (Photos: Lesego Matlala and Drieke Fourie, North-West University).

According to the perineal-pattern morphology (Figure 3.3A), females belonging to *M. javanica* had a circular to oval shape with medium high dorsal arch and apex squarish to broadly round. Lateral lines were distinct as double lines, and areas above these lines were not bulged out while rectal punctuations were also visible (Table 3.1). The general oesophageal identification (Figure 3.3B) was made upon an ovoid metacorpus lining being present.

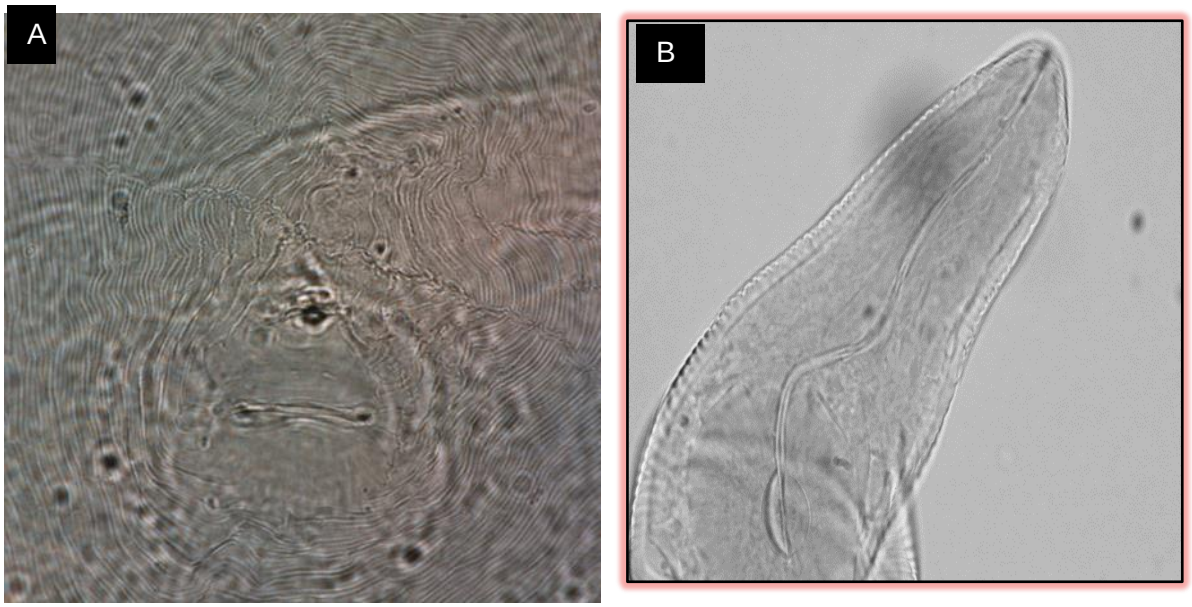

Figure 3.3: A perineal pattern (from Vlakfontein net house) that resembles a (A) *Meloidogyne javanica* female specimen (50x magnification using a stereomicroscope) and the oesophageal region (B) of the same specimen (50x magnification using a stereomicroscope) showing the lumen of the oesophagus (Photos: Lesego Matlala and Drieke Fourie, North-West University).

According to the perineal-pattern morphology (Figure 3.4A), females belonging to *M. incognita* had an oval shape with high dorsal arch and apex broadly squarish. The tail area terminus was clear/with some disordered phasmids (Table 3.1). The general oesophageal identification (Figure 3.4B) was made upon the presence of a spheroid metacorpus lining.

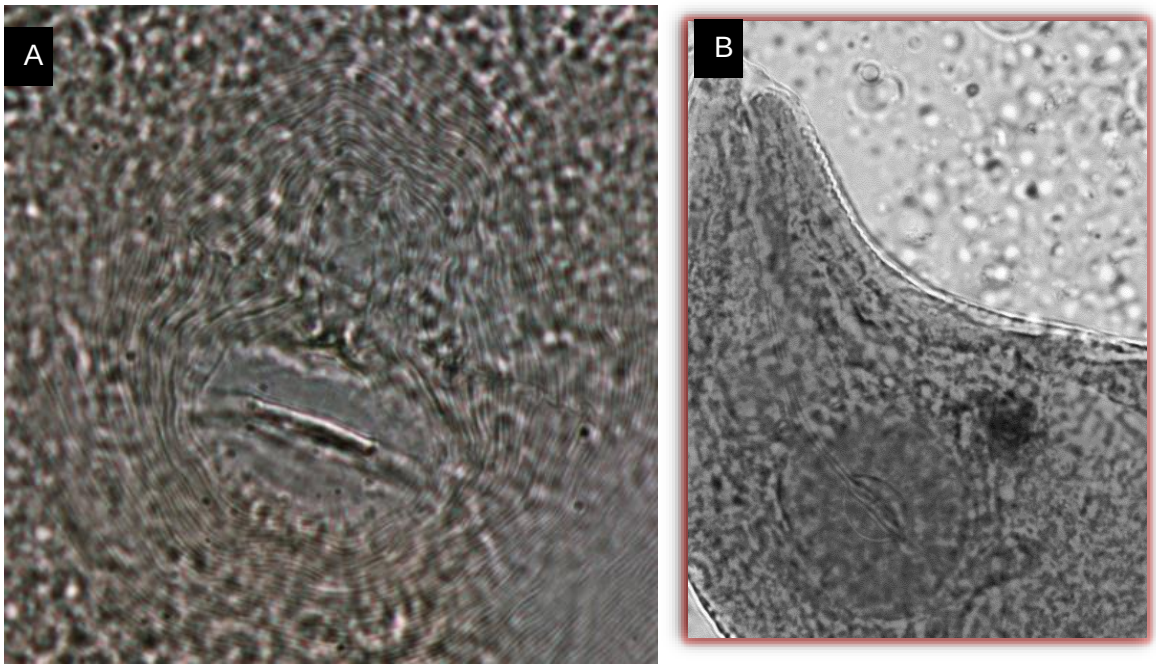

Figure 3.4: A perennial pattern (from Denneland net house) that resembles (A) a *Meloidogyne incognita* female specimen (50x magnification using a stereomicroscope) and the oesophageal region (B) of the same specimen (50x magnification using a stereomicroscope) showing the lumen of the oesophagus (Photos: Lesego Matlala and Drieke Fourie, North-West University).

According to the perineal-pattern morphology (Figure 3.5A), females belonging to *M. enterolobii* had an oval shape with high dorsal arch and apex broadly squarish. Distinct phasmids in the tail-terminus, with striae found around phasmids. Striae on ventral area of pattern generally finer and smoother (Table 3.1). The general oesophageal structures (Figure 3.5B) could not be used for identification since no information is available for this species.

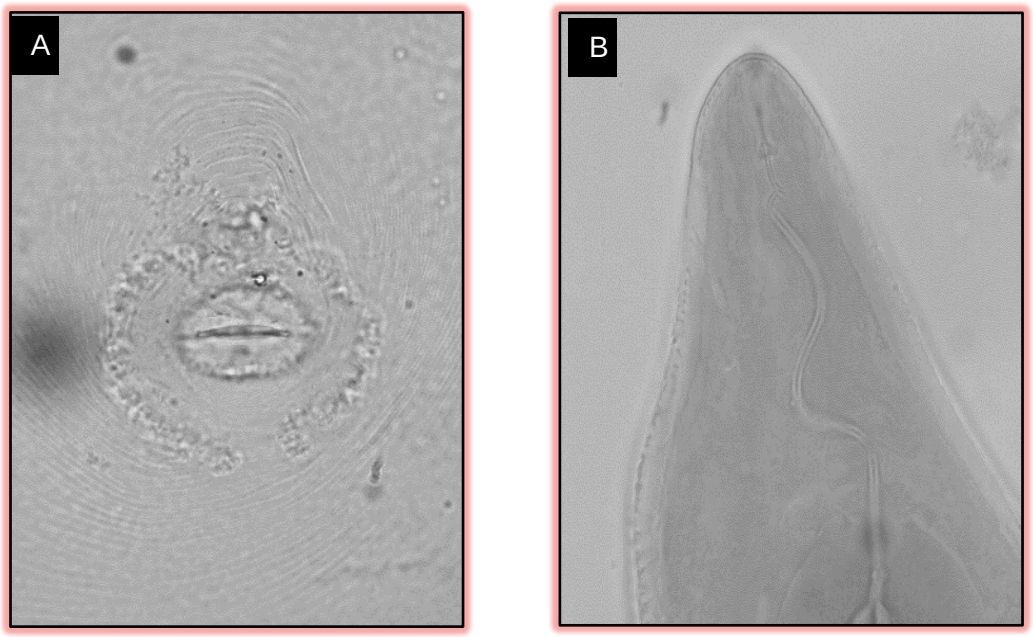

Figure 3.5: A perennial pattern that resembles (A) a *Meloidogyne enterolobii* female specimen (50x magnification using a stereomicroscope) and the oesophageal region (B) of the same specimen (50x magnification using a stereomicroscope) showing the lumen of the oesophagus (Photos: Drieke Fourie, North-West University).

3.3.2 Molecular species identification

The SCAR-PCR method also identified four *Meloidogyne* spp. (*M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica*) infecting tomato roots in the six net houses investigated in this study (Figure 3.6). These results verify the outcome of the morphological approach (Paragraph 3.3.1) revealing the identity of the same four species.

The PCR with the *M. arenaria* specific, forward and reverse SCAR primers resulted in the amplification of a 420 bp fragment for female DNA from five net houses (Vlakfontein, Mellet, Makeppies, Denneland and Ferreira) (Figure 3.6A) (Table 3.2; Zijlstra et al., 2000). The length of the SCAR fragments of these females mirrored those of the *M. arenaria* standard used and confirmed their identity (Table 3.3).

The DNA of the *M. incognita* females from two net houses (Denneland and Ferreira) resulted in the amplification of a 1 200 bp SCAR fragment following the PCR with the *M. incognita* species-specific primers (Table 3.2; Zijlstra et al., 2000); the lengths of

these SCAR fragments coincided with that of the standard *M. incognita* population (Table 3.3) verifying their identity.

Meloidogyne enterolobii was present in six net houses (Vlakfontein, Mellet, Makeppies, X-land, Denneland and Ferreira) with the 250 bp SCAR fragment being amplified by PCR using the species-specific markers (Table 3.2; Long et al., 2006). The lengths of the SCAR fragments of these *M. enterolobii* populations mirrored that of the standard *M. enterolobii* population (Table 3.3) verifying their identity.

Meloidogyne javanica was present in the six net houses (Vlakfontein, Mellet, Makeppies, X-land, Denneland and Ferreira) with the DNA of the females resulting in the amplification of the 700 bp SCAR fragment when the PCR was performed with the species-specific primers (Table 3.2; Zijlstra et al., 2000). The identity of the 14 populations containing *M. javanica* also corresponded with the DNA fragment of the *M. javanica* standard that was used as a positive control (Table 3.3).

All the net houses contained mixed *Meloidogyne* spp. that infected the tomato roots. Mixed communities of the three species *M. arenaria*, *M. enterolobii* and *M. javanica* were identified from the Vlakfontein 2, Mellet 6 and Makeppies 2 net houses. For X-land net house, a mixed community of *M. enterolobii* and *M. javanica* was identified, while in the Denneland 2 and Ferreira 2 net houses communities containing all four species were detected (Figure 3.6A and 3.6B; Table 3.4).

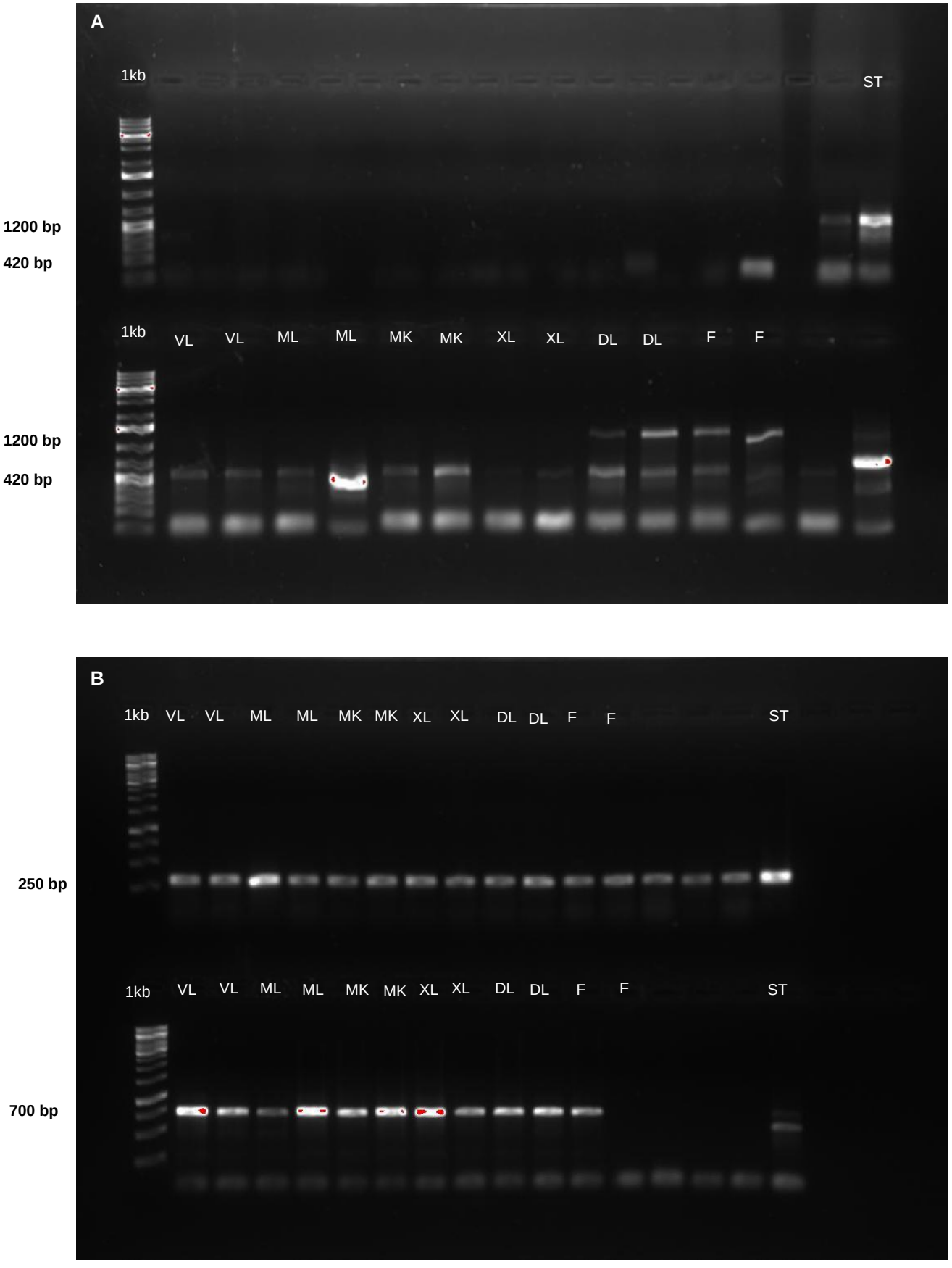

Figure 3.6: Gel photographs of DNA-fragment amplification products of *Meloidogyne* spp. females obtained from six net houses in which tomato crops were grown in the

Limpopo Province, South Africa using the Sequence Characterised Amplified Region – Polymerase Chain Reaction (SCAR-PCR). (A) included *M. arenaria* and *M. incognita*, (B) included *M. enterolobii* and *M. javanica;* A 1kb DNA ladder was included and ST denotes the DNA of females from the standard populations that were included for each of the four species identified. VL= Vlakfontein 2, ML= Mellet 6, MK= Makeppies 2, XL= X-land, DL= Denneland 2, F= Ferreira 2.

3.3.3 Molecular and morphological comparison

Comparison of results showed a 33% similarity between morphological and molecular identifications (Table 3.4). The abundance of the species differed per net house as can be seen by the percentage based on the 21 females identified per net house (Table 3.4).

According to both methods *M. javanica* was the most prevalent species occurring in all net houses, with a frequency varying from 8 to 83% (Table 3.4). *Meloidogyne arenaria* was detected in 5 of the 6 net houses using molecular techniques, whereas, the morphological methods showed it be present in all net houses (with frequencies varying from 8 to 50%).

Meloidogyne enterolobii was recovered from 4 of the 6 net houses (with frequencies varying from 5 to 33%) using the morphological methods compared to all net houses using the molecular techniques.

In contrast, *M. incognita* was identified in all net houses (with frequencies varying from 4 to 33%) using morphological methods compared to only 2 net houses when using the SCAR-PCR technique.

Table 3.4: Comparison of results obtained from morphological characteristics and molecular characterisation from six net houses.

Net houses	Species identified according to morphological data	Species identified according to molecular techniques	Comparison of results
Vlakfontein 2	*M. arenaria* (13%) *M. incognita* (4%) *M. javanica* (83%)	*M. arenaria* *M. enterolobii* *M. javanica*	Different
Mellet 6	*M. arenaria* (11%) *M. enterolobii* (5%) *M. incognita* (21%) *M. javanica* (63%)	*M. arenaria* *M. enterolobii* *M. javanica*	Different
Makeppies 2	*M. arenaria* (50%) *M. incognita* (33%) *M. javanica* (17%)	*M. arenaria* *M. enterolobii* *M. javanica*	Different
X-land	*M. arenaria* (18%) *M. enterolobii* (32%) *M. incognita* (18%) *M. javanica* (32%)	*M. enterolobii* *M. javanica*	Different
Denneland 2	*M. arenaria* (8%) *M. enterolobii* (8%) *M. incognita* (17%) *M. javanica* (67%)	*M. arenaria* *M. enterolobii* *M. incognita* *M. javanica*	Similar
Ferreira 2	*M. arenaria* (50%) *M. enterolobii* (13%) *M. incognita* (29%) *M. javanica* (8%)	*M. arenaria* *M. enterolobii* *M. incognita* *M. javanica*	Similar

3.4 Discussion

The four *Meloidogyne* spp. identified in this study, namely *M. arenaria*, *M. enterolobii*, *M. incognita*, and *M. javanica*, have previously been reported to occur in tomato fields in South Africa (Keetch and Buckley, 1984; Kleynhans et al.,1996; SAPPNS, 2020[1]) and other countries across the world (Kiewnick et al., 2013; Hallmann and Meressa, 2018). For example, the same four species were found to infect tomato in Mexico

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(Carrillo-Fasio et al., 2000; Cid del Prado et al., 2001; Martínez-Gallardo et al., 2015) In selected Sub-Saharan Africa (SSA) countries (Kenya, Nigeria, Tanzania and Uganda) these four species were also listed to parasitize tomato, while *Meloidogyne izalcoensis* Carneiro, Almeida, Gomes and Hernandez, 2005 was also reported for Tanzania (dos Santos et al., 2018). Interestingly is that *M. arenaria* was recovered for the first time from tomato roots in the Mooketsi area as a result of the present net house study and surprisingly parasitized the crop in all the net houses investigated.

Furthermore, all net houses investigated contained mixed communities of *Meloidogyne* spp.; representing two to four combinations of species. This is in agreement with a survey report by Rashidifard et al. (2019b) indicating that a mixed community of *M. enterolobii, M. incognita* and *M. javanica* occurred in tomato fields from the Mooketsi area where some of the net houses in the current study are located. In other SSA countries (Kenya, Nigeria, Tanzania and Uganda), single species populations dominated in peri-urban sites where tomato crops are grown, while two to three species were recorded to also occur in combinations; but to a lesser extent (dos Santos et al., 2018). In Mexico, up to two species occurred in combination in glasshouses where tomato was grown (Carrillo-Fasio et al., 2018). The presence of mixed species infecting a crop is not unusual since two or more root-knot nematode species are known to occur in one field (Karssen et al., 2013; Visagie et al., 2018; dos Santos et al., 2018; Rashidifard et al. 2019a, b).

Morphological identification enabled relatively easy distinction between *M. arenaria* and *M. javanica* females based on investigation of the oesophageal and perineal-pattern areas. However, identification of *M. enterolobii* females using perineal-pattern morphology was challenging; particularly to distinguish it from *M. incognita* females. This species shares similarities in perineal-pattern morphology with those of *M. incognita* which made positive identification in most cases very difficult (Brito et al., 2004; Hunt and Handoo, 2009). Nevertheless, to discriminate between the two species, the following in terms of the oesophageal area was considered for *M. enterolobii*: female stylet knobs are rounded, slightly sloping backwards and divided longitudinally by distinct grooves so each knob appears as two. Unfortunately, no distinctive characteristics have been published for *M. enterolobii* females regarding the lumen of the oesophagus; a characteristic that has been published by Kleynhans

(1991) to aid in discriminating among South African *Meloidogyne* spp. females. *Meloidogyne enterolobii* also seems to have an atypical perineal pattern, usually medium to high, with a pronounced square-like dorsal arch like that of *M. incognita* (Rashidifard et al., 2019a). However, the similarity in perineal-pattern morphology for *M. enterolobii* and *M. incognita* makes distinction particularly difficult and it is suggested as the reason why *M. enterolobii* was detected to a greater extent in the molecular than the morphological identification approaches in this study. Therefore, most probably *M. enterolobii* females in this study might have been misidentified as *M. incognita* using the perineal-pattern approach.

Molecular results verified the morphological outcome and also indicated that all four species were present in the net houses investigated in this study; but *M. incognita* was found less frequent relative to the other three species. This difference may be explained by *M. incognita* not occurring to a great extent (as was found with the morphological identifications) in this area and/or as a result of using only 15 females per net house for molecular analyses. The real extent of species' occurrence might also have been masked this way. The use of J2 and/or eggs that can be extracted from infected roots in high numbers and from which DNA can be extracted, may contribute towards obtaining a more accurate result (Visagie et al., 2018). It is also possible that other RKN species could be present since species-specific primers of only the four most commonly encountered species in the tropical and subtropical regions (*M. arenaria, M. enterolobii, M. incognita* and *M. javanica*) (Hallmann and Meressa, 2018) were used for the current study. The presence of the cryophilic *Meloidogyne hapla* Chitwood, 1949 was, for example, detected in tomato fields in Ethiopia, while the presence of the thermophile species *M. arenaria*, *M. incognita* and *M. javanica* was also confirmed using a combination of molecular and biochemical techniques (Seid et al., 2019).

Comparison of the SCAR-PCR method and morphological characterisation illustrated the efficiency and necessity of using both methods for accurate identification. However, while molecular methods have been recorded to give accurate and more precise diagnosis (Hunt and Handoo, 2009; Rashidifard et al., 2018), results from this study confirmed that using only morphological or only molecular analyses might produce inadequate/incomplete results. This was also observed in studies conducted

by Visagie et al. (2018) and Rashidifard et al. (2019a) where these author groups emphasised the importance of using both techniques to optimize results.

Meloidogyne arenaria, *M. incognita* and *M. javanica* were reported by Marais et al. (2017a) to be common in South African fields in most crops including tomato. The occurrence of *M. arenaria* (26%) and *M. incognita* (21%) in the present net house study was low relative to that of *M. javanica* (43%), which dominated according to molecular results. Similar trends were indicated by Marais et al. (2017a); *M. javanica* (7.4%) was more dominant, and in contrast to this study, however, they reported *M incognita* (5%) to be more prevalent in crop fields of South Africa than *M. arenaria* (0.4%). The predominance of *M. javanica* in crop fields in the northern parts of South Africa amount to 23% out of the 44% *Meloidogyne* spp. present in the province (SAPPNS, 2020[1]), hence agrees with results from this net house study where *M. javanica* prevailed in more net houses than did the other species. The adverse effect of this species to tomato production has been demonstrated in Spain where an initial population density of 4 750 J2 / 250 cm^3 resulted in 61% yield reduction under plastic-house conditions (Verdejo-Lucas et al., 1994). Coinciding with results of the present net house study that *M. javanica* dominated in tomato crops grown in net houses are results from a Sub-Suharan Africa (SSA) study that also reported *M. javanica* to dominate since it occurred at 52% of the peri-urban sites where tomato was grown. However, the SSA study was different from our study since the predominant prevalence of *M. javanica* was followed by *M. incognita* (26%), *M. arenaria* (16.5%), *M. enterolobii* (4%), *Meloidogyne iscoelensis* Carneiro, Almeida, Gomes and Hernández, 2005 (0.8%) and an undisclosed species (0.8%) (dos Santos et al., 2018).

Meloidogyne arenaria, known as the peanut root-knot nematode is considered amongst the four economically most important nematodes, and in South Africa it has been found in various agricultural fields, *viz*. potato (*Solanum tuberosum* L.), maize (*Zea mays* L.), soybean (*Glycine max* (L.) Merr.) (Fourie et al., 2017; Jones et al., 2017; Mc Donald et al., 2017; Visagie et al., 2018; SAPPNS, 2020[1]) and other plant

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species including *Bromus inermis* Leyss., *Citrullus lanatus* (Thunb.) Matsum. and Nakai (watermelon), *Crotalaria lanceolata* E. Mey. (Keetch and Buckley, 1984; Ntidi et al., 2012; SAPPNS, 2020[1]). This species is known to occur in warm areas; thus, it is not surprising that *M. arenaria* was found in the Mooketsi area (Limpopo Province) in tomato net houses.

Infection of tomato by *M. arenaria* may have not been regarded as severe as that by its thermophilic counterparts (*M. enterolobii*, *M. incognita* and *M. javanica*). However, the frequency of its occurrence as shown in the study indicates the ability of *M. arenaria* to cause severe damage to tomato crops. Since this is the first report indicating the presence of *M. arenaria* infecting tomato in the area the question arises where it came from. The species may have been introduced into the tomato fields by irrigation water or the planting of *M. arenaria* contaminated peanut pods obtained from other peanut-producing areas where it has been infected with this species (Kolte, 2017).

Meloidogyne enterolobii, has been spread widely in South Africa since it has been first identified from guava (*Psidium guajava* L.) roots in the Mpumalanga Province in the late 1990s (Willers, 1997). Although it was originally found on roots of guava trees it has now been recovered from a wide range of crops including tomato, potato, pepper (*Capsicum annum* L) maize, peanut (*Arachis hypogaea* L.) and spinach (*Spinacia oleracea* L.) (Onkendi et al., 2014; Visagie et al., 2018; Rashidifard et al., 2019b). According to Visagie et al. (2018) and Rashidifard et al. (2019b), this species poses more damage when it occurs together with other species in mixed communities. Although the local species may have a lower reproductive potential than their counterpart thermophile species (Rashidifard et al., 2019b), they have shown to render resistance traits in various other plant species that are resistant to *M. arenaria*, *M. incognita* and *M. javanica* ineffective and is referred to as a virulent species (Kiewnick et al., 2009). This species was present in all the net houses in the present study and, although it may not be as abundant, it is identified by Brito et al. (2007) as the most

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injurious species when compared to other thermophilic species. Interestingly, Carrillo-Fasio et al. (2018) reported that *M. enterolobii* dominated in terms of its prevalence in tomato-growing sites in Mexico; followed by *M. incognita*.

3.5 Conclusion

The approach of using both techniques (molecular and morphological) to identify *Meloidogyne* spp. has shown the importance to address inconsistencies between the outcomes of both methods. It is thus, recommended that both methods be used in combination to ensure that correct and adequate *Meloidogyne* spp. identifications can be done. To further optimise the consistency of both methods, the use of male and vermiform J2 life stages as part of morphological characterisation will be beneficial.

Findings from the study have generated accurate insight on the identity and distribution of *Meloidogyne* spp. in the net houses where tomato are grown in the Mooketsi area. The presence of mixed communities of *Meloidogyne* spp. in tomato net houses suggests the severe damage they can cause. This information will help the producer to take appropriate management actions which include resistant or more tolerant rootstocks. Furthermore, it will guide them to use complementary cropping practices (e.g. off-season cover/trap crops, biological control agents, and others) to keep *Meloidogyne* spp. densities low in tomato net houses to enable sustainable crop production.

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Chapter 4

Investigating the effect of pre-plant, crop production strategies on population densities of *Meloidogyne* and *Pratylenchus*: a microplot study

Abstract

Tomato production, under net house conditions, by South Africa's largest producer ZZ2 is limited due to nematode damage. Due to a shortened fallow period between the end of the previous and the next crop cycle, a build-up of nematode population densities became imminent. However, following the 'Natuurboedery/Nature Farming' principle of adding compost, organic material (OM), fermented plant extracts and other organic products, nematode infection only becomes evident 8-12 weeks after planting the follow-up tomato crop. The objective of the study was to determine which pre-plant strategies undertaken by ZZ2 producers to prepare net houses for a next tomato crop cycle delays infection in tomato roots. The trial layouts were randomized, complete block designs with 18 replicates and five treatments representing i) the end of crop cycle, ii) fallow, iii) ridge disking, iv) addition of organic material (compost, compost tea, fermented plant extracts and other organic products), and v) addition of OM combined with inoculation of the soil with ±2 000 infective, second-stage juveniles (J2) of *M. javanica*. Soils, representative of the treatments above, naturally infested with *Meloidogyne* and/or *Pratylenchus* were obtained from net houses located in a cold semi-arid (BSK) and a warm subtropical (CWA) area and transferred to 4-L pots that were arranged in a microplot setup. Soil from BSK net houses contained both *Meloidogyne* spp. and *Pratylenchus* spp. individuals, and those from CWA net houses only *Meloidogyn*e spp. individuals. One tomato seedling was transplanted to each pot and the effect of the treatments evaluated. Addition of OM to the potted BSK and CWA net house soils reduced *Meloidogyne* spp. densities by 84 and 40% in tomato roots, and by 54 and 39% in the soil, respectively. Addition of OM inoculated with ±2 000 infective *M. javanica* J2 and fallow (applied to soil from both net houses) showed a similar trend with reductions of *Meloidogyne* densities of 67 and 8% in tomato roots, and by 34 and 1% in the soil, respectively. Fallow and addition of OM resulted in a direct reduction of *Meloidogyne* spp. densities. *Pratylenchus* spp. densities were not affected by the treatments.

Keywords: Cultural practice, nature farming, monocropping, tomato.

4.1 Introduction

Tomato (*Solanum lycopersium* L.) is among the most popular vegetable crops grown and consumed worldwide (OECD, 2017). However, production of this crop is challenged by many pests and diseases. One of the major pests is root-knot nematodes (*Meloidogyne* Göldi, 1889), as they are the most common and damaging plant-parasitic nematodes (PPN) (Jones et al., 2013). They are polyphagous plant pathogens, able to cause damage in more than 5 500 plant species (Hussey and Janssen, 2002; Hallmann and Meressa, 2018). In tomato, yield losses can amount to 50% worldwide, whilst in South Africa, losses amounting to $US 216 million has been recorded (Nemlab, 2012). Annual yield losses world-wide in other crops is estimated to range between 12-20% although it can amount to 80% in some vegetable crops (Sasser and Freckman, 1987; Özarslandan et al., 2019). Their polyphagous feeding ability enables root-knot nematodes to be responsible for significant losses especially under intensive cultivation of susceptible crops, when precautions against population build up are not taken (Hallmann and Meressa, 2018; Özarslandan et al., 2019).

The four main *Meloidogyne* spp. found worldwide include: *Meloidogyne arenaria* (Neal 1889) Chitwood, 1949, *Meloidogyne incognita* (Kofoid and White, 1919), Chitwood, 1949, *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 and *Meloidogyne hapla* Chitwood, 1949 (Rashidifard, 2019). These species account for about 90% of total damage caused by this genus (Dammini Premachandra and Gowen, 2015). *Meloidogyne arenaria*, *M. incognita* and *M. javanica* are commonly found in tropical areas (and referred to as thermophiles), whereas, *M. hapla* is more common in temperate and/or cold areas (Oka, 2010; Renčo, 2013) and referred to as a cryophile (Jones et al., 2013). In South Africa, *M. javanica* and *M. incognita* are predominant and can cause severe damage in vegetable cultivation (Onkendi et al*.,* 2014; Jones et al., 2017).

The short life cycle and abundant reproductive ability of *Meloidogyne* spp. as well as their wide host range are the main reasons why control is so difficult once it has invaded the vegetable fields (Hallmann and Meressa, 2018). The damage observed on the host plant root system is measured by the galling, hence, it is a general indication of *Meloidogyne* spp. population density in the rhizosphere (soil and roots) (Charegani et al*.,* 2012).

Numerous nematode control strategies have been used to minimize the effect of nematode pests to crop yield/quality losses, with effective results obtained by using methods such as nematicides, crop rotation, resistant varieties, non-hosts, organic amendments, antagonistic crops and biological control (predators and parasites of nematode pests) (Dutta et al., 2019). Important, however, is to take into consideration that each method has limitations regarding its implementation and efficacy (Renčo, 2013). And, it is accepted that the different control methods should be integrated into a crop management system to enable optimal protection of plants against nematode pests (Massawe, 2010; Collange et al*.,* 2011).

Environmental and human concerns have brought forth a decline in the use of chemical nematicides, thus resulting in an increased search for alternative control measures to manage nematode pests (Collange et al*.*, 2011). Crop rotation is one of the popular methods used for nematode management, even though it has limitations due to the wide host range of particularly *Meloidogyne* spp., the need of various cropping practises, equipment requirements, specific climatic requirements for specific crops and the specific market value of a crop (Kratochivil et al., 2004; Chen and Tsay, 2006; Manneh, 2016). The efficiency of this method is dependent on knowledge of the target species present in the field in order to select a poor- or non-host plant (Hill, 1988). Chen and Tsay (2006) observed that rotating crops should not only focus on reducing densities of the target nematode pest, but also other PPN present. They reported that including maize (*Zea mays* L.) in a strawberry- (*Fragaria ananassa* Duchesne) based system reduced root-knot nematode densities but increased those of root-lesion nematodes.

The use of organic amendments is another strategy that have been used widely to control PPN. They are also popular due to their benefit of improving soil structure and fertility, releasing nemato-toxins and increasing populations of fungal and bacterial organisms (Thoden et al., 2011; Seid et al., 2015; Manneh, 2016). Soil micro-organisms are generally stimulated by the addition of organic materials, thus reducing the population densities of PPN (Renčo, 2013; Lutuf et al., 2018). The use of manure amendments, including cattle manure, chicken manure and castor bean (*Ricinus communis* L.) extract, have for example been widely studied for their nematicidal

properties (Massawe, 2010; Oka, 2010; Renčo and Kovacik, 2012; Renčo, 2013). Manneh (2016) also reported on the effect of fresh sweet orange (*Citrus sinensis* L.) and cassava (*Manihot esculenta* Crantz) peels that were effective in reducing root-knot nematode densities.

Another alternative approach to combat nematode pests is the use of compost; it provides soil with nutrients and organic matter, also aiding in suppressing soil-borne diseases (Oka, 2010). Micro-organisms in compost play a role in plant disease management, but the efficacy of these amendments in reducing nematode population densities will vary with each nematode species, type and content of the amendments used. Also, the time allowed after application is crucial since some composts will release nemato-toxins slowly while others might release it quicker (Oka 2010; Renčo, 2013).

With continuous monocropping, which is commonly practiced under intensive agricultural production, it is often impossible to apply many of the traditional, often short-term, nematode management strategies because of time and space constraints, and ultimately financial considerations. Soil biodiversity is often unbalanced under monocropping and pests such as PPN are likely to become more abundant (Malherbe, 2016). Nevertheless, due to human population increases that are directly coupled with a concurrent higher demand for food, as well as the combined problem of land shortage, monocropping seems to be the only solution for producers.

Although extensive monocropping is practised by the largest tomato producer in South Africa, Bertie Van Zyl (Pty) Ltd., also known as ZZ2, the farming practices of this enterprise are aimed at abandoning some of the deleterious aspects of industrialised farming. The principle of ‘Natuurboedery/Nature Farming’ followed by ZZ2 addresses the decline of soil health through the use of, amongst other things, biologically active compost, compost tea and effective microbes (EM). As part of their intergrated pest management approach, the use of fermented plant extracts (FPE) manufactured from plants with medicinal properties such as, *Lantana* L., is promoted to replace harsh chemicals for the control of nematode pests. Carbon-loaded fertiliser and organic materials are also used to deter the harmful effects of fertiliser on soil microbes (Taurayi, 2011).

In the farming system used by ZZ2, it was observed that, regardless of high root-knot nematodes recorded at the end of a crop cycle, the first infected plants in a follow-up cycle were only observed two months after planting. Hence, pre-plant strategies followed when preparing soils for the next planting were suggested to have an impact on the development and reproduction of root-knot nematodes and other plant-parasitic genera. Thus, the objective of this study was to determine which pre-plant strategies are effective in delaying the build-up of root-knot and root-lesion nematode densities by means of micro-plot experiments.

4.2 Materials and methods

4.2.1 Experimental site

The study was conducted under microplot conditions at the Natuurboerdery Research Centre, Bertie Van Zyl Pty Ltd (ZZ2) Mooketsi, Limpopo Province (23° 36' 11.459" S, 30° 6' 18.133" E) (Figure 4.1). This area is geographically defined as a warm, dry, subtropical area (CWA).

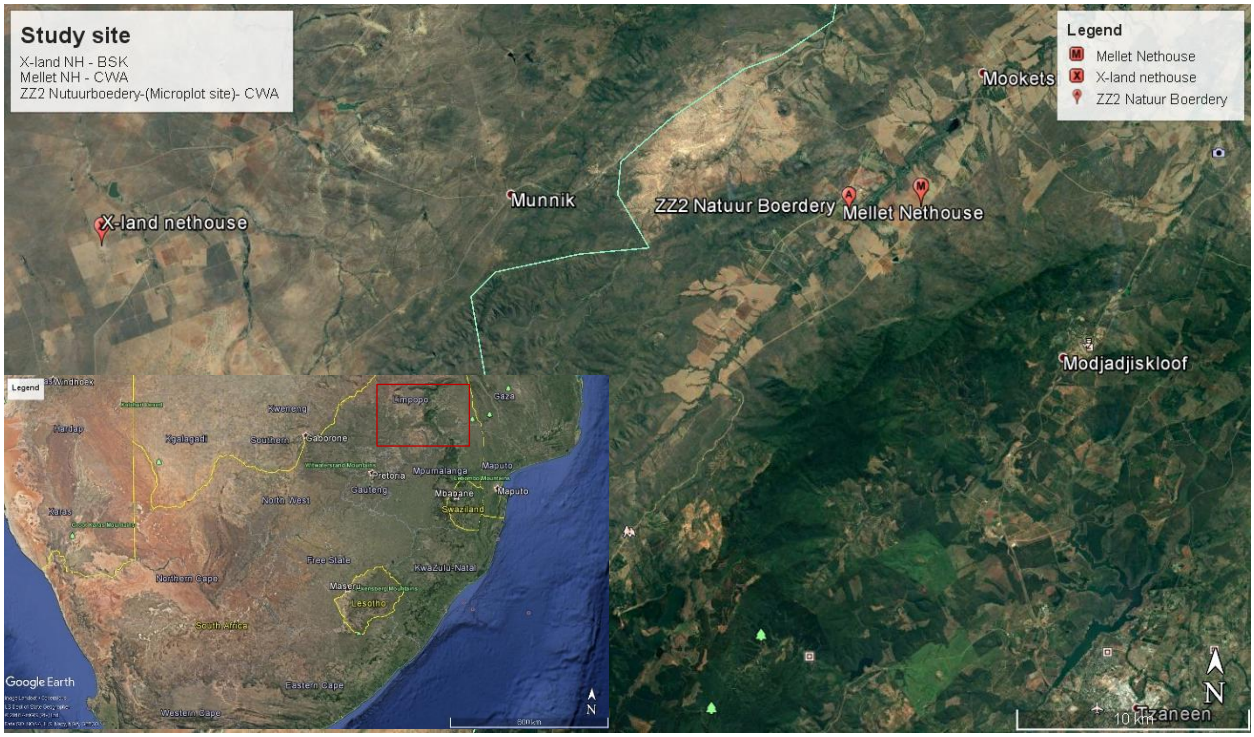

Figure 4.1: Map of Limpopo Province, South Africa, depicting the study area at ZZ2 Natuurboedery and two net houses (X-land and Mellet) where soils were collected.

4.2.2 Experimental procedure, treatments and design

Soil naturally infested with *Meloidogyne* spp. was collected from X-land net house (23° 39' 34.87" S 29° 42' 01.50" E) situated in the cold semi-arid area (BSK) and Mellet net house (23° 38' 27.41" S 30° 04' 25.57" E) situated in the dry, warm subtropical area (CWA) (Table 4.1; Figure 4.1). The different pre-plant strategies practiced in these net houses are as follows: tomato plant rests are removed at the end of each crop cycle, after which the soil is left fallow until soil preparation for next cycle starts (normally constituting a period of about three months). Soil preparation includes ploughing, discing and finally just prior to planting, addition of compost and other organic materials [(fermented plant extract, compost tea, effective micro-organisms (EM)]. Treatments for the microplot trial mimicked the different pre-plant strategies with soil obtained, every time at the same place, from the net houses and transferred to individual pots. The treatments hence represented the following:

1. Treatment 1: Soil collected at the end of the crop cycle during removal of tomato plant rests were planted immediately with one tomato seedling per pot. This treatment represented the control.
2. Treatment 2: Soil collected at the end of the crop cycle during removal of tomato plant rests was left fallow in the microplots and one tomato seedling per pot was only planted when Treatment 4 and 5 commenced (16 weeks after Treatment 1 commenced).
3. Treatment 3: Soil collected after disking of ridges but before any organic material/product was added to the soil, was planted with one tomato seedling per pot immediately after collection of the soil (nine weeks after Treatment 1).
4. Treatment 4: Soil collected a day before planting in the net house when compost, mulch and other organic components had been added, with one - tomato seedling per pot planted immediately after collection of the soil (16 weeks after Treatment 1).
5. Treatment 5: Soil collected a day before planting in the net house when compost, mulch and other organic components had been added, was planted with one tomato seedling per pot immediately after collection of the soil, but this soil was inoculated with ±2 000 J2 of *M. javanica* per pot a week after planting (16 weeks after Treatment 1) (*M. javanica* were added to investigate the effect of organic components specifically on root-knot nematodes since other pre plant practices also have an effect on root-knot nematode).

The soils for each of these treatments were added to 4L capacity plastic pots which were buried in elevated soil ridges (closed off with concrete walls) to represent a micro-plot setup (Figure 4.2). Pots were placed at 8 cm intra-row and 7 cm inter-row spacings. One six-week old root-knot nematode susceptible tomato seedling (cultivar Romanita obtained from Hishtil Nursery, South Africa) (Stevens, 2018) was transplanted into each pot at time intervals as indicated in the treatment section above. The pots were irrigated with 250 mL tap water as needed, which was usually twice a day. MULTIFEED® P 5:2:4 (43) (at the prescribed dosage rate: 4-6 kg/ha) was applied to each pot every two weeks using a drench application. The layout of both experiments (BSK and CWA) was a randomized complete block design (RCBD) with 18 replicates for each treatment. Six replicates each were used for evaluation at 6, 12 and 18 weeks after transplanting, respectively. Soils from both net houses were collected prior trial initiation (at the end of crop cycle) and nematode population were determined (Table 4.1).

Table 4.1. Information about two tomato net houses (located in two distinct climatic areas in the Mooketsi area in the Limpopo Province) from which soils were obtained for a microplot study and the status of population densities of nematode genera present in soils to investigate the effect of pre-plant production practices on *Meloidogyne* and *Pratylenchus* densities.

	X- land net house	**Mellet net house**
Climatic region	**Cold semi-arid (BSK)**	**Warm, dry sub-tropical (CWA)**
GPS coordinates	23˚ 39’ 34.87” S	23˚ 38’ 27.41” S
	29˚ 42’ 01.50” E	30˚ 04’ 25.57” E
Average temperature (ºC)	17.86	20.96
Average rainfall (mm)	297.8	852.37
Soil texture	Course sand	Loamy sand
Altitude (m)	1183	770
Previous crop cycles (years)	5	4
Plant-parasitic nematodes		
Criconema	0	2000*
Meloidogyne	4500	6500
Pratylenchus	9000	0
Beneficial, non-parasitic nematodes		
Acrobeloides	1500	1500
Panagrolaimus	1500	500

Tylenchus	1500	0

*Although *Criconema* was found in high numbers in the pre-plant soil sample, *Criconema* number were very low in the trial and therefore not considered.

Figure 4.2: A microplot setup of soil, naturally infested with plant-parasitic nematodes, obtained from two tomato net houses (located in two distinct climatic areas in the Mooketsi area in the Limpopo Province) and used for a microplot study to investigate the effect of pre-plant production practices on *Meloidogyne* and *Pratylenchus* densities. (Photo: Lesego Matlala, ZZ2 Natuurboedery).

4.2.3 Preparation and application of *Meloidogyne javanica*

4.2.3.1 Rearing *Meloidogyne javanica*

The colony of a *M. javanica* population was obtained from tomato roots of 'Money maker' from the Green Technologies Research Centre, University of Limpopo, South Africa (23° 53′ 10″ S, 29° 44′ 15″ E). The egg masses were colonised and reared in 30, 25 cm^3 plastic pots in roots of a *M. javanica* susceptible tomato cultivar Romanita (Stevens, 2018) in a net house at the Natuurboedery/Nature Farming research centre of the ZZ2 farm.

4.2.3.2 Extraction of Eggs and second-stage juvenile (J2)

Eggs and second-stage juveniles (J2) of *M. javanica* were extracted from roots of net house grown susceptible tomato plants (cultivar 'Romanita') by using the adopted NaOCl method (Riekert, 1995). The root systems were cut off from the aerial parts of each infected plant and gently rinsed free of soil and adhering debris under running tap water. Roots were then cut into 1-cm pieces and thoroughly mixed after which root samples were shaken for 4 min in 500 ml of a 1% NaOCl solution. The NaOCl mixture, containing eggs and J2, was decanted on a 250-aperture mesh sieve that was nested on a stacked range of sieves, which from top to bottom consisted of the 250-, 38- and 25-µm-mesh sieves. Root fragments on the top 250-µm-mesh sieve were washed thoroughly for about 4 min with running tap water. Eggs and J2 were collected on the 38-µm and 25-µm aperture mesh sieves (solution combined) and dispensed into a 100-ml sample beaker. A Peter's counting slide (Marais et al., 2017) and Nikon light microscope set at 10x and 40x magnification were used to count the eggs and J2 to prepare the inoculum.

4.2.3.3 Inoculation of *Meloidogyne javanica* to potted soil

Soil samples collected as treatment 5 described in Paragraph 4.2.2 were inoculated by creating holes around the root system and dispensing approximately ±2 000 eggs and J2 of *M. javanica* using a pipette. After dispersal, holes created were covered with soil.

4.2.4 Data collection

Six plants from each treatment were removed at 6, 12, 18 weeks after tomato seedlings were transplanted (see Paragraph 4.2.2). Variables *viz*, plant height; stem diameter; fresh and dry shoot mass; and fresh root mass were measured for each plant at each termination interval. Plant height was measured from the soil surface to the tip of the flag leaf, shoots were cut at the soil surface and stem diameters measured 5 cm above the severed ends using a Mac-Afric 150mm digital Vernier caliper (manufactured by Mac-Afric). Shoots were oven-dried at 70 °C using Series 2000 scientific oven (manufactured by Scientific) for 24 hours and weighed. The root system of each plant was removed from each of the pots and immersed in water to remove soil particles and debris. The RKN gall index was rated as percentage galls present on the root system. Nematodes from roots (30 g) and 250 cm^3 rhizosphere

soil were extracted from each root system using the sugar-flotation (for roots) and decanting and sieving, followed by the sugar-flotation technique (for soil) as described by Swart and Marais (2017).

4.2.5 Counting of nematodes and eggs

The nematode-water suspension in the beaker was adjusted to 100 mL by adding (depending on the initial volume of the suspension) tap water to each suspension. The suspension was homogenized by blowing air into it using a pipette. One millilitre (1 mL) aliquot of the nematode-water suspension was collected from each sample bottle using a pipette, placed onto a Peter's counting slide and counted using Nikon light microscope. For each sample, two aliquots of 1 mL were counted as explained above; the means of the two counts multiplied by 100 were then calculated and extrapolated to obtain the number of nematodes and eggs per 30 g of roots and number of nematodes per 250 cm^3 of soil.

4.2.6 Statistical analyses

Data of both experiments, an initial and a repeat, were first analysed using Analyses of Variance (ANOVA). Thereafter, the dataset containing the data of the two experiments were subjected to Factorial Analysis of Variance (Statistica, version 10; www.statsoft.com) and means were separated with Tuckey's HSD Test ($P \leq 0.05$). Treatments represented Factor 1 and net house locations (BSK and CWA) Factor 2. Nematode counts were $\log_{10}(x+1)$ transformed before statistical analysis to reduce variation. Significant variables were further assessed using relative impact, which was expressed as: Relative impact (RI) = $(\frac{Nematode\ per\ cultural\ practice}{Nematode\ of\ end\ of\ crop\ cycle} - 1) \times 100$, where an increase in nematode densities is depicted through positive (+) values and a decrease through negative (-) values.

4.3 Results

4.3.1 Nematode densities in roots from BSK net house

Tomato roots grown in potted soil obtained from the BSK net house data showed significant differences ($P \leq 0.05$) among the pre-application practices/treatments for both *Meloidogyne* and *Pratylenchus* population densities (Table 4.2). For *Meloidogyne*, fallowing (Treatment 2) as well as the addition of organic material with

and without the addition of additional *M. javanica* inoculum (Treatments 5 and 4, respectively) resulted in significantly lower *Meloidogyne* densities compared to the ridge disking (Treatment 3) and the control treatment (Treatment 1: soils obtained at the end of the crop cycle). Addition of organic material without the addition of *M. javanica* J2 inoculum (Treatment 4) was superior in reducing *Meloidogyne* densities by 84%, followed by addition of organic material with *M. javanica* inoculum (67%) and fallow (49%).

For *Pratylenchus*, none of the pre-application practices/treatments resulted in a reduction in population densities of this genus compared to the control (Treatment 1; soil obtained at the end of crop cycle), which had the lowest (Table 4.2). By contrast, the application of all treatments showed an increase in lesion nematode densities with Treatment 4 (addition of organic material without the addition of *M. javanica* J2 inoculum) showing the lowest increase in densities (63%) which was significantly ($P \leq 0.05$) lower than that of all the other treatments, except the control. Ridge disking (Treatment 3) showed the highest increase in lesion nematode densities (157%).

The number of eggs per 30 g roots was not significantly different among the treatments although ridge disking (Treatment 3) reduced it by 15% and the addition of organic material with, and without the addition of *M. javanica* J2 inoculum (Treatment 4 and 5) by 23 and 7%, respectively (Table 4.2). Treatment 2 (fallow) resulted in a numerical (but not significant) increase in *M. javanica* egg numbers.

Table 4.2. Numbers of *Meloidogyne, Pratylenchus* and eggs in 30 g roots ($log_{10}(x+1)$ transformed) and percent reduction (compared to the untreated control from log transformed nematode mean data) for the different treatments applied to soil collected from the BSK net house.

Treatment	***Meloidogyne***		***Pratylenchus***		**Eggs**	
	Population density (30 g roots) [y]	% Relative Impact: increase (+) or decrease (-) [z]	Population density (30 g roots)	% Relative Impact: increase (+) or decrease (-)	Population density (30 g roots)	% Relative Impact: increase (+) or decrease (-)
1 - End of crop cycle (control)	2.84* (969** ± 1.09***) a	-	1.41 (439 ± 0.99) c	-	2.70 (953 ± 0.36) a	-
2 - Fallow	1.44 (305 ± 1.09) b	-49	3.45 (3 496 ± 0.99) a	+145	2.88 (2 852 ± 0.36) a	+7
3 - Ridge disking	2.77 (1 862 ± 1.09) a	-3	3.62 (5 101 ± 0.99) a	+157	2.29 (4 355 ± 0.36) a	-15
4 - Addition of organic material	0.47 (42 ± 1.09) b	-84	2.30 (824 ± 0.99) b	+63	2.08 (1 093 ± 0.36) a	-23
5 - Addition of organic material + ±2 000 *M. javanica* J2	0.94 (209 ± 1.09) b	-67	3.42 (3 500 ± 0.99) a	+143	2.52 (1 437 ± 0.36) a	-7
P-value	**0.001**		**0.001**		0.0986	

*Log_{10} (x+1) transformed nematode mean data; **Real means in parenthesis followed by the ***Standard Deviation; [Y]Letters that differ for treatments indicate a significant difference at $P \leq 0.05$ according to Tukey HSD-test according ANOVA, [Z]Relative Impact = [(cultural practice/end of crop cycle – 1) *100].

4.3.2 Nematode densities in roots from CWA net house

Tomato roots obtained from CWA net house showed significant ($P \leq 0.05$) differences among the pre-application practices (i.e. treatments) for *Meloidogyne* population density and egg numbers (Table 4.3). Fallowing (Treatment 2) as well as the addition of organic material with ±2 000 *M. javanica* egg and J2 inoculum (Treatment 5) resulted in significantly lower *Meloidogyne* population densities relative to ridge disking (Treatment 3), but not compared to the control (Treatment 1: soil obtained at the end of crop cycle). Addition of organic material without the addition of *M. javanica* J2 inoculum (Treatment 4) was most effective in significantly reducing *Meloidogyne* densities by 40%, followed by addition of organic material with *M. javanica* inoculum (8%) and fallow (5%).

Pratylenchus numbers in this net house were very low, therefore, this data warrant no further discussion.

The number of eggs was significantly ($P \leq 0.05$) different for the pre-application practices (Table 4.3). Only addition of organic material without *M. javanica* inoculum (Treatment 4) reduced the egg densities by 13%. Fallowing (Treatment 2), ridge disking (Treatment 3) and addition of organic material with the addition of *M. javanica* J2 inoculum (Treatment 5) showed no significant changes in egg numbers.

Table 4.3. Numbers of *Meloidogyne, Pratylenchus* and eggs in 30 g roots ($log_{10}(x+1)$ transformed) and percent reduction (compared to the untreated control from log transformed nematode mean data) for the different treatments applied to soil collected from the CWA net house.

Treatment	***Meloidogyne***		***Pratylenchus***		**Eggs**	
	Population density (30 g roots) [y]	% Relative Impact: increase (+) or decrease (-) [z]	Population density (30 g roots)	% Relative Impact: increase (+) or decrease (-)	Population density (30 g roots)	% Relative Impact: increase (+) or decrease (-)
1 - End of crop cycle (control)	3.26* (2 545** ± 0.64***) ab	–	0.27 (17 ± 0.16) a	-	2.48 (5 439 ± 0.35) ab	-
2 - Fallow	3.11 (3 756 ± 0.64) b	-5	0a	-100	2.67 (60 117 ± 0.35) ab	+8
3 - Ridge disking	3.71 (18 475 ± 0.64) a	+14	0a	-100	3.14 (17 473 ± 0.35) a	+27
4 - Addition of organic material	1.97 (916 ± 0.64) c	-40	0a	-100	2.17 (2 723 ± 0.35) b	-13
5 - Addition of organic material + ±2 000 *M. javanica* J2	2.99 (1 809 ± 0.64) b	-8	0.32 (14 ± 0.16) a	+19	2.56 (3 818 ± 0.35) ab	+3
P-value	**0.001**		0.0705		**0.0153**	

*Log_{10} (x+1) transformed nematode mean data; **Real means in parenthesis followed by the ***Standard Deviation; [Y]Letters that differ indicate a significant difference between treatments at $P \leq 0.05$ according to Tukkey HSD-test ANOVA, [Z]Relative Impact = [(cultural practice/end of crop cycle – 1) *100].

4.3.3 Interaction between nematodes in roots from BSK and CWA

Significant interactions ($P \leq 0.05$) were observed for treatments*weeks for *Meloidogyne* spp. population densities for both net houses (X-land, BSK and Mellet, CWA), while a the same was evident for treatments*weeks*location (when data of the two net houses were analysed together) (Table 4.4).

Significant interactions ($P \leq 0.05$) were observed for treatments*weeks for *Pratylenchus* for the X-land (BSK) net house, but not for the Mellet (CWA) net house, while a siginficant ($P \leq 0.05$) interaction existed for treatments*weeks*location (when data of the two net houses were analysed together) (Table 4.4).

For eggs signifcant interactions were observed for treatments*weeks for both net houses, while the same was evident for treatments*weeks*location (when data of the two net houses were analysed together) (Table 4.4).

Table 4.4. Interaction data for *Meloidogyne* and *Pratylenchus* numbers ($log_{10}(x+1)$ transformed) per 30 g roots to evaluate the effect of pre-plant production practices and their abundance in soils obtained from two tomato net houses (located in two distinct climatic areas in the Mooketsi area in the Limpopo Province) in a microplot study.

		Meloidogyne		***Pratylenchus***		**Eggs**	
		F-value	*P*	F-value	*P*	F-value	*P*
X-land nethouse (BSK - cold semi-arid area)	**Treatment[1]**	18.81	**0.0001**	53.23	**0.0001**	2.04	0.0986
	Weeks[2]	4.97	**0.0098**	10.81	**0.0001**	10.87	**0.0001**
	Treatments*Weeks	2.92	**0.0077**	16.79	**0.0001**	11.05	**0.0001**
Mellet nethouse (CWA - warm, dry sub-tropical area)	**Treatments**	25.04	**0.0001**	2.26	0.0705	3.30	**0.0153**
	Weeks	55.68	**0.0001**	0.17	0.8412	164.85	**0.0001**
	Treatments*Weeks	10.10	**0.0001**	1.86	0.0788	2.71	**0.0113**
X-land x Mellet nethouses	**Treatments**	36.99	**0.0001**	30.83	**0.0001**	3.40	**0.0111**
	Weeks	18.42	**0.0001**	6.28	**0.0025**	73.24	**0.0001**
	Location	127.31	**0.0001**	1168.91	**0.0001**	2.01	0.1582
	Treatments*Weeks*Location	4.40	**0.0001**	13.75	**0.0001**	7.78	**0.0001**

[1]Treatments = pre-plant production practices; [2]Weeks = duration of trial until termination (6 week interval x 3).

4.3.4 Nematode densities in soil from BSK and CWA net houses

Potted soil from BSK net house showed no significant differences ($P \leq 0.05$) among the pre-application practices for *Meloidogyne* population densities (Table 4.5). While it may appear that *Meloidogyne*, in pre-application treatment 2 (fallow), 4 and 5 (addition of organic material without and with the addition of *M. javanica* J2 inoculum) reduced the population by 58, 53 and 34%, respectively, these were not significantly different to the control (Treatment 1). Treatment 3 (ridge disking) increased the population density by 34%.

For *Pratylenchus,* however, an increase in population densities for all the pre-application practices was evident (Table 4.5). The control (Treatment 1; soil obtained at the end of the crop cycle) had the lowest numbers differing significantly ($P \leq 0.05$) from the fallow treatment (Treatment 2), but not from the other treatments.

Potted soils from CWA showed significant differences ($P \leq 0.05$) in *Meloidogyne* populations amongst the pre-application practices (Table 4.5). Treatments 2 (fallow) and 3 (ridge disking) increased population densities by 4 and 57%, respectively. On the contrary, treatments 4 and 5 (addition of organic material with J2 inoculum and without the addition of *M. javanica* J2 inoculum, respectively) reduced population densities by 36 and 1%.

Pratylenchus numbers were very low or specimens of the genus was not present at all and therefore this data warrant no further discussion.

Table 4.5. Numbers of *Meloidogyne, Pratylenchus* in 250 cm^3 soil ($log_{10}(x+1)$ transformed) and percent reduction (compared to the untreated control from log transformed nematode mean data) for the different treatments applied to soil collected from BSK and CWA net house in a microplot study.

Treatment	BSK				CWA			
	Meloidogyne		***Pratylenchus***		***Meloidogyne***		***Pratylenchus***	
	Population density (250 cm^3 soil) [y]	% Relative Impact: increase (+) or decrease (-) [z]	Population density (250 cm^3 soil)	% Relative Impact: increase (+) or decrease (-)	Population density (250 cm^3 soil)	% Relative Impact: increase (+) or decrease (-)	Population density (250 cm^3 soil)	% Relative Impact: increase (+) or decrease (-)
1 - End of crop cycle (control)	0.92* (61** ± 0.37***) a	-	0.55 (28 ± 0.35) b	-	1.76 (483 ± 0.59) b	-	0.02 (0.1 ± 0.06) a	-
2 - Fallow	0.39 (28 ± 0.37) a	-58	1.56 (122 ± 0.35) a	+184	1.83 (867 ± 0.59) a	+4	0 a	-100
3 - Ridge disking	1.23 (189 ± 0.37) a	+34	0.91 (50 ± 0.35) ab	+66	2.76 (3 219 ± 0.59) a	+57	0a	-100
4 - Addition of organic material	0.43 (28 ± 0.37) a	-53	0.88 (61 ± 0.35) ab	+60	1.12 (222 ± 0.59) b	-36	0a	-100
5 - Addition of organic material + ±2 000 *M. javanica* J2	0.61 (50 ± 0.37) a	-34	1.17 (78 ± 0.35) ab	+113	1.74 (272 ± 0.59) b	-1	0.13 (11 ± 0.06) a	+550
P-value	0.0797		0.0507		**0.001**		0.465	

*Log_{10} (x+1) transformed nematode mean data; **Real means in parenthesis followed by the ***Standard Deviation; [Y]Letters that differ indicate a significant difference at P ≤ 0.05 between the treatments according to Tuckey HSD-test according to ANOVA, [Z]Impact = [(cultural practice/end of crop cycle – 1) *100

4.3.5 Interaction between nematodes in soil from BSK and CWA

There was no significant interaction (*P*≤0.05) observed for the X-land net house (BSK) for both *Meloidogyne* and *Pratylenchus* regarding treatment*weeks (Table 4.6).

From CWA, a significant interaction (*P*≤0.05) was observed for treatments*weeks for *Meloidogyne* but not for *Pratylenchus* (Table 4.6).

When both net house (BSK and CWA) were compared significant differences (*P*≤0.05) were observed for treatments, weeks and locations for both genera but no significant interaction (*P*≤0.05) between treatment*weeks*location was observed (Table 4.6).

Table 4.6. Interaction data for *Meloidogyne* and *Pratylenchus* numbers ($log_{10}(x+1)$ transformed) per 250 cm^3 soil to evaluate the effect of pre-plant production practices and their abundance in soils obtained from two tomato net houses (located in two distinct climatic areas in the Mooketsi area in the Limpopo Province) in a microplot study.

		Meloidogyne		***Pratylenchus***	
		F-ratio	*P*	F-ratio	*P*
X-land net house (BSK - cold semi-arid area)	**Treatment[1]**	2.19	0.0797	2.51	0.0507
	Weeks[2]	2.76	0.0709	5.18	**0.0082**
	Treatments*Weeks	1.91	0.0731	1.68	0.1193
Mellet net house (CWA - warm, dry sub-tropical area)	**Treatments**	7.97	**0.0001**	0.91	0.4650
	Weeks	39.71	**0.0001**	0.82	0.4449
	Treatments*Weeks	2.10	**0.0469**	0.99	0.4484
X-land x Mellet net houses	**Treatments**	7.68	**0.0001**	2.66	**0.0353**
	Weeks	22.14	**0.0001**	5.80	**0.0038**
	Location	59.39	**0.0001**	83.05	**0.0001**
	Treatments*Weeks*Location	1.71	0.1024	1.59	0.1338

[1]Treatments = pre-plant production practices; [2]Weeks = duration of trial until termination (6 week interval x 3)

4.3.6 Plant data

Significant differences (*P*≤0.05) were observed for tomato potted in soil obtained from both the X-land (BSK) and the Mellet (CWA) net houses amongst the pre-application practices for plant height, stem diameter, fresh shoot mass, dry shoot mass, fresh root mass and RKN gall index (Table 4.7). From the X-land nethouse (BSK), tomato plants of Treatment 1 (soil obtained at the end of the crop cycle; control) showed superior

increases in plant height, stem diameter, fresh and dry shoot mass, fresh root mass and gall index. For the RKN gall index, Treatment 4 (addition of organic material without the addition of *M. javanica* J2 inoculum) showed the lowest rating that differed significantly ($P \leq 0.05$) from the control (Treatment 1) and ridge disking (Treatment 3).

At CWA, Treatment 1 (soil obtained at the end of the crop cycle; control) had significantly ($P \leq 0.05$) higher stem diameter, fresh and dry shoot mass, fresh and dry root mass values compared to the other treatments. The other treatments did not differ from one another for these parameters except for fresh shoot mass for Treatment 3 (ridge disking) being significantly ($P \leq 0.05$) higher. Gall index showed significant ($P \leq 0.05$) differences with Treatment 3 (ridge disking) having a significantly higher gall index than the other treatments except from Treatment 1 (soil obtained at the end of crop cycle; control). Treatment 4 (addition of organic material without the addition of *M. javanica* J2 inoculum) had the lowest gall index (Table 4.7).

Table 4.7. Influence of pre-plant production practices to potted tomato cv. Romanita from two tomato net houses (located in two distinct climatic areas in the Mooketsi area in the Limpopo Province) in a microplot study**.**

Treatments	**PH (cm)**	**SD (mm)**	**FSM (g)**	**DSM (g)**	**FRM (g)**	**Gall index (%)**
X-land net house (BSK)						
1 - End of crop cycle (control)	96.9 a	8.7 a	131.0 a	28.6 a	25.7 a	19.7 a
2 - Fallow	73.6 b	6.8 b	47.7 b	20.0 a	13.9 b	2.4 b
3 - Ridge disking	81.2 b	7.7 b	60.1 b	18.6 b	13.9 b	25.5 a
4 - Addition of organic material	71.2 b	7.0 b	40.5 b	18.0 b	9.4 b	0.9 b
5 - Addition of organic material + ±2 000 *M. javanica* J2	75.2 b	7.1 b	45.1 b	18.9 b	11.7 b	6.6 b
P-value	**0.001**	**0.001**	**0.001**	**0.0128**	**0.001**	**0.001**
Mellet net house (CWA)						
1 - End of crop cycle (control)	87.0 a	8.1 a	140.2 a	22.5 a	26.1 a	42.1 ab
2 - Fallow	73.7 b	6.8 b	49.5 c	13.3 b	16.1 b	39.5 b
3 - Ridge disking	75.2 ab	6.7 b	97.2 b	14.9 b	14.1 b	64.2 a
4 - Addition of organic material	76.5 ab	6.7 b	49.3 c	13.4 b	14.2 b	11.2 c
5 - Addition of organic material + ±2 000 *M. javanica* J2	74.6 b	6.9 b	51.6 c	12.9 b	13.4 b	28.9 bc
P-value	**0.0146**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**

PH: plant height, SD: stem diameter, FSM: fresh shoot mass, DSM: dry shoot mass, FRM: fresh root mass

4.4 Discussion

Treatments applied in this study were selected based on practices followed by the ZZ2 farmers in terms of the soil preparation for the planting of new/follow-up tomato crops in the net houses. At the end of the crop cycle, severly galled, RKN infected tomato roots were present in the net houses. However, when investigating roots two months after planting, RKN galling was substantially lower than anticipated, indicating that the farmland preparation adversely influenced nematode development.

Two net houses where RKN were previously causing severe problems and high root galling was observed, were selected for the trials. These net houses were situated in two different climatic regions with similar pre-plant strategies followed in both net houses. Each step represented a different treatment. Nematode analyses of soil obtained at the end of crop cycle, representing Treatment 1, as denoted in Table 4.1 demonstrated the nematode community composition at both sites. This diversity and composition may be influenced by tomato cultivars planted prior to trial initiation, while the fluctuations may also have resulted from the different soil types, climate (Table 4.1) and rootstocks used. Adediran et al. (2005) and Grabau et al. (2017) emphasized the importance of environmental factors influencing nematode population densities and diversity in the soil. Another reason soils from BSK had more root-lesion nematodes, is that *Pratylenchus* prefers cooler temperatures (Smiley, 2010), while *M. javanica* is a cryophilic species preferring warmer temperature for reproduction (Chavez et al., 2014). Soil from the BSK net house was incidentally collected during winter months when temperatures were lower, favouring reproduction of root-lesion nematode.

The first step in soil preparation between two crop cycles is a fallow period of 16 weeks. This treatment seemed to reduce *Meloidogyne* population densities significantly in roots and soil of the BSK net house while in the CWA net house it was less clear. The efficacy of bare fallow as denoted by Duncan (1986), is dependent on the climatic and soil conditions, and nematode species which might explain why the level of reduction in *Meloidogyne* population densities was not the same in soil collected in these two net houses. Population densities of *Pratylenchus* from BSK net house were not reduced by the bare fallow treatment in both root and soil (Table 4.2 and 4.5). Kroese et al. (2016) showed that *Pratylenchus penetrans* (Cobb, 1917)

Filipjev and Schuurmans Stekhoven,1941, was able to survive in old root material for up to 8 months.

The second step in the soil preparation is ridge disking. Ridges (30 cm high) are made after which disking cuts open these ridges when preparing land for planting the new crop. Soil samples taken during this stage showed high population densities of *Meloidogyne* in both trials and high *Pratylenchus* densities from the BSK net house in both roots and soil (Table 4.2, 4.3 and 4.5). The high population densities is suggested to result from nematodes that had migrated to deeper layers but were brought to the surface by the ridge discing. Miigration of lesion nematodes into deeper layers could either be due to irrigation that enabled the movement of nematodes deeper into the soil profile or deep tillage practices that transposed soil layers. Results of the current study conform to those of Özarslandan et al. (2019) who demostrated that nematodes survived in deeper soil layers (>20 cm), after soil solarisation, but migrated upwards in the next cropping cycle in reaction to root exudates of green pepper (*Capsicum* L.).

Manneh (2016) indicated that organic amendments applied to soil, enhanced crop productivity by stimulating soil micro-organisms and reducing PPN population densities as well as improving soil fertility; influencing its chemical, physical and biological properties. Addition of organic material to the potted BSK and CWA net house soils reduced *Meloidogyne* spp. densities by 84 and 40% in tomato roots, and by 53 and 36% in the soil. Even with the artificial inoculation of ±2 000 infective, J2 of *M. javanica,* the OM treatment was able to keep *Meloidogyne* population levels lower than those in the control treatment; with 67 and 8% reduction respectively. Fallow in both net houses showed a similar trend. The effects of Treatment 4 (addition of organic material without the addition of *M. javanica* J2 inoculum) on *Pratylenchus* populations densities were superior in BSK net house, resulting in the lowest increase in densities (63%) compared to all the other treatments.

The efficacy of organic material application varies with the nematode pest species present together with the dosage and length of the application (Massawe, 2010; Collange et al., 2011). A report from Thoden et al. (2011) illustrated that addition of compost had no effect on populations of root-lesion nematodes while in some cases population densities increased. Their results are in agreement with results of the

present study with respect to soil collected from the BSK net house. *Pratylenchus* species numbers were not inhibited by the addition of organic material, on the contrary, their population was enhanced. In the soil collected from the CWA net house, *Pratylenchus* numbers were too low to draw any conclusions.

Once decomposition of the organic material has taken place, organisms that are antagonistic or predatory to nematodes increase and they release toxic susbtances to which are spread through the soil pore spaces. At each irrigation schedule soluble fractions and other products released from plants move through the pore spaces killing nematodes (Youssef and El-Nagdi, 2010). The mechanism discussed by Youssef and El-Nagdi (2010) could explain observations made in our study . Even when *M. javanica* was added to the soil with organic material its reproduction was hindered.

Root gall indices are used to indicate the level of host susceptibility of the tomato plants in reaction to the treatments and determine the level of *Meloidogyne* infection. In the present study, soil obtained at the end of crop cycle (Treatment 1) and ridge disking (Treatment 3) showed high root galling relative to other treatments, indicating increased initial RKN populations for both treatments (Table 4.7). The results are in accordance with those of Charegani et al. (2012), who illustrated that increased initial densities of RKN results in an increased number of eggs and galls per root in cucumber (*Cucumis sativus* L.) and tomato.

Soil obtained at the end of crop cycle (Treatment 1) resulted in better plant growth relative to the other treatments at 6, 12 and 18 weeks after planting. Although, all the treatments were terminated at the same amount of weeks after planting, the planting dates differed, with Treatment 1 being planted 16 weeks prior to Treatments 2, 4 and 5, due to current farm practices. This resulted in a difference in climatic conditions, specifically, minimum and maximum temperatures. This could have played a role in the superior growth reaction of this treatment.

4.5 Conclusions

Management of PPN require an integrated control approach. Results from this study showed that each step taken as part of land preparation impacted on the population development of PPN, particularly for *Meloidogyne*. The bare fallow that followed the

end of crop cycle treatment (Treatment 1) was able to reduce RKN population densities. The additon of organic material as a standard practice followed by ZZ2 farmers showed to have substaintially delayed *Meloidogyne* infection of tomato roots. This practice showed tob e the most effective measure. However, since this step follows the bare fallow and ridge disking, the outcome is probably an effect of a combination of all the steps undertaken.

The principle of ‘Natuurboedery/Nature Farming’ adopted by ZZ2 is of great importance especially under net house production with limited fallow periods and a tomato production period of 35 to 40 weeks. Highly galled roots, due to RKN parasitism, at the end of a crop cycle and reinfection only observed 8 to 12 weeks after transplant does indicate that the farmers are doing something right. Futhermore, the next step to follow this is to find out which of the added material compost, compost tea, EM, fermented plant extracts (Garneem, Lantana) is responsible for the delayed RKN infection.

4.6 Reference

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Chapter 5

5.1 Conclusions and Recommendations

Production of tomato over the years has been moving towards protected structures due to its beneficial advantages of prolonged harvest, improved quality of fruits, efficient use of resources and limited incidences of pests and diseases (Sharma et al., 2009; Ramasamy and Ravishankar, 2018). However, despite the many economic benefits, most of the soilborne pests such as plant-parasitic nematodes (PPN), dominate especially under extensive monocropping (Sabir and Walia, 2017). The commonly known PPN under tomato production is root-knot nematodes (RKN), which are very aggressive and have a wide host range (Jones et al., 2017). This pest has been observed in the largest tomato production area grown under net house structures by ZZ2 farmers, in the Limpopo Province, South Africa. Thus, the focus of this study was therefore to determine the identity of RKN species and their population dynamics in these net houses over a period of three years and to investigate the effect of pre-plant strategies on RKN development under tomato production systems.

The importance of determining PPN with special reference to RKN and their population development in tomato production over time was highlighted in Chapter 2 of this dissertation, using physio-chemical soil, cultivar, planting date and climatic data. Species identification of RKN from these net houses was further done using morphological (perineal pattern and oesophageal structure) and the molecular sequence derived amplified region – polymerase chain reaction (SCAR-PCR) approaches (Chapter 3). Farmer's pre-plant strategies were further investigated in Chapter 4 as each practice was analysed in a microplot study to identify the most effective approach.

There were 56 nematode genera, of which 11 represented PPN and 45 NPN, two NPN families (Criconematidae and Dolichodoridae) and two NPN sub-families (Dorylaiminae and Dolichodoridae) identified from pre-plant soil samples of ZZ2 net houses representing a baseline study. Regardless of the biodiversity, RKN was the most abundant genus and did not seem to be influenced by competition of other

genera present in the rhizospheres of tomato plants. The study revealed that RKN occured more in sandy soils, an observation that agrees with reports from Ferji et al. (2005) for banana (*Musa* L.) and Kim et al. (2017) for carrot (*Daucus carota* L.). Furthermore, RKN were abundant in soils that had higher percentages of P, Mg:K and Na:K ratios. Reduced soil infestion and root infection by RKN was associated with soil that had higher K, Ca, pH and acid saturation levels. Ngeno et al. (2019) illustrated that high levels of P and Mg were associated with high population densities of *Meloidogyne exigua* Göldi, 1887, while high levels of Ca in the soil were associated with lower nematode damage to crop roots confirming our results. On the contrary, *Helicotylenchus* spp. were associated with higher K and Ca contents in soil as was also observed in a study on sugarcane by Cadet et al. (2002). The % RKN infection relating to rootstock and scion selection suggested that RS2, RS3 and RS13 were tolerant, whereas, ZZX125, RS11, ZZX132 and ZZX64 were more susceptible. Planting date did not seem to have any influence on the buildup of nematode populations. However, over the three years of this study, there was a progressive increase in RKN population densities in all net houses.

Morphological identification (oesophageal areas and perineal-patterns) and molecular identifcation (SCAR-PCR) successfully identified four thermophilic *Meloidogyne* spp. *viz*. *M. arenaria*, *M. enterolobii*, *M. incognita*, and *M. javanica* from the infected tomato roots sampled from the net houses. There was a 33% similarity between morphological and molecular identifications indicating the efficiency and necessity of using both methods for accurate identification. Morphological identification of *M. incognita* and *M. enterolobii* was challenging due to species similarities, thus it is possible that *M. enterolobii* was misidentified before. Other authors have, however, also indicated differences in results between morphological and molecular identifications (Visagie et al., 2018; Rashidifard et al., 2019). In the six net houses a mixture of *Meloidogyne* spp. (ranging from two to four) was present. This is also not surprising as many authors have found *Meloidogyne* to occur in mixed communities (dos Santos et al., 2018; Visagie et al., 2018; Rashidifard et al., 2019).

Both the morphological and molecular approaches revealed *M. javanica* to be the most abundant species, which was not surprising as it has been recorded as the most

prevalent species in Limpopo Province among *Meloidogyne* spp. (SAPPNS, 2020[1]) The spatial distribution of *M. arenaria* in the Mooketsi area is also notable, especially because it has never been reported before from this area (SAPPNS, 2020[1]).

The delayed infection of tomato roots by mixed RKN species, after transplanting the seedlings, necesitated the analyses of pre-plant strategies that are commonly followed by ZZ2 farmers. Two net houses with different physio-chemical soil characteristics and climatic conditions were thus considered. Infested soil from X-land net house, located in the cold semi-arid area (BSK), had a mixture of RKN and *Pratylenchus* spp., while Mellet net house located in the warm subtropical area (CWA) only had RKN. From both net houses, it was observed that addition of organic amendments was effective in delaying RKN infection of tomato roots. However, since this step is preceeded by bare fallow and ridge disking, a combination of all the practice contributed to the delay.

The overall spatial distribution of PPN in ZZ2 net houses has also been revealed in the study. There are various factors that contribute to the population build up and increased infestation levels of PPN in these net houses. The highest infestions of RKN were correlated with sandy soil and high ratios of Mg:K, Na:K and %Mg. The highest incidence of *M. arenaria* was found in Makeppies NH, which was comprised of sandy soil. Furthermore, reduced RKN infestation levels were associated with sandy loam soil, also having the highest Ca, K, pH and acid saturation levels. These net houses were dominated by *M. javanica*.

It is worth noting that none of the four RKN species identified as a result of this study were found on its own, but occurred in combination. The dominance of particular species may depend on many factors of which rootstock and scion selection, presence and abundance of other PPN and temperature regimes are just a few.

[1]Dr Mariette Marais of the Nematology Unit, Biosystematics Division, Agricultural Research Council–Plant Protection Research is thanked for the use of data from the South African Plant-Parasitic Nematode Survey (SAPPNS) database; E-mail: maraism@arc.agric.za

Recommendations:

Since pre-plant practices are mandatory at ZZ2, farmers can improve on the selection of organic amendments to increase the tolerance or resistance of tomato plants to economically important PPN, particularly RKN. Knowledge of the RKN species present will help in rootstock selection, since some species are resistant to the *Mi* gene; rootstocks exhibiting this gene may hence not be planted in some extreme environments as they may not be effective (Grabau et al., 2017). Additionally, cover crops and trap crops can be implemented in the waiting period between a harvested and fallow-up crop (Collange et al., 2011). It is also important to optimally perform farm management practices such as irrigation and humidity control to reduce stress to the tomato plants which makes them more susceptible to RKN infections (Ornat and Sorribas, 2008).

Future Studies:

The pre-plant strategies tested in Chapter 4 had an influence on the RKN re-infection of tomato roots, and it is suggested that each of the organic amendments added to the net houses should be studied in isolation to identify which one is more effective against RKN species. Identification of the use of different combinations of organic amendments is another option since this approach maybe even be more effective in reducing RKN population densities in nethouses.

Furthermore, in the current study, only six net houses were used for identification of the RKN species. A more intense study including more net houses might reveal other RKN species or mixtures of RKN communities and might confirm the most abundant *Meloidogyne* species. While only four thermophilic species (*M. arenaria*, *M. enterolobii*, *M. incognita*, and *M. javanica*) were identified, more species including some cryophilic species might be present but in this study, we only tested using SCAR-PCR for the presence of the four above-mentioned species.

Rootstock screening for tolerance and/resistance to the four identified RKN species should be done on a continuous basis as this is one of the major pillars in RKN control and will help in nematode management.

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Addendum 1

Table 1: Abundance and frequency of occurrence of plant-parasitic and non-parasitic nematode genera in 250 cm^3 soil samples from 26 net houses located in the Limpopo Province (South Africa) over a 3-year period.

Genus/ Family/ Subfamily	Mean population densities	%Frequency of occurrence
Meloidogyne	45890	27.7
Criconematidae	4890	28.1
Mesorhabditis	3320	16.9
Helicotylenchus	2530	20.6
Acrobeles	2290	30.4
Acrobeloides	2205	30.7
Aphelenchus	2000	30.4
Tylenchorhynchus	1895	19.6
Tylenchus	1710	16.6
Cruznema	1665	10.1
Aphelenchoides	1375	18.6
Cephalobus	1215	20.6
Chiloplacus	1050	17.2
Panagrolaimus	1005	13.2
Ditylenchus	985	17.9
Seleborca	940	13.5
Zeldia	830	11.5
Scutellonema	825	13.5
Nanidorus	770	12.5
Pratylenchus	570	12.9
Elaphonema	520	7.1
Dorylaiminae	465	14.9
Aporcelaimellus	320	9.8
Eucephalobus	235	4.1
Stegella	205	5.4
Tobrilus	196	2.7
Discolaimoides	185	7.8
Discolaimium	160	4.4
Rotylenchus	155	3.0
Tylencholaimus	145	5.1
Plectus	145	3.7
Rhabditis	145	2.7
Dorylaimellus	125	3.7
Psilenchus	105	3.4
Aporcelaimus	95	5.1
Alaimus	80	2.0
Butlerius	70	1.0
Cervidellus	70	1.7

Eudorylaimus	70	3.7
Mylonchulus	70	2.4
Discolaminae	65	3.4
Paraxonchium	60	2.7
Xiphinema	40	0.7
Geocenamus	35	1.0
Xiphinema	30	1.4
Leptonchus	25	1.0
Diploscapter	25	0.3
Celeborca	20	0.3
Koeerneria	20	0.3
Mononchus	20	0.7
Thornema	20	1.0
Dolichodoridae	15	1.0
Fictor	15	1.0
Longidorus	15	0.6
Xiphinemella	10	0.7
Tyleptus	10	0.7
Hoplolaimus	5	0.3
Pungentus	5	0.3
Anaplectus	5	0.3
Monhystera	5	0.3
Prodorylaimus	5	0.3

Appendix A

DECLARATION OF LANGUAGE EDITING

Language editing statement

TO WHOM THIS MAY CONCERN

I, Prof. Hendrika (Driekie) Fourie, hereby declare that the dissertation titled: **"Population dynamics of *Meloidogyne* spp. and the effect of pre-plant practices on the pest in tomato net houses in South Africa"** by F.L. Matlala has been edited for language correctness and spelling by the supervisors. No changes were made to the academic content or structure of this work.

21 May 2020

Prof. Driekie Fourie

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Appendix B

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ETHICS APPROVAL LETTER OF STUDY

Based on the review by the **Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC)**, the Committee hereby clears your study as no ethical risk. This implies that the FNASREC grants permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

Study title: Population dynamics of Meloidogyne spp. and the effect of pre-plant practices on the pest in tomato net houses in South Africa
Study Leader/Supervisor: Prof D Fourie
Student: FL Matlala

Ethics number: NWU-01410-20-A9
(Institution | Study Number | Year | Status)

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application type: Single **Risk Category:** No Risk
Commencement date: 01/02/2020
Expiry date: 01/04/2021

General conditions:
The following general terms and conditions apply:

- *The commencement date indicates the date when the study may be started.*
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The FNASREC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the FNASREC or the NWU-SCRE for any further enquiries or requests for assistance.

Yours sincerely,

Prof Roelof Burger
Chairperson Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC)

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Appendix C

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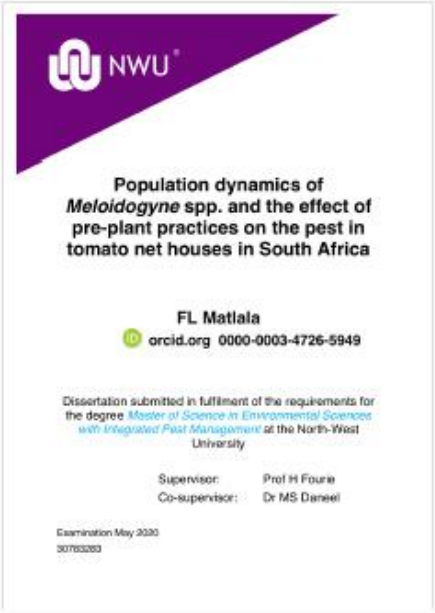

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Population dynamics of *Meloidogyne* spp. and the effect of pre-plant practices on the pest in tomato net houses in South Africa

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Dissertation submitted in fulfilment of the requirements for the degree *Master of Science in Environmental Sciences with Integrated Pest Management* at the North-West University

Supervisor: Prof H Fourie
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