

The host status of selected soybean cultivars to *Meloidogyne enterolobii* under field and greenhouse conditions

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ABSTRACT

Soybean is a popular oilseed crop in South Africa with an increase in production and hectares that doubled in last 10 years. Crop production is predicted to increase with 41% by 2030. The crop is infected and damaged by a series of pests and diseases of which plant parasitic nematodes are among the most important pests. In South Africa, root-knot nematodes (RKN), *Meloidogyne* spp., are the most serious pests of soybean, and growers who have a RKN problem find it difficult to control the nematode and its damage. In the Mpumalanga region, soybean farmers suffer from serious yield losses due to infection of soybean by RKN. Among the different *Meloidogyne* spp., the more pathogenic *Meloidogyne enterolobii*, that is difficult to control, was previously found in maize and potato in the Mpumalanga region. The study's objectives are to identify the RKN species present at the field trial site using morphological and molecular techniques, to determine the host status of selected soybean genotypes commonly grown in the Highveld region of the Mpumalanga province to the RKN species occurring in a field site with a high natural infestation and verify the results in a glasshouse trial where inoculation with the RKN species took place. The soybean genotypes evaluated under field conditions, at intervals of 30, 56 and 100 days after planting confirmed the results of the glass house studies terminated at 56 days after RKN inoculation. Yield of the genotypes at the field plot was also analyzed to determine whether some genotypes were tolerant to *M. enterolobii*. Results showed that genotype P61T38R and P48T48R were able to suppress the reproduction of RKN under greenhouse conditions significantly compared to the susceptible control while in the field trials, genotype P48T48R had a significantly lower infestation compared to the susceptible control. Genotype P59T33R showed tolerance as it produced a good yield even under high *M. enterolobii* population densities in the field site. Informing farmers about soybean genotypes that are poor hosts or tolerant to *M. enterolobii* and that can be cultivated to reduce its population densities, can be a great shift in ensuring food security.

KEYWORDS:

Grain crops, host plant resistance, *Meloidogyne enterolobii*, root-knot nematodes (RKN)

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Soybean (*Glycine max* L. Merr) is considered an important source of protein and oilseed worldwide (Miransari, 2016), and it is a relatively cheap source of good quality protein feed (Atnafu, 2021). The area cultivated to the crop in South Africa is expanding continuously (GrainSA, 2022). For the past 10 years, the hectares (ha) planted nearly doubled; increasing from 418 000 ha (2010/2011) to an estimated 827 100 ha (2020/2021). The 62% increase in grain/seeds produced during this period further accentuates the interest by local producers to grow the crop. More interesting is that a further 41% increase in ha is projected to be planted by 2030 compared to the average that was reported for 2019-2021 seasons (BFAP, 2022).

The biggest production of soybean is found in the following provinces in South Africa, namely Free State and Mpumalanga provinces contributing 74.3% of the total crop during 2021, followed by North West, 8.8%, Kwazulu-Natal, 6.8%, Gauteng, 5.6%, Limpopo, 3.8%, Eastern Cape, 0.5% and Northern Cape 0.2% of the crop and produced 1,890.45 MMT (GrainSA, 2022). Although the expansion of soybean production has the benefit of providing more protein and oil to address animal and human needs, it also has a negative consequence, namely that the crop is exposed to new and existing diseases and particularly to pests that have the potential to seriously hamper its production. Plant-parasitic nematodes, particularly root-knot nematodes (RKN), known also as *Meloidogyne* Göldi, 1887, are considered the most damaging genus (Jones *et al.*, 2013) and are a serious constraint globally (El-Borai & Duncan, 2005; Lima *et al.*, 2017). RKN are also the predominant nematode genus infecting soybean crops in local fields and cause substantial yield losses that can result in total crop devastation (Fourie *et al.*, 2015).

Above-ground symptoms of soybean plants infected with RKN may not be visible but may represent stunted, yellowish, and wilted plants given in Figure 1.1. Such aerial symptoms of infected plants are usually absent or are generally confused with those caused by other

abiotic and biotic pathogenic factors, i.e., drought, excessive heat, and insufficient or poor nutrient uptake (Fourie *et al.*, 2017).

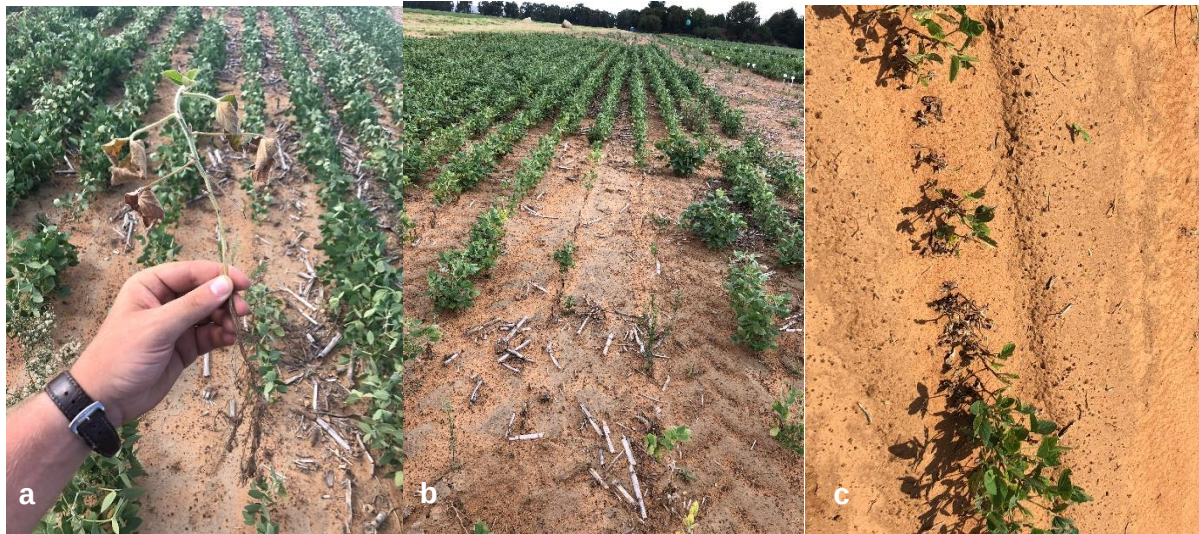


Figure 1.1: a, b & c. RKN infected soybean plants showing aerial symptoms of stunted plants, wilted upper leaves, and dried under leaves in the Middelburg (a), Hendrina (b) and Bethal (c) areas situated in the Highveld of the Mpumalanga province of South Africa; the second biggest soybean production area after the Free State (Photo's by P.R. Janse van Rensburg, NWU).

Below-ground symptoms, typically visible as knots or galls as shown in Figure 1.2 a, b & c are easily seen on uprooted roots of infected soybean plants (Fourie *et al.*, 2017). This genus causes substantial yield losses, estimated between 25 and 70%, and in severe infestations total plant destruction (Fourie *et al.*, 2010).



Figure 1.2: a, b & c. RKN infected soybean roots showing severe galling in the Middelburg (a), Hendrina (b) and Bethal (c) areas situated in the Highveld of the Mpumalanga province of South Africa; the second biggest soybean production area after the Free State (Photo's by P.R. Janse van Rensburg, NWU).

The wide distribution of root-knot nematodes in South African summer grain farmlands, including those on which soybean are grown (Engelbrecht *et al.*, 2020; Fourie *et al.*, 2001; Mbatyoti, *et al.*, 2020) in rotation with maize, pose a major problem to local producers. The presence of the four known economically important RKN species, *Meloidogyne arenaria* (Neal, 1889; Chitwood, 1949), *Meloidogyne incognita* (Kofoed & White, 1919; Chitwood, 1949), and *Meloidogyne javanica* (Treub, 1885; Chitwood, 1949), as well as the more pathogenic species (Moens *et al.*, 2009) *Meloidogyne enterolobii* Yang and Eisenback, 1983, supports data from diagnostic reports (personal communication: Prof Driekie Fourie, January 2023, Potchefstroom) that local soybean producers are experiencing high population build-ups of this genus which potentially predicts that higher yield losses are likely to be experienced.

1.2 Problem statement

According to the United Nations (UN), an immediate increase in the world population is estimated to increase from 6,143,493,823 to 7,986,276,100 during 2000–2022 and by 2050 it will be 9,735,033,990 (Worldometer, 2022). The population of South Africa (RSA) has increased by more than 15 million people (44,967,708 to 61,058,803) in the last 22 years, from 2000 – 2022 based on the most recent data released by the UN on 6 November 2022 (Worldometer, 2022).

Taking the quick rise in numbers of the world human population into consideration, it is suggested that in sub-Saharan Africa (SSA) the food demand can increase by more than three times by the year 2050 (Ten Berge *et al.*, 2019; Van Ittersum *et al.*, 2016). A logic deduction is therefore that the demand for protein rich food such as soybean will increase accordingly, with a projected 514 million tons estimated to be produced globally by the year 2050 (WWF, 2020).

Globally, production of the crop is hampered by various diseases such as bacteria, fungi (Faske *et al.*, 2014), viruses (Heinrichs & Muniappan, 2018) and pests (Gaur & Mogalapu, 2018) with RKN generally being the predominant nematode pest (Lima *et al.*, 2017). In South Africa, RKN also represent the most abundant genus occurring throughout the grain crop production area (Ngobeni *et al.*, 2011). Since soybean is widely rotated with

maize (*Zea mays* L.), though it was not generally accepted that maize crops were good hosts of RKN, research done during the 1900s proved that maize plants served as hosts to these pests (Riekert, 1995). Hence, crops included in rotation schemes with RKN susceptible maize and soybean crops, are generally heavily infected (Tiwari *et al.*, 2009) since species of the latter genus have a wide host range (Jones *et al.*, 2013; Moens *et al.*, 2009). Thus, growers experiencing RKN problems may find it difficult to control these pests, and the damage they inflict, by practicing traditional crop rotation.

The increased trend of rotating soybean with maize by South African producers (especially in the Mpumalanga and the Free State provinces), is attributed to the increased available nitrogen left in the soil after soybean plantings (Acevedo-Siaca & Goldsmith, 2020; Nel, 2013). However, the major attraction for grain producers to replace maize with soybean is the distinctly higher price (R10 090) received for a ton of soybean compared to the price (R4 370) received for a ton of maize (February price drawn on the 15th of February 2023) (SAFEX, 2023).

Another contributing factor to the soybean-nematode problem experienced in South Africa is that producers are still to a great extent un- or misinformed about plant-parasitic nematodes and the damage they cause to soybean crops. This is especially the case in the Highveld region of the Mpumalanga province where soybean crops are severely infected by RKN as is demonstrated in Figure 1.1. The nematode problem in local soybean production areas was until 2021 even more problematic since no nematicide was registered locally for use on the crop (Fourie & De Waele, 2019). Registration of Velum® 1G (Bayer, 2021), containing the active ingredient (a.i.) fluopyram, however, made it possible from the 2020/2021 growing season for local producers to reduce the population densities of plant-parasitic nematodes, especially RKN, in rhizospheres of soybean crops. But, applying a single nematode management strategy is not the answer to contain the problem and using different management strategies in combination is a more viable approach to combat nematode pests and mitigate their damage (Mbatyoti, *et al.*, 2020). Moreover, knowledge about the host status of South African genotypes of soybean (Fourie *et al.*, 2015) and the impact of growing such genotypes on the reproduction rates of RKN is becoming scarcer since such research to identify poor- and

resistant genotypes is not a priority anymore. The use of poor-host and/or resistant or even tolerant genotypes is known to contribute to the management of this nematode pest genus and assist producers to sustainably produce soybean and maize crops. Undeniably, the use of resistant genotypes is a cost-effective and environmentally friendly management tool that can easily be incorporated into existing pest management programs (Singh *et al.*, 2017).

The challenges and problems discussed above and faced by local producers to grow soybean sustainably in nematode infested soil to ensure food security (in the form of sufficient and optimal calorie and nutrient intake) and prevent conditions resulting from malnutrition and famine initiated this study. The focus of this study was investigating the host status of selected, local soybean genotypes that are commonly grown in the Highveld region of the Mpumalanga province, representing the second biggest soybean production area after the Free State province. This way producers will be capacitated to use it as an Integrated Pest Management (IPM) tool to mitigate damage caused by RKN and enable the sustainable production of crops in soils infested with lower population densities of this pest genus.

1.3 Hypothesis

It is hypothesized that:

- the host status of soybean genotypes to *M. enterolobii* will be elucidated in glasshouse trials,
- genotypes that are classified as poor hosts (compared to the susceptible and resistant standards) based on reproduction factor values (R_f) where $R_f < 1$ will be validated in the field trial where *M. enterolobii* prevails and
- that the prevalent root-knot nematode species' population densities will adversely impact on yield of different soybean genotypes grown in the field trial.

1.4 Research aims and objectives

Aim

The overall aims of the study were to:

- i) verify the presence of *M. enterolobii* at the field trial site and to assess the host status (resistant, poor and susceptible hosts) of soybean genotypes under glasshouse conditions and at an infested field conditions in the Highveld region of the Mpumalanga province, South Africa) and
- ii) assess yield of the genotypes at the field site to determine whether they were tolerant to *M. enterolobii*.

Specific objectives

The objectives of this study were to:

- verify the identity of the RKN species present at the field trial site as *M. enterolobii* using selected morphological and molecular techniques,
- determine the host status of selected soybean genotypes, commonly grown in the Highveld region of the Mpumalanga province, to *M. enterolobii* in glasshouse trials and in an on-farm field study, and
- determine the effect that *M. enterolobii* have on the yields of different soybean genotypes used in the study.

The outcomes of this study will be shared with producers and the industry to:

- inform them about the commercially available soybean genotypes that were identified as resistant, poor or tolerant hosts to *M. enterolobii* and that such genotypes can be cultivated sustainably as part of an IPM approach and contribute towards food security.

1.5 Chapter layout

The following chapters are contained in this dissertation:

Chapter 1 – Introduction

Chapter 2 – Literature review

Chapter 3 – Materials and methods

Chapter 4 – Results and discussion: RKN species identification, determination of the host status of selected soybean genotypes under field and glasshouse conditions

Chapter 5 – Conclusion and future recommendations

CHAPTER 2: LITERATURE REVIEW

2.1 Soybean Origin and Products used

The origin of soybean (*Glycine max* L. Merr) can be traced back to Japan and dates back to 1597. The word soybean is derived from the word 'shoyu' (Hymowitz, 2008). Soybean is an important leguminous food source for a large number of people worldwide (Miransari, 2016). According to estimates by the United States Department of Agriculture (USDA), soybean oil and cake (the two main ingredients) represent 87% of global production, with soybean cake used nearly entirely as animal feed (Fig 2.1) (USDA, 2022). Only 6% of global soybean produce is used as food sources for human consumption (e.g., edamame beans, tofu, soybean milk, and soybean sauce).

Soybean is mainly used as a source of vegetable oil and protein for humans and is the largest protein source for animal consumption (Miraj & Kiani, 2016). Soybean seeds consist of two main ingredients, namely oil and protein (meal). The seeds contain 18-23% oil which is converted to mayonnaise, margarine, salad dressing, and oils for human consumption. The meal constitutes 38-44% of the seed and is primarily used as a high-protein source that is used in feeds to produce protein-rich animal products for human consumption such as pork meat, beef, fish, poultry, eggs and milk. Protein concentrates, isolates, and texturized protein from soybean meal can also be used for human consumption (Hymowitz, 2008).

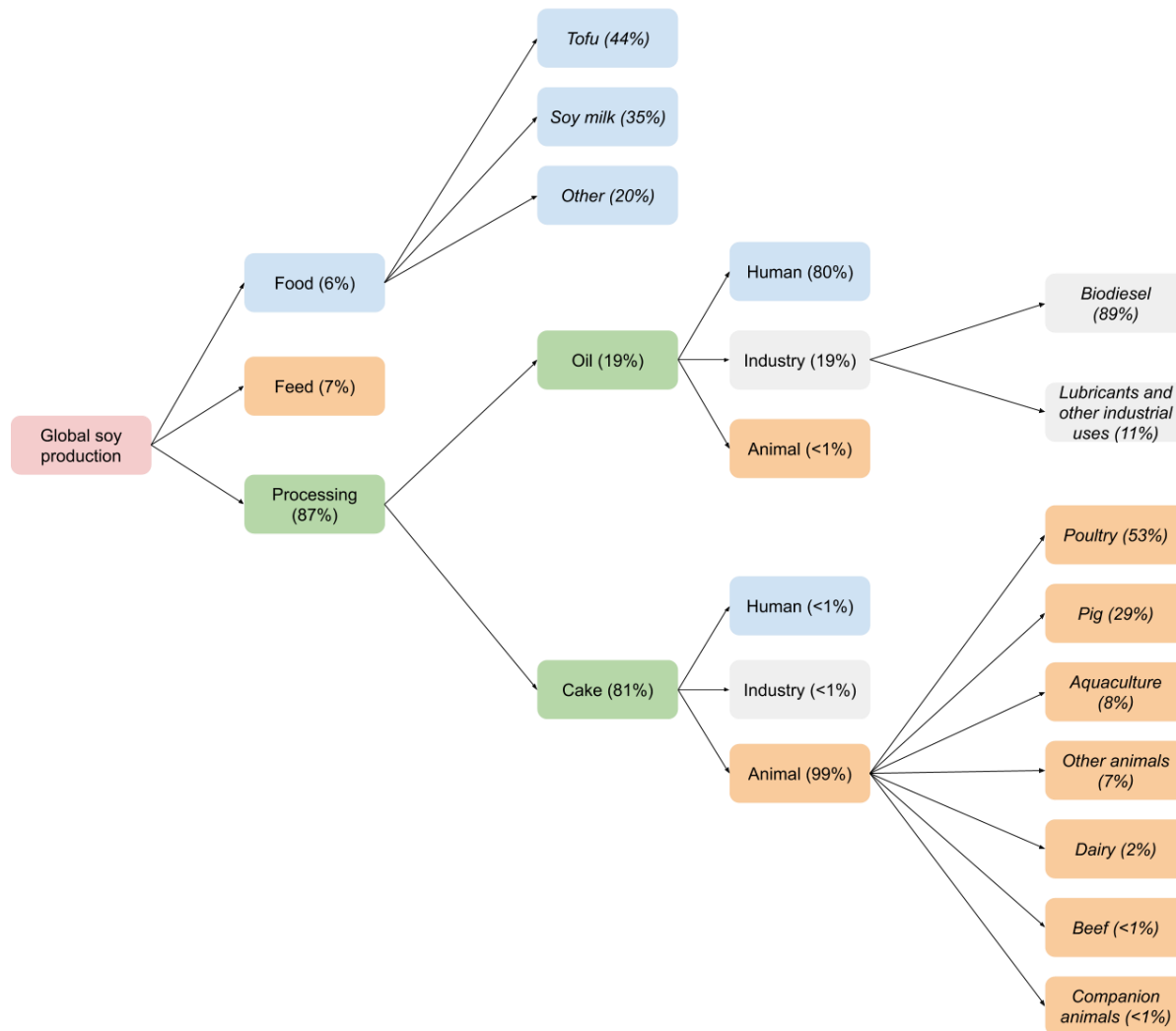


Figure 2.1: The soybean food flowchart illustrating the main uses and ingredients of soybean seeds, and how and for what it is used. This figure is provided by the Food Climate Research Network (FCRN) (Fraanje & Garnett, 2020).

2.2 Soybean Production

Worldwide, approximately 390.989 million metric tons (MMT) of soybean were produced in the year 2022 (USDA, 2022). The top three countries producing soybean worldwide during 2022 were Brazil with 152 (MMT), followed by the United States of America (USA) with 117 MMT and Argentina with 51 MMT; these three countries are responsible for 82% of global production (USDA, 2022).

The first-time soybean was grown in South Africa (SA) was during 1902-1903 (Khojely *et al.*, 2018; Shurtleff & Aoyagi, 2019). Currently, SA is listed 13th worldwide in terms of production of the crop producing 1.890 MMT during 2020/21 (<1% of world production) grown on 827 100 hectares (ha) (GrainSA, 2022). Up to 2021, soybean was produced in all provinces of SA except for the Western Cape (GrainSA, 2022). The three biggest producers of soybean locally are the Free State (FS), Mpumalanga (MP) and North-West (NW) provinces are listed in Table 2.1.

Table 2.1: Hectares on which soybean were produced in the three top producing provinces in SA over the last 10 years, with the number of Metric tons (MT) as well as the hectares (ha) produced (GrainSA, 2022).

	Year	2011/12	2012/13	2013/14	2014/15	2015/16	2016/17	2017/18	2018/19	2019/20	2020/21
Province (MT)	FS	192,500	225,750	352,000	366,000	148,000	504,000	552,000	391,300	519,750	766,500
	MP	263,050	369,000	335,000	389,900	408,000	554,300	672,700	511,500	416,000	638,000
	NW	31,500	17,600	32,000	52,500	14,100	52,900	61,200	48,600	80,000	166,800
Province (Ha)	FS	175,000	215,000	201,000	305,000	174,000	240,000	345,000	301,000	315,000	365,000
	MP	200,000	205,000	203,000	245,000	240,000	241,000	310,000	310,000	260,000	290,000
	NW	21,000	22,000	16,000	35,000	15,500	23,000	36,000	36,000	40,000	69,500

Land-use practices coupled with climatic variability are contributing factors towards the instability of soils (Laker, 2013). Furthermore, soil organic carbon content, which is typically low in South African farmlands, is reduced through mechanical soil disturbance (Du Preez & Van Huyssteen, 2020) that further increases soil erosion rates and contributes to soil degradation (Cardoso *et al.*, 2013). Intensive tillage formed part of the crop production regime of commercial farmers due to mechanization during the industrial revolution of the 1930s (Sithole *et al.*, 2016). During this time, the prolonged reoccurrence of droughts, together with continuous tillage, were responsible for major soil losses of up to 91 million ha in mid-western America (Hobbs, 2007; Hobbs *et al.*, 2008). In addition, conventional farming is considered to have a large ecological impact resulting from its high rate of mechanization, intensive energy input, and ongoing depletion of natural resources (Du Preez & Van Huyssteen, 2020; Van der Laan *et al.*, 2017). The salinization of irrigated farmlands, environmental pollution, acidification of soil profiles, soil organic matter losses, and freshwater aquifer depletion are all further consequences of conventional farming practices (Van der Laan *et al.*, 2017). Such occurrences have been

projected to cause major yield losses in the future (Sarkar *et al.*, 2020). It is not only the above-mentioned farming systems but also extensive and unregulated livestock farming that is now impacting up to 2 million ha of land in South Africa by causing subsoil compaction through the unmanaged grazing of maize and wheat (*Triticum* sp.) residues of dryland or irrigation production farmlands (Du Preez & Van Huyssteen, 2020). Furthermore, more than 64% of South Africa's groundwater is now utilized for irrigation purposes and the country's groundwater utilization for agricultural purposes is drastically increasing (Du Preez & Van Huyssteen, 2020).

2.2.1 Increased soybean production

A big driver for the substantial increase in soybean production was the release of genetically modified (GM) glyphosate-tolerant soybean genotypes in 1996 which resulted in less input costs for producers. The use of broad-spectrum herbicides that contain glyphosate as an active ingredient (a.i.) replaced or substantially decreased the need for tillage, making weed control easier and more profitable for farmers (Bindraban *et al.*, 2009; Fraanje & Garnett, 2020). Zero-tillage farming is hence linked to the production of genetically engineered crops as well as the usage of glyphosate-based broad-spectrum herbicides which control a wide range of weeds (Fraanje & Garnett, 2020). The combination of using glyphosate-based herbicides and GM soybean made it easier to establish new soybean production in areas with a high level of vegetation where the weed levels are high (Bindraban *et al.*, 2009; Fraanje & Garnett, 2020). Unfortunately, despite the benefits it holds for animals and humans, soybean cultivation is one of the biggest causes of deforestation in the Brazilian Amazon region (Sauer, 2018) and it is also a direct driver of deforestation in the Argentine Chaco region (Fehlenberg *et al.*, 2017).

Genetically modified soybean genotypes also had a big impact on the market. Since 1999, activities involving genetically modified organisms (GMOs) have been regulated under the Genetically Modified Organisms Act, 1997 (Act No.15 of 1997) administered by the Department of Agriculture, Forestry and Fisheries. SA is one of the sub-Saharan Africa (SSA) countries that has approved GM soybean genotypes for commercial production (Khojely *et al.*, 2018), while resistance to such technologies exists in other countries in the region such as Zimbabwe (Zamasiya & Kefasi, 2018). The first GM,

Roundup-Ready® genotype (A5409RG) was registered in SA 21 years ago with more than 90% of all soybean genotypes produced in 2017 being glyphosate tolerant (Khojely *et al.*, 2018). The current soybean genotype trials in SA are also all glyphosate tolerant soybean genotypes (De Beer *et al.*, 2022).

Including soybean in crop rotation systems represents another factor that contributed to increased production of the crop. Increased yields and easier, and more cost-effective management practices due to advances in agricultural biotechnology enabled commercial farmers to easily switch to soybean because it is a good rotation crop for other grain crops to maximize profits (Dlamini *et al.*, 2014). Traditional production practices showed that SSA producers commonly cultivated monoculture maize or rotated it with other crops such as legumes, like dry bean (*Phaseolus vulgaris* L.) or soybean (Kermah *et al.*, 2017). South African farmers quickly realized that crop rotation had benefits since maize/soybean rotations led to increases of approximately 7% on soybean yields compared to just soybean monoculture; for maize an average yield benefit of >20% has been recorded (Behnke *et al.*, 2018). Another benefit associated with rotation of soybean and maize includes increased soil fertility, particularly due to higher nitrogen (N) levels prevailing in the soil after soybean cultivation. Soybean rotations are generally considered a sustainable method to increase soil fertility in agricultural systems due to the crop's ability to capture atmospheric N into the soil (Acevedo-Siaca & Goldsmith, 2020; Khojely *et al.*, 2018). This is explained by soybean roots developing a symbiotic association with rhizobia such as *Bradyrhizobium japonicum*, from which most of the essential N for growth and yield production are acquired by the plant (Franke *et al.*, 2018; Uzoh *et al.*, 2019). Ultimately soybean rotations can lead to reduced biotic pressure which is attributed to lower disease pressure (Franke *et al.*, 2018; Uzoh *et al.*, 2019). Crop rotations have long been recognized as a useful strategy for reducing pest and disease pressure because they interrupt disease cycles and can minimize increases in pest population densities over time. Farmers have the chance to diversify their agricultural production systems and increase their resilience to damaging biotic factors by using soy-maize rotations (Acevedo-Siaca & Goldsmith, 2020). Crop rotation systems that constitute diverse crops, impact substantially on weed control using herbicides. It provides the opportunity to rotate herbicides with different modes of action and reduce

the risk of herbicide-resistant weeds developing (Beckie & Reboud, 2009; Simić *et al.*, 2016).

Due to the new genotypes that are better adapted to the environmental conditions, it is now possible to grow soybean with a much lower risk for the farmer than before. For example, soybean usually requires between 500 mm to 900 mm of water per growing season. Soybean genotypes with a low transpiration rate have a large implementation potential in dry areas and can be valuable in marginal areas with water shortages or where low rainfall occurs (Foyer *et al.*, 2019; Sinclair *et al.*, 2014). Another factor that contributes towards increased soybean production is improvement in terms of minimizing pod shattering. Pod shattering is the opening of pods along both its dorsal and ventral sides (Bhor *et al.*, 2014; Tukamuhabwa *et al.*, 2002). Seed losses of 50-100% have been reported in susceptible genotypes as a result of delayed harvesting after physiological maturity (Tefera, 2011). Studies have shown that the seed number per plant was the most important factor affecting soybean yield (Jin *et al.*, 2010; Miransari, 2016). Today's improved soybean genetics provides farmers with a longer time from physiological maturity to harvest before the pods start to shatter (Kataliko *et al.*, 2019).

It is expected that the rise in demand for vegetable oil for human consumption will contribute to further growth of soybean production, which is mainly used in animal feed (Fraanje *et al.*, 2020).

2.2.2 Soybean genotype selection

Knowledge of the genotype and its adaptation to the specific region where it is to be grown is invaluable for soybean growers and breeders. Using many different localities to test genotypes on a multiple-year basis is a key approach for identifying and selecting high-yielding and stable soybean genotypes adapted to specific environmental conditions in SSA (Krisnawati & Adie, 2019; Tefera *et al.*, 2009).

Soybean genotypes are divided into different maturity groups (MG). In the past, soybeans were only divided into groups 4 to 7 in SA. By contrast, the groups are more precisely categorized in the USA, with designations such as 5.3 or 5.7. The genotype is closer to maturity group 5 according to the 5.3, and to maturity group 6 according to the 5.7

(Blignaut & Taute, 2010). Currently, the genotypes are classified according to their exact growth length, and this varies from 4.8 MG to 7.1 MG for the genotypes that are registered and available for SA according to the data of the Agricultural Research Council (ARC) of soybean genotype trials in SA (De Beer *et al.*, 2022).

The MG of a soybean genotype describes the overall length of the growing season and the day length responsiveness of a specific genotype (Ortel *et al.*, 2020). The MG selection in term of the genotype as well as the correct planting date can result in a significant increase in soybean yields (Salmerón *et al.*, 2016). In SA, the first soybean trials in which a wide variety of genotypes belonging to the different MG were initiated in 1978/79 by the then Oil and Protein Seed Centre (now the Agricultural Research Council-Grain Crops or ARC-GC, Potchefstroom) under government directives to support and stimulate the local soybean industry. The trials are still done with results being published annually (De Beer *et al.*, 2022) with sufficient data being collected over the years to inform producers which MG genotype performs optimally in specific regions by means of a South African soybean production map (Blignaut & Taute, 2010).

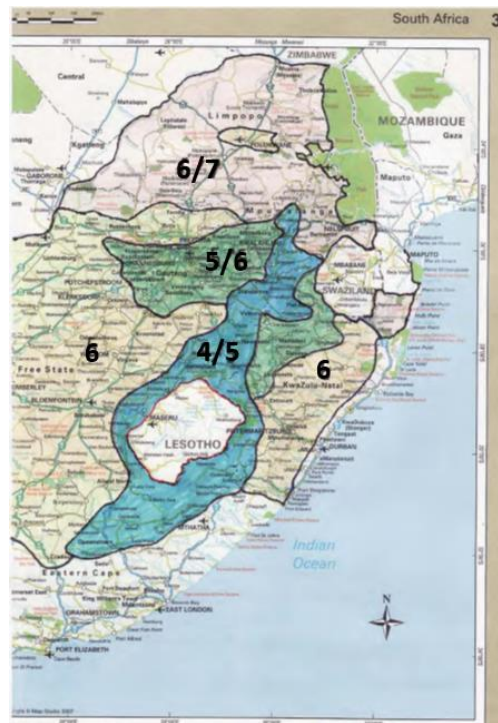


Figure. 2.2: Graphic representation of South Africa's soybean production areas showing growth class groupings which is determined according to each region's climate and grow length of the season (Blignaut & Taute, 2010).

The genotype trials are conducted in three environmental regions namely cold, moderate, and warm and in rainfed and irrigated areas. All the genotypes are planted across the three regions each year and from these results, recommendations can be made about which MG performs best in which specific area while it also shows the improved yields of the newer genotypes over time (De Beer *et al.*, 2022). The length of the growing season is the determining factor in deciding which genotype to plant from the appropriate maturity group. The recommended planting window for soybean in SA is in early to mid-November (Khojely *et al.*, 2018).

2.2.3 Challenges faced by soybean producers

Traditionally the average soybean yield obtained by SA producers was substantially lower than that of their counterparts in other countries. For example, average yields in SA are recorded as 1.7 Metric Tonnes (MT)/ha compared to the international average of 2.8 MT/ha (Cornelius & Goldsmith, 2020).

The low soybean yields reported for SA can be largely ascribed to poorly performing genotypes, while the lack of sustained addition of a *Rhizobium* inoculant and fertilizers contributes further towards this situation especially to farmers for whom soybean production is still relatively new (Khojely *et al.*, 2018; Woome *et al.*, 2014). The South African National Seed Organization (SANSOR) estimates that up to 80% of soybean plantings in SA are, for example, done with farm-saved seed or seed that has been withheld by the farmer from the previous production season (SANSOR, 2019). It is a widespread practice around the world to save a portion of the harvest of open-pollinated crops to plant again the following season.

This scenario poses a challenge to producers and the industry since seeds of newly released and improved genotypes are not used optimally by local producers. This situation furthermore results in producers being uninformed about improved characteristics of newly released genotypes; for example, higher yield and disease

resistance that such genotypes may exhibit to enable sustainable production (SANSOR, 2019).

As SA's soybean production expands, the risk exposing the crop to infection by more pests and diseases also increases (Engelbrecht *et al.*, 2020). Pests and diseases can have a significant negative impact on soybean yields (Roth *et al.*, 2020). Soybean pests can attack at any growth stage, from seedlings to mature plants, with damage being caused either directly (causing wounds during feeding and hence exposing plants to attacks by other pathogens) and/or indirectly (by transmission of viruses and other pathogens) that can lead to a total crop loss (Heinrichs & Muniappan, 2018). Pathogens and pests infect and attack all parts of the plants from roots to seeds, and the extent of economic plant damage depends upon many factors including host susceptibility (Chawla *et al.*, 2013). Insect pests on soybean plants are classified into pests that damage stems, and leaves by sucking, total defoliation or destruction of pods (Gaur & Mogalapu, 2018).

Pod borer, *Helicoverpa armigera* (Hübner, 1808) and stink bugs such as the Southern green stink bug, *Nezara viridula* (Linnaeus, 1758) are the most economically important insect pests of soybean because they feed directly on pods (Gaur & Mogalapu, 2018; Greene, 2017).

The most prevalent bacterial disease that affects soybeans, known as bacterial blight (*Pseudomonas savastanoi*), is found worldwide in all places that produce soybeans. Anthracnose is a fungal disease and is caused by *Colletotrichum* spp. that occurs worldwide in soybean production and reduces plant stand in soybeans. All regions of the world where soybeans are produced are affected by the soybean mosaic virus (SMV). In some production systems, yield loss ranges from 8% to 35%, reaching a maximum loss of 94% (Faske *et al.*, 2014).

Nematode pests are a major concern for producers since such pests can lead to major or total crop losses in soybean plantings (Heinrichs & Muniappan, 2018). The major plant-parasitic nematode genera infecting and parasitizing soybean worldwide are the root-knot nematodes (RKN) or *Meloidogyne* Göldi, 1892 (Karssen, 2002) and lesion nematodes

(LN) or *Pratylenchus* Filipjev, 1936 (Bajaj & Bhatti, 1984). These two genera are also the most important nematode pests of soybean crops in SA (Fourie *et al.*, 2017).

Heterodera glycines Ichinohe, 1952, a soybean cyst nematode that significantly reduces yields of soybeans in Brazil, the USA, and other Asian nations (Bridge & Starr, 2007) has not yet been reported in SA (Fourie, H., North-West University, Potchefstroom, North-West, SA – personal communication, 2022).

The damage caused by nematode infections in crop roots is more prone to secondary infection by soilborne fungi and bacterial pathogens and can lead to plant death (Lima *et al.*, 2017). The nematode problem experienced by soybean producers in SA is real (Engelbrecht *et al.*, 2020) and is often exacerbated by the following factors that have been reported by De Waele and Elsen (2007):

a) Farmers 'lack of awareness of nematodes' existence, b) the microscopic nature of nematodes, c) the general lack of above-ground symptoms caused by RKN, except in cases where extremely high population densities exist, d) the occurrence of multi or mixed species communities, e) the association of RKN with other pathogens, f) the lack of trained taxonomists, g) the complexity of planning and executing nematode surveys, and h) the lack of logistic and financial resources (De Waele & Elsen, 2007).

Opposed to high-income cash crops, e.g., potato (*Solanum tuberosum* L.), soybean, like maize, has a lower monetary market value and nematode control is by many producers regarded as a non-critical input component of cultivation. Inputs that are not immediately linked to profit (improved yields and quality) represent financial risk, such as the usage of a nematicide under difficult rain-fed conditions (Fourie, 2022).

2.3 Root-knot nematodes (RKN) - *Meloidogyne* spp.

RKN are seen as the most important plant-parasitic nematode genera infecting and parasitizing the roots and/or other below-ground parts of a wide range of crops and have been identified as one of the most serious threats to global agriculture (Jones *et al.*, 2013).

Meloidogyne is a Greek term that means "apple-shaped female" (Moens *et al.*, 2009). The genus *Meloidogyne* is classified using the standard classification system for plant-parasitic nematode genera reported from SA: Phylum Nematoda (Rudolphi, 1808) Lankester, 1877, Class: Secernentea von Linstow, 1905, Subclass: Diplogasteria Maggenti, 1982, Order: Tylenchida Thorne, 1949, Suborder: Tylenchina Chitwood, 1950, Superfamily: Tylenchoidea Örley, 1880, Family: Heteroderidae Filipjev and Schuurmans Stekhoven, 1941, Subfamily: Meloidogyninae Skarbilovich, 1959 (Van den Berg *et al.*, 2017).

To diagnose the problem and provide adequate nematode control, it is important to identify the RKN species correctly (Elling, 2013). Worldwide the genus *Meloidogyne* consists of more than 100 species, and they parasitize almost every species of vascular plants (Jones *et al.*, 2013). In SA, the number of RKN species that have been identified and reported to infect soybeans mainly include thermophilic species such as *Meloidogyne incognita* (Kofoed & White, 1919; Chitwood, 1949) and *Meloidogyne javanica* (Treub, 1885; Chitwood, 1949). Nonetheless, the presence of *Meloidogyne ethiopica* (Whitehead, 1968) and the cryophilic *Meloidogyne hapla* (Chitwood, 1949) have also been reported in local production areas (Fourie *et al.*, 2017). The emerging threat species, *Meloidogyne enterolobii* (Yang and Eisenback, 1983) (also a thermophile), has recently been found twice in a soybean production area in the Highveld of Mpumalanga namely in potato (Visagie *et al.*, 2018) and maize (Pretorius, 2018). Thermophile and cryophile RKN species are two separate groupings of species defined by their ability to withstand lipid-phase transitions that occur below 10°C; cryophiles can sustain soil temperatures below 10 °C, whilst thermophiles do not (Perry *et al.*, 2009).

There are different methods that can be used for identification of *Meloidogyne* spp. The classical technique (morphological characteristics) (Karssen, 2002) uses morphometrical traits but several of the characteristics overlap between species leading to misidentification (see Chapter 3 paragraph 3.3.1). The North Carolina differential host test (Hartman & Sasser, 1985) has limited usage since *Meloidogyne* spp. can share similar host responses (Ye *et al.*, 2013).

Isozyme phenotyping (Esbenshade & Triantaphyllou, 1985), esterase phenotyping (Dos Santos *et al.*, 2019), molecular diagnostics (Hunt & Handoo, 2009; Moens *et al.*, 2009) and genetic approaches (Rashidifard *et al.*, 2019; Rashidifard *et al.*, 2018) are examples of procedures to follow for accurate *Meloidogyne* spp. identification and should be combined with the classical morphometric identification to ensure accuracy. Collett (2020) listed parameters and techniques with references of morphological, molecular, and genetic methods used to identify *M. enterolobii*. Only the techniques used in the study are discussed below.

Since the late 1940s, perineal patterns of females have been used as a diagnostic feature to distinguish *Meloidogyne* spp. (Chitwood, 1949). The morphological and morphometrical properties of the i) perineal-pattern area (and its related features) and ii) oesophageal area of adult females have been used to identify *M. enterolobii* (Kleynhans, 1991; Rashidifard *et al.*, 2019). Further, Rashidifard *et al.* (2019) discovered three traits that can be utilised to distinguish *M. enterolobii* populations in SA. These include big phasmids encircled by fine striae, fine striae on the lateral edges of the vulva, and the adult female perineal pattern with a moderate to high square-like dorsal arch.

The presence of multiple *Meloidogyne* spp. in one area challenges reliable species identification (Hunt & Handoo, 2009) and due to intraspecies diversity, this approach can be difficult (Da Cunha *et al.*, 2018). Misidentification of *M. enterolobii* and *Meloidogyne inornata* Lordello, 1956, as *M. incognita* is a classic example, as their perineal-pattern morphology is very similar (Carneiro *et al.*, 2016). Due to a lack of taxonomic and molecular-trained experts, it remains challenging (Rashidifard *et al.*, 2019). *Meloidogyne* spp., in particular, rendered perineal pattern morphology insufficient for distinguishing between some species therefore molecular identification is also necessary for accuracy.

Molecular diagnostics were presented by Curran *et al.* (1985) as an early example of the application of DNA-based technologies, specifically restriction fragment length polymorphisms (RFLPs). Using the Polymerase Chain Reaction (PCR) approach, this molecular diagnosis of nematodes has grown significantly (Nega, 2014). Several

polymerase chain reaction (PCR)-based approaches are currently in use for the identification of nematode species (Collett, 2020; Philbrick *et al.*, 2020; Pretorius, 2018).

The technique used in this study was the Sequenced Characterized Amplified Region (SCAR)-PCR technique. The SCAR primers were developed from the Random Amplified Polymorphic DNA (RAPD) (Cenis, 1993) to amplify diagnostic repetitive sequences that identify various *Meloidogyne* spp. (Blok & Powers, 2009; Zijlstra *et al.*, 2000).

The diagnostic repetitive areas were discovered by analyzing isolates of seven *Meloidogyne* spp. with short RAPD primers (8 to 10 nucleotides) (Blok & Powers, 2009). These primers were found to be less sensitive than RAPDs and to create fewer banding patterns for contaminants (e.g., microbiological contaminants) (Zijlstra *et al.*, 2000). The SCAR-PCR approach is a faster and more accurate method of identifying *Meloidogyne* spp. However, in today's fast-paced world, knowledge of nematode DNA sequencing must be regularly updated in order to develop more efficient primers, making SCAR-PCR an expensive operation (Zijlstra *et al.*, 2000). This method of identifying *Meloidogyne* spp. is addressed further in Chapter 3 paragraph 3.3.2.

2.3.1 Life stages and the life cycle

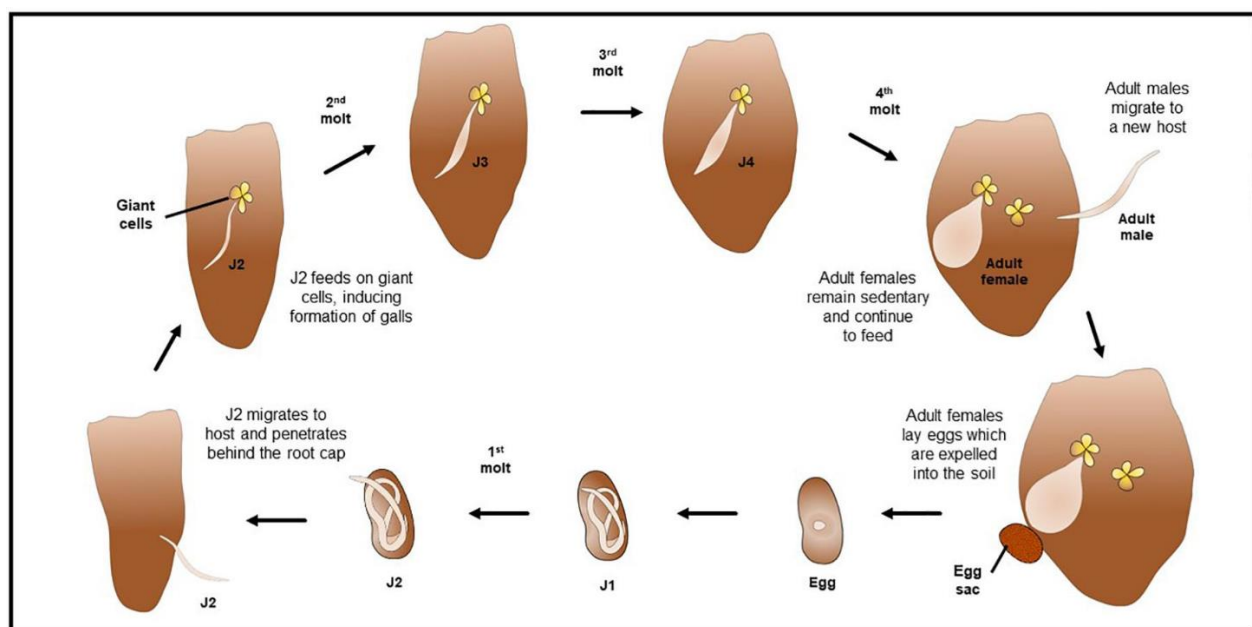


Figure 2.3: Illustration of the life cycle and root galling of *Meloidogyne enterolobii* (Philbrick *et al.*, 2020).

Meloidogyne spp. individuals are sessile endoparasites that feed in plant tissues by piercing of plant cells with their stylets (Bridge & Starr, 2007). This plant-parasitic nematode genus has evolved specific characteristics, such as a hollow protrusible stylet and oesophageal glands, that allow them to penetrate plant cells and feed on its contents, namely the cytoplasm. Infectious second-stage juveniles (J2s) of some RKN species are attracted to root exudates and move to root tips, infiltrating beneath the root cap at the elongation zone (Davis *et al.*, 2000).

Meloidogyne spp. can reproduce through amphimixis, facultative meiotic parthenogenesis, and obligate mitotic parthenogenesis (Chitwood & Perry, 2009). *Meloidogyne enterolobii* reproduces through obligate mitotic parthenogenesis or obligatory asexual reproduction in which the nucleus divides into two daughter nuclei with the same genetic material as the original (Chitwood & Perry, 2009). Males are not essential for reproduction, but severe environmental circumstances may stimulate their growth in individuals genetically destined to become females (Chitwood & Perry, 2009).

Meloidogyne spp. individuals moults four times before reaching maturity. The small, vermiform first-stage juvenile (J1) moults in the egg to become the infective J2, which then hatches from the egg to infect plant tissues of a suitable host plant and establishes a permanent feeding site. The infective, migratory J2 enlarge and moult into a non-feeding third-stage juvenile (J3) that again develops into a non-feeding fourth-stage juvenile (J4). Males revert to a vermiform morphology, whereas female-destined juveniles remain swollen. During feeding by J2 and mature females, about 6-12 plant cells are converted into giant cells in roots/other below-ground plant tissues of susceptible hosts, which serve as the feeding location and sole source of nutrients for the rest of its life nematode's cycle (Perry *et al.*, 2009).

A mature female lays her eggs in an egg sac or egg mass consisting of a gelatinous matrix produced by her rectal glands (Mahmud, 2014). This matrix holds the eggs together and protects them from enemies and severe environmental conditions (Moen *et al.*, 2009). Under favorable conditions, which are mainly dictated by moisture and temperature, J2 hatch from eggs (Goodell & Ferris, 1989) and immediately search to find tissue of the host plant to infect. The J2 penetrate usually closely behind the root tip by

using the protrusible stylet (Abad *et al.*, 2003) and the life cycle mentioned above is repeated.

Most *Meloidogyne* spp., including *M. enterolobii*, have a 30-35-day life cycle under ideal conditions in roots of tomato (*Solanum lycopersicum* L.), and each female can produce up to 600 eggs during such a cycle (Da Silva *et al.*, 2019; Philbrick *et al.*, 2020). However, Collett (2020) found that *M. enterolobii* females produced eggs 15 days after J2 inoculation of soybean roots of the genotype DM5953RSF (Collett, 2020). Throughout the growth season, several generations of the life cycle may occur (Chitwood & Perry, 2009) which will determine the number of offspring produced by a female. RKN are economically significant because they can exist in large quantities in the soil, produce many offspring, and have a broad range of hosts which results in high damage potential (Mitkowski & Abawi, 2003).

2.3.2 Symptoms of RKN on soybean

Below-ground symptoms

The vernacular name of RKN comes from the formation of galls (knots) on the surface of infected below-ground parts of the host, which is induced by feeding of motile J2 and *Meloidogyne* females (Jones *et al.*, 2013). RKN-infected roots of soybean plants are shown in Figure 2.4 a & b.



Figure 2.4: a & b. Heavily infected and galled roots of soybean, caused by RKN infection, from a field in the Mpumalanga Highveld of SA (Photo: P.R. Janse van Rensburg, North-West University, Potchefstroom).

Galled roots have limited ability to absorb water and nutrients and transport them to the rest of the plant; severely infected plants can wilt even in the presence of enough soil moisture and show nutrient deficiency symptoms (Mitkowski & Abawi, 2003). Galls formed by RKN parasitism may furthermore vary in size and shape and it is influenced by the type of plant that respond differently to *Meloidogyne* infection (Mahmud, 2014), species of RKN and the infestation densities (Rahman, 2003; Ralmi *et al.*, 2016). *Meloidogyne hapla* galls are for example generally smaller than those caused by other species; however, such visual observations should not be used in attempting species identification.

There is, however, an important difference between galls caused by RKN and the nodules formed by *Bradyrhizobium* or *Rhizobium* spp. (Engelbrecht *et al.*, 2020). The nitrogen-fixing nodules can be easily removed from the root system (Figure 2.5 a & b), compared to the RKN galls that form part of the root system (Bridge & Starr, 2007). RKN have been found to also reside in the rhizobium nodules (Figure. 2.6). *Meloidogyne* spp. for example

has been found to reduce the ability of soybean plants to bind atmospheric nitrogen (Coyne & Oyekanmi, 2007).



Figure 2.5: a & b, Healthy soybean roots, showing no RKN galls, but nitrogen-fixing nodules visible (Photos P.R. Janse van Rensburg, North-West University, Potchefstroom).



Figure 2.6: A RKN infected, galled soybean root with RKN visible in rhizobium nodules (Photo by Suria Bekker, EcoNemaria, Potchefstroom).

Above-ground symptoms

Above-ground symptoms in fields where high population densities of RKN occur may include delayed growth or stunted plants with yellowing leaves (Fourie *et al.*, 2015). Text-book examples of severe RKN infection of soybean plants in a field near Bultfontein, Free State province, SA are illustrated in Figure 2.7. Severe RKN damage was found to be inversely related to top shoot and seed mass. Severely damaged soybean plants that were infected with RKN showed a 36.5% decrease in top shoot mass and a 44.7% decrease in seed mass (Korayem & Mohamed, 2018). A graphical representation of a healthy soybean plant in Figure. 2.8 a compared to plants infected with RKN Figure 2.8 b & c aims to demonstrate the impact that this genus can have on crop growth and yield.



Figure 2.7: Severe RKN infection of soybean plants in a field near Bultfontein, Free State province, SA are illustrated (Photo by Driekie Fourie, North-West University, Potchefstroom).

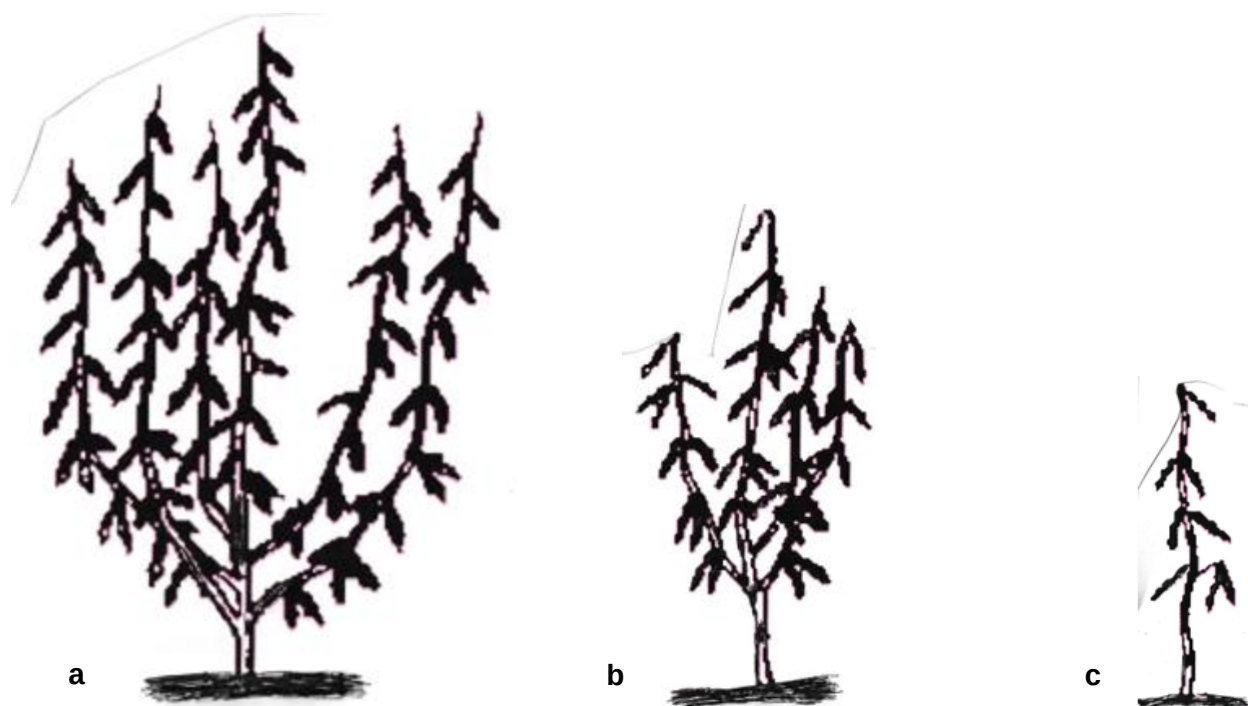


Figure 2.8: a, b & c. A graphical representation of stunted growth of soybean plants infected with RKN with a) representing a healthy soybean plant, with multiple pods, and infected with low numbers of RKN; b) a stunted soybean plant infected with higher population numbers of RKN (compared to a) and with less pods; and c) a severely stunted soybean plant with low numbers of pods and infected with high population densities of RKN (compared to both a and b) (Sketch made by P.R. Janse van Rensburg, North-West University, Potchefstroom).

2.3.3 Dissemination of RKN

Plant-parasitic nematodes are mostly aquatic and require free moisture to develop and live in the hygroscopic water film that surrounds soil particles and plant tissues (Bridge & Starr, 2007). Causes of RKN spread are infected roots that can be transported on machinery implements (Minton, 1986) to new, uninfected areas of another or the same field. Other ways are running water causing soil erosion in a field as well as animals and humans that walk in or across fields transporting nematodes that cling to their feet or shoes to new areas (Rahman, 2003). Nematodes can also be carried by the wind (especially in sandy-soil areas where erosion commonly occurs because of windstorms) (De Rooij-van der Goes *et al.*, 1997) or animals and disseminated to new geographical locations where they can infect new hosts (Otranto & Deplazes, 2019).

2.3.4 Damage potential

Meloidogyne spp. are defined as r-strategists since they represent small nematodes with relatively short life cycles and high numbers of offspring (Bongers & Bongers, 1998). The damage potential of nematode pests is influenced by a variety of abiotic and biotic factors, as well as the duration of their life cycles (Mc Donald *et al.*, 2017).

Worldwide *M. incognita*, *M. javanica* and *M. arenaria* (Neal, 1889; Chitwood, 1949), are among the most rapidly spreading and widely occurring nematode pests (Bebber *et al.*, 2014). The rapid spread of the tropical RKN species may have been facilitated by their wide host range (including many non-crop plants), high fecundity, protective gelatinous matrix in which eggs are laid, microscopic size which makes them difficult to detect by means of quarantine protocols and because their mainly parthenogenetic reproduction strategy (Singh *et al.*, 2013).

Yield losses of soybean due to RKN infection are difficult to determine precisely, with estimates ranging on a global scale from USD 157 billion (Abad *et al.*, 2008) to USD 173 billion (Elling, 2013) on an annual basis. Soybean crop losses resulting from RKN infection in the USA and Brazil have been recorded as an average crop yield loss of 10–15% (Lima, *et al.*, 2017). For SA, crop loss figures reported for soybean range from 25–70% and can cause total crop failure (Fourie *et al.*, 2015). However, multiple nematode species infections combined with disease infections, as well as poor awareness by farmers and extension officers/chemical and seed representatives are challenging factors that limit reports about crop losses in SSA (Coyne *et al.*, 2018).

Speculation about the damage potential and distribution of RKN is anticipated to be influenced by climate change. Variation in the host suitability and infection rate of crops by nematode pests makes it, however, difficult to predict population responses to environmental variables, which will also linked to changes in crop root exudate profiles (Badri & Vivanco, 2009) that promote nematode hatching and attraction (Fudali *et al.*, 2012). Temperature has a direct effect on nematode development, with slower

development rates at cooler soil temperatures and faster development rates at warmer soil temperatures (Nisa *et al.*, 2021). Increased temperatures are projected to result in earlier and faster crop development, as well as higher pest damage at the earlier sensitive stages of crop development (Skendžić *et al.*, 2021).

Water stress is another abiotic factor that can cause increased crop damage and lower yields because of RKN and is the main constraint for producers in rain-fed production areas of SA, as most crops are grown under high input conditions. Smallholders in the rest of SSA grow crops in high-stress conditions with limited nutrients where major biotic factors prevail and where frequent droughts occur (Franke, 2021). A study reporting on the impact of drought and nematode resistance on maize growth in SSA showed an interaction between irrigation x genotypes. It also showed that lesion nematodes were more likely to reproduce under conditions of water scarcity, 50 times more than RKN, which only increased in most inbreds under optimal irrigation conditions (Kagoda *et al.*, 2015).

2.4 Nematode management strategies

One of the most difficult tasks for nematologists is to find specific, practical, and long-term solutions for growers to protect grain crops in such a way that plant parasitic nematode populations remain below pest threshold levels (McDonald *et al.*, 2017).

Preventing the introduction and spread of plant parasitic nematodes is the most effective nematode management strategy, but other corrective strategies exist since prevention is rarely achieved by producers. According to Soares & Nascimento (2022), prior knowledge of the population density of the dominating pest nematode species, as well as its occurrence and distribution is essential for designing integrated nematode management (INM) strategies to be implemented to reduce nematode densities and increase crop yield (Soares & Nascimento, 2022). The INM approach was first described by Bird (1981) as the integration of research, development, technology transfer, and implementation that is required to manage one or more nematode pest species using two or more control strategies.

2.4.1 Integrated nematode management

A good guideline to use for nematode management is the advice by expert nematologists as provided in the book 'Integrated Nematode Management (INM): State-of-the-Art and Future Visions'. This book is a practical guide to nematode management that focuses on current INM tactics and strategies, as well as a vision of targeted research needed to better manage nematodes now and in the future (Sikora *et al.*, 2021). According to Sikora *et al.* (2021), INM is a term that can replace Integrated Pest Management (IPM).

According to Singh *et al.* (2017), IPM refers to the careful consideration of all available plant protection methods and the subsequent integration of appropriate measures that discourage the growth of harmful organism populations, keep the use of plant protection products and other forms of intervention to economically and ecologically justified levels, and reduce or minimize risks to human health and the environment (Singh *et al.*, 2017). This concept advocates the growth of a healthy crop with the least amount of disruption to agroecosystems feasible, as well as the use of natural pest control techniques with the focus being the prevention of disease and pest problems. It includes keeping track of pest populations, identifying pests, and deciding on a strategy to keep pest populations under control. The four basic elements of IPM according to the FAO (2022) are natural control, sampling, economic levels, and insect biology and ecology, while five components of this approach encompass chemical control, physical and mechanical methods, biological control, host plant resistance and regulatory control (Morillo-Rejesus & Rejesus, 1992).

Plant-parasitic nematodes are a part of the so-called 'big giveaway' when it comes to global food insecurity (Sikora *et al.*, 2017). Therefore, it is important that all management practices must be in place to improve soybean yield averages, especially in SA where low yields are still being recorded (GrainSA, 2022). Loss of seed is the primary factor influencing soybean yield, i.e. fewer seeds equals less yield (Jin *et al.*, 2010). It is hence extremely important that soybean producers are introduced to INM in order to produce soybeans in the presence of nematode pests, specifically RKN that prevails in soils in all production areas (Engelbrecht *et al.*, 2020). Therefore, it is important to apply nematode control strategies that aim at reducing population densities of RKN to levels that cause

minimal plant damage and yield (Heinrichs & Muniappan, 2018). To ensure sustainable food production and food security, the need exists to exploit strategies to reduce population densities of *Meloidogyne* spp. that infect and damage soybean crops. A combination of the five components of an IPM approach are required to control RKN pests in a cost-effective way and provide sustainable crop production with minimum adverse effects on animals, humans, and the environment (Mitkowski & Abawi, 2003).

As advocated by IPM, farmers should start with the management approaches to manage diseases and pests and protect their crops that will have the least adverse environmental effects (Dara, 2019). The use of genotypes with genetic host plant resistance, for example, to negative impact on nematode pest densities should be considered as the first line of defence (Dara, 2019). Genetic host plant resistance (GHPR) against plant parasitic nematodes, such as RKN, has been identified as one of the most cost-effective and ecologically friendly management strategies to minimize damage and yield loss of soybean crops (Bridge & Starr, 2007; Sikora *et al.*, 2021).

2.4.2 Genetic host plant resistance

Since the main aim of this study was to screen soybean genotypes for their host suitability to the RKN species occurring in a major soybean production area in SA, the emphasis is on this strategy as is elaborated on below.

Due to the immobile lifestyle of plants, they have developed sophisticated molecular strategies to prevent and/or limit pathogen invasion and the damage they cause (Dangl & Jones, 2001). One such strategy employed by plants is GHPR that is described as the inability of the target nematode pest to produce optimal and functional feeding sites in the host plant after invasion, and subsequently to develop and reproduce optimally. A host plant that exhibits GHPR for example to RKN hampers the optimal development and reproduction of such nematode pests and minimize their damage potential in soybean crops (Fourie *et al.*, 2015).

Due to restrictions on the use of chemical pesticides, research into alternative nematode management strategies, in this case developing host plants that exhibit resistance to

diseases and pests, has grown in popularity (Bernard *et al.*, 2017). Plant breeders find it attractive and practical to incorporate plant genotypes that have multiple resistance to various diseases and pests (Bernard *et al.*, 2017) by using a pest-resistant or pest-tolerant genotype generated through traditional breeding or genetic engineering (Nelson *et al.*, 2018).

Unfortunately, there are soybean genotypes that are resistant or tolerant to only one RKN species (Sikora *et al.*, 2018). In the United States, certain soybean genotypes such as Henderson, show moderate resistance to *M. incognita* but are susceptible to *M. arenaria* and *H. glycines* (Fourie & De Waele, 2019; Weaver & Sharpe, 2013). Some Edamame soybean breeding lines in the USA have also been found to have *M. incognita* resistance, and these traits can be transferred into commercial genotypes (Wilkes & Kirkpatrick, 2020). The USA soybean genotype PI 567516C was discovered as an effective source of resistance because it is resistant to not only *M. incognita* but also to the soybean cyst nematode and reniform nematode (*Rotylenchulus reniformis* Linford and Olivera, 1940) (Jiao *et al.*, 2015).

A significant amount of research has been done in SA to determine the host status of soybean genotypes to RKN (Fourie *et al.*, 2015; Mbatyoti *et al.*, 2021). Fourie *et al.* (2015) discovered that a few soybean genotypes exhibited varying levels of resistance to several RKN species and races, whereas Mbatyoti *et al.* (2021) did a similar study but only for *M. incognita*. However, genotypes identified as a result of these studies are not necessarily available for commercial purposes. Moreover, the development of transgenic resistant genotypes is done by private industry, and its high cost may increase the price of seed therefore breeding for nematode resistance is not a priority in SA.

Feeding of the RKN female causes root galling and for quick field assessment to see if plants are resistant, they can be evaluated for RKN damage based on the percentage and types of roots galled (Bridge & Page, 1980). Bridge and Page (1980) provided guidelines for accurate results. Many factors influence the size of the galls, including the species, host status of the infected genotype and the number of infecting RKN (Karssen *et al.*, 2013). The other method is to measure the Egg Laying Female numbers (E.L.F.)

that are present in the roots (Hussey & Boerma, 1981). This protocol was used in this study with the index scale is discussed in Chapter 3, paragraph 3.4.5.

The main benefit of GHPR against PPN, such as RKN and lesion nematodes, has been discovered as one of the most cost-effective and ecologically pest management options in soybean (Bridge & Starr, 2007) in both high- and low-value agricultural systems, as the direct cost to growers is minimal (Molinari, 2011). Resistance has been an effective management technique for increasing crop yields, decreasing nematode population densities, and promoting the development of successful rotation systems (Starr *et al.*, 2002), although the main objective of resistance as a control approach is to protect the yield potential. As a result, an applicable use of resistance must cause minimum crop damage and be related with nematode tolerance, which may differ from the recognized ability of resistant plants to limit pest reproduction (Molinari, 2011).

The disadvantages of using RKN-resistant genotypes include for example their limited availability to commercial farmers, as explained above for SA, as well as the process of breeding resistance genes into commercial genotypes with acceptable agronomic traits (El-Deeb *et al.*, 2018; Hassanin *et al.*, 2020) may result in yield penalty or other unfavorable characteristics (Fuller *et al.*, 2008). This is mainly due to the fact that it takes time and efforts to develop new resistance genes into desirable crop genotypes. Another difficulty experienced during breeding RKN resistant soybean genotypes is the presence of numerous species and genera in soil ecosystems in SA grain growing areas (Pretorius, 2018; Visagie *et al.*, 2018).

As a result, in fields where many *Meloidogyne* spp. are present, resistance to the whole *Meloidogyne* community is essential for optimal control (Coyne *et al.*, 2018). Resistance to a single species will not solve problems in soybean fields where numerous species coexist. Another concern is the widespread usage of resistant genes, which may make the use of resistant genotypes ineffective at specific locations (Molinari, 2011). The potential for success in releasing resistant genotypes appears to be very promising, given the numerous available sources of nematode resistance in a wide range of plant families, as well as rapid advances in techniques such as in-embryo rescue, somatic hybridization,

and direct gene transfer, which should promote more efficient genetic transfer across conventionally difficult biological barriers (e.g., sexual incompatibility, polyploidy, unacceptable gene linkages) (Davies & Elling, 2015; Molinari, 2011).

2.4.3 Chemical Control

Soybeans form part of a low-value food legume crop grown for dry grain. According to Roberts (2022), there is a slim difference between the expenses of input costs, such as INM technologies, and the profits earned from crop production. Only when other/alternative methods of pest control fail, are pesticides employed to keep pest populations below economically destructive levels (Singh *et al.*, 2017). Chemical nematicides have been effectively used to reduce at-planting nematode populations and maximize yields, but the cost of doing so on soybean production in the USA is expensive (Hartman *et al.*, 2015). SA has only recently registered one chemical nematicide for use on soybean crops, Velum® 1GR (Bayer Crop Science South Africa, 2021).

A more economical option is to use a nematicide as a seed coat. Several nematicide seed treatments have been approved for use on soybeans. Seed treatments typically reduce early root penetration of young seedlings and appear to have a varied influence on yield, but they rarely provide season-long control to protect the following crop in the rotation (Sikora, 2022). It is far less expensive than conventional nematicides, provides a more targeted strategy, and drastically reduces environmental pollution and human and animal exposure to toxic nematicides (Fourie, 2022).

2.4.4 Cultural control

To protect crops against pests, several cultivation and management techniques can be employed. These, amongst others, include crop rotation, fallowing, adjusting planting and harvesting dates, altering plant and row spacing, and removing old crop residues. A cover crop can also be used as a green manure, incorporating nitrogen (N) and organic matter into the soil to benefit the following crop (Singh *et al.*, 2017). Pest infestations can also be influenced by plant density or row spacing (Dara, 2019).

Crop rotation

Crop rotation, ideally choosing non- or poor hosts of RKN, should be the major pillar of an INM strategy, as it is cost-effective, ecologically friendly, and widely available to farmers (Fourie, 2022).

When rotating maize (cv. PAN6043) with oilseed crops in a sandy soil in the Free State Province, Riekert and Henshaw (1998) emphasized the negative effects of crop rotation. When cowpea (cv. Glenda) and soybean (cv. Knap) were included once in a maize-based rotation sequence over four consecutive growing seasons, significant increases (up to 189-fold) in levels of a mixed *M. incognita* and *M. javanica* populations (70:30 ratio) were demonstrated (Riekert & Henshaw, 1998). Therefore, it is crucial that whenever possible, producers must use a genotype with a low reproduction value when planting maize (Soares & Nascimento, 2022).

Physical or mechanical controls - Tillage

According to the findings, disc-ploughed areas were able to reduce RKN populations under maize by 96% when compared to zero tillage plots, although the yield of zero tillage was 35% higher than disc plough (Kankam & Adomako, 2014). In areas with a high RKN infestation, it is however critical to mechanically destroy the previous crop's roots by turning the soil at least twice. This strategy seeks to break the nematode life cycle. Soil turning can also help with the management of other nematodes. On the other hand, this strategy promotes nematode spread (Soares & Nascimento, 2022).

Many weeds that are commonly found in SA in crop production fields are susceptible to RKN and serve as reservoirs of these pests (Ntidi *et al.*, 2015; Ntidi *et al.*, 2012). Therefore, it is also very important to control the weeds, with chemical control or tillage, because successful weed control can limit RKN infestations, for the following crop.

2.4.5 Biological controls

Chemical pesticides are being used less frequently due to the high demand for organic agriculture and environmental concerns. Grain farmers are very interested in alternative pest management methods, such as using biological treatments (Bernard *et al.*, 2017). The effectiveness of hematophagous bacteria and fungi in controlling certain nematode

pests, such as cyst nematodes and RKN, has been well documented (Meyer, 2003). During a glasshouse experiment, *Bacillus firmus* in an aqueous suspension reduced egg hatching in *M. incognita* between 98 to 100% 24 days after treatment, whereas gall and number of eggs were reduced on roots of tomato seedlings (Terefe *et al.*, 2009).

2.5 Conclusion

More research is needed to determine the impact of RKN in particular in soybean-growing regions in Africa. The knowledge that will hence emanate from this study regarding GHRP of soybean genotypes and the efficacy of a resistant soybean genotype to reduce *Meloidogyne* spp. numbers will be important information that can be applied when decisions are made for managing this pest using an INM approach.

CHAPTER 3: MATERIAL AND METHODS

3.1 Introduction

In this chapter the methodology used to assess the host status of selected soybean (*Glycine max* L. Merr) genotypes supplied by Pioneer Seed South Africa, a seed division of Corteva Agriscience RSA (Pty) Ltd and Agricol (who has a trade agreement with DonMario Seeds, Argentina) to a root-knot nematode (RKN) (*Meloidogyne*) species that was present in high population densities in a farmer's field near Bethal in the Highveld of Mpumalanga, South Africa was described. Firstly, identification of the target RKN species (using classical/morphological and molecular approaches) was elaborated on. This was followed by describing the assessment of the host status of the selected genotypes in two glasshouse trials and one field trial. The techniques used to plant the soybean seeds, perform nematode sampling, conduct extraction and counting are described. Information about the study site (location, environmental conditions, soil type and farmer practices followed) where the field trial was done is also provided. Ultimately, parameters (nematode and plant parameters) used to measure the RKN reproduction in rhizospheres of the selected genotypes are discussed, while the statistical tests (SAS) applied to analyse the data are referred to as well.

3.2 RKN species identification

3.2.1 Morphological/classical

To enable identification of the RKN species present at the field trial site, the classical / morphological approach as described by Marais *et al.* (2017), was done by using selected characteristics represented in the tail areas of 10 mature females, e.g., the perineal-pattern, dorsal arch/apex, striae formation, phasmids, and lateral lines. For such identification, the publication of Kleynhans (1991) was used to enable discrimination between RKN species females such as *Meloidogyne incognita* (Kofoed & White, 1919; Chitwood, 1949) and *Meloidogyne javanica* (Treub, 1885; Chitwood, 1949) females, while protocols by Visagie *et al.* (2018), Brito *et al.* (2004) and Rashidifard *et al.* (2019) were consulted to identify *Meloidogyne enterolobii* (Yang and Eisenback, 1983) females (Brito

et al., 2004; Kleynhans, 1991; Rashidifard *et al.*, 2019; Visagie *et al.*, 2018). Ten mature females were isolated from infected roots. The species were mass-reared, for the identification of the RKN species used in this study.

The identification of a species based on the morphology of a perineal pattern needs the successful detection of the characteristics of the apex, dorsal arch, lateral lines, phasmids, striae, and tail regions as shown in Table 3.1. for the species.

Table 3.1. The perineal pattern morphology of the species *Meloidogyne enterolobii*, according to Kleynhans (1991); Brito *et al.* (2004); Karssen *et al.* (2013); Rashidifard (2019).

	<i>Meloidogyne</i> spp.		
Characteristics	<i>M. enterolobii</i>	<i>M. javanica</i>	<i>M. incognita</i>
General shape	Round to ovoid	Round to oval	Round to oval
Dorsal arch	Moderately high to high	Low to medium high	Medium high to high
Apex	Round, and square in some specimens	Squarish to broadly rounded	Broadly rounded or squared
Phasmids	Distinct phasmids in tail-terminus	Not clear, but visible	Distorted phasmids
Tail region	Tail tip terminus visible	Tail tip terminus visible	Tail tip terminus visible
Lateral lines	Not distinct	Clearly visible as double lines	Not visible
Striae	Finer pattern on ventral side; none present in peri-vulval area	Smooth to wavy	Smooth, wavy, and zigzag pattern
Miscellaneous	Perivulva region free of striae	Distinct rectal punctations	Whorl around tail terminus

The sodium-hypochlorite-acid-fuchsin method (Byrd *et al.*, 1983) was used to stain the RKN females present in the infected soybean roots. This step facilitated removal of

stained females for dissection of the anterior and posterior parts for identification purposes (Figure 3.1 a). Such staining was done by first rinsing after the infected soybean roots with tap water and submerging it into a 1: 5 NaOCl : tap water solution for 6 min. The roots were then rinsed under running tap water for 1 min and submerged into 400 ml tap water for 15 min. The root systems were then removed and rinsed under running tap water for 1 min and transferred to a fume hood where the roots were submerged for 30 s into a boiling solution containing 40 ml tap water and a 1-ml acid-fuchsin solution (3.5 g acid-fuchsin dissolved in 250 ml acetic acid). After leaving the roots in this solution for 30 min (cooling down to room temperature), it was washed for 1 min with running tap-water and transferred to a sample bottle containing 10 ml glycerol. The stained roots containing the females were transferred from the fume hood to the microscope where 10 stained, mature females were isolated from infected and stained roots using a scalpel and tweezers.

A scalpel with a new blade was then used to prepare the obese female's cuticle by first puncturing it just behind the neck area (Figure 3.1 b). This was then followed by making a horizontal incision that divided the posterior and anterior parts of the body. The posterior part of the body containing the reproductive organs and the perineal cuticular pattern was then placed in lactic acid that dissolved the internal structures and organs. The cuticular area of the posterior part of the female's body was then trimmed with the scalpel to prepare the perineal area that was mounted on a microscope slide in a drop of glycerine (Figure 3.1 c). The anterior region of each female was also isolated to study the stylet, lumen of the oesophagus and medium bulb.

The posterior region was isolated and by applying slight pressure to the globular structure the content was removed (Figure 3.1 d). The cuticle was then cut with two vertical incisions and the small part of the cuticle containing the perineal pattern was situated facing upwards. Pressure was carefully applied again to remove any remaining internal tissue, then the cuticle with the perineal pattern was mounted in a drop of glycerol onto a glass microscope slide and covered with a glass cover slide. The same process was followed for the anterior part. Further investigation was done using a Nikon Eclipse 50i Light microscope (100x magnification) with dedicated NIS-Elements D3.10, sp3 software.

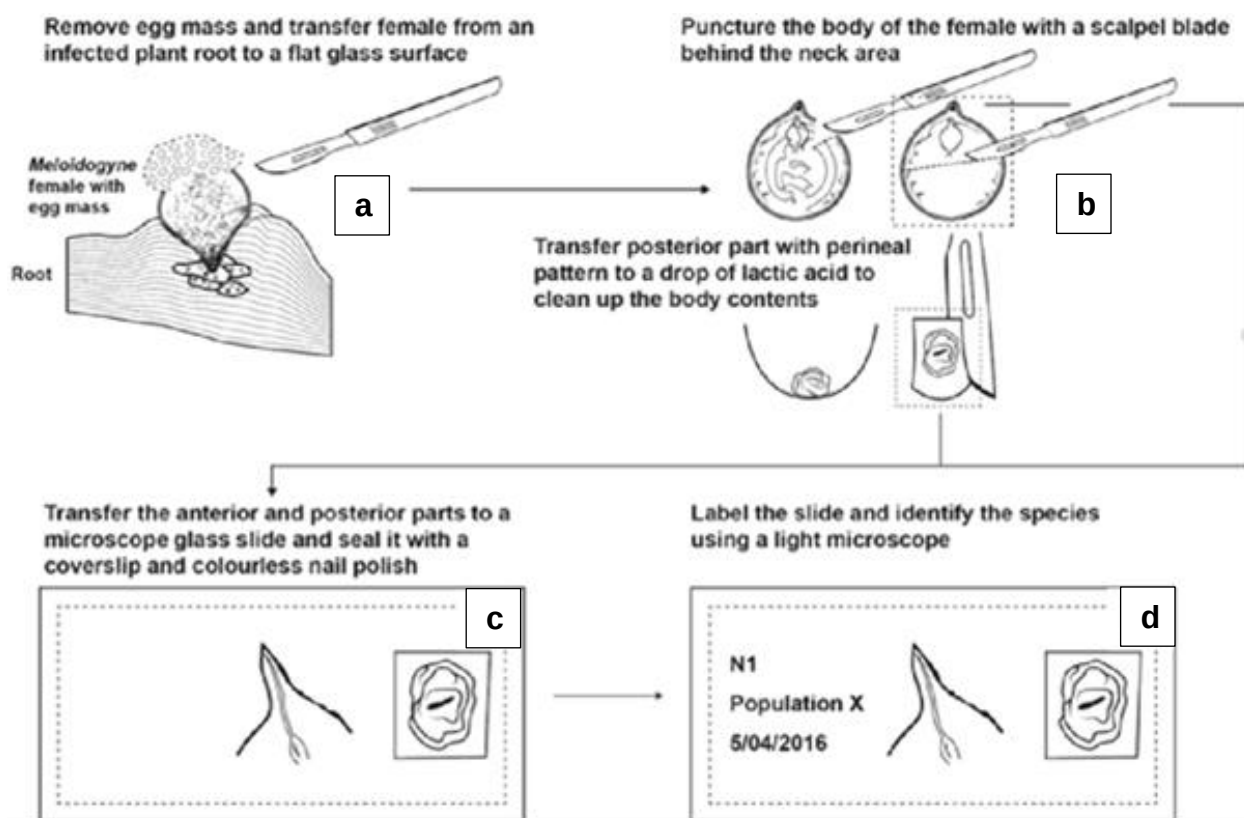


Figure 3.1: An illustration of the technique used to obtain the anterior and posterior parts of a mature RKN female by: (a) removing it from an infected root piece; (b) puncturing of the globose female body and then cutting it in half (the body content is also gently removed and the cuticle where the perineal pattern is situated, trimmed); (c) mounting of the oesophageal part and the perineal pattern of the female in glycerol on a glass microscope slide; and (d) labelling the slide containing the mounted body parts (Photo: Marais *et al.*, 2017).

3.2.2 Molecular

To obtain deoxyribonucleic acid (DNA) bands (expressed on an electrophoresis gel for identification purposes) for the RKN species present in the field site, the sequence characterised amplified region – polymerase chain reaction (SCAR-PCR) was applied.

Information about the RKN standard populations used, the origin of these populations and the hosts from which below-ground plant parts the females were sourced are tabulated in Table 3.2.

Table 3.2. Locality and hosts of the three South African *Meloidogyne* spp. populations used in the study.

Species	Locality	Host
<i>Meloidogyne arenaria</i>	Western Cape Province	Litchi (<i>Litchi chinensis</i> Sonn.)
<i>Meloidogyne enterolobii</i>	Mpumalanga Province	Guava (<i>Psidium guajava</i> L.)
<i>Meloidogyne incognita</i>	Western Cape Province	Potato (<i>Solanum tuberosum</i> L.)
<i>Meloidogyne javanica</i>	Western Cape Province	Potato (<i>Solanum tuberosum</i> L.)

In Table 3.3. information is listed about the SCAR-PCR primer sequences (Forward/F and Reverse/R) used for the RKN species used as standards and tested for in the soybean roots obtained from the field trial site.

Table 3.3. The primer sequences used for identification of South African populations of *Meloidogyne enterolobii*, *Meloidogyne incognita* and *Meloidogyne javanica* applying the sequence characterised amplified region – polymerase chain reaction (SCAR-PCR).

Primer code for <i>Meloidogyne</i> sp.	Primer sequence (5' → 3')	Species & Fragment size	Reference
Ma-F	TCG GCG ATA GAG GTA AAT GAC	<i>M. arenaria</i> (420 bp)	Zijlstra <i>et al.</i> (2000)
Ma-R	TCG GCG ATA GAC ACT ACA ACT		
Me-F ¹	AACTTTTGTGAAAGTGCCGCTG	<i>M. enterolobii</i> (250 bp)	Long <i>et al.</i> (2006)
Me-R ²	TCAGTTCAGGCAGGATCAACC		
F-inc	CTCTGCCCAATGAGCTGTCC	<i>M. incognita</i> (1 200 bp)	Zijlstra <i>et al.</i> (2000)
R-inc	CTCTGCCCTCACATTAGG		
Fjav	GGTGCGCGATTGAACTGAGC	<i>M. javanica</i> (700 bp)	Zijlstra <i>et al.</i> (2000)
Rjav	CAGGCCCTTCAGTGGA ACTATAC		

¹F = Forward; ²R = Reverse.

The SCAR-PCR procedure, originally described by Musapa *et al.* (2013), and adapted by Rashidifard *et al.* (2019), which was used in this study to identify the RKN species present in the field trial site near Bethal, is described below:

- i) RKN female isolation for DNA extraction

Infected soybean roots, obtained from the field trial site for nematode analysis during the first sampling interval (30 DAE) were used to isolate 20 mature females which was transferred to two sterilized Eppendorf tubes (10 females per tube). The two Eppendorf tubes with the females were stored in a fridge at 4 °C until DNA extraction commenced.

ii) DNA extraction

The 10 females in each tube were homogenised using a pestle, sterilised in a 96% alcohol solution (to prevent cross-contamination between the two Eppendorf tubes), and vortexed. Next, 20 µl chelex (5% w/v) and 3 µl proteinase K (20 mg.ml⁻¹) were added to each of the two Eppendorf-tubes containing the homogenised females and the tubes incubated for 2 h at 56 °C, followed by 10 min incubation of 95 °C; this step enabled the release and purification of the DNA extracts from the females. These two Eppendorf tubes were put aside until needed.

iii) DNA amplification of DNA (Vacutec thermocycler: www.vacutec.co.za)

The following substances were added to four other sets of Eppendorf tubes which represented the four RKN species to be tested for (*M. arenaria*, *M. enterolobii*, *M. incognita* and *M. javanica*): 25 µl PCR mix, 5.5 µL ddH₂O, 12.5 µl master mix (Promega Corporation, USA), 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM) of each of the standard RKN species used (see Table 3.2). Finally, 5 µl of extracted DNA from the RKN females obtained from the field trial site and present in the two tubes that were put aside (see above under ii) were added to the four sets of Eppendorf tubes.

The RKN species-specific primers (see Table 3.3) was then used during the amplification processes: 5 µl of each primer to the content of the Eppendorf tubes as prepared in the step above. The Eppendorf tubes were transferred to PCR machines; one PCR machine used for each of the four RKN species tested for. Next, the polymerase chain reaction (PCR) amplification profiles for each RKN species were applied using the information listed in Table 3.4. This was done from the process of denaturation temperature until the final extension temperature step to enable and allow time for the production of the PCR products for each of the four RKN species tested for.

iv) Gel electrophoresis

Solidification of an 1% agarose gel solution containing TAE (Tris – Acetate – EDTA) was done using a characteristic rectangular form container used for gel analyses; this container had individual wells at the top in which the PCR products of RKN species tested for were loaded. However, before loading the RKN PCR products, a 1 base pair (bp) ladder was loaded into the first; this ladder indicates the size of each DNA fragment size that were generated for the species determined. Next, 4 µl of PCR product from each the Eppendorf tubes prepared for the RKN species tested for and of which the content was subjected to the PCR process, were obtained with a micropipette from each of the Eppendorf tubes, carefully mixed with 1 µl GelRed and loaded in wells 2, 3, 4 and 5. After loading these PCR products into the wells of the gel, the same procedure was repeated with 4 µl droplets of the standards' DNA used for each of the three species. The DNA fragments of the RKN females were then separated with an electrical current running through gel for approximately 30 – 35 min. After this step, the gel was exposed and inspected using an ultraviolet transilluminator to determine the size of the DNA fragments expressed. The RKN species standards and the 1 bp ladder were used as guidelines to determine the identity of the species, visible as a DNA band(s) (see Chapter 4, Figure 4.3).

Table 3.4. Polymerase chain reaction (PCR) amplification profiles used with the species-specific primers for identification of *Meloidogyne enterolobii*, *Meloidogyne incognita* and *Meloidogyne javanica*. (Long *et al.*, 2006; Zijlstra *et al.*, 2000).

Primer pair	Denaturation temperature and time	Cycle	Denaturation temperature and time	Annealing temperature and time	Extension temperature and time	Final extension temperature and time
Ma-F/Ma-R	94°C, 2 min	35	94°C, 30 s	61°C	72°C, 1 min	72°C, 5 min
Me-F/Me-R	94°C, 2 min	35	94°C, 30 s	64°C, 30 s	72°C, 1 min	72°C, 5 min
F-inc/R-inc	94°C, 2 min	45	94°C, 30 s	54°C, 30 s	72°C, 1 min	72°C, 5 min
F-jav/R-jav	94°C, 2 min	45	94°C, 30 s	64°C, 30 s	72°C, 1 min	72°C, 5 min

3.3 Glasshouse study

3.3.1 Location and preparation of seedlings

The glasshouse trials were conducted on the premises of the EkoRehab facility of the North-West University, Potchefstroom. The ambient temperature range in the glasshouse for the duration of the trial was 19 – 20±1 °C (min) and 25 - 27±1 °C (max). The seven soybean genotypes tested for their host status to the prevailing RKN species in the glasshouse trial. Pioneer Seed South Africa supplied the seeds of the selected genotypes included in this study. Five soybean genotypes (P48T48R, P59T33R, P61T38R, P64T39R and P71T74R) were selected for this study. The five selected genotypes contained relatively new genetics and produced good yields across SA's soybean production areas. In addition, the RKN susceptible (S) genotype DM5953RSF and the

poor-host genotype DM6.8IRR (resistant standard) (Mbatyoti, *et al.*, 2020), were selected as the susceptible and poor-host standards.

The seeds of each soybean genotype were inoculated with *Bradyrhizobium japonicum* (Soycap by Soygro; <http://www.soygro.co.za/rhizobium>) during planting at a rate of 250 g packet Soycap per 25 kg seed to stimulate and promote germination and nitrogen fixation.

To prepare soybean seedlings, seven 10-L capacity plastic pots were filled with sandy soil [97.6% sand, 1.2% silt, 1.2% clay and 0.06% organic C, with a pH of 5.73 (H₂O)] provided by Witpoort Sand & Stone (Ventersdorp, North West Province). Analyses were done by the Eco Analytica Laboratory of the North-West University, Potchefstroom. Soil-borne pathogens were eliminated from the soil by fumigating it with Telone® II (97,5% active ingredient 1,3-dichloropropene) at a dosage of 150 L.ha⁻¹ 40 days before the glasshouse trial commenced.

A nutrient solution, 100 ml of a 1 g.L⁻¹ solution of Nutrifeed (Stark Ayres), was applied to the pots at seedling transplanting and comprised Ca (7%); K (13%); Mg (2,2%); N (6,5%); P (2,7%); S (7,5%) and <0,1% B, Cu, Fe, Mn, Mo, and Zn. Application of the same nutrient dosage was done 30 days after seedling emergence.

3.3.2 RKN inoculum

RKN infected roots were obtained from the field trial site and RKN eggs and J2 extracted using the adapted NaOCl method of Riekert (1995) which was also used for the field trial (see Paragraph 3.2.9.1). The eggs and J2 obtained were counted using a De Grisse counting dish (De Grisse, 1963) by means of the following method:

- the extracted eggs and J2 were collected in tap water in a glass beaker, placed on a magnetic stirrer, and gently stirred throughout the inoculation process.
- five aliquots of 10 µl each were collected with a pipette, poured into a counting dish and eggs and J2 counted to check for a minimum of 95% accuracy level. The contents of the five aliquots was then poured back into the glass beaker. The desired number of eggs and J2 to be inoculated per soybean seed were suspended in tap water after calculations were made with $\pm 2 \times 10^6$ eggs and J2

and ± 2191 eggs and J2, respectively, used to inoculated each soybean genotype for the first and second/repeat trial.

3.3.3 Planting and inoculation of soybean genotypes

Twenty seeds of each of the seven genotypes (Table 3.2) were planted in individual 10-L capacity pots to enable germination of seedlings. Fourteen days after planting, one seedling of each genotype was transplanted into a hole made in the soil in each 4-L capacity pot (filled with the same soil as described above for seedling rearing) (see Figure 3.2), and RKN eggs and J2 of the same species prevailing at the field site were inoculated on the exposed roots of each soybean seedling. The inoculum was suspended evenly on the roots of the seedling and the sample bottle containing the inoculum was then also rinsed with tap water and decanted onto the roots to ensure all eggs and J2 were inoculated. The roots of each seedling were covered with the soil used to make the hole after RKN inoculation took place.

The soybean seedlings were watered with equal amounts of tap water every second day for the duration of the trial which was 56 days after nematode inoculation.



Figure 3.2: a & b. Two randomized complete soybean glasshouse trials with seven replicates of each genotype: (b) first trial and (b) second/repeat trial in which seven soybean genotypes were assessed for their host status to the RKN species identified from the field trial site near Bethal, Highveld Region of Mpumalanga, South Africa (Photo: P.R. Janse van Rensburg, Pioneer Seeds South Africa & North-West University).

3.3.4 Harvesting of soybean roots, egg mass ratings and nematode extraction

The root systems of the soybean plants were uprooted at termination of the experiment (56 days after nematode inoculation), excised directly above the roots and the root systems of each genotype removed carefully so that no roots broke off. The whole root system of each genotype was washed free of surrounding soil, weighed and immersed into a 0.1% Phloxine B solution for 20 min to enable staining of RKN egg masses (Hussey & Boerma, 1981). This was done by submerging the roots of each genotype in a plastic container filled with 200 ml of water and the pink-stained egg masses were counted under a bright light using a commercial hand-held magnifying glass (Figure 3.3). Next, the egg-laying-female index (E.L.F.) for each genotype's root system was calculated.

Soybean root galls are related to the amount of egg masses. The more egg masses, the more galls will be present on roots. A gall rating chart for root knot damage can also be used on many crops and is based on the percentage and types of roots galled. According to Bridge & Page (1980) guidelines are provided for accurate results.

The species, host status of the genotype infected, the quantity of infectious second-stage juveniles (J2) that developed into third- (J3) and fourth-stage juveniles (J4), and finally mature females or males, all play a role in determining the size of the galls (Karssen *et al.*, 2013).

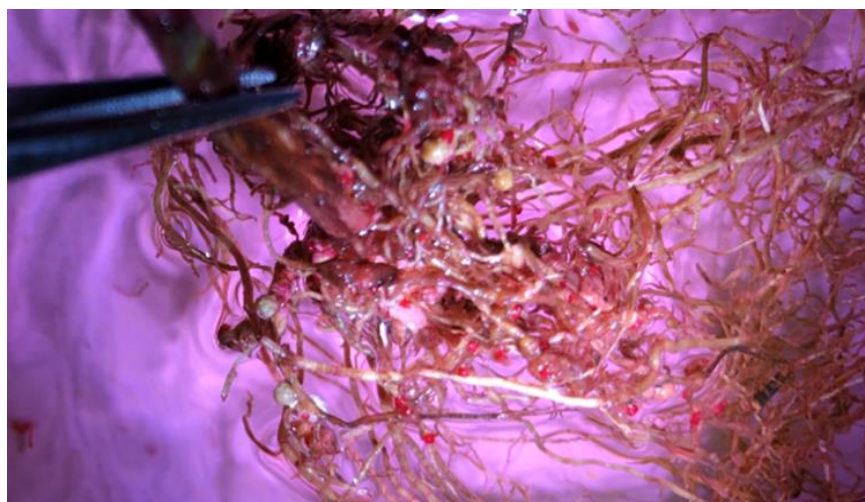


Figure 3.3: Pinkish-red stained egg masses produced by females on soybean roots.

After E.L.F. indices were determined, the whole root system of each genotype was then blotted on paper towel and cut into 0.5-cm pieces to enable extraction of eggs and J2 extracted with the adapted NaOCl method (Riekert, 1995).

3.3.5 Nematode extraction of 20 g roots

The extraction of nematodes from soil and plant tissue is an important step in determining the population densities of plant-parasitic nematode species or genera during the various stages of crop growth (Marais *et al.*, 2017).

3.3.6 Adapted NaOCl method (Riekert, 1995)

The four roots sampled and bagged per plot were removed, rinsed with a gentle stream of tap water to get rid of the surrounding soil and cut into 0.5 mm pieces. The root pieces were mixed thoroughly, and a 20 g composite sample was taken and analysed. This procedure was followed for every sample obtained from each plot (Figure 3.4).



Figure 3.4: Preparation of roots for nematode extraction, with 20 g being weighed per sample and placed in a labelled, plastic container (Photo: Ané Loggenberg, North-West University).

A 1% sodium hypochlorite (NaOCl) solution was prepared, and 400 ml thereof added to a plastic container in which the root pieces of a sample was placed. The NaOCl solution covered all the root pieces. The container with the NaOCl solution and root pieces were then placed on an orbital shaker for 4 min to dissolve the gelatinous matrix surrounding the egg masses and enable the release of the eggs in the solution.

The contents of the container were decanted onto a 250- μm mesh sieve, which was stacked on a 75- μm , 63- μm and 20- μm mesh sieve (from top to bottom) and washed with a gentle stream of tap water. The contents of the all the sieves were discarded except for that on the bottom 20- μm mesh sieve which was poured into a 50-ml capacity centrifuge tube. Each sample was prepared using the procedure explained above and then subjected to the sugar-centrifugal-flotation method described below.

3.3.7 Sugar-centrifugal-flotation method (Marais et al., 2017)

Each centrifuge tube containing the nematodes suspended in water was filled with tap water until they were nearly full. Kaolin (5 g) was then added to each centrifuge tube and the suspension thoroughly mixed. The teaspoon used for adding the kaolin was rinsed well in water before using it to add the product to the next centrifuge tube to avoid cross contamination between tubes/samples.

Four centrifuge tubes, containing four different samples, were put in the centrifuge for 5 min at 3500 rpm. The supernatant of each tube was discarded after centrifugation. Next, a sugar-tap water solution was prepared by dissolving 1.6 kg of sugar in 2 L of tap water. The sugar-water solution with a specific gravity of 1.15 - 1.30 g cm^{-3} specific gravity, which is higher than that of water (1 g cm^{-3}) was then added to each centrifuge tube and the pellet at the bottom of each tube thoroughly mixed with the sugar-water using a spatula (which was rinsed between individual samples to prevent contamination). The tubes containing the sugar-water solution were centrifuged for 1 min at 3500 rpm. This way, the nematodes remained suspended and were separated from the roots and soil particles. A time limit of 1 min was adhered to since nematodes may disintegrate and sustain structural damage due to plasmolysis, resulting from high osmotic pressure if left for longer in sugar water. To obtain the nematodes from the sugar solution, the contents of

the tubes were washed through a 20 µm-mesh sieve with tap water. The nematodes were then rinsed from the 20-µm mesh sieve into sample bottles, ± 20 ml, and stored at 6 – 10 °C in a labelled sample container before counting commenced. The nematode life stages extracted from the roots and soil samples were counted and identified to genus level using a Nikon SMZ 1 500 Nikon stereo microscope (60x magnification).

3.3.9 Nematode and plant parameters

The nematode parameters were determined at termination of the glasshouse trials:

- i) the E.L.F. indices for each genotype's root system were determined using Hussey and Boerma's (1981) zero to 5 scale method, where 0 = 0 egg masses; 1 = 1 to 2 egg masses; 2 = 3 to 10 egg masses; 3 = 11 to 30 egg masses; 4 = 31 to 100 egg masses and 5 = more than 100 egg masses/root system.
- ii) Males, eggs and J2 abundance per root system that were extracted from the root system of each genotype using the adapted NaOCl method of Riekert (1995) and counted using a De Grisse counting dish (De Grisse, 1963).
- iii) the reproduction potential of the RKN species for each of the genotypes was calculated using Oostenbrink's reproduction factor (Rf): $Rf = \text{final egg and J2 numbers (Pf)} / \text{initial egg and J2 numbers (Pi)}$ (Windham & Williams, 1987).

Only the root mass (g) of each genotype was measured, using a laboratory balance, in terms of plant parameters.

3.3.9 Experimental design and statistical analyses

The layouts of the first and second/repeat experiments were representative of randomised completed block designs (RCBD), with eight replicates for each of the soybean genotypes included.

The males, egg and J2 data were subjected to a Factorial Analysis of Variance (ANOVA) (SAS, Version 15.2) with trial as the main effect and genotypes as the sub-factor. If a significant interaction was evident between the two trials, data for each trial were subjected to an ANOVA and analysed separately.

3.4 Field study

3.4.1 Study site

The trial was planted at the Uitendacht Farm near Bethal (located in the Highveld region of the Mpumalanga province, South Africa), with the following site coordinates: Latitude: 26°19'8.25"S Longitude: 29° 30'36.70"E (Figure 3.5).

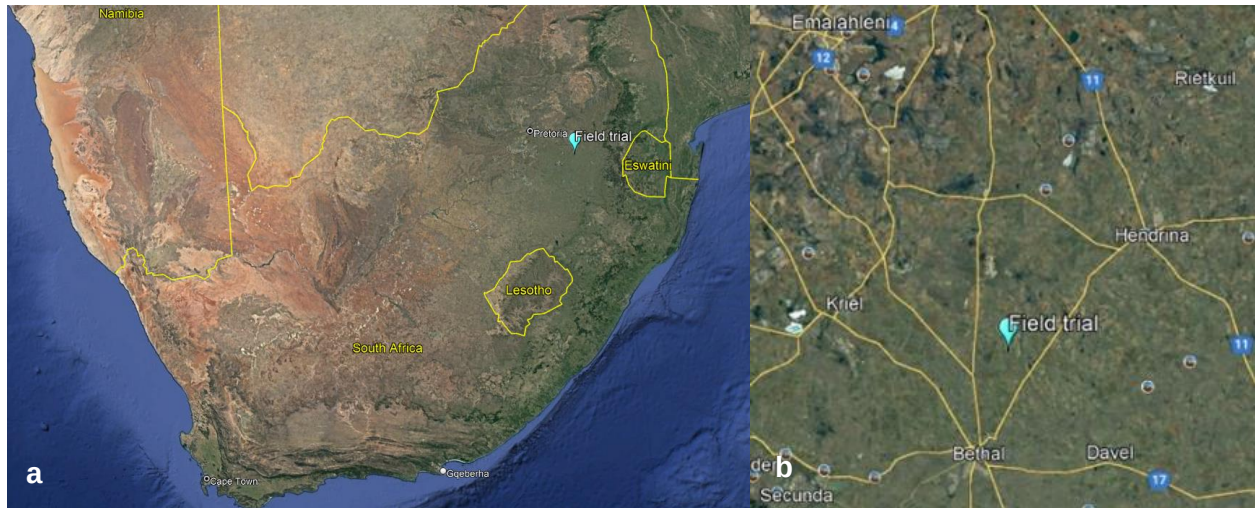


Figure 3.5: a & b. (a) South African map, with the location of the study site near Bethal in the Highveld region of the Mpumalanga province (north-eastern part of South Africa) and (b) Location of the field trial, the farm Uitendacht which is located 17 km north of Bethal where this nematode-based study was conducted (Map created by P.R. Janse van Rensburg, Pioneer Seeds South Africa & North-West University Source: Google Earth Pro: <https://earth.google.com/web/>)

3.4.2 Environmental conditions

Bethal is in the western side of the Mpumalanga province of South Africa; altitude above sea level is 1 690 m and the climate description is as follows: Summers in Bethal are long. The warm season last for 4.9 months (usually ranging from October 25 to March 23) while winters are short (the cold season last for 1.9 months) ranging from June 2 to July 29. Throughout the year, the temperature typically ranges from -0.5 °C to 24 °C, with temperatures rarely falling below -4 °C in winter or rising above 28 °C in summer. The average annual rainfall is between 700-800 mm (WheatherSpark, 2022), and is well

spread throughout the summer months with most rain falling in the months of November to January (Phalane, 2021). Winters are thus mostly dry due to this area receiving rain mainly in the summer.

3.4.3. Soil physicality and site selection

To obtain chemical and physical parameters of the soil, samples were randomly collected over the trial site before planting, combined and sent for soil classification. Texture, soil pH and organic carbon was analysed. That was done by personnel of the Omnia Fertilizer, a division of the Omnia Group (Pty) Ltd, Fourways, Sandton, South Africa. These samples were analysed by Omnia's Chemtech Laboratories. The word soil texture refers to the particle size range in the soil, i.e., whether the particles that make up a given soil are primarily large, small, or of some intermediate size or range of sizes. Results showed that the soil at the trial site contained 89% sand, 0.75% silt, 10% clay and 0.25% organic carbon, and had a pH of 4.55 (H₂O).

3.4.4 Farmer's practices

For more than half a decade the farmer owning this field has been engaged in grain crop rotation by growing maize (*Zea mays* L.) and soybean interchangeably during summer growing seasons.

The farmer sprays herbicides containing glyphosate as active ingredient twice during the summer growing season to ensure good weed control since he plants Roundup Ready® (RR) genotypes.

3.4.5 Soybean genotypes

Pioneer Seed South Africa supplied the seeds of the selected genotypes included in this study. Five soybean genotypes (P48T48R, P59T33R, P61T38R, P64T39R and P71T74R) were selected for this study. The five selected genotypes contained relatively new genetics and produced good yields across SA's soybean production areas. In addition, the RKN susceptible (S) genotype DM5953RSF and the poor-host genotype DM6.8IRR (resistant standard) (Mbatyoti, *et al.*, 2020), were selected as the susceptible

and poor-host standards. The seeds were not treated with any fungi-, herbi- or pesticides since it was requested to be used in nematode experiments.

Table 3.5: Plant growth stage progress and description from one to the next (Nlelya et al., 2019).

Stage Number	Title	Description
Vegetative Stages		
VE	Emergence	Cotyledons growing above the soil's surface.
VC	Cotyledon	Unifoliate leaves unfurl so that the leaf margins do not come into contact.
V1	1st trifoliate	The first trifoliate completely developed and opened.
V2	2nd trifoliate	Plants have three nodes and two trifoliates that are unfolded.
V3	3rd trifoliate	Plants have four nodes and three completely unfolded trifoliates.
V4	4th trifoliate	
V5	5th trifoliate	
V6	6th trifoliate	
V(n)	nth trifoliate	N is the number of the most recently fully grown trifoliate leaf.
Reproductive Stages		
R1	Beginning flowering	Any node on the main stem can have one open bloom.
R2	Full bloom	An open flower with a fully grown leaf at one of the two upper nodes on the main stem.
R3	Beginning pod	A 0.5 cm length pod on one of the four top nodes.
R4	Full pod	A pod 2 cm long at one of the main stem's four topmost nodes.
R5	Beginning seed	The seed is 3 mm long and is found in one of the four topmost nodes on the main stem.
R6	Full seed	A pod carrying a green seed that fills the pod cavity at one of the main stem's four topmost nodes.
R7	Beginning maturity	One normal pod on the main stem has matured pod colour (tan or brown).
R8	Full maturity	95% of pods have matured pod colour.

Table 3.6: A list of the soybean genotypes evaluated for their host status to RKN in a field trial near Bethal (Highveld of the Mpumalanga province, South Africa) with the growth stages of each soybean genotype

during each sampling time (days after emergence: DAE). (Illustration: P.R. Janse van Rensburg, Pioneer Seeds South Africa & NWU)

Genotype	MG	30 DAE	56 DAE	100 DAE
P48T48R	4.8	V6	R3	R8
DM5953RSF	4.8	V6	R3	R8
P59T33R	5.9	V5	R2	R7
P61T38R	6.1	V5	R1	R6
P64T39R	6.4	V5	R1	R6
DM6.8IRR	6.8	V4	V12	R5
P71T74R	7.1	V4	V12	R5

3.4.6 Planting of soybean trial and layout

Before planting, the rows (52 cm apart) were marked by the farmer with his precision equipment where the soybean seeds were to be planted. Each plot consisted of 4 rows (each row was 5 m long).

The soybean seeds of each genotype selected for this study were planted with a hand planter see demonstration in Figure 3.6, with interrow spacing of 6 cm enabling 320 000 seeds to be planted per hectare (ha). This is also the commercial soybean seeding rate which the farmers plant in the area (Personal communication, Emile van den Berg National Agronomy Manager for Pioneer seeds South Africa, October 2021). The seeds were furthermore planted 4 cm deep into the soil profile which is the recommended planting depth for soybean to produce more uniform stands and higher yields (Figure 3.7) (Lawson *et al.*, 2009).



Figure 3.6: Use of a manually operated planter, equipped with a soybean plate for seed spacing, as well as a pressure wheel at the front and back to keep the depth control of the seed the same throughout the trial site (Photo: Hannes Swanepoel, Pioneer Seeds South Africa).



Figure 3.7: A photo indicating soybean seedling emergence 7 days after planting the side rows (next to the trial) show good germination and was uniform in size. The interrow spacing was also accurate (± 6 cm) (Photo: P.R. Janse van Rensburg, Pioneer Seeds South Africa & North-West University).

The seeds were inoculated with *B. japonicum* during planting as explained for the glasshouse trials (see Paragraph 3.3.1).

3.4.7 Nematode sampling intervals

Sampling took place three times throughout the summer growing season at 30, 56 and 100 days after emergence (DAE) see in Table 3.6. At each sampling interval, the soybean plants of each genotype were at a different growth stage, due to each variety having a specific growth length which is expressed as the Majority Group (MG).

The samples were taken at regular intervals throughout the crop cycle since it provides information on the target nematode's population dynamics, which were RKN at the Bethal trial site (Marais *et al.*, 2017).



Figure 3.8: a-c. Photos of the trial site showing: (a) soybean plants 30 days after emergence (first sampling interval) not growing optimally due to high RKN infestation and too much rain (waterlogged conditions) occurring early in the season; (b) Soybean plants showed signs of recovery 56 days after emergence although some plants died due to poor nutrient availability (sandy soil), unfavourable environmental conditions (waterlogging) and the high RKN infestation; (c) At 100 days after emergence poor plant stand was again evident due high RKN infestation, with plants of the short growing maturity groups reaching physiologically maturity and began to die of - plants of the longer growing season maturity groups were still green (Photo's: P.R. Janse van Rensburg, Pioneer Seed South Africa & North-West University).

3.4.8 Nematode sampling pattern

Rhizosphere samples, root, and soil for nematode analysis, were systematically taken in a 'zig-zag' pattern (Figure 3.9) only on the outer two rows of each plot for each of the genotype (Marais *et al.*, 2017). This was done during each sampling time. Four samples were taken from each plot during each sampling time and then combined and mixed to obtain one composite sample per plot that was further analysed.



Figure 3.9: A schematic representation of soybean plants 30 days after emergence with a red line indicating where four samples were taken for each genotype in each plot for further nematode analysis (P.R. Janse van Rensburg, Pioneer Seed South Africa & North-West University).

The four samples per plot were put in labelled plastic bags and kept in cooler boxes while they were transported to the Nematode Diagnostic Laboratory of the North-West University, Potchefstroom Campus. At the laboratory, the bagged samples were stored in a cold room at 6 - 10 °C before extraction. Roots were stored with the rhizosphere soil, to prevent the roots from drying out. The extraction of nematodes from the rhizosphere samples took place within 2-3 days after arrival at the laboratory to ensure that accurate data be obtained.

3.4.9 Nematode extraction of 20 g roots

The extraction of nematodes from soil and plant tissue is an important step in determining the population densities of plant-parasitic nematode species or genera during the various stages of crop growth (Marais *et al.*, 2017). The methodology has been described in Paragraph 3.3.5.

3.4.10 Nematode and plant parameters

The nematode parameters determined were:

- i) *Meloidogyne* egg and J2 abundance / 20 g roots 30, 56, and 100 DAE
- ii) Yield per soybean genotype: kilogram / hectare.

The only plant parameter measured was yield, expressed as kg/ha per plot, which was obtained during harvesting. The soybean pods of the middle two rows only of each plot were harvested by hand when the grain moisture was in the range of 11.5 - 12.5%. A kernel moisture level of 12.5% for soybean seed is adhered to by farmers in South Africa according to contracts provided by cooperatives (e.g., Senwes, Afgri and NWK's) since it is safe for storage after harvesting. Gloves were used to remove the pods from the plants (Figure 3.10 a), which were then transferred into a plastic bag. The plastic bag was shaken to enable the pods to pop open and release the seeds. The pod shells were then removed by manually blowing it off and away to separate the seeds and were weighed (Figure 3.10 b). The genotypes were not ready to be harvested at the same time due to the different MG they belong to (Figure 3.10 c) and therefore the moisture levels of soybean seeds were taken for each soybean genotype to indicate whether it was ready to be harvested (Figure 3.10 d).



Figure 3.10 a-d: Photo' indicating actions during the harvesting of soybean with: (a) gloves being used to strip the soybean pods from the soybean plant; (b) separation of seed from pod material and placing it in a container weighing it on a portable balance; (c) harvest ready soybeans (d) and the percentage moisture of each genotype's seeds were taken with a Wile 55 moisture tester at harvest time (Photo: P.R. Janse van Rensburg, Pioneer Seeds South Africa & North-West University).

3.4.11 Experimental design and statistical analysis of data

The trial layout represented a randomized complete block design (RCBD) with each genotype being replicated eight times. The nematode data were subjected to a Factorial Analysis of Variance (ANOVA) (SAS, Version 15.2) with the genotypes as the main effect and the three sampling times as the sub-factor.

3.5 Field trial hypotheses

It is hypothesized that the genotypes will differ in terms of their host status to the RKN species present at the field trial site as will be indicated by data obtained for the susceptible and resistant standards regarding the abundance of this nematode pests per 20 g roots. Furthermore, it is foreseen that differences in yield will be evident for the genotypes used and that a tolerant genotype may be identified.

3.6 Glasshouse trials hypotheses

It is expected that high tolerance and low tolerance of the different genetics will be highlighted and differ meaningful from each other. It is also expected that the numbers

will be very high because conditions are much more favourable for the nematodes to multiply under glasshouse conditions.

CHAPTER 4: RESULTS AND DISCUSSION: ROOT-KNOT NEMATODE SPECIES IDENTIFICATION, DETERMINATION OF THE HOST STATUS OF SELECTED SOYBEAN GENOTYPES UNDER FIELD AND GLASSHOUSE CONDITIONS

4.1 Introduction

This chapter deals with the determination of host status of commercial soybean genotypes under glasshouse and field conditions because limited sources of root-knot nematode (RKN) resistance are available in SA. Moreover, genotypes that were identified with resistance previously are no longer available on the market due to new genetics with improved agricultural traits such as yield, replacing them. The study also aimed to make growers aware of the presence of plant-parasitic nematodes (with an emphasis on *Meloidogyne* spp.) in soybean producing areas, which may increase the risks of reduced yields and/or quality of soybean crops.

The five commercially available genotypes selected to be evaluated for their host status are listed in Table 3.6 (Chapter 3) and are owned by Pioneer Seeds South Africa. They were selected since they are popular due to their preferable agricultural traits and were made available to the farmer by his local crop supplier, namely Pioneer Seeds ([Soybean pioneer.com](http://Soybean.pioneer.com)). A susceptible genotype (DM5953RSF) for *Meloidogyne incognita* (Kofoid & White, 1919; Chitwood, 1949) and resistant (DM6.8IRR) standard were also included (Mbatyoti *et al.*, 2021). These two genotypes are commercially available and genetically modified, Roundup® Ready (RR). They are owned by the seed company Don Mario and supplied by Agricol who has a trade agreement with Don Mario Seeds, Argentina.

The five Pioneer genotypes and the two standards were first evaluated for their host status to *Meloidogyne enterolobii*, Yang and Eisenback, 1983 in two separate glasshouse trials under regulated temperature conditions (see Chapter 3, paragraph 3.3) whereafter results were verified in a field trial. The field trial site was selected in the Mpumalanga province, near Bethal (see Chapter 3, paragraph 3.4.1) due to the presence of high *M. enterolobii* densities in roots of the susceptible genotype DM5953RSF. During the

flowering stage of the 2019 growing season, nematode population numbers ranged between 10 000 and 50 000 eggs & second-stage juveniles (J2) / 50 g roots (Janse van Rensburg & Fourie, 2021). The high population density and severity of *M. enterolobii* infection in soybean, represented as severe root galling and poor performance of plants (genotype DM5953RSF) is visible at this trial site as shown in Figure 4.1 a, b & c. This locality was also chosen because no treatment has been done to suppress the RKN population densities before this study commenced.

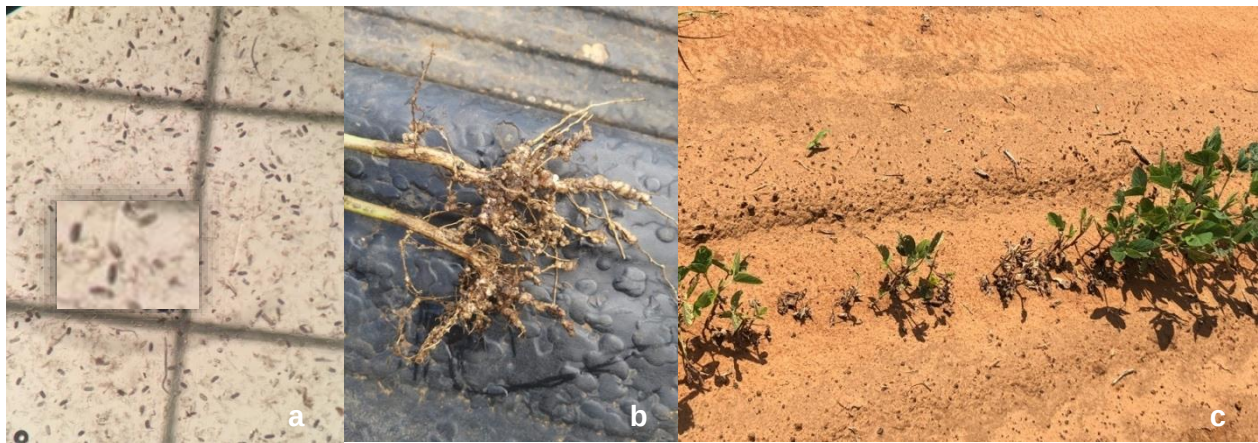


Figure 4.1 a, b & c: a) Eggs visible as blackish, elongated oval structures, of *Meloidogyne* spp. extracted from the roots of the infected soybean plant visible in a Grisse counting dish (Grisse, 1969) using a stereomicroscope (60x magnification), b) severe root galling of a soybean plant (cv. DM5953RSF) grown at the infested field trial site near Bethal, and c) stunted and dying soybean plants at the chosen field trial site due to high RKN infection (Photo's: P.R. Janse van Rensburg, Pioneer Seeds South Africa & North-West University).

Although gall ratings and egg mass indices have been routinely utilised to determine RKN resistance in soybean (Bridge & Starr, 2007; Moens *et al.*, 2009), egg production is viewed as a more reliable criterion (Moens *et al.*, 2009; Windham & Williams, 1988). The nematode parameters were determined at termination of the glasshouse trials (see Chapter 3, paragraph 3.3.6) and the field trial (see Chapter 3, paragraph 3.4.10). A randomised completed block design for the layout of the trials was used and statistical analyses were done by Factorial Analysis of Variance (ANOVA) (see Chapter 3, paragraph 3.3.7 and 3.4.11).

4.2 Morphological/Classical Identification of *Meloidogyne* spp.

The distinction between the three species was and still is difficult for the inexperienced and untrained eye. However, according to Table 3.1 in Chapter 3, each species possesses a set of defined characteristics that are used to distinguish between them and most of the characteristics corresponded to those of *M. enterolobii*, therefore the females were identified as *M. enterolobii*.

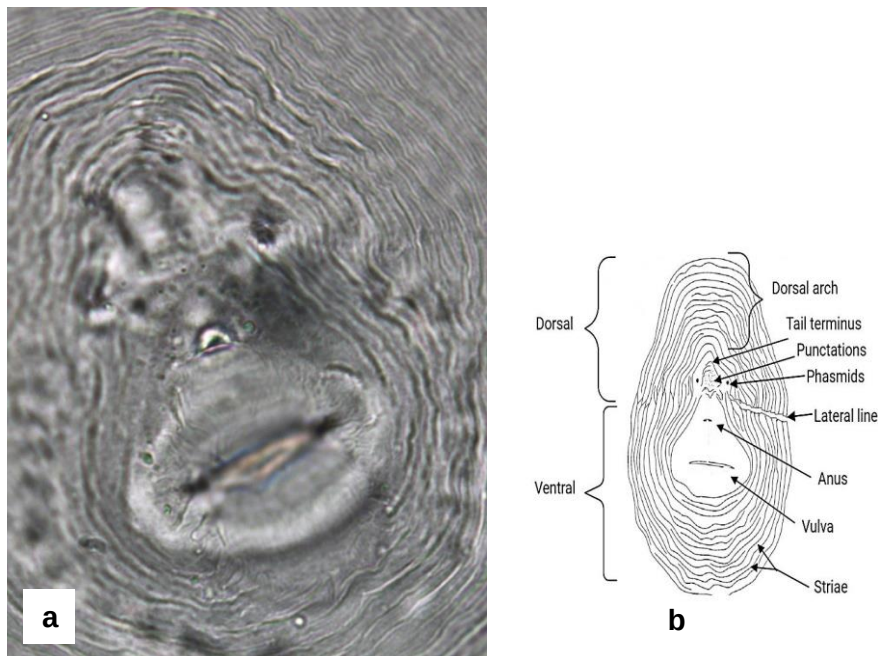


Figure 4.2 a & b: a) Perineal patterns of a mature female of *Meloidogyne enterolobii*. Image taken at 100x magnification, b) compared to perineal pattern diagram indicating characteristics of interest (Photo: Raymond L. Collett, North-West University, Potchefstroom).

4.3 Molecular identification

The identity of the species was confirmed (Figure 4.3) using the SCAR-PCR technique (Zijlstra *et al.*, 2000). The species-specific primers for the identification of four *Meloidogyne* spp. were used to identify *M. enterolobii*, *M. incognita*, *Meloidogyne javanica* (Treub, 1885; Chitwood, 1949) and *Meloidogyne arenaria* (Neal, 1889) (See Chapter 3; Table 3.3).

For *M. enterolobii*, the DNA results from the SCAR-PCR method indicated a 250 bp fragment being amplified, present in the 5th well, which is similar in bp size of the fragment to that Long *et al.* (2006) reported for this species. The DNA fragments from our samples placed in the 2nd, 3th, 4th wells indicated the presence of *M. enterolobii* (Figure 4.3). No fragments were amplified when the DNA of this species was subjected to the SCAR-PCR primers of *M. arenaria*, *M. incognita* and *M. javanica* (data not showed).

The *Meloidogyne* spp. was successfully identified as *M. enterolobii*, using both morphological and molecular approaches, to allow an investigation of the host status of selected soybean genotypes to *M. enterolobii* under field and greenhouse conditions.

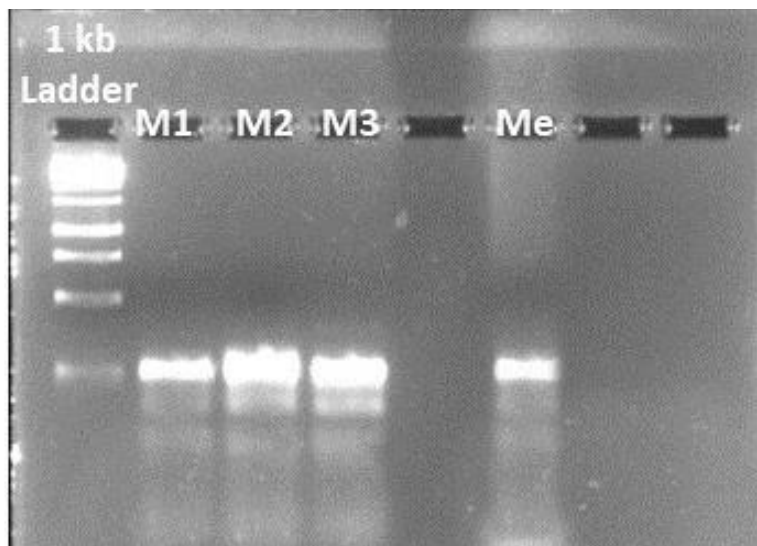


Figure 4.3. Gel electrophoresis results indicating DNA fragments of specific DNA sizes associated with specific *Meloidogyne* spp.; (*Me* – well 5) indicating the presence of *Meloidogyne enterolobii*; (*St. M1, M2, M3* – well 2, 3 and 4) compared to the standard *Meloidogyne enterolobii* standard in well 5 (Photo: Gerhard Engelbrecht, North-West University, Potchefstroom).

4.4 Glasshouse study root data

Fifty-six days after nematode inoculation of the roots of the genotypes, plants were uplified and *M. enterolobii* eggs and J2 extracted from excised and washed roots.

Inoculation of soybean seedlings with *M. enterolobii* eggs and J2 was successful as demonstrated by high E.L.F. indices, egg and J2 densities and Rf values per root system for the susceptible standard DM5953RSF (Table 4.1). High RKN infection of soybean seedlings were also evidenced in the galls formed on the roots (Figure. 4.4 a), while visible aerial symptoms representing yellowing of leaves were evident too (Figure 4.4 b).



Figure 4.4. a & b: a) Severe root galling caused by *Meloidogyne enterolobii* infection of the susceptible standard DM5953RSF (right side) compared to no visible root galling of the resistant DM6.8IRR (left side); b) aboveground chlorosis of leaves of an infected soybean plant (Photos: P.R. Janse van Rensburg, Pioneer Seeds South Africa).

Table 4.1. Nematode parameter data per root system of seven selected soybean genotypes owned by Pioneer Seeds South Africa and a susceptible standard¹ evaluated for their host status to *Meloidogyne enterolobii*.

Genotype	Trial 1	Trial 2	Trial 1		Trial 2		Trial 1			Trial 2			Trial 1		Trial 2	
	Root mass (g)		Egg-laying female (E.L.F.) Index				Males, Eggs and J2						Reproduction factor (Rf)			
P61T38R	6.25	7.16	2.7	aAB	2.8	abABC	8.04*	(3243)	aA	9.25*	(11527)	aABCD	1.62	aA	5.76	aABC
P48T48R	6.57	6.40	3.2	bABCDE	3.3	bcBCDE	8.33*	(4686)	abAB	9.56*	(19998)	abcBCDE	2.34	abA	10.00	aABC
DM6.8IRR	9.43	8.23	2.3	aA	2.5	aAB	8.5*	(5779)	abcAB	9.35*	(12930)	abBCD	2.89	abAB	6.46	aABC
P59T33R	7.12	5.80	2.8	aABC	3.0	abABCD	8.78*	(7262)	abcABC	9.33*	(12243)	abABCD	3.63	abAB	6.12	aABC
P64T39R	10.45	8.52	3.7	bCDE	4.0	cE	9.78*	(22268)	bcdBCDE	10.43*	(35003)	cdDE	11.13	abcABC	17.5	abCD
¹ DM5953RSF	6.42	6.78	4.0	cE	4.0	cE	9.86*	(25377)	cdCDE	10.79*	(55513)	dE	12.69	bcABC	27.76	bD
P71T74R	11.87	7.58	3.7	bCDE	3.8	cDE	10.27*	(31134)	dDE	10.16*	(27043)	bcdDE	15.57	cBCD	13.52	abABC
P Value			0.001		0.001		0.001			0.001			0.001		0.001	
F Value			31.06		12.32		7.58			10.8			6.018		8.77	
Interaction data: Trials																
P Value			0.1				0.001						0.001			
F Value			2.78				36.56						12.38			
Interaction data: Genotypes																
P Value			0.001				0.001						0.001			
F Value			22.5				13.46						14.57			
Interaction data: Trial x Genotype																
P Value			0.99				0.071						0.066			
F Value			0.14				2.04						2.09			

*Log(x+1) transformed data; Real nematode means data in parenthesis. Different small letters in the same column indicate a significant difference at $P \leq 0.05$ among the genotypes in each trial (Trial 1 and Trial 2) according to One-Way ANOVA; different capital letters in the same column indicate significant differences ($P \leq 0.05$) among the two trials in in each segment, for Egg-laying female (E.L.F.) Index, for *Meloidogyne* Males, Eggs and J2 and for the Reproduction factor (Rf) according to the Tukey HSD Test.

No significant interactions were observed between genotype x sampling interval regarding E.L.F index, mean population densities of *M. enterolobii* per 20 g roots and reproduction values (Table 4.1). Significant interactions were however evident for the E.L.F index, mean population densities of *M. enterolobii* and Rf value among the seven genotypes while the latter two parameters also showed a significant interaction between the two trials (Table 4.1)

4.4.1 ELF indices

The E.L.F. index values ranged from 2.3 to 4 for Trial 1 and from 2.5 to 4 for Trial 2 (Table 4.1). The resistant standard DM6.8IRR had the lowest E.L.F. index of 2.3 in Trial 1 and 2.5 in Trial 2. In Trial 1, it did not differ significantly from those of genotypes P59T33R, P61T38R but it was significantly lower compared to the susceptible standard DM5953RSF and Pioneer genotypes P48T48R, P64T39R and P71T74R. In Trial 2, it did not differ significantly from those of genotypes P61T38R, P48T48R and P59T33R but it was significantly lower compared to the susceptible standard DM5953RSF, P64T39R and P71T74R. When the trial data was combined, significant differences between DM6.8IRR P59T33R and P61T38R on the one side and DM5953RSF on other side were obvious.

4.4.2 *Meloidogyne* Males, Eggs and J2

The P61T38R genotype had the lowest numbers namely 3243 in Trial 1 and 11527 in Trial 2. In both trials, it did not differ significantly from those of genotypes P59T33R, DM6.8IRR and P48T48R, but it was significantly lower compared to the susceptible standard DM5953RSF and Pioneer genotypes P64T39R and P71T74R in both trials.

4.4.3 Reproduction factor (Rf)

The Rf values ranged from 1.62 to 15.57 for Trial 1 and from 5.76 to 27.76 for Trial 2. Genotype P61T38R had the lowest Rf values for of 1.62 in Trial 1 and 5.76 for Trial 2, while the P71T74R had the highest Rf for Trial 1 and the susceptible standard DM5953RSF had the highest value for Trial 2 (Table 4.1).

In Trial 1, genotype P61T38R had a significantly lower Rf value than DM5953RSF and P71T74R, while in Trial 2, DM5953RSF had a significantly higher Rf value than P61T38R, P48T48R, P59T33R and the resistant genotype DM6.8IRR.

4.5 Field study: Nematode data in 20g roots

Roots of each genotype were collected at 30, 56 and 100 days after emergence (DAE), (see Chapter 3, paragraph 3.4), kept cool and taken to the EcoRehab premises of the North-West University, Potchefstroom, South Africa. Eggs and J2 were extracted from excised and washed roots as explained in Chapter 3, paragraph 3.3. The nematode parameters and data are listed in Table 4.2.

High nematode infection of roots of soybean seedlings was observed early in the growing season. Plants did not appear to be growing well at any stage and remained yellow and stunted at every sampling interval (see Chapter 3, Fig. 3.8). Infection of RKN eggs and J2 in soybean roots was successful as demonstrated by high egg and J2 densities per 20 g roots for the susceptible standard DM5953RSF (Table 4.2).

4.5.1 Root-knot nematodes (*Meloidogyne enterolobii*)

Table 4.2. Mean population densities (MPD) of *Meloidogyne enterolobii* in 20 g root samples of seven soybean genotypes grown at one location with each interval at 30 DAE, 56 DAE and 100 DAE respectively.

Genotype	30 DAE			56 DAE			100 DAE		
P61T38R	7.72*	(3347)	aAB	9.13*	(13178)	abABC	8.74*	(11683)	abcABC
P48T48R	7.38*	(2109)	aA	8.04*	(4715)	aABC	7.52*	(2645)	aA
DM6.8IRR	7.68*	(2748)	aAB	8.49*	(7084)	aABC	7.784*	(3368)	abAB
P59T33R	8.52*	(7459)	abABC	9.79*	(28644)	cC	9.03*	(12425)	bcABC
P64T39R	7.9*	(3881)	aAB	8.70*	(7834)	aABC	8.36*	(6084)	abcABC
DM5953RSF	8.96*	(11915)	bABC	9.71*	(24135)	bcC	9.34*	(15347)	cBC
P71T74R	7.69*	(2800)	aAB	8.03*	(4626)	aABC	8.13*	(5196)	abABC
P value	0.0126			0.0003			0.0584		
F value	3.13			5.44			2.23		
Interaction data: Sampling interval									
P value	0.001								
F value	5.8								
Interaction data: Genotype									
P value	0.001								
F value	16.05								
Interaction data: Genotype x Sampling interval									
P value	0.734								
F value	0.71								

*Log(x+1) transformed nematode data; **real nematode means in parenthesis; different small letters in the same column indicate a significant difference at $P \leq 0.05$ among the genotypes for each sampling interval (30, 56 & 100 DAE) according to One-Way ANOVA; different capital letters indicate significant differences ($P \leq 0.05$) among the sampling intervals according to the Tukey HSD Test.

The MPD ranged from 2109 to 11915 for sample interval 30 DAE, from 4626 to 28644 for sample interval 56 DAE, and from 2645 to 15347 for sample interval 100 DAE (Table 4.2).

No significant interactions were observed between genotype x sampling interval regarding mean population densities of *M. enterolobii* per 20 g roots (Table 4.2). Significant differences were however evident for the mean population densities of *M. enterolobii* among the three sampling intervals, and for the seven genotypes.

Significant differences between the genotypes per sampling interval were observed with all genotypes except P59T33R differing significantly from the susceptible standard DM5953RSF for the 30 DAE sampling interval. For the 56 DAE sampling interval, all genotypes except P61T38R and P59T33R differed significantly from the susceptible standard DM5953RSF regarding their MPD. At 100 DAE, only genotypes P48T48R, DM6.8IRR and P71T74R differed significantly from the susceptible standard DM5953RSF regarding their MPD.

4.6 Soybean Yield

Table 4.3. Mean yield kg/ha of seven soybean genotypes evaluated for their host status to *Meloidogyne enterolobii* in a field trial in the Mpumalanga province near Bethal.

Genotype	Soybean yield	
LSD(p=0.05)	475.27	
P71T74R	1402.3	a
P61T38R	1337.7	a
P64T39R	1288.9	a
DM6.8IRR	1190.3	ab
P59T33R	995.2	ab
DM5953RSF	947.3	ab
P48T48R	725.2	b
P value	0.0183	
F value	2.77	

Real mean yield (kg/ha) values indicate significant differences ($P \leq 0.05$) among the genotypes at harvesting.

ANOVA analysis indicated significant differences between the yield of the genotypes. Yield kg/ha ranged between 1402.3 kg/ha (P71T74R) to 725.2 kg/ha (P48T48R) (Table 4.3). A significant difference was evident for yield between P48T48R and genotypes P71T74R, P61T38R and P64T39R. The susceptible genotype, DM5953RSF, had the second lowest yield (947.3 kg/ha), but did not differ significantly from P71T74R with a yield of 1402.3 kg/ha.

4.7 Discussion

Identification of *Meloidogyne* spp.

An important finding from this study is the identification of *M. enterolobii* from the field site. Several publications have advocated for the use of morphological and molecular identification for *Meloidogyne* spp., particularly those belonging to the *M. incognita* group, to avoid species misidentification. For example, this was done for South African *Meloidogyne* spp., some of which were represented by *M. enterolobii* (Rashidifard *et al.*, 2019; Visagie *et al.*, 2018). In many countries, *Meloidogyne* spp. is regarded as the most economically important group of nematodes on soybeans (Bridge & Starr, 2007). Although *M. incognita* and *M. javanica* occur as the Top-2 species in local soybean production areas (Fourie *et al.*, 2017), *M. enterolobii* can be added to the list of species associated with the crop in SA. Another species identified in local soybean production areas is *Meloidogyne ethiopica* Whitehead, 1968 in association with soybean in SA (Fourie *et al.*, 2001).

Meloidogyne spp. identification is critical because *M. incognita* and *M. javanica* are known to produce problems in local maize (Mc Donald *et al.*, 2017) and soybean (Fourie *et al.*, 2017) producing areas. *Meloidogyne enterolobii*, on the other hand, was just recently found from South Africa's second largest soybean production area (the Highveld of Mpumalanga) (Pretorius, 2018), where the crop is often rotated with maize. Furthermore, the distribution of *M. enterolobii* has lately been reported from more crops farmed in South Africa (Visagie *et al.*, 2018; Rashidifard *et al.*, 2019).

The prevalence of mixed RKN species in soybean-based cropping systems (Fourie *et al.*, 2017), as well as the prospect of discovering novel species, necessitates the adoption of multiple identification methods to assure accurate identification. The use of the sequence-characterized amplified region-polymerase chain reaction (SCAR-PCR) approach, which

employs *Meloidogyne* spp.-specific primers, should be the preferred method for distinguishing *Meloidogyne* spp. for local studies, in addition to morphological and morphometric analysis. It was clear by studying the females' oesophageal morphology and perineal patterns, to identify *M. enterolobii* was difficult. This agrees with findings that discriminating between the latter and other species in the *Mi* group is challenging, even for experienced and trained taxonomists (Carneiro *et al.*, 2016; Carneiro & Cofcewicz, 2008). Thus, by employing molecular approaches such as the SCAR-PCR method (Zijlstra *et al.*, 2000), as explained in Chapter 3, Paragraph 3.2.2 misinterpretation of the identity of the *Meloidogyne* spp. used in this study was prevented.

By combining the morphological approach with the SCAR-PCR molecular results, that yielded species-specific DNA fragments of a particular bp length (250 bp and were similar to the positive standards included for the respective species), accurate identification of the *Meloidogyne* spp. was achieved and *M. enterolobii* was confirmed to be used for the glass house trials as well as being present in the field trial.

Glasshouse study

A study by Kim *et al.* (2017) proved that nematode penetration, gall, and egg-mass formations were significantly higher in 100% sand than in the other soil mixtures. Sand-like soil types further increase damage from infection since they have higher plant-water and nutrient deficits and are frequently associated with RKN infestations (Roberts, 2022). Soil used in the glass house experiments consisted of 97.6% sand which probably contributed to the high infection rate.

Data of experiments may vary, for example Glasshouse 1's data for the total *M. enterolobii* numbers were substantially lower than that of Trial 2, namely the egg and J2 numbers ranging from 11527 for cv P61T38R to 55513 for cv DM5953RSF in Trial 2 compared to 3243 for cv P61T38R to 31134 for cv DM5953RSF in Trial 1. An explanation can be the difference in the temperature range in the glasshouse for the two trials. Although the trials were planted in the same glasshouse and the temperatures ranged from 19 – 20±1 °C (min) and 25 - 27±1 °C (max), the first glasshouse trial was conducted during the spring months (September to November) while the second glasshouse trial was conducted during the summer months (November to January). Even with the temperature being regulated in the glasshouse, sunlight hours and outside temperature tend to play a role on the fecundity of the nematodes with the first glass house trial having shorter daylight

hours and colder outside temperatures whereas the opposite was true for the second glasshouse trial that was planted during summer. Data from this study agrees with findings of Mbatyoti *et al.* (2021) as they also found a substantial difference in egg and J2 data for *M. incognita* between the two glasshouse trials. According to Nardacci and Barker (1978), Rf of *M. incognita* in soybean roots was greatly impacted by temperature, with the lowest nematode numbers at temperatures ranging from 16 to 20 °C and the greatest at 30 °C. Several environmental conditions, such as humidity and temperature, are known to influence how nematodes interact with their hosts.

However, valuable information about the host status of soybean cultivars to the predominant endoparasitic plant-parasitic nematode *M. enterolobii* was generated during the study.

Important is that all genotypes proved to be highly susceptibility to *M. enterolobii* when data were pooled across the two glasshouse trials. Cultivar P61T38R, however, maintained the lowest infection of only 10% of the population density of the most susceptible cultivar DM5953RSF for Trial 1, and 20% for Trial 2.

Field trial study

In the field trial, *Meloidogyne* dominated in 20 g roots of the glyphosate-tolerant soybean genotypes due to a combination of high population densities and a high frequency of occurrence. Nematodes are known to have a patchy distribution (Abd-Elgawad, 2021), but in this trial all the plots were highly infested with *Meloidogyne*. It is known that RKN thrive in warm, moist, sandy soils (Olsen, 2011), which the soil of the field site chosen for this study is representative of since it contains 89% sand therefore it can also be attributed to good infestation of root-knot nematodes at this site.

Meloidogyne enterolobii egg and J2 densities per 20 g roots were the highest in roots of all seven soybean genotypes sampled during 56 DAE. Riekert (1995) discovered that the mean population densities (MPD) of plant-parasitic nematodes peaked during maize flowering which is probably the same with soybean. Thirty days after emergence, *M. enterolobii* densities were the lowest while at 100 days the numbers were lower than the 56 days which is suggested to be because of the build-up of the population at physiological maturity of the genotype while there were no longer enough roots available

as a food source for them at that time. Other factors include lower temperatures and moisture which could play a role later in the season (Das & Perry, 2010).

Significant differences between the genotypes per sampling interval were observed with P48T48R having the lowest nematode densities, differing significantly from the susceptible standard DM5953RSF at the three sampling intervals. Although the Tukey HSD test indicates this significance, it was not mirrored in the P-value ($P < 0.05$) of 0.0584 for the 100 DAE. This is suggested to be the result of variability in data within the replicates of each genotype. The other tested genotypes did not differ significantly from one another except for P59T33R which had the highest MPD at 56 DAE, where it differed significantly from most of the genotypes tested (Table 4.2). At 100 DAE, P59T33R still had a significantly higher MPD than P48T48R but not compared to the other genotypes. Although, there is a growth length difference in the genotypes, P48T48R is classified as a 4.8 growth stage cultivar, meaning that it reaches maturity and ripening faster, compared to P59T33R which is classified as a 5.9 growth length cultivar; the latter taking a longer period to grow through its life stages, see the description of the different majority of soybean genotypes in Table 3.6 in Chapter 3.

Field results generally agreed with those of the glasshouse study, indicating that DM5953RSF (susceptible) maintained the highest egg and J2 numbers / 20 g roots at 30 DAE (11915) and 100 DAE (15347) and second highest at 56 DAE (24135). However, inconsistent findings for glasshouse and field experiments is not uncommon (Mbatyoti *et al.*, 2021) and were observed for cultivars such as P61T38R which showed some resistance in the glasshouse, but not in the field trial. On the other hand, P48T48R had the lowest *M. enterolobii* numbers in each sampling interval namely 30 DAE (2109), 56 DAE (4715) and 100 DAE (2645) but in the glasshouse trial, it did not perform as good since P61T38R had consistently the lowest nematode numbers varying from 3342 to 11527 per root system. An additional explanation for the observation is that *M. enterolobii* population densities were substantially higher during the glasshouse trial compared to the field trial which could have been due to the impact of various other abiotic and biotic environmental factors that were not recorded. However, according to the data collected from the field trial, no genotype can be identified as a resistant genotype since all the genotypes tested had Rf values higher than 1. They are therefore all classified as susceptible to *M. enterolobii*.

Generally, the genotype DM5953RSF is highly recommended due to its good yield potential. However, in our trial due to the high *M. enterolobii* numbers, it did not perform optimally. Each genotype handles the impact of plant-parasitic nematodes differently, which means that threshold levels for genotypes will differ. Soybean genetics have the ability to compensate for stress factors such as droughts, hail damage and pest damage and still maintain a good yield (Personnel communication: Emile van den Berg, National Agronomy Manager of Pioneer. 22 April 2022).

Due to severe waterlogging conditions experienced during the early growing season, as well as the high *M. enterolobii* population density at the site, soybean production was lower than expected. However, P59T33R produced a fairly good yield (1.08t/ha) despite high *M. enterolobii* infection levels (16358/20 g roots).

Resistant or poor hosts genotypes can be grown to reduce population densities of *M. enterolobii* and should be a viable approach to combat RKN and limit their damage. It is, however, crucial that soybean genotypes must be evaluated not only for yield potential, but also for disease and insect susceptibility and sensitivity, including plant-parasitic nematodes. Although the results of various South African glasshouse studies on the host response of soybean genotypes to RKN have been reported (Fourie *et al.*, 1999, Fourie *et al.*, 2001; Fourie *et al.*, 2015; Mbatyoti *et al.*, 2021) it is crucial to conduct such studies regularly since genotype entries to the market change every year.

Based on the glasshouse and field results of this study, farmers can use the genotype with the lowest host status to reduce *M. enterolobii* populations. However, this approach will have to be complemented by other control strategies. According to findings in Ghana, disc ploughed field were able to reduce RKN populations under maize by 96% when compared to zero tillage plots, although the yield of zero tillage was 35% higher than disc plough (Kankam & Adomako, 2014). This maybe a strategy that can be used by local soybean farmers not practising conservation or regenerative agriculture.

Another important aspect of nematode control is reducing weed populations in the field. Weeds that are commonly found in South African crop production fields are susceptible to RKN and serve as reservoirs of these pests (Ntidi *et al.*, 2015; Ntidi *et al.*, 2012). Although all cultivated soybeans are glyphosate tolerant, resistance against glyphosate is increasing on certain weeds (Heap & Duke, 2018).

Farmers have implemented crop rotations since maize and soybean rotation did increase the soybean yield by about 7% compared to monoculture soybean production and maize-soybean rotation. This showed an average yield benefit of more than 20% of maize yield (Behnke *et al.*, 2018) whereby they rotate maize with soybeans. However, the maize cultivars used in SA are all considered susceptible to RKN and will therefore not suppress nematode populations (Fourie *et al.*, 2015). The RKN problem in grain production areas are hence aggravated by such crop rotation systems and ultimately limit sustainable crop production.

It is clear that the soybean industry does not have an effective solution beyond using a nematicide to control nematode numbers and resistance screening will be necessary to help the farmers in the future.

CHAPTER 5: CONCLUSION AND FUTURE RECOMMENDATIONS

5.1 Conclusion

At the beginning of the study, it was hypothesized that:

- the host status of soybean genotypes to *M. enterolobii* will be elucidated in glasshouse trials,
- genotypes that are classified as poor hosts (compared to the susceptible and resistant standards) based on reproduction factor values (R_f) where $R_f < 1$ will be validated in the field trial where *M. enterolobii* prevails and
- that the prevalent root-knot nematode species' population densities will adversely impact on yield of different soybean genotypes grown in the field trial.

This study revealed the presence of *Meloidogyne enterolobii* (Yang and Eisenback, 1983) in an important soybean production area of South Africa and the susceptibility of the popular, commercially- available glyphosate-tolerant local soybean genotype planted in the Highveld Mpumalanga to *Meloidogyne*.

The glyphosate-tolerant soybean genotypes used during this study were identified as susceptible to *M. enterolobii* and can, hence, support high population densities of this species. The first two parts of the hypothesis were supported by both the glasshouse and field studies conducted during this MSc project. It can thus be concluded that this RKN species has a high reproduction potential on the local glyphosate-tolerant soybean genotypes grown. Hence, these nematode genera especially pose a challenge to producers since nematicides are expensive to apply for the production on soybean in South Africa.

Moreover, only a few cultivars with resistance to *M. incognita* are available (Mbatyoti *et al.*, 2021), which makes the local industry vulnerable to these pests. Additionally, genetic progress is very fast in soybeans for high yield and most of those genotypes identified as poor hosts or as resistant are no longer planted by the farmers.

Inconsistent findings for glasshouse and field experiments whereby P61T38R provided the best results in the glass house trials but seemed to be susceptible to *M. enterolobii* in

the field, is not unexpected since such deviations have been reported from other studies and other resistant genotypes (Mbatyoti *et al.*, 2021).

The importance of obtaining yield data is an aspect that should be accentuated and hence data from this study supported the third part of the hypothesis. All cultivars produced yields above 1 ton/ha except for the susceptible standard, P59T33R and P48T48R that showed higher sensitivity to high *M. enterolobii* population densities. Non-optimal yields attained indicate the adverse effect of this RKN species on the soybean cultivars evaluated under prevailing environmental conditions at the trial site.

The fact that these genotypes showed no resistance to *M. enterolobii*, confirmed that these genotypes should preferably not be used at sites where *M. enterolobii* is the prevalent RKN species. If a farmer wishes to use these cultivars, he or she should have an additional control strategy in place, such as registered nematicides.

Lastly, it is emphasised that the host response testing of local genotypes to *M. enterolobii* and other *Meloidogyne* spp. that are prominent in South African soybean-based production areas should be done on a regular basis to offer producers with the most up-to-date information.

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SUPPLEMENTARY MATERIAL

ADDENDUM 1A: Tables for the different parameters in the Glasshouse trials of the *Meloidogyne enterolobii* extracted from the soybean roots;

Table A1a. the number of the (E.L.F) index of *Meloidogyne enterolobii*, 56 (days after inoculation: dai) in roots of soybean genotypes according to the One-Way ANOVA; $P \leq 0.05$ among the genotypes in Glasshouse 1

Tukey HSD test; variable <i>Meloidogyne</i> E.L.F index (Glasshouse Trial 1) Homogenous Groups, alpha = ,05000 Error: Between MS = 34,613, df = 30,000 Interaction: Genotypes P=0.001, F ratio=31.06					
Cell No.	Genotype	E.L.F. Index	a	b	c
6	DM6.8IRR	2.3	****		
3	P61T38R	2.7	****		
4	P59T33R	2.8	****		
5	P48T48R	3.2		****	
1	P71T74R	3.7		****	
2	P64T39R	3.7		****	
7	DM5953RSF	4			****

Table A1b. the number of the (E.L.F) index of *Meloidogyne enterolobii*, 56 (days after inoculation: dai) in roots of soybean genotypes according to the One-Way ANOVA; $P \leq 0.05$ among the genotypes in Glasshouse 2

Tukey HSD test; variable <i>Meloidogyne</i> E.L.F index (Glasshouse Trial 2) Homogenous Groups, alpha = ,05000 Error: Between MS = ,18019, df = 29,000 Interaction: Genotypes P=0.001, F ratio=12.32					
Cell No.	Genotypes	ELF index	a	b	c
2	DM 6.8IRR	2.5	****		
5	P61T38R	2.8	****	****	
4	P59T33R	3.0	****	****	
3	P48T48R	3.3		****	****
7	P71T74R	3.8			****
6	P64T39R	4.0			****
1	DM5953RSF	4.0			****

Table A1c. factorial table for the (E.L.F) index of *Meloidogyne enterolobii*, 56 (days after inoculation: dai) in roots of the 2 Glasshouse trials in roots of the soybean genotypes according to a three-way factorial analysis of variance (statistica 13.3) with means separated at $p \leq 0.05$ according to the Tukey HSD Test.

Tukey HSD test; Meloidogyne E.L.F index (Exp 1 & 2) Homogenous Groups, alpha = ,05000
(Non-Exhaustive Search) Error: Between MS = ,19517, df = 69,000

Interaction:

Trials (1 & 2), P=0.1, F ratio=2.78

Genotypes, P=0.001, F= 22.50

Trials & Genotypes. P=0.990, F=0.14

Cell No.	Exp no.	Genotype	ELF index	A	B	C	D	E
6	1	DM 6.8IRR	2.3	****				
13	2	DM 6.8IRR	2.5	****	****			
3	1	P61T38R	2.7	****	****			
10	2	P61T38R	2.8	****	****	****		
4	1	P59T33R	2.8	****	****	****		
11	2	P59T33R	3	****	****	****	****	
5	1	P48T48R	3.2	****	****	****	****	****
12	2	P48T48R	3.3		****	****	****	****
2	1	P64T39R	3.7			****	****	****
1	1	P71T74R	3.7			****	****	****
8	2	P71T74R	3.8				****	****
7	1	DM5953RSF	4					****
14	2	DM5953RSF	4					****
9	2	P64T39R	4					****

Table A1d. The total number *Meloidogyne enterolobii*, 56 (days after inoculation: dai) in roots of soybean genotypes according to the One-Way ANOVA; $P \leq 0.05$ among the genotypes in Glasshouse 1.

Tukey HSD test; variable <i>Meloidogyne</i> total*Log(x+1) transformed (Glasshouse Trial 1) Homogenous Groups, alpha = ,05000 Error: Between MS = ,55221, df = 30,000 Interaction: Genotypes P=0.001, F ratio=7.58						
Cell No.	Genotype	Mel tot Log(x+1	a	b	c	d
3	P61T38R	8.04103	****			
5	P48T48R	8.33478	****	****		
6	DM6.8IRR	8.49617	****	****	****	
4	P59T33R	8.77886	****	****	****	
2	P64T39R	9.77900		****	****	****
7	DM5953RSF	9.85722			****	****
1	P71T74R	10.26926				****

Table A1e. The total number *Meloidogyne enterolobii*, 56 (days after inoculation: dai) in roots of soybean genotypes according to the One-Way ANOVA; $P \leq 0.05$ among the genotypes in Glasshouse 2

Tukey HSD test; variable <i>Meloidogyne</i> total*Log(x+1) transformed (Glasshouse Trial 2) Homogenous Groups, alpha = ,05000 Error: Between MS = ,19869, df = 29,000 Interaction: Genotypes P=0.001, F ratio=10.80						
Cell No.	Genotype	Mel tot Log(x+1	a	b	c	d
2	P61T38R	9.25406	****			
5	P59T33R	9.32521	****	****		
4	DM6.8IRR	9.35000	****	****		
3	P48T48R	9.55916	****	****	****	
7	P71T74R	10.16335		****	****	****
6	P64T39R	10.42943			****	****
1	DM5953RSF	10.78558				****

Table A1f. factorial table for the total number of *Meloidogyne enterolobii*, 56 (days after inoculation: dai) in roots of the 2 Glasshouse trials in roots of the soybean genotypes according to a three-way factorial analysis of variance (statistica 13.3) with means separated at $p \leq 0.05$ according to the Tukey HSD Test.

Tukey HSD test; variable Mel tot Log(x+1) Transformed (Glasshouse 1 & 2) Homogenous Groups, alpha = ,05000 (Non-Exhaustive Search) Error: Between MS = ,40322, df = 69,000 Trials (1 & 2), P=0.001, F ratio=36.56 Genotypes, P=0.001, F= 13.46 Trials & Genotypes. P=0.71, F=2.04									
Cell No.	Exp	Genotype	Mel tot Mean no.	Mel tot Log(x+1)	A	B	C	D	E
3	1	P61T38R	3242.5	8.04103	****				
5	1	P48T48R	4686	8.334781	****	****			
6	1	DM6.8IRR	5779	8.496169	****	****			
4	1	P59T33R	7262	8.778859	****	****	****		
10	2	P61T38R	11527	9.254059	****	****	****	****	
11	2	P59T33R	12243	9.325215	****	****	****	****	
13	2	DM 6.8RR	12929.5	9.350004		****	****	****	
12	2	P48T48R	19997.67	9.559165		****	****	****	****
2	1	P64T39R	22267.67	9.778998			****	****	****
7	1	DM5953RSF	25376.5	9.857222			****	****	****
8	2	P71T74R	27043.17	10.16335				****	****
1	1	P71T74R	31134.33	10.26926				****	****
9	2	P64T39R	35003.17	10.42943				****	****
14	2	DM5953RSF	55512.5	10.78558					****

Table A1g. The Reproduction (factor Rf) *Meloidogyne enterolobii*, 56 (days after inoculation: dai) in roots of soybean genotypes according to the One-Way ANOVA; $P \leq 0.05$ among the genotypes in Glasshouse 1.

Tukey HSD test; variable (Reproduction factor) Rf Meloidogyne (Glasshouse Trial 1) Homogenous Groups, alpha = ,05000 Error: Between MS = 33,498, df = 30,000 Interaction: Genotypes P=0.001, F ratio=6.018					
Cell No.	Genotype	Rf Mel	a	b	c
3	P61T38R	1.62125	****		
5	P48T48R	2.34300	****	****	
6	DM6.8IRR	2.88950	****	****	
4	P59T33R	3.63100	****	****	

2	P64T39R	11.13383	****	****	****
7	DM5953RSF	12.68825		****	****
1	P71T74R	15.56717			****

Table A1h. The Reproduction (factor Rf) *Meloidogyne enterolobii*, 56 (days after inoculation: dai) in roots of soybean genotypes according to the One-Way ANOVA; $P \leq 0.05$ among the genotypes in Glasshouse 2.

Tukey HSD test; variable (Reproduction factor) Rf Meloidogyne (Glasshouse Trial 1) Homogenous Groups, alpha = ,05000 Error: Between MS = 43,580, df = 29,000 Interaction: Genotypes P=0.001, F ratio=8.770				
Cell No.	Genotype	Rf Mel	a	b
5	P61T38R	5.76350	****	
4	P59T33R	6.12150	****	
2	DM6.8IRR	6.46475	****	
3	P48T48R	9.99883	****	
7	P71T74R	13.52158	****	
6	P64T39R	17.50158	****	****
1	DM5953RSF	27.75625		****

Table A1i. factorial table for the Rf of *Meloidogyne enterolobii*, 56 (days after inoculation: dai) in roots of the 2 Glasshouse trials in roots of the soybean genotypes according to a three-way factorial analysis of variance (statistica 13.3) with means separated at $p \leq 0.05$ according to the Tukey HSD Test.

Tukey HSD test; variable Reproduction factor (Rf) Meloidogyne (Glasshouse 1 & 2) Homogenous Groups, alpha = ,05000 (Non-Exhaustive Search) Error: Between MS = 40,247, df = 69,000 Trials (1 & 2), P=0.001, F ratio=14.57 Genotypes, P=0.066, F= 13.46 Trials & Genotypes. P=0.71, F=2.09							
Cell No.	Genotype	EXP no.	Rf Mel	A	B	C	D

5	P61T38R	1	1.62125	****			
9	P48T48R	1	2.343	****			
11	DM 6.8IRR	1	2.8895	****	****		
7	P59T33R	1	3.631	****	****		
6	P61T38R	2	5.7635	****	****	****	
8	P59T33R	2	6.1215	****	****	****	
12	DM 6.8IRR	2	6.46475	****	****	****	
10	P48T48R	2	9.998833	****	****	****	
3	P64T39R	1	11.13383	****	****	****	
13	DM5953RSF	1	12.68825	****	****	****	
2	P71T74R	2	13.52158	****	****	****	
1	P71T74R	1	15.56717		****	****	****
4	P64T39R	2	17.50158			****	****
14	DM5953RSF	2	27.75625				****

ADDENDUM 1B: tables for the different parameters in the glasshouse trials of the *Meloidogyne enterolobii* extracted from the soybean roots for the different time intervals 30 DAE, 56 DAE and 100 DAE

Table B1a. The Mean numbers of *Meloidogyne enterolobii*, 30 (days after emergence DAE) in roots of soybean genotypes according to the One-Way ANOVA; $P \leq 0.05$ among the genotypes in the field trial.

Tukey HSD test; One way Anova 30 Days after emergence (DAE)						
Homogenous Groups, alpha = ,05000						
Genotypes, P=0.0126, F= 3.13						
Cell No.	Genotypes	DAE	Mel tot Mean no	Mel tot Log(x+1)	a	b
21	P48T48R	30	2109.375	7.379474	****	
10	DM6.8IRR	30	2747.875	7.675634	****	
13	P71T74R	30	2800	7.694702	****	
15	P61T38R	30	3346.875	7.721638	****	
20	P64T39R	30	3881.25	7.89671	****	
14	P59T33R	30	7459.375	8.524711	****	****
6	DM5953RSF	30	11915.13	8.963611		****

Table B1b. The Mean numbers of *Meloidogyne enterolobii*, 56 (days after emergence DAE) in roots of soybean genotypes according to the One-Way ANOVA; $P \leq 0.05$ among the genotypes in the field trial

Tukey HSD test; One way Anova 56 Days after emergence (DAE) Homogenous Groups, alpha = ,05000 Genotypes, P=0.0003, F= 5.44							
	Genotypes	DAE	Mel tot Mean no	Mel tot Log(x+1)	a	b	c
9	P71T74R	56	4626	8.029364	****		
12	P48T48R	56	4714.625	8.04222	****		
1	DM6.8IRR	56	7084.375	8.486671	****		
16	P64T39R	56	7834.375	8.704723	****		
18	P61T38R	56	13178.13	9.129198	****	****	
17	DM5953RSF	56	24134.5	9.708159		****	****
2	P59T33R	56	28643.75	9.787544			****

Table B1c. The Mean numbers of *Meloidogyne enterolobii*, 100 (days after emergence DAE) in roots of soybean genotypes according to the One-Way ANOVA; $P \leq 0.05$ among the genotypes in the field trial

Tukey HSD test; One way Anova 100 Days after emergence (DAE) Homogenous Groups, alpha = ,05000 Genotypes, P=0.0584, F= 2.23							
	Genotype	DAE	Mel tot Mean no	Mel tot Log(x+1)	a	b	c
19	P48T48R	100	2644.75	7.521275	****		
4	DM6.8IRR	100	3368.375	7.840253	****	****	
7	P71T74R	100	5195.875	8.132044	****	****	
11	P64T39R	100	6084.125	8.362658	****	****	****
8	P61T38R	100	11683.38	8.740145	****	****	****
3	P59T33R	100	12424.75	9.027676		****	****
5	DM5953RSF	100	15346.5	9.339021			****

Table B1d. factorial table for the real mean numbers and Log(x+1) Transformed of *Meloidogyne enterolobii*, 30 DAE, 56 DAE and 100 DAE sample intervals in roots of the Field trials in roots of the soybean genotypes according to a three-way factorial analysis of variance (statistica 13.3) with means separated at $p \leq 0.05$ according to the Tukey HSD Test.

Tukey HSD test; variable Meloidogyne total, Sampling interval Days after Emergence (DAE) data Log(x+1) Transformed (Field Trial) Homogenous Groups, alpha = ,05000 (Non-Exhaustive Search) Error: Between; Within; Pooled MS = ,98200, df = 129,57 Samling interval (30 DAE, 56 DAE & 100DAE), P=0.001, F ratio=16.05 Genotypes, P=0.066, F= 5.80 Trials & Genotypes. P=0.737, F=0.71							
Cell No.	Genotype	DAE	Mel tot Mean no.	Mel tot Log(x+1)	A	B	C
21	P48T48R	30	2109	7.379474	****		
19	P48T48R	100	2645	7.521275	****		
10	DM6.8IRR	30	2748	7.675634	****	****	
13	P71T74R	30	2800	7.694702	****	****	
15	P61T38R	30	3347	7.721638	****	****	
4	DM6.8IRR	100	3368	7.840253	****	****	
20	P64T39R	30	3881	7.896710	****	****	
9	P71T74R	56	4626	8.029364	****	****	****
12	P48T48R	56	4715	8.042220	****	****	****
7	P71T74R	100	5196	8.132044	****	****	****
11	P64T39R	100	6084	8.362658	****	****	****
1	DM6.8IRR	56	7084	8.486671	****	****	****
14	P59T33R	30	7459	8.524711	****	****	****
16	P64T39R	56	7834	8.704723	****	****	****
8	P61T38R	100	11683	8.740145	****	****	****
6	DM5953RSF	30	11915	8.963611	****	****	****
3	P59T33R	100	12425	9.027676	****	****	****
18	P61T38R	56	13178	9.129198	****	****	****
5	DM5953RSF	100	15347	9.339021		****	****
17	DM5953RSF	56	24135	9.708159			****
2	P59T33R	56	28644	9.787544			****

Table B1e. Soybean genotypes yield, with infected *Meloidogyne enterolobii*, according to the One-Way ANOVA; $P \leq 0.05$ among the genotypes in the field trial.

Least Significant difference LSD ($p=0.05$) 475.27				
	Cultivar	Yield	a	b
1	P71T74R	1402.3	****	
2	P61T38R	1337.7	****	
4	P64T39R	1288.9	****	
5	DM6.8IRR	1190.3	****	****
3	P59T33R	995.2	****	****
6	DM5953RSF	947.3	****	****
7	P48T48R	725.2		****

