



Effects of new-generation nematicides on nematode assemblages in soybean rhizospheres

S Esterhuizen

 **orcid.org 0000-0002-0309-479X**

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Supervisor: Prof MS Daneel
Co-supervisor: Prof H Fourie
Co-supervisor: Prof DGMA de Waele

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ABSTRACT

The root-knot nematode (*Meloidogyne* spp.) is known to cause severe yield losses in agricultural crops, especially in soybean. The number of nematicides available in the current market is reducing while the environmental impacts of these nematicides have proven to be harmful. Research to identify effective biological control agents of nematodes is becoming increasingly important while introducing new-generation nematicides can reduce the environmental impacts.

In this study, the effect of new-generation nematicides was evaluated in both *in vitro* and *in vivo* experiments. *In vitro* bioassays were conducted using eight different biological products (T3C, Oligo, Ses-A, Aba-5, Graphite, BM-T, Green liquid, and Oxa-40). The most effective products were then evaluated in a micro-plot experiment, together with DT-200, RV42-20, a commercial fertilizer (Bio-Green) and a commercial nematicide (Velum® 1GR). Tunnel as well as field experiments were also conducted to evaluate the reduction of root-knot nematode populations under natural conditions, using the commercial fertilizer (Bio-Green and the commercial nematicide Velum® 1GR).

The most effective new-generation nematicide in the *in vitro* trial was the Oligo-product. However, all the evaluated products significantly reduced the motility of J2 *Meloidogyne* spp. The micro-plot experiments resulted in no significant differences between the nematode population densities of the different treatments after 112 days after emergence (DAE). Both the tunnel and field trials of the commercial products, Velum® 1GR and Bio-Green granule significantly reduced nematode densities, while a combination of both the products had the most significant reduction in nematode population densities after 112 DAE.

In conclusion, new-generation nematicides have the potential to reduce root-knot nematode population densities. However, natural conditions influence the effectiveness of these nematicides, and further studies should be conducted to improve the efficacy of these products during different environmental conditions and for a variety of crops.

Keywords: *Meloidogyne*, *Pratylenchus*, soybean, biocontrol.

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1 Introduction

1.1 Description and background of the problem

Soybean (*Glycine max* (L.) Merr) is one of the 10 most important food crops globally (Pagano and Miransari, 2016). The top three soybean production countries in the world are currently Brazil, which produces 37% of the global soybean production, followed by the United States of America (USA) and Argentina (Ritchie and Roser, 2022). The African continent contributes less than 1% of the global soybean production of which 39% is produced in South Africa (SA). The total soybean production in SA has increased by 29% over the past decade, with a record harvest of 2,7 million tons in the 2022/2023 season (Grain-SA, 2023). This increase in production emphasises the importance of soybean for the national economy. One of the many biotic stresses soybean crops experience is infection by plant-parasitic nematodes (PPN). The economically most important group of nematodes affecting soybean crop production worldwide are the root-knot nematodes (RKN) (Jones *et al.*, 2013). RKN are widely distributed and can cause substantial damage to soybean plants which may lead to high yield losses (Fourie *et al.*, 2017). The predominant RKN species that prevails in soybean production areas globally as well as in SA is *Meloidogyne incognita* (Kofoed and White, 1919) Chitwood, 1949.

RKN are successful pathogens because of their wide host range and the lack of efficient management strategies, including none or only a few registered nematicides in many African countries (Hartman *et al.*, 2015). The application of a nematicide raises the production cost and lowers profit margins significantly. Consequently, nematicides are mostly applied to high-value crops (Desaeger *et al.*, 2020). Moreover, since the 1970's, the negative environmental side effects due to nematicide application have become an important concern due to the high and broad-spectrum toxicity of nematicides which may affect non-target organisms, including humans and has led to a decrease in biodiversity (Oka, 2020). The recent interest in the development of more environment-friendly and sustainable nematode management strategies, including novel nematicides, is mainly due to the increasing regulatory pressure to either limit or ban the use of popular but hazardous nematicides such as methyl bromide, fenamiphos, and aldicarb (Oka, 2020).

Nematicides, can be divided into old-generation nematicides and new-generation nematicides (Desaeger *et al.*, 2020). Many of the old-generation nematicides (organophosphate and carbamate groups) have already been banned. New-generation nematicides are mainly different from the old-generation nematicides in three aspects: *i*) they are less toxic, *ii*) they are safer to use and *iii*) they are more selective and only target a specific nematode pathogen (Desaeger *et al.*, 2020).

Velum® 1 GR is a nematicide that is used by soybean producers in the USA in states such as Arizona, California as well as Hawaii and was first introduced to the SA market in 2021 (Engelbrecht *et al.*, 2020). This product can be considered a new-generation nematicide. Its active ingredient is fluopyram, which has a low toxicity to both invertebrates and vertebrates (Oka, 2020). Velum® 1 GR is easy to apply, highly effective in reducing RKN population densities, long-lasting as it reduced the population densities of PPN for up to 238 days after application according to Oka (2020). And the product is safe for the applicators and the environment but is very costly (Engelbrecht *et al.*, 2020; Oka, 2020).

In addition to chemicals, biological products, such as plant- and microorganism-derived products and biological-based fertilisers and/or bio stimulants, can also be applied for the management of PPN. Several biological products have been tested to determine their effect on RKN. For instance, Osman *et al.* (2021) concluded that a bio-agent containing *Bacillus weidmanni* NR221 applied with commercial microbial fertilisers effectively controlled *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949, in peanuts. Saeedizadeh *et al.* (2020) evaluated the effect of four different fertilizers (chemical, organic, semi-organic and bio-fertiliser) on *Meloidogyne* spp. populations as well as on the plant growth of tomato plants. The fertilizers decreased *in vitro* egg hatching and increased the mortality of the second-stage juveniles (J2). Although the fertilizers reduced hatching, they were not as effective as synthetic nematicides. However, with the development of new fertilisers that have nematocidal properties, RKN populations could be suppressed effectively without having negative environmental impacts.

An interesting candidate to examine the effect of fertilizers on RKN is Bio-Green granules. This fertilizer is commercially available and includes nutrient content such as N (14 g/kg), Ca (178 g/kg), Mg (4 g/kg), S (26 g/kg), Zn (4g/kg) and C (262 g/kg). This pelletised vermicast is also enriched with beneficial fungi namely mycorrhizae and a well-known biological control agent containing *Trichoderma* spp. Previous studies have shown that a combination of mycorrhizae and *Trichoderma* spp. reduced J2 populations by 55% (Nafady *et al.*, 2022). Therefore, the Bio-Green granules have the potential to reduce nematode populations as well as improve plant growth.

1.2 General aim and specific objectives

The general aim of our study was to determine the nematicidal effects of selected environmental-friendly, plant-derived products (Oligo, T3C, Ses-A, Aba-5, Graphite, BM-T and Oxa-40) as well as the fertilizer Bio-Green granules on *Meloidogyne incognita*.

The specific objectives of our study were:

- i) to evaluate *in vitro* the effect of eight selected experimental bio-nematicides on the motility of J2 of *M. incognita*

ii) to evaluate the effect of these experimental bio-nematicides and a bio-fertiliser in suppressing the population densities of *M. incognita* on soybean grown under tunnel, in a microplot and field conditions.

1.3 Hypothesis

Experimental bio-nematicide products from Elim Kunsmsis will negatively impact the biology (motility of second-stage juveniles: J2) of *M. incognita*, while application of the latter products, as well as that of the bio-fertilizer Bio-Green granule, will reduce RKN population densities significantly in soybean rhizospheres under microplot and field conditions.

2 Literature study

2.1 Soybean

2.1.1 Morphology and growth stages

Soybean (*Glycine max* (L.) Merr.) is an annual legume of the family Fabaceae. Classification:

Phylum Magnoliophyta

Class Magnoliopsida

Family Fabaceae Lindl., 1836

Genus *Glycine* Willd., 1802

Species *Glycine max* Merr., 1917

Soybeans require approximately 120 days to reach their dry-harvesting stage, however, the development of soybeans can be delayed due to several environmental factors. Soybeans can grow optimally in full sunlight at temperatures between 22 and 35°C on well-drained soils with a pH of 6.5 and higher (Coleman *et al.*, 2021). Several varieties have been developed; therefore, the seed shape and size are extremely variable, but most cultivars have an oval outline. These characteristics are controlled by genetics as well as environmental impacts.

Soybean has various growth stages from vegetative emergence (VE) to full maturity which is referred to as reproductive stage 8 (R8) when 95% of the pods are brownish in colour (Fig. 2.1). Soybean used for fresh produce can grow into the full seed (R6) in 100 days, however, full maturity (R8) for a dry harvest may take 160 days (Purcell *et al.*, 2014). VE is the emergence stage when cotyledons push through the soil surface; VC is the vegetative cotyledon stage when the untrifoliate leaves unfold. The vegetative stages (V) of development continue with the unfolding of trifoliate leaves. The reproductive stages (R) start with R1 when the plant starts flowering. R3 is the reproductive stage when pods are formed, and when the plant is in full seed (R7) the pods contain green seeds which fill the capacity of the pods (Fig.2.1). R7 is a critical stage in the development of soybean and will influence the final yield potential. Successful pod

fill can be ensured through proper plant nutrition, soil health and fertility as well as the surrounding environmental conditions.

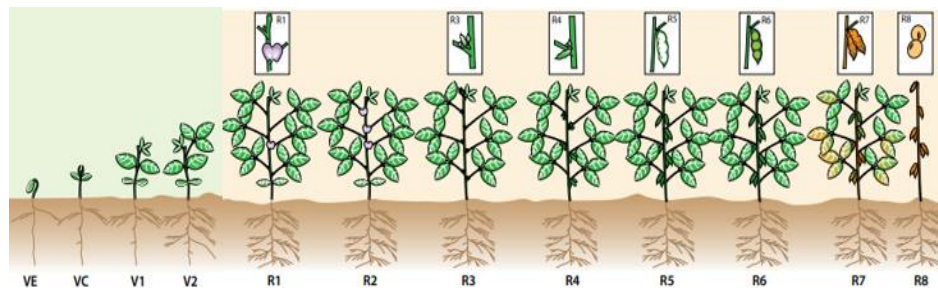


Figure 2.1. Growth stages of soybean (Purcell *et al.*, 2014)

2.1.2 Importance

Soybean has a good nutritional profile for both human and animal consumption. A soybean seed consists of approximately 35% protein and 20% oil. It is the most important legume as well as a source of food in terms of monetary value, with 70% of the soybean production being used for animal feed, 20% for human consumption and 4% for industries producing biodiesel as well as lubricants (Ritchie and Roser, 2023). The world soybean production has increased substantially over the past 50 years (Fig. 2.2). Brazil is the leading soybean producer in the world as of 2022, followed by the USA and Argentina with a total global world production of 388 million metric tonnes (t).

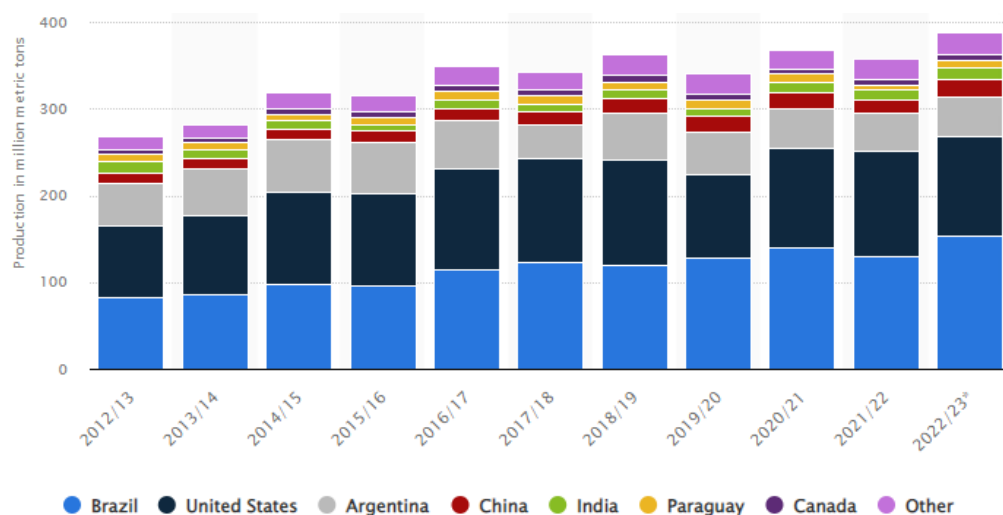


Figure 2.2. Global soybean production from 2013 until 2023 (Ritchie and Roser, 2023)

South Africa (SA) contributes approximately 0.5% of the annual global soybean production (Grain-SA, 2023). This production is sufficient for national consumption. The commercial demand for soybean for the production season 2022/2023 was estimated at 2,065,400 t; the production was 2,353,400 t with a total surplus of 59,800 t and a total import of 14,000 t (Grain-SA, 2022). The annual surface area planted with soybean in the 2022/2023 season was 1,148,300 ha; the

average yield was 2.36 t/ha which led to a total production of 2,710,150 t representing a record harvest for local soybean production (Grain-SA, 2023) (Fig. 2.3). The production estimate for the 2022/2023 season was 356,750 t below the actual yield, which was due to the increased production area and favourable environmental conditions. For the 2022/2023 growing season, the surface area planted with soybean increased by 19.4% and production increased with 480,150 t compared to the 2021/2022 season, indicating the increasing popularity of this crop. The leading province producing soybean in SA is Free State, followed by Mpumalanga and North-West with contributions of 49.2%, 26.6% and 13.5% of the total local soybean production for the 2022/2023 season, respectively.

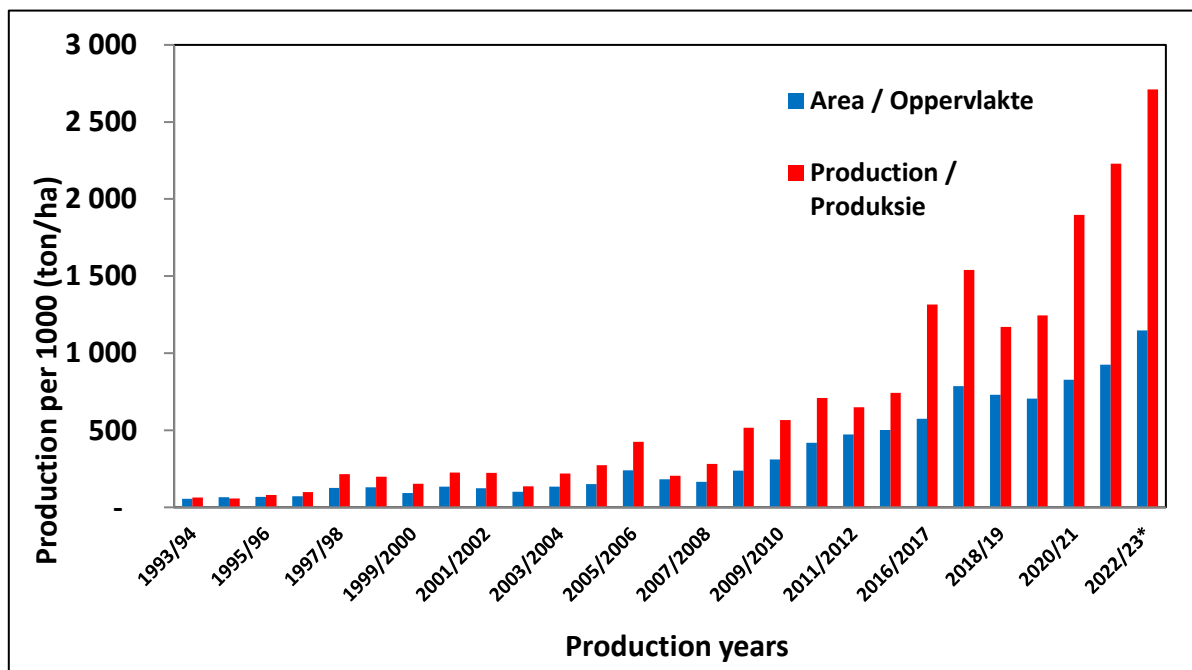


Figure 2.3. Area planted and production of soybean in SA for the 2022/2023 season (Grain-SA, 2023)

2.2 Plant-parasitic nematodes

2.2.1 General

Wilson (2003) described nematodes as the most abundant animals in the world: four out of five animals are nematodes and if all earthly matter would disappear except nematodes a ghostly outline of all matter would still be visible. Nematodes belong to the phylum Nematoda (Rudolphi, 1808) Lankester, 1877, class Secernentea von Linstow, 1905, order Rhabditida Chitwood, 1933 (Decraemer and Hunt, 2006).

Nematodes have transparent, durable and thick cuticles, and move with longitudinal muscles; they are cylindrical and have no segments (Decraemer and Hunt, 2006). Nematodes are highly successful animals inhabiting a variety of habitats and fulfilling many functions (Sikora *et al.*, 2018). Despite their ecological diversity, all nematodes have a similar structure. Nematodes

can be classified based on their mode of feeding into bacterivores, fungivores, predators, omnivores, and plant-parasitic nematodes (PPN) or herbivores (Perry and Moens, 2013). PPN only contribute 10% to the total nematode population on earth (Sikora *et al.*, 2018). They can be identified by a spear-like mouth part known as a stylet (Fig. 2.4). There are three main types of stylets (stomatostylet, onchiostylet and odontostylet); however, not only PPN have stylets. Of the beneficial nematodes, fungivores and omnivores also have stylets while predators have a tooth-like structure used to feed on other microorganisms (Brantley, 2017). Tylenchina is the group of PPN that cause the most damage globally and is therefore economically the most important group of PPN (Sikora *et al.*, 2018).

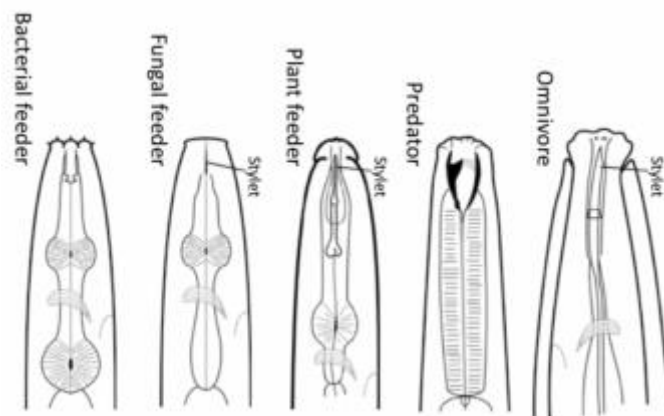


Figure 2.4. Mouth part structure of different trophic groups of nematodes (Brantley, 2017)

Nematodes are essentially aquatic animals that require a film of water to survive and move. Many species die when exposed to drought, but some can survive in an anhydrobiotic state (Sikora *et al.*, 2018). Soil structure is a determining factor for nematodes as the pore size influences their mobility: sandy soils are a favourable habitat for most nematode species whereas clay soils can be a limiting factor for some species (Fig. 2.5).

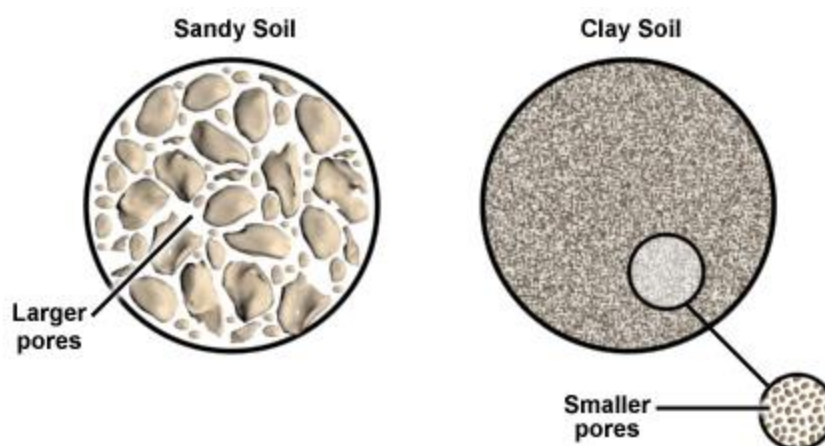


Figure 2.5. Mobility of nematodes are greatly influenced by soil types and the pore size (source: <https://extension.unr.edu/publication.aspx?PubID=2161>)

Plant-parasitic nematodes can penetrate plant parts using several different methods depending on the nematode species. There are three main feeding types of PPN, viz. ectoparasites, semi-endo parasites and endoparasites (Figs. 2.6 and 2.7). (Sikora *et al.*, 2018)

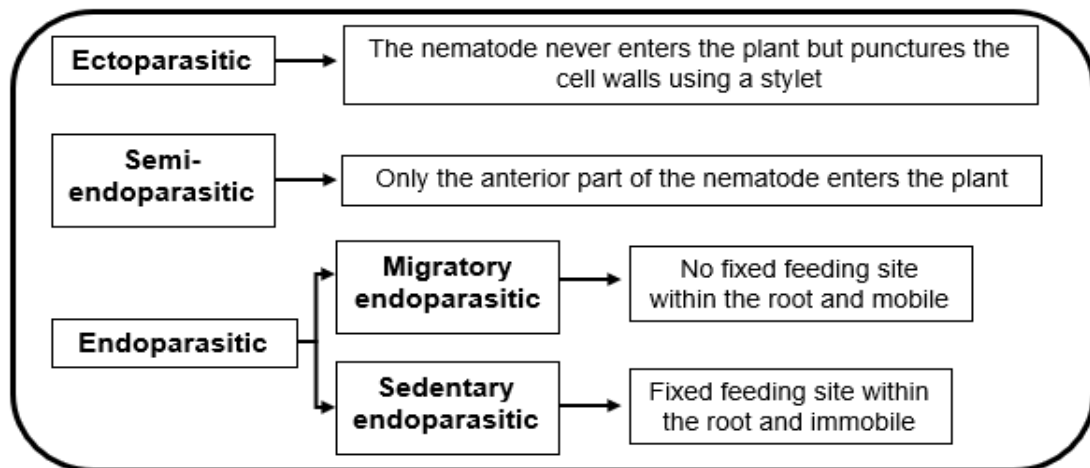


Figure 2.6. Feeding types of plant-parasitic nematodes (S. Esterhuizen, NWU)

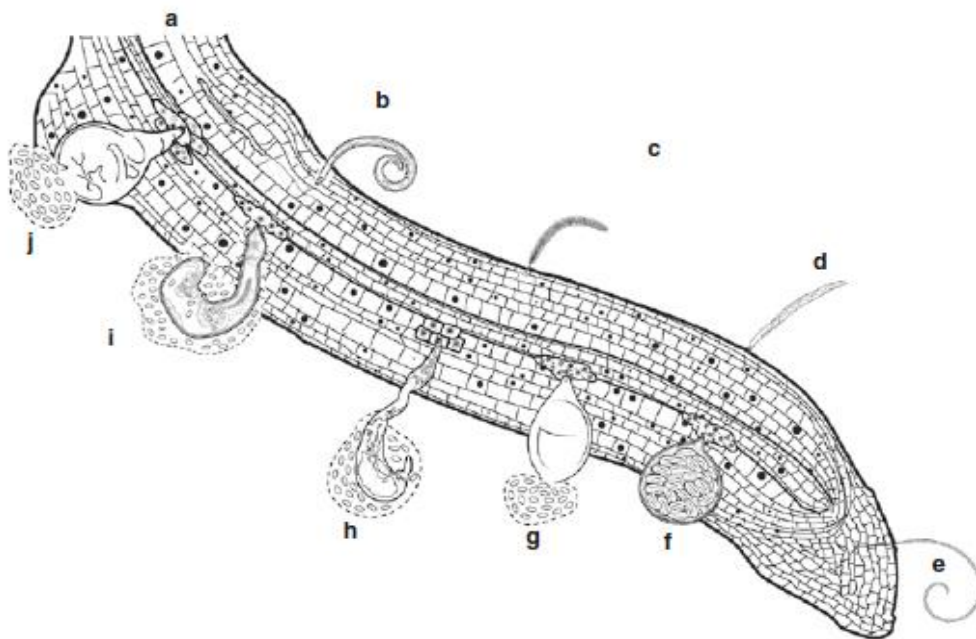


Figure 2.7. Specific feeding sites of several different nematodes within a plant root: (a) migratory endoparasite (b) semi-endo-ecto-parasite (c-e) ectoparasite (f-j) sedentary endoparasite (Fourie *et al.*, 2017)

The feeding process of PPN can cause major damage to the roots or other below-ground parts of their host plants, thus limiting the absorption of water and nutrients by the plant (Lambert and Bekal, 2002). The above-ground symptoms that PPN cause are limited and when visible resemble symptoms caused by water and nutrient deficiency such as wilting, stunted plant growth

and yellowing of the leaves, and in severe cases plant death (Lambert and Bekal, 2002). Root galling and the presence of visible cysts are the most noticeable physical signs, respectively, of infection by root-knot and cyst nematodes. In most fields however, nematode infestation goes unnoticed.

2.2.2 Plant-parasitic nematodes affecting soybean production

The annual loss of soybean production resulting from nematode infection globally is estimated at R1,3 trillion with an average crop yield loss of 15% (Lima *et al.*, 2017). Worldwide, the top three economically most important groups of PPN that infect, damage and limit soybean production are cyst nematodes (SCN; *Heterodera glycines* Ichinohe, 1952), root-lesion nematodes (RLN; *Pratylenchus* Filipjev, 1936) and root-knot nematodes (RKN; *Meloidogyne* Goeldi, 1892).

Heterodera glycines is known as a highly destructive nematode species infecting soybean in some of the countries with the highest soybean production, e.g., the USA, Brazil and Argentina (Sikora *et al.*, 2018). In the USA, SCN causes more yield loss than any other soybean disease. A 20-30% yield loss can occur without any symptoms, and a higher yield loss occurs when symptoms, such as yellowing of the leaves and stunting, and cysts on roots are visible (Fig. 2.8) (Sikora *et al.*, 2018). Although this nematode is considered the most important nematode species of soybean, neither the South African Plant-Parasitic Nematode Survey (SAPPNS) nor the National Collection of Nematodes (NCN) databases contain any record of the presence of *H. glycines* in either South Africa (SA) or the South African Development Community (SADC) region until now (Mariette Marais, personal communication).



Figure 2.8. Yellowish cysts of *Heterodera glycines* on a soybean root (Hunt, 2008)

Root-lesion nematode (RLN) species can also cause considerable damage to soybean crops. Several RLN species have been associated with soybean in SA. Current research in South Africa is focused on the specie *P. hippeastri*, a survey conducted in 2018 determined that the RLN was found in 85% of apple orchards in South Africa (Knoetze *et al.*, 2019).

Other species currently identified in South Africa include *Pratylenchus brachyurus* (Godfrey, 1929) Filipjev and Schuurmans Stekhoven, 1941, *Pratylenchus crenatus* Loof, 1960, *Pratylenchus flakkensis* Seinhorst, 1968, *Pratylenchus neglectus* (Rensch, 1924) Filipjev and Schuurmans Stekhoven, 1941, *Pratylenchus scribneri* Steiner, 1943, *Pratylenchus penetrans* (Cobb, 1917) Filipjev and Schuurmans Stekhoven, *Pratylenchus teres* Khan and Singh, 1974, *Pratylenchus thornei* Sher and Allen, 1954, *Pratylenchus zeae* Graham, 1951, and *Pratylenchus vulnus* Allen and Jensen, 1951 (Engelbrecht *et al.*, 2020). The most important RLN species infecting and damaging soybean worldwide are *P. brachyurus* and *P. zeae* which can cause yield reductions of up to 50% (Sikora *et al.*, 2018). Some symptoms of RLN infection in soybean include stunting and chlorosis, as well as visible blackish lesions on roots (Figs 2.9A and B).



Figs 2.9A and B. A) poorly developed soybean plant (in the white rectangle) with less roots, foliage and pods that did not fill with seeds due to being heavily infected with root-lesion nematodes (a) compared to a non-infected plant with a well-developed root system and abundant foliage with seed-filled pods (b). B) damaged roots infected with RLN (A: Driekie Fourie, NWU and B: Suria Bekker, NWU)

2.2.3 Root-knot nematodes

Root-knot nematodes are, generally, regarded as the economically most important genus that infect crops globally (Jones *et al.*, 2013). The most damaging species are *Meloidogyne arenaria* Neal, 1889, *Meloidogyne incognita* and *Meloidogyne javanica* (Sikora *et al.*, 2018). In addition, *Meloidogyne enterolobii* Yang and Eisenback, 1983 has also been listed to infect and damage soybean (Sikora *et al.*, 2018). The three former species are also known to infect soybean in SA (Mbatyoti *et al.*, 2020), with *M. enterolobii* recently being added to the list (Engelbrecht, 2022).

2.2.3.1 Morphology and life cycle

The mature female has a rounded- or pear-shaped body, a total body length between 350 and 3,000 μm and a maximum body width ranging from 300 to 700 μm (Fig. 2.10; Perry and Moens, 2013). The head region is slightly set off with a visible cephalic framework. The stylet ranges from 10 to 25 μm in length with three distinctive basal bulbs and a dorsally curved cone. A substantial metacarpus is connected to the pharyngeal glands which vary in size as well as

shape, according to the species. The pharyngeal gland overlaps the intestine ventrally. The reproductive region is didelphic with convoluted gonads composed of an ovary containing a germinal and growth zone, an oviduct as well as lobed spermatheca and a uterus (Perry and Moens, 2013).

The male and J2 have a vermiform body shape throughout all their developmental stages. The male and J2 have similar structures although they differ significantly in size. Both are non-sedentary, and clearly annulated (Figs 2.11A and E). The body length of the male ranges from 600 to 2,500 μm . The anterior region contains a head cap with a spherical labial disc fused with four medial lips. The stylet is well-developed and similar to the stylet of the female; however, the metacarpus is relatively smaller in size (Manzanilla-López, 2012).

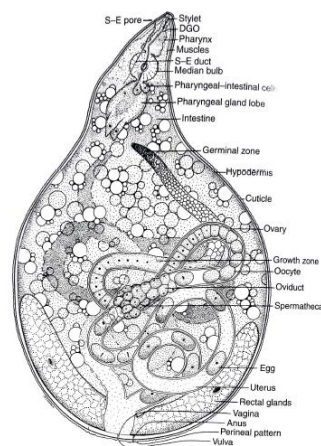


Figure 2.10. A drawing of *Meloidogyne* female (Perry and Moens, 2013)

The pharyngeal gland has a ventral overlap with the intestines. The reproductive region of the male has a single testis that is relatively long with no bursa and slim spicules between 20 to 40 μm long with a gubernaculum of approximately 10 μm . The J2 has a body length ranging from 250 to 600 μm , visibly smaller than the male. The stylet is more delicate compared with the male and ranges from 9 to 16 μm (Manzanilla-López, 2012).

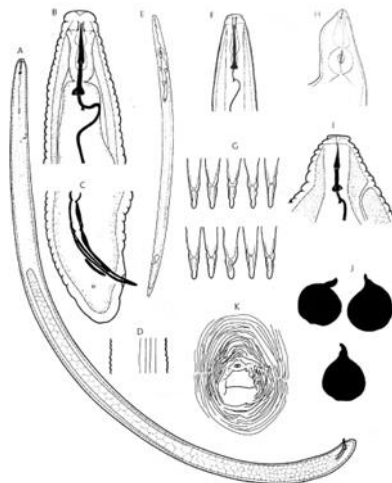


Figure 2.11. General morphology of *Meloidogyne* (Manzanilla-López, 2012). a) anterior region of the J2, b) anterior region of the J2, c) male posterior region, d) lateral field, e) J2, f) anterior region of the J2, g) J2 tail region, h) and i) female anterior region, j) female and k) perineal pattern (Manzanilla-López, 2012)

The second-stage juvenile (J2; Fig. 2.12) of RKN is the infective stage. The hatching of J1 into J2 does not depend on a host plant stimulus but rather on a suitable moisture content and temperature (Jones *et al.*, 2013). RKN are sedentary endoparasites. The host plant is infected by the J2 behind the root cap which is penetrated using the stylet while cellulolytic and pectolytic enzymes facilitate the breakdown of the plant's cell wall. The J2 then migrate intercellularly to the apical meristem and into the vascular cylinder to the zone of differentiation where they establish their feeding site (Jones *et al.*, 2013).

After establishing a permanent feeding site, the surrounding cells start to expand/increase in size after 2 to 3 days (Agrios, 2005). Cells continue to increase in size for 14 to 21 days, creating visible galls caused by the enzymes contained by the saliva secreted by nematodes during feeding. Mature females produce a gelatinous matrix in which the eggs are deposited. The damage the nematode causes not only limits the absorption of water and nutrients by the root system of the host plant but also generates weakened root tissues, allowing several secondary pathogens such as fungi to infect the root system (Fig. 2.12; Agrios, 2005) (Perry and Moens, 2013).

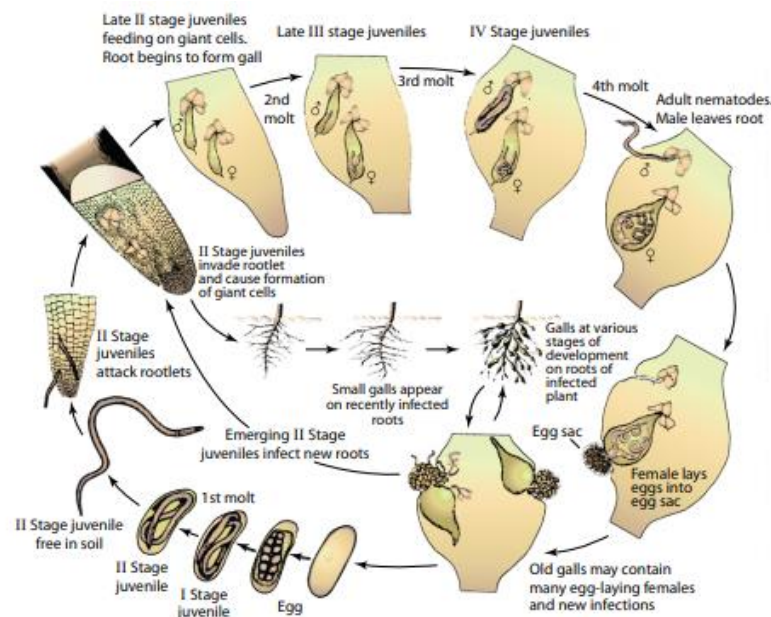


Figure 2.12. Life cycle of root-knot nematodes (Agrios, 2005)

2.2.3.2 Symptoms, damage and yield loss

Above-ground damage caused by RKN can include symptoms such as stunted growth, wilting and leaf discoloration (Fig. 2.13A; Fourie *et al.* 2017).

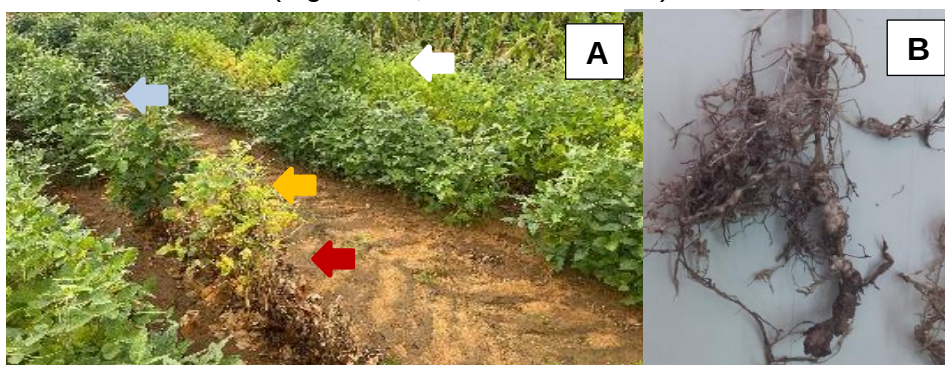


Figure 2.13A and B. A soybean field in the Bultfontein area with a severe *Meloidogyne incognita* infection showing chlorotic plants (white arrow), dead plants (red arrow), stunted plant (orange arrow) as well as healthy plants (blue arrow) in one small area (a) and galls on an infected soybean root system of a soybean plant (A: Driekie Fourie, NWU; B: Surietha Esterhuizen, NWU)

Above-ground symptoms are limited and can often be misinterpreted for nutrient deficiency. The harvest quantity as well as the quality of the soybean crop produces can be substantially reduced for nematode-infected plants, however, it seldom causes the plant to die except in the case of extremely high infections (Fig. 2.13A). Mature plant roots may also show necrosis and root rot (Agrios, 2005). According to Perry and Moens (2013), the symptoms are typically related to the number of J2 that have infected the host plant. Usually, it is only late in the development of the soybean crop that damage caused by RKN can be observed and recognised as damage caused by RKN. Often at that stage in the development of the soybean crop, options for treatment are limited or not possible anymore (Lima *et al.*, 2017).

Due to the lack of distinctive symptoms, described above, as well as a lack of awareness about the damage RKN can cause to soybean, the damage caused by RKN on soybean is still widely underestimated. No annual loss calculations are available for SA and most African countries due to a lack of comprehensive assessments of the economic damage caused by RKN (Onkendi *et al.*, 2014).

2.3 Management strategies of plant-parasitic nematodes

2.3.1 General

Several management practices such as cover crops, crop rotation, plant resistance, organic amendments, biological control, and chemical control have been developed to limit the damage caused by PPN to crops, with the use of synthetic nematicides still being the predominant

strategy applied globally (Perry and Moens, 2013). Nematicides are usually very costly, and their application will lower the profit margins of the crop.

2.3.2 Nematicides

2.3.2.1 Chemical nematicides

Chemical nematicides have a 30% share in the global pesticide market (Manzanilla-López, 2012), and nematicides that target RKN have a 48% total value share in the market of nematicides. Nematicides are mainly used in vegetables (38% market share) and potatoes (25% market share) (Manzanilla-López, 2012).

The most frequently used nematicides in the USA to control nematodes in soybean contain aldicarb and terbufos as active ingredients (Vitti *et al.*, 2014). Furthermore, nematicide use in Brazil has grown exponentially over the last decade, whereby approximately 13.4 million ha (64%) of soybean crop land was treated with nematicides in 2022 (Gottens, 2023). A total of 75% of these nematicides were biological nematicides and the other 25% were chemical nematicides. This trend is seen globally and a future increase of 4.6% CAGR (compound annual growth rate) is predicted from 2022 – 2029 (Gottens, 2023). Several seed treatments containing abamectin have been used for the control of soybean in Brazil and the USA (Vitti *et al.*, 2014). The increase in nematicide use is due to an increase in intensive agricultural production systems. Although the market for nematicides is increasing, SA has only one registered nematicide for nematode control on soybean: Velum® 1G with the active ingredient fluopyram. This product is readily available in all major soybean-producing countries such as the USA, Brazil, and Argentina (Engelbrecht *et al.*, 2020). In addition, a new-generation, chemical-based seed-treatment product with primarily nematocidal and secondarily fungicidal properties, VICTRATO® 500FS developed by Syngenta ([Syngenta, 2022](#)), has been registered in countries such as Argentina, Australia, Brazil, El Salvador, Zambia and others however, in SA the product is not yet registered.

2.3.2.2 Bionematicides

Biological nematicides contain biological agents, e.g., plants, bacteria, or fungi, and are environmentally friendly products that, while controlling PPN infestations effectively, also improve soil and plant health (Wilson and Jackson, 2013).

Several nematicides of organic origin have been tested and found effective for the successful control of nematodes on soybean in a number of countries (Wilson and Jackson, 2013). Brazil has several registered nematicides that have active ingredients that occur naturally or are derived from secondary metabolites of biocontrol agents to control several RKN species. These agents include *Lactobacillus plantarum*, *Bacillus subtilis* and *Enterococcus faecium* (Fontana *et al.*, 2021), *Bacillus licheniformis*, *B. subtilis* and *Trichoderma longibrachiatum*

(Miamoto *et al.*, 2017) as well as *Pochonia chlamydosporia* (Nasu *et al.*, 2018). Nigeria has three biological agents registered for the control of RKN on soybean; *Trichoderma harzianum* (Izuogu and Abiri, 2015) as well as *Pleurotus ostreatus* and *Pleurotus tuberregium* (Okorie *et al.*, 2011).

2.3.3 Host plant resistance

Growing resistant plant cultivars is an economical method for the control of PPN infecting both low- and high-cash crops as nematicides are very expensive and the growing of resistant plant varieties does not require additional input costs (Clifton *et al.*, 2018). Host plant resistance is mostly found against PPN that have a highly complex plant-host interaction such as RKN and CN (Clifton *et al.*, 2018). Globally, several soybean cultivars with resistance against nematodes such as *M. incognita*, *M. javanica* and *H. glycines* have been developed (Schwarz and Gorny, 2023).

2.3.4 Crop rotation

Crop rotation is a well-known agricultural practice, that farmers use by planting different crops successively on the same area. Crop rotation is known to improve soil health, optimize nutrient cycling and suppress pests and weeds in fields. Pests such as RKN are difficult to control as this group of PPN has a wide host range (Karuri, 2022)). Several studies have shown this management strategy to be ineffective in soybean-maize rotation across sub-Saharan Africa, as both these crops are good hosts of both RKN and RLN (Acevedo-Siaca and Goldsmith, 2020). However, with careful consideration, the identification of poor hosts of RKN might be possible, considering that the rotating crop does not increase the population densities of other PPN such as RLN or that of other genera (Karuri, 2022)).

2.3.5 Other methods for nematode control

2.3.5.1 Organic amendments

Organic amendments are comprised of different organic substances emanating from biomass (Maiti and Ahirwal, 2019). Organic amendments include green manure, compost, biochar, straw, and others. Organic amendments are known to increase soil fertility, alter microclimatic conditions and improve microbial growth (Roskopf *et al.*, 2020). Several organic amendments are being commercially produced and used as nematicides as they have proven to exhibit great nematocidal activity. The decomposition of organic amendments releases compounds such as methyl isothiocyanates (MITCs) which is a broadly used soil fumigant with non-specific biocidal activity (Roskopf *et al.*, 2020). Rich and Rathi (1995) concluded in their study on *Crotalaria juncea* L. powdered seeds that the exudates from the plant tissue contained monocrotaline and pyrrolizidine alkaloids, known to be nematotoxic to nematode species such as *M. incognita* and *M. javanica*.

2.3.5.2 Cover crops

Cover crops have proven to be an important part of an integrated nematode management strategy (Gill *et al.*, 2022). Cover crops have several advantages however, RKN has a wide host range and cover crops are often susceptible to the infestation of RKN. Therefore, finding the right cover crop has proven to be a challenge. However, nematodes cannot move over long distances and a non-susceptible host may prevent nematodes from completing their life cycles due to starvation, which helps to manage PPN population densities (Sobhy *et al.*, 2020). Gill *et al.*, (2022) studied the effect of cover crops on the development of several *Meloidogyne* spp. and concluded that egg masses of *Meloidogyne* spp. were significantly reduced in roots of cover crops such as cowpea (*Vigna unguiculata* (L.) Walp.), castor bean (*Ricinus communis* L.) and showy rattlegod (*Crotalaria spectabilis* Roth).

2.3.5.3 Fallow

Fallowing is an agricultural management practice whereby the fields are intentionally not used to grow crops or any other plants, this management practice is used to allow soil to recover and reduce pests by removing the host plants for a season. Fallowing can reduce populations of nematodes as they cannot reproduce for a season and might starve to death due to the lack of a host plant, however, several nematode species can survive during long periods of dormancy and are not affected by this management strategy, provided the field is weed-free (McSorley, 2003).

2.3.5.4 Fertilizers

Plant fertilizers are known to increase the supply and availability of nutrients to crops as well as improve the growth of crops (Youssef and Eissa, 2014). The growth, yield and several other quality parameters of crops are improved by biological and chemical fertilizers and stimulants. Fertilizers are primarily applied to improve the nutrient quality of the soil for plants to take up while stimulants are applied primarily to improve plant growth (Delgado *et al.*, 2016). Chemical and biological fertilizers and stimulants can be distinguished by the origin, chemical fertilizers are synthetically derived while biological fertilizers are derived from biological materials such as botanicals, organic minerals, and micro-organisms (Delgado *et al.*, 2016).

Fertilizers containing nitrogen fixing bacteria, phosphates and potassium solubilizing bacteria have proven to reduce the population of *Meloidogyne* spp. (Youssef and Eissa, 2014). In their research, the fertilizers were able to reduce the total number of eggs and juveniles on the roots of chilli (*Capsicum frutescens* L.), tomato (*Solanum lycopersicum* L.) and sunflower (*Helianthus annuus* L.). However, the population densities of SCN were not reduced using fertilizers containing 157 kg N ha⁻¹ (ammonium nitrate), 49 kg P ha⁻¹ (triple super phosphate), and 103 kg K ha⁻¹ (potash) on maize and soybean in a study done by Grabau *et al.* (2017).

However, a significant decrease in the population of SCN were observed when a fertilizer and a nematicide were combined (Grabau *et al.*, 2017).

Nutrients play an important role in plant growth but also in nematode physiology. A study conducted by Melakeberhan *et al.* (1987) concluded that an increase in nematode populations, decreased the nutrients concentration within the plant which led to chlorosis and early leaf abscission. The change in potassium (K) concentration due to increased infection of nematodes also led to a decrease in photosynthesis by influencing the CO₂ uptake and the osmotic potential of the plant (Melakeberhan *et al.*, 1987). A lower K concentration also resulted in bigger root-galls, giant cell sizes and increase metabolic activity of the nematode *M. incognita*. Nitrogen (N) increased while photosynthesis and the chlorophyll contents declined at later stages of nematode infection (Melakeberhan *et al.*, 1987). Therefore, providing evidence that nutrients such as K and N play a crucial role in nematode infection as the nematodes make use of these nutrients for their own development and deprive the plant from these elements. Increasing nutrients through fertilizers will increase additional nutrients to plants infected with nematodes. The effect of three phosphonate fertilizers CPO(OH)₂, Calphos (a.i. calcium phosphonate), Magphos (a.i. magnesium phosphonate and potassium phosphonate) and Phosphoros (a.i. potassium phosphonate) were evaluated *in vitro* for the reduction of *M. incognita* and *M. javanica* (Habash and Al-Banna, 2011). Habash and Al-Banna (2011) found no significant effect of Phosphoros application on the nematode populations but a significant decrease in egg hatching and J2 survival was observed when applying Calphos and Magphos to the nematode populations in petri dishes. This effect may be the result of the low pH (2,2 and 3,46 respectively) in both fertilizers as nematodes prefer a pH of 6,5 as well as a high salinity content (Habash and Al-Banna, 2011).

Elim Kunsmis is a company based in Brits, SA, that produces a bio-fertilizer consisting of compost from the Johnson-Su bioreactor which was then fed to earthworms (vermicast) with added mycorrhizae and *Trichoderma* spp. spores. This fertilizer may also have the potential to decrease the population density of *Meloidogyne* spp. in soybean.

3 Materials and methods

3.1 *In vitro* bioassay: effect of several experimental biological products on the motility and viability of J2 *Meloidogyne* spp.

The *in vitro* bioassay was carried out during March 2022 in the nematology laboratory at the Potchefstroom Campus of North-West University (NWU). The objective of this bioassay was to examine *in vitro* the effect of several experimental biological products on the motility and viability of freshly hatched J2 of *Meloidogyne* spp. Each of the products was tested in a 24-well culture plate, each well had a 1.4 ml capacity (only 1 ml was utilised). Approximately 50 J2 were then

exposed to these products and evaluated after 24, 48 and 72 h. An untreated control (H₂O) and an upper standard control (ethanol) were also included.

3.1.1 Products included in the study

The products included in the *in vitro* bioassay with full name, concentration, active ingredient and use are listed in Table 3.1.

Table 3.1. Treatments included in the *in vitro* bioassay, active ingredient, and use

Treatment	Full name, concentration and active ingredient	Use
T3C	100% Garlic oil	Nematicide
T3C-1	1% Garlic oil	Nematicide
T3C-5	5% Garlic oil	Nematicide
T3C-10	10% Garlic oil	Nematicide
T3C-20	20% Garlic oil	Nematicide
T3C-30	30% Garlic oil	Nematicide
Oligo-1	1% Chitosan oligo saccharide	Growth stimulant
Oligo-2	2% Chitosan oligo saccharide	Growth stimulant
Oligo-5	5% Chitosan oligo saccharide	Growth stimulant
Oligo-10	10% Chitosan oligo saccharide	Growth stimulant
Oligo-15	15% Chitosan oligo saccharide	Growth stimulant
Ses-A-5	5% Sesame oil	Nematicide
Ses-A-10	10% Sesame oil	Nematicide
Ses-A-20	20% Sesame oil	Nematicide
Ses-A-30	30% Sesame oil	Nematicide
Aba-5	5% Abamectin	Nematicide
Graphite	Graphite	Bio-stimulant
BM-T	Bontera® BM-T, a variety of soil microbes	Bio-fertilizer
Green liquid	Green Liquid, amino acids and carbon	Fertilizer
Oxa-40	Oxamyl (40%)	Nematicide
Alcohol	-	Positive standard
dH ₂ O	-	Negative standard

3.1.2 Experimental setup

a) Collection of eggs and second-stage juveniles (J2) of the *Meloidogyne* populations

Egg masses of the three *Meloidogyne* species included in our study (*M. incognita*, *M. javanica* and *M. enterolobii*) were obtained from infected 30-day-old tomato (*Solanum lycopersicum* L. cultivar Rodade) plants grown in soil in pots in a glasshouse. The *Meloidogyne* populations were originally obtained from infected tomato roots from the Letsitele area, Limpopo Province (SA). The egg masses were dislodged from the roots and eggs were obtained using the method described by Riekert (1995). Surface-sterilised eggs were placed on the surface of a 20-µm-aperture sieve, the sieve was placed in a flat 5-L container and filled with approximately a 3 cm layer of dH₂O. The container was then incubated at 25 ± 2°C for 24 h. To ensure optimal

viability, the J2 that had hatched during the first 24 hours–were discarded. J2 that had hatched during the subsequent 48 hours were used in the bioassay.

b) Preparation of the treatments

The treatments were all in liquid form in 500 ml amber glass bottles to protect the products against light exposure. The products Oligo, T3C (garlic oil) and Ses-A (sesame oil) were already prepared at different concentrations in the amber glass bottles (Table 3.1). The treatments were prepared in a 100-ml capacity glass beaker whereby 22.5 ml dH₂O was mixed with 2.5 ml product to have 0.1 ml product for every ml of dH₂O using a magnetic stirrer. This process was repeated for all the products mentioned in Table 3.1.

c) Exposure of J2 of *Meloidogyne* spp. to treatments

For each treatment, a different 24-well culture plate was used. In each well, 0.9 ml of the product mixture was pipetted and 18 wells per culture plate were filled. There were six repetitions for each time interval (24 h, 48 h and 72 h). Two controls per treatment were included in the bioassay; the untreated control contained 0.9 ml dH₂O and the upper standard control contained 0.9 ml of 50% ethanol. The freshly hatched J2 were decanted into a 250 ml beaker and filled with water until 100 ml. The J2 were counted and approximately 50 ± 4 J2 per 0.1 ml were pipetted into each well. The J2 were then exposed to the product.

d) Effect on J2 motility

Each treatment was assigned to one culture plate to avoid contamination including the possibility of chemical fumes of the treatments influencing each other (Proença *et al.*, 2021). The culture plates were then covered with lids, sealed with parafilm and placed in a completely randomised design with six replicates per treatment per time interval in an incubator at $25 \pm 2^\circ\text{C}$ in the dark.

After 24, 48 and 72 h, the content of each well was transferred into a Peters' counting slide and the number of immobile J2 was counted using a stereomicroscope. The J2 were considered immobile when no movement was observed after 60 s and after being prodded with a needle. Concentrations were considered motile-inhibitive when significantly more J2 became immobilised compared with the control (Wuyts *et al.*, 2006).

3.1.3 Statistical analysis

The bioassay was repeated, and the data of both experiments were compared for statistical differences. Since there were no statistical differences, the data sets of the two experiments were pooled. The number of immobile J2 was transformed into percentages and subjected to Repeated Measures Analysis of Variance (TIBCO Statistica, Version 13.2; www.tibco.com). Treatment means were statistically compared using Fisher's protected least significant LSD [Least significant difference] test ($P \leq 0.05$).

3.2 Microplot - *in vivo* experiment: effect of bio-fertilizers on the motility of *Meloidogyne* spp. J2's

The microplot experiment was initiated after the *in vitro* bioassays. The soybean seeds were planted on the 1st of December 2022. The treatments showing the best nematocidal effects during the *in vitro* bioassays were used as treatments in the micro-plot experiment. This experiment was conducted at the Potchefstroom campus of North-West University (NWU). The objective of this experiment was to evaluate if the nematocidal effects observed in the *in vitro* bioassays could be confirmed by assessing *in vivo* nematode reproduction.

3.2.1 Plant material

The *Meloidogyne incognita*-susceptible soybean cultivar P57T19R was used in this experiment.

3.2.2 Experimental setup

a) Microplots

The microplots were installed over a drainage system and consisted of fiberglass tubes (1 m in diameter and 1 m deep (Fig. 3.1)). The plots were filled with soil containing 86.4% sand, 13.3% clay and 1.18% organic material according to an analysis by personnel from the EcoAnalitica Laboratory of NWU. The pH of the soil was 6.78 (H₂O) and the CEC was 13.13.



Figure 3.1. Microplot experiment (picture: D. Fourie, North-West University)

b) Planting

A total of 35 seeds with 3 cm intra-row spacing of cultivar P57T19R were planted in each of the microplots at a depth of 2 cm in a complete randomized block design (Fig.3.2). The rows were 50 cm apart. The soil was irrigated with approximately 25 mm of borehole water (pH 6.7) the day prior to planting to ensure that the soil was moist.

R4 T4	R4 T8	R4 T6	R4 T1	R4 T5	R4 T9	R4 T3	R4 T2	R4 T7
R3 T4	R3 T1	R3 T2	R3 T9	R3 T7	R3 T5	R3 T6	R3 T8	R3 T3
R2 T6	R2 T8	R2 T3	R2 T5	R2 T9	R2 T2	R2 T4	R2 T1	R2 T7
R1 T1	R1 T2	R1 T3	R1 T4	R1 T5	R1 T6	R1 T7	R1 T8	R1 T9

Figure 3.2. Randomised block design used for the microplot experiment

c) Inoculation

Nematode inoculation commenced with eggs and J2 of a single-species *M. incognita* population and mixed life stages of an unidentified *Pratylenchus* species (referred to as *Pratylenchus x* until its identification is completed). *Pratylenchus x* was used as it was found consistently during the past three summer growing seasons from maize and soybean roots obtained from the grain crop production areas of SA. The single-species population of *M. incognita* used for this experiment was reared and maintained in roots of an *M. incognita*-susceptible tomato variety ('Moneymaker') (Jones *et al.*, 2017) in a glasshouse at an ambient temperature range of 20-26 °C for 40 days. This population was originally established from root-knot nematode-infected material collected from roots of infected maize sampled during a survey done in 2020. The single-species *Pratylenchus x* population was obtained from Dr Rinus Knoetze (ARC-Infruitec, Stellenbosch) after it had been reared *in vitro* on carrot discs at 30 °C for 90 days (as described by Fourie *et al.*, 2003). This species is currently under investigation to enable its identification using morphological and molecular identification tools.

The various life stages of the two nematode species (*Meloidogyne* and *Pratylenchus*) were evenly applied across the seeds in each furrow to enable inoculation of approximately 1 800 eggs and J2 of *M. incognita* and 850 individuals (eggs and motile life stages) of *Pratylenchus x* per seed. After nematode inoculation of the seeds, *Bradyrhizobium japonicum* was applied to the seeds to enable nitrogen fixation (Boote, 2018). The recommended rate of 250 g Soyapac® per 25-50 kg soybean seed was used. After application of *B. japonicum*, the seeds were covered with the rest of the soil removed when making the furrows.

d) Application of treatments

The treatments (see Table 3.2) were applied to the microplots according to the layout shown in Fig. 3.1. Treatment application was done across the surface of each microplot, as broadcast applications of 5 L/ha, and the applied products were immediately washed in with approximately 5-7 mm water through drip irrigation. The untreated control microplots did not

receive any product, while a nematicide standard Velum® 1G was included (dosage rate of 1.5 g/100 m row) as registered for soybean in South Africa (www.cropscience.bayer.africa).

Table 3.2. Treatments included in the microplot experiment, the organic compounds, active ingredient, use and formulation.

Treatment	Full name and active ingredient	Use
Oligo-5	5% Chitosan oligo saccharide	Growth stimulant
Graphite	Graphite	Bio-stimulant
BM-T	Bontera® BM-T, a variety of soil microbes	Bio-fertilizer
Green liquid	Green Liquid, amino acids, and carbon	Fertilizer
DT-200	Ditera® DF, <i>Myrothecium verrucaria</i> (AARC-0255)	Nematicide
RV42-20	RhizoVital 42, <i>Bacillus amyloliquefaciens</i> (FZB42)	Bio-stimulant
Velum® 1GR	Velum, fluopyram	Nematicide
Bio-Green	Bio-Green Granule, vermicast, micro-nutrients, mycorrhizae, and <i>Trichoderma</i>	Fertilizer
Untreated control	-	-

3.2.3 Collection of the eggs and J2 of the nematode population

a) 20 g root extraction

The root samples were obtained using an adapted 20 g root extraction method described by Riekert (1995). The roots were cut into small pieces of 2-3 mm and placed into a container filled with a solution of 3.5% NaCOL (bleach). The container was then sealed with an airtight lid and shaken for 4 min. The container was then rinsed into a blender and blended for 20 seconds. The solution and the roots were subsequently transferred to a 250 µm sieve stacked on a 20 µm sieve and then rinsed. After the content of the 20 µm sieve was thoroughly rinsed, it was poured into a centrifuge tube. The tube was then filled with tap water. The centrifuge tubes were then subjected to the sugar centrifugal flotation method (Coolen and D'Herde 1972; Hooper *et al.*, 2005).

The sugar centrifugal flotation method

Nematodes were separated from organic materials in the root samples using the sugar flotation method. After the nematodes were extracted, the separation made the samples clearer to count. For this method, 5 ml of kaolin was added to the centrifuge tubes, which was thoroughly mixed (ensuring that the mixing object is cleaned after each sample to avoid contamination) after which it was transferred to the centrifuge for 5 min at 3000 rpm (Fourie *et al.*, 2017). The supernatant was decanted and discarded. A solution of 1,6 kg of sugar and 2 L tap water was then used to fill up each centrifuge tube and thoroughly mixed while avoiding contamination.

These tubes were then centrifuged for 1 min at 4,000 rpm. The centrifuge tubes were then transferred separately to a 20 µm sieve and rinsed into a clearly marked sample beaker for counting and identification (Fourie *et al.*, 2017).

3.2.4 Assessment of nematode reproduction

Root extractions were done at 56 and 112 days after planting (DAP). At each time interval, eight plants from each treatment were removed. The final number of eggs of the mixed *Meloidogyne* and *Pratylenchus x* populations were determined. In addition, population densities of motile J2, sessile J2, sessile third stage (J3) and fourth stage (J4) and females of *Meloidogyne* were counted as well as motile life stages of *Pratylenchus x*. This was done by counting these different nematode life stages in a De Grisse counting dish (De Grisse, 1963).

3.2.5 Statistical analysis

Nematode data, population densities for the various life stages (for both 56 and 112 DAP) from the root extraction, were recorded and $\log_{(x+1)}$ was transformed to compensate for variation. The data was then subjected to one-way ANOVA (each sampling interval) and Repeated Measures ANOVA (both sampling intervals) using the TIBCO Statistica Version 14.0.1 software (www.tibco.com). Treatment means were compared using Tukey's HDS test [HDS = honest significant difference] ($P \leq 0.05$).

3.3 Tunnel and field trials - *in vivo* experiment: effect of Velum® 1G nematicide and Bio-Green bio-fertilizer on nematode populations

The tunnel and field trials commenced in the soybean season of 2021/22. The tunnel trial was conducted on the premises of North-West University (NWU) in Potchefstroom (GPS coordinates: 26°45'17.69" S; 27°06' 20.27" E) and the field trial was conducted near Bultfontein, Free State province, SA (GPS coordinates: 26° 23' 54" S; 28° 04' 80" E). The objective of these trials was to evaluate the effect of the Bio-Green granular bio-fertilizer and a commercially available nematicide Velum® 1G on the reproduction of *Meloidogyne* spp.

3.3.1 Plant material

Commercially available soybean seed (cultivar DM 6.9 RR) was used in both the tunnel and field trials. The cultivars differ from the micro-plot experiment because the seeds weren't available the next season, however, the characteristics of the seeds remain similar.

3.3.2 Experimental setup

a) Planting

The soil in the tunnel trial was collected from the infested site at Bultfontein and transported to the tunnel trial site. The soil was supplemented with vermiculite (50:50 ratio) to

ensure that the pots were filled up to 85%. The soybean seeds were planted 3 cm apart in each pot for each treatment at a total of 32 seeds per pot.

Approximately 270,000 seeds were planted per hectare for the field trial; for each treatment, four rows 76 cm apart and 25 m long were planted.

b) Inoculation

For the tunnel trial, the soil infested with *Meloidogyne* spp. and other plant-parasitic nematodes (PPN) was added to 50 L-capacity pots. To ensure a successful infestation of PPN, 2,000 *Meloidogyne* spp. eggs per seed were additionally inoculated 7 days after planting (DAP). The egg masses of the three *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) were obtained from infected tomato plants (cultivar Rodade) grown in soil in pots in a glasshouse. The *Meloidogyne* populations were originally obtained from infected tomato roots in the Letsitele area in the Limpopo province (SA). Rieker's (1995) extraction method was used to obtain eggs from the egg masses. The eggs were counted under a stereomicroscope in a De Gisse counting dish, then placed in a beaker, and gently mixed with a magnetic stirrer.

The field trial was naturally infested with PPN, and no additional inoculation was done.

c) Application of treatments

The treatments used in both tunnel and field trials are listed in Table 3.3, with Treatment 1 (T1) an application of Velum® 1G (active ingredient fluopyram/pyridinylethylbenzamide) 10 g/kg at the registered dosage of 150 g/100-meter row with 20 replicates. Treatment 2 (T2) was an untreated control with 10 repetitions. Treatment 3 (T3) was an application of both Velum® 1 GR and the Bio-Green granule with five replicates and Treatment 4 (T4) consisted of only the Bio-Green granule with five replicates (Owner company Elim Kunsmis). After the application of the treatments, no additional fertilizers were applied to the soybean, the field trials were rainfed and the tunnel trials were watered regularly (3 times per week).

Table 3.3. Treatments included in the tunnel and field trials, active ingredient, use and formulation

Treatment No	Treatment	Active ingredient	Use
1	Velum	Fluopyram	Nematicide
2	Untreated control	-	-
3	Velum® 1GR + Bio-Green	Fluopyram, vermicast, micro-nutrients, mycorrhizae, <i>Trichoderma</i> , microfine gypsum, carbon and kelp	Nematicide and fertilizer
4	Bio-Green	Vermicast, micro-nutrients, mycorrhizae, <i>Trichoderma</i> , microfine gypsum, carbon and kelp	Fertilizer

A complete randomised block design was used (Fig. 3.3), with the treatments placed in no discernible pattern within the block to avoid the influence of sun/light and temperature on the four different treatments.

R5 T1	R5 T2	R5 T1	R5 T3	R5 T1	R5 T4	R5 T1	R5 T2
R4 T1	R4 T1	R4 T4	R4 T2	R4 T1	R4 T1	R4 T3	R4 T2
R3 T1	R3 T3	R3 T1	R3 T1	R3 T4	R3 T2	R3 T2	R3 T1
R2 T2	R2 T2	R2 T1	R2 T3	R2 T1	R2 T4	R2 T1	R2 T1
R1 T1	R1 T2	R1 T1	R1 T1	R1 T1	R1 T2	R1 T3	R1 T4

Figure 3.3. Randomised block design used for the tunnel and field trials

3.3.3 Collection of the *Meloidogyne* egg population

The 20 g root extraction protocol was used as described in 3.2.3

3.3.4 Assessment of nematode reproduction

Root extractions were done at 14, 28, 56 and 112 days after emergence (DAE). At each time interval, eight plants from each treatment were removed and 20 g root were extracted (Riekert, 1995). The final number of eggs, *Meloidogyne* spp. and *Pratylenchus* spp. populations were determined. This was done by counting these different nematode life stages in a De Grisse counting dish (De Grisse, 1963).

3.3.5 Statistical analysis

Nematode data, population densities for the various life stages (for 14, 28, 56 and 112 DAP) from the root extraction were recorded for each of the tunnel and field trials. The data for each of the tunnel and field studies were then subjected to one-way ANOVA (each sampling interval) and to Repeated Measures ANOVA (both sampling intervals) using the TIBCO Statistica Version 14.0.1 software (www.tibco.com). Treatment means were compared using Tukey's HDS test [HDS = honest significant difference] ($P \leq 0.05$).

4 Results

4.1 *In vitro* bioassay: effect of several experimental biological products on the motility and viability of J2 *Meloidogyne* spp.

4.1.1 T3C product

The different concentrations of T3C resulted in a significantly higher ($P=0.00$) percentage immobility of J2 compared with the untreated control (dH₂O) for the three time intervals (Fig. 4.1.1A). No dosage response was observed, and the percentage of immobility did not increase over time (Table 4.1).

Table 4.1. Percentage immobile second-stage juveniles (J2) of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) after treatment with garlic oil (T3C) at different concentrations, a negative (alcohol) and positive (H₂O) control after 24, 48 and 72 h exposure in an *in vitro* bioassay

Treatment	Immobile J2 (%)		
Exposure period	24 h	48 h	72 h
T3C^	97.72 kl	92.02 ghi	93.31 hij
T3C-1	76.64 b	90.49 efgh	90.81 efgh
T3C-5	88.07 cdef	86.74 cd	90.34 efgh
T3C-10	93.17 hji	95.17 ijk	91.03 fgh
T3C-20	89.16 defg	88.71 cdefg	95.84 jk
T3C-30	87.45 cdef	87.39 cde	85.37 c
Alcohol	100.00 l	100.00 l	100.00 l
dH ₂ O	7.33 a	4.33 a	6.06 a
P-value	0.00*	0.00*	0.00*
F-value	3.46	7.49	8.58

^Concentrations used were 100, 1, 5, 10, 20 and 30 % of garlic oil. Percentages followed by a different letter are significantly ($P \leq 0.05$) different according to Fisher's protected least significant difference (LSD) test.

4.1.2 Oligo product

The Oligo-product with concentrations ranging from 1 to 15 % chitosan oligo saccharide produced a significantly higher ($P=0.00$) percentage immobility of the J2 compared to the untreated control (dH₂O) for the three time intervals (Fig. 4.1.2; Table 4.2). Percentage immobility ranged from 81 to 100%, apart from Oligo-5 at 24 h. No dosage response was observed.

Table 4.2. Percentage immobile second-stage juveniles (J2) of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) after treatment with chitosan oligo saccharide (Oligo) at different concentrations, a negative (alcohol) and positive (H₂O) control, after 24, 48 and 72 h exposure in an *in vitro* bioassay

Treatment	Immobile J2 (%)		
Exposure period	24 h	48 h	72 h
Oligo-1^	81.86 c	91.24 d	100.00 g
Oligo-2	90.00 d	92.99 de	96.52 f

Oligo-5	75.88 b	96.40 f	96.41 f
Oligo-10	96.28 ef	100.00 g	100.00 g
Oligo-15	97.67 fg	100.00 g	100.00 g
Alcohol	100.00 g	100.00 g	100.00 g
dH ₂ O	7.33 a	4.33 a	6.06 a
<i>P</i> -value	0.00*	0.00*	0.00*
<i>F</i> -value	3.93	12.19	19.28

^Concentrations used were 1, 2, 5, 10 and 15 % of chitosan oligo polysaccharides. Percentages followed by a different letter are significantly ($P \leq 0.05$) different according to Fisher's protected least significant difference (LSD) test.

4.1.3 Ses-A product

The Ses-A (sesame oil) concentrations tested produced a significantly higher ($P=0.00$) percentage immobility for all three time intervals compared to the untreated control with immobility ranging from 89 to 97% (Table 4.3). No dosage response was observed.

Table 4.3. Percentage immobile second-stage juveniles (J2) of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) after treatment with sesame oil (Ses-A) at different concentrations, a negative (alcohol) and positive (H₂O) control, after 24, 48 and 72 h exposure in an *in vitro* bioassay

Treatment	Immobile J2 (%)		
Exposure period	24 h	48 h	72 h
Ses-A-5^	92.31 defg	91.41 cde	96.95 hi
Ses-A-10	88.69 c	95.02 fgh	95.29 gh
Ses-A-20	93.19 efg	94.40 efgh	91.45 cde
Ses-A-30	89.63 cd	96.98 hi	91.61 cdef
Alcohol	100.00 i	100.00 i	100.00 i
dH ₂ O	7.00 b	1.67 a	7.67 b
<i>P</i> -value	0.00*	0.00*	0.00*
<i>F</i> -value	10.60	12.55	5.16

^Concentrations used were 5, 10, 20 and 30% of sesame oil. Percentages followed by a different letter are significantly ($P \leq 0.05$) different, according to Fisher's protected least significant difference (LSD) test.

4.1.4 Aba-5 product

A significantly higher ($P=0.00$) percentage immobility between the product Aba-5 and the untreated control (dH₂O) was observed for the three time intervals with percentage immobility ranging from 89 to 94% (Table 4.4). The J2 observed in this bioassay showed immobility, but no dosage response was visible.

Table 4.4. Percentage immobile second-stage juveniles (J2) of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) after treatment with 5% Abamectin (Aba-5), a negative (alcohol) and positive (H₂O) control, after 24, 48 and 72 h exposure in an *in vitro* bioassay

Treatment	Immobile J2 (%)		
Exposure period	24 h	48 h	72 h
Aba-5	89.05 d	93.74 c	93.77 c
Alcohol	100.00 b	100.00 b	100.00 b

dH ₂ O	4.33 a	6.32 a	4.33 a
P-value	0.00*	0.00*	0.00*
F-value	14.26	28.62	38.73

Percentages followed by a different letter are significantly ($P \leq 0.05$) different, according to Fisher's protected least significant difference (LSD) test.

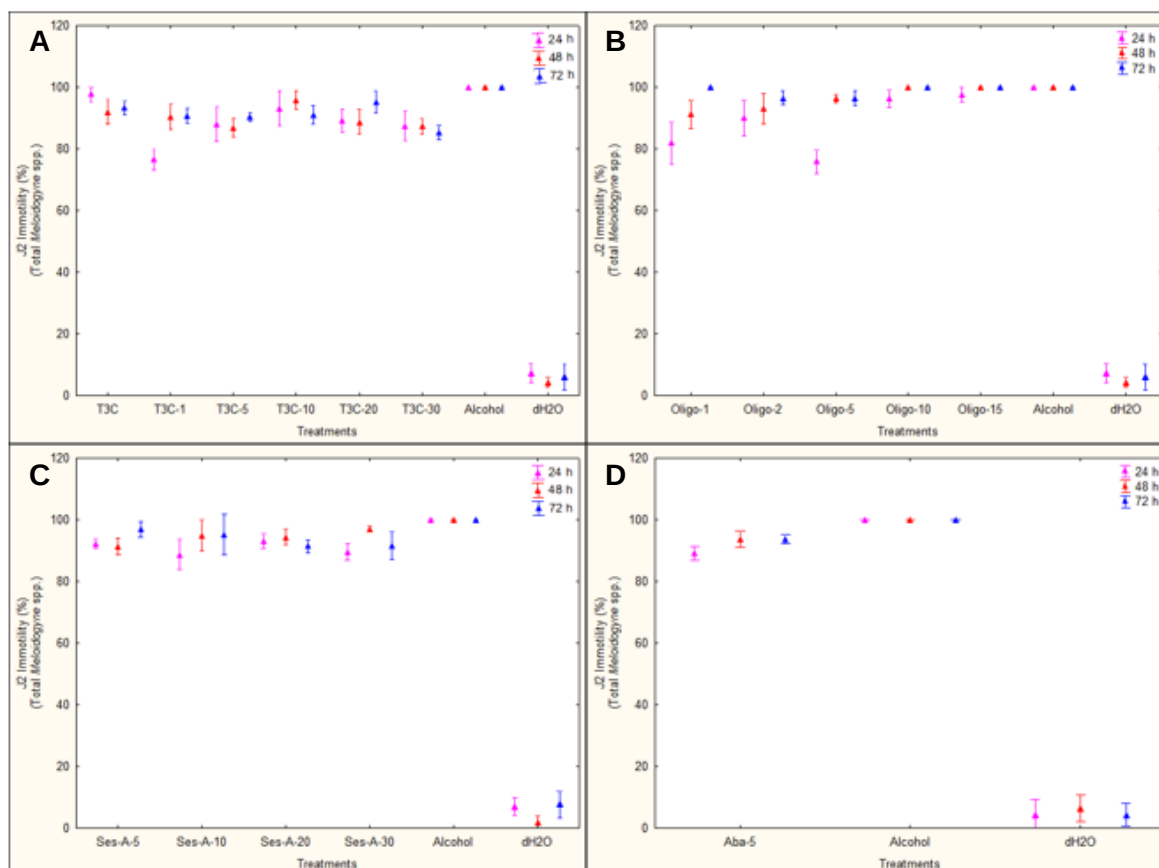


Figure 4.1.1. *In vitro* bioassay. Effect of different concentrations of T3C product (A), Oligo product (B), Ses-A product (C) and Aba-5 product (D), and a negative (alcohol) and positive (H₂O) control on the motility of second-stage juveniles (J2) of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) after 24, 48 and 72 h exposure. Vertical bars indicate the confidence interval (CI = 95%)

4.1.5 Graphite product

A significantly higher ($P=0.00$) percentage immobility was observed for the graphite product compared to the untreated control (dH₂O) at the three time intervals (Fig. 4.1.2 A). Percentage immobility ranged from 93.5 to 100% (Table 4.5). After the suspension of the product, clear degradation of the J2 could be observed after 24 h.

Table 4.5. Percentage immobile second-stage juveniles (J2) of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) after treatment with graphite, a negative (alcohol) and positive (H₂O) control, after 24, 48 and 72 h exposure in an *in vitro* bioassay

Treatment	Immobile J2 (%)		
Exposure period	24 h	48 h	72 h
Graphite	93.56 c	96.14 bc	99.12 ab

Alcohol	100.00 a	100.00 a	100.00 a
dH ₂ O	4.33 d	6.32 d	4.33 d
<i>P</i> -value	0.00*	0.00*	0.00*
<i>F</i> -value	15.36	21.95	24.80

Percentages followed by a different letter are significantly ($P \leq 0.05$) different, according to Fisher's protected least significant difference (LSD) test.

4.1.6 BM-T product

A significantly higher ($P=0.00$) percentage immobility was observed for the product BM-T compared to the untreated control (dH₂O) for the three time intervals with percentage immobility ranging from 81 to 92% (Figure 4.2.1 B; Table 4.6).

Table 4.6. Percentage immobile second-stage juveniles (J2) of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) after treatment with Bontera® BM-T, a negative (alcohol) and positive (H₂O) control, after 24, 48 and 72 h exposure in an *in vitro* bioassay

Treatment	Immobile J2 (%)		
Exposure period	24 h	48 h	72 h
BM-T	80.63 b	91.70 c	89.97 c
Alcohol	100.00 d	100.00 d	100.00 d
dH ₂ O	4.33 a	6.32 a	4.33 a
<i>P</i> -value	0.00*	0.00*	0.00*
<i>F</i> -value	21.30	18.77	24.68

Percentages followed by a different letter are significantly ($P \leq 0.05$) different, according to Fisher's protected least significant difference (LSD) test.

4.1.7 Green-liquid product

The Green-liquid product resulted a significantly higher ($P=0.00$) percentage immobility of J2 compared to the untreated control with values ranging from 78 to 92% for the three time intervals (Table 4.7).

Table 4.7. Percentage immobile second-stage juveniles (J2) of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) after treatment with Green-liquid, a negative (alcohol) and positive (H₂O) control, after 24, 48 and 72 h exposure in an *in vitro* bioassay

Treatment	Immobile J2 (%)		
Exposure period	24 h	48 h	72 h
Green-liquid	88.03 c	92.22 c	78.45 b
Alcohol	100.00 d	100.00 d	100.00 d
dH ₂ O	3.67 a	3.67 a	1.00 a
<i>P</i> -value	0.00*	0.00*	0.00*
<i>F</i> -value	21.72	22.67	3.45

Percentages followed by a different letter are significantly ($P \leq 0.05$) different, according to Fisher's protected least significant difference (LSD) test.

4.1.8 Oxa-40 product

Oxa-40-product resulted in significantly higher ($P=0.00$) percentage immobility of J2 for all three sampling intervals compared to the untreated control (dH₂O) (Fig.4.1.2 D). The percentage immobility for Oxa-40 ranged between 88% and 95% (Table 4.8).

Table 4.8. Percentage immobile second-stage juveniles (J2) of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) after treatment with 40% Oxamyl (Oxa-40), a negative (alcohol) and positive (H₂O) control, after 24, 48 and 72 h exposure in an *in vitro* bioassay

Treatment	Immobile J2 (%)		
Exposure period	24 h	48 h	72 h
Oxa-40	89.42 c	94.66 b	88.45 c
Alcohol	100.00 a	100.00 a	100.00 a
dH ₂ O	3.67 d	3.67 d	1.00 d
P-value	0.00*	0.00*	0.00*
F-value	43.13	30.50	43.97

Percentages followed by a different letter are significantly ($p \leq 0.05$) different, according to Fisher's protected least significant difference (LSD) test.

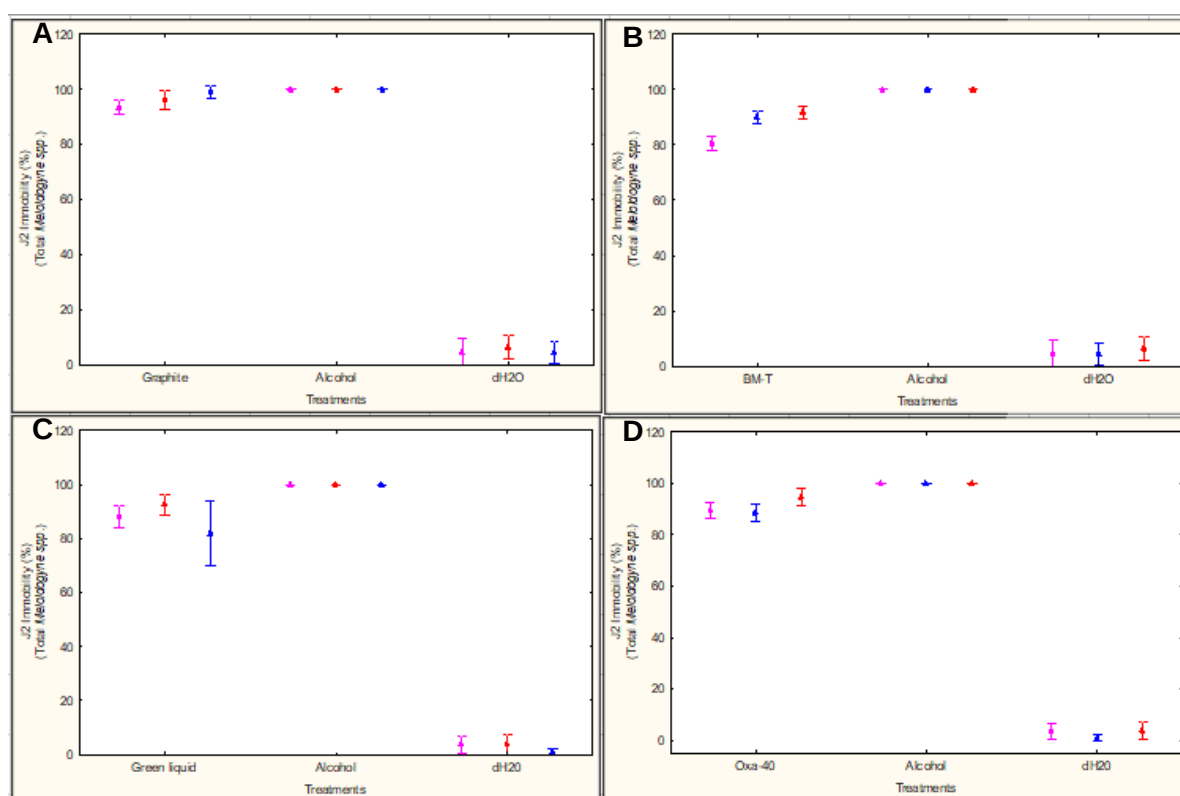


Figure 4.1.2. *In vitro* bioassay. Effect of different concentrations of Graphite product (A), BM-T product (B), Green-liquid product (C) and Oxa-40 product (D) as well as a negative (alcohol) and positive (H₂O) control on the motility of second-stage juveniles (J2) of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) after 24, 48 and 72 h exposure. Vertical bars indicate the confidence interval (CI = 95%)

4.2 Microplot - *in vivo* experiment: evaluating the effect of bio-fertilizers on the motility and reproduction of *Meloidogyne* and *Pratylenchus* species.

With the first extraction of the roots 56 DAP (20 g per treatment), high population numbers were recorded for all three nematode variables (eggs numbers, *Meloidogyne* juvenile and mature life stage numbers, and *Pratylenchus* motile life stage numbers). Therefore, it was evident that the inoculation had been successful. The second root extraction of the roots at 112 DAP had no significant difference between the treatments and three nematode varieties.

4.2.1 Egg numbers

The egg numbers differed significantly among treatments ($P=0.02$) (Table 4.9). At 56 DAP, RV42-40 had a significantly lower number of eggs than the untreated control (Fig. 4.2.A). However, the untreated control, DT-200 and Graphite treatment did not differ significantly from one another (Table 4.9). At 112 DAP, no significant differences were observed among the treatments ($P=0.72$) (Fig. 4.2A).

4.2.2 *Meloidogyne* juvenile and mature life stages

No significant differences were observed among the different treatments for the *Meloidogyne* motile stages at any time interval ($P=0.06$ at 56 DAP and $P=0.34$ at 112 DAP) (Table. 4.9).

4.2.3 *Pratylenchus* spp. numbers

Pratylenchus motile life stage numbers differed significantly among the treatments at 56 DAP ($P=0.00$) (Table 4.9), all treatments had significantly lower numbers compared to the untreated control (Fig. 4.2C). At 112 DAP, no significant difference was observed among the treatments ($P=0.06$) (Table 4.9).

4.2.4 Interaction data

Significant interactions were observed between the egg numbers and the treatments ($P=0.03$) and egg numbers and sampling intervals ($P=0.00$), but no significant interaction ($P=0.16$) was recorded between egg number and treatments x sampling intervals. The only significant interaction ($P=0.00$) for *Meloidogyne* motile life stages was between the nematode numbers and sampling intervals. The only significant interaction ($P=0.00$) for *Pratylenchus* motile life stages was between the nematode numbers and the treatments (Table 4.9).

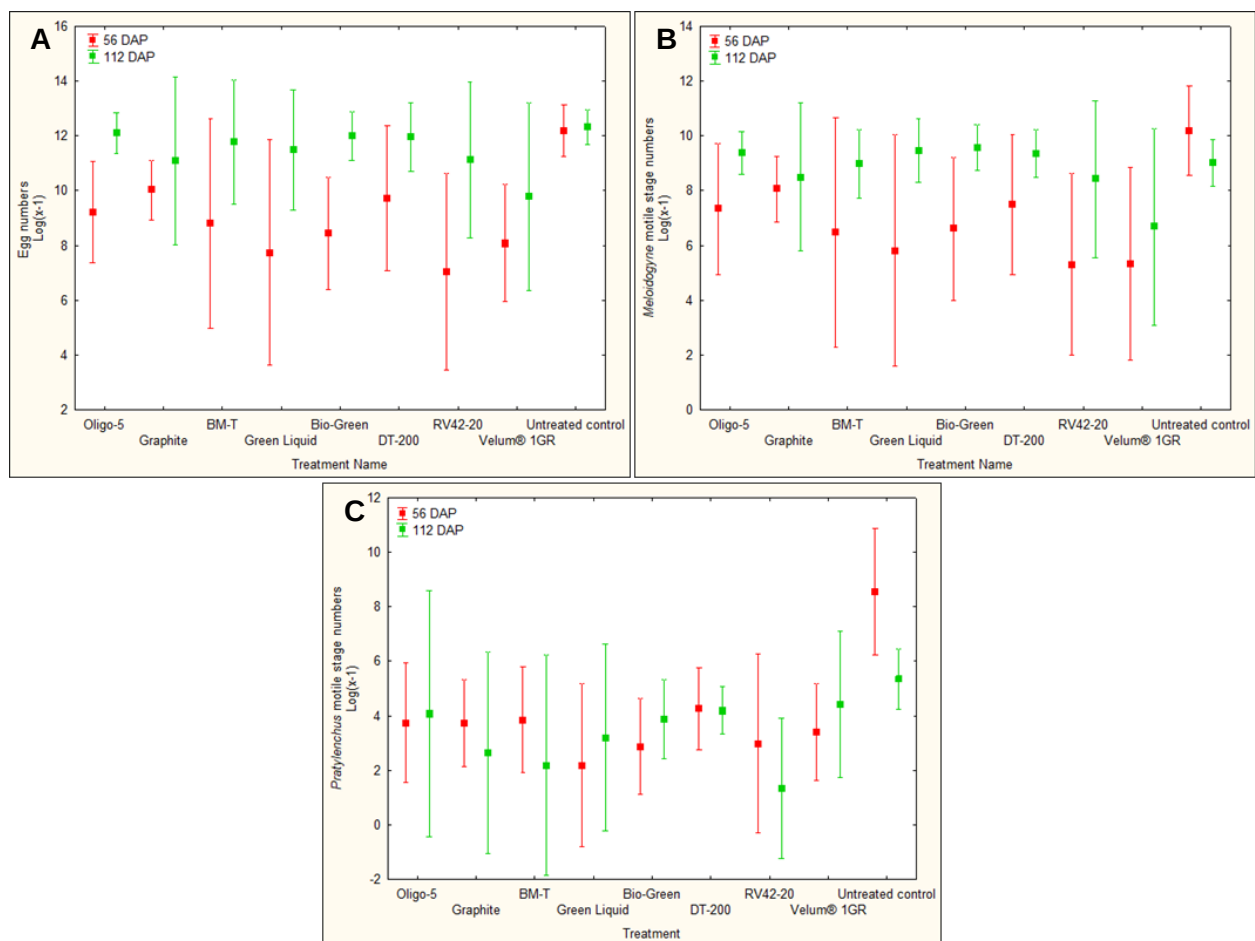


Figure 4.2. The effect of seven experimental treatments, as well as an untreated control and standard nematicide (Velum® 1GR) in a microplot experiment. The log transformed ($\text{Log}(x-1)$) number of eggs (A), *Pratylenchus spp.* motile stages (B) and *Meloidogyne incognita* motile stages (C) in the roots of the susceptible soybean cultivar (P57T19R) for two sampling intervals after 56 and 112 DAP (days after planting). Vertical bars indicate the confidence interval (CI = 95%)

Table 4.9. Microplot experiment. Effect of seven products, and the nematicide Velum® 1GR on the reproduction of a mixed population of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) and *Pratylenchus* species (unknown species) (individuals/20 g roots) extracted from roots of soybean on the susceptible soybean cultivar P57T19R, 56 and 112 DAP (DAP = days after planting) after inoculation with 1,800 eggs + J2 of *Meloidogyne* and 850 eggs + motile life stages of *Pratylenchus* per seed

Treatments	Eggs per 20 g roots	<i>Meloidogyne</i> motile stages per 20 g roots	<i>Pratylenchus</i> motile stages per 20 g roots
56 DAP			
Oligo-5	9.21 ¹ (15,351 ² ± 6,643 ³) bcDEF	7.34 ¹ (2,851 ± 1,339) aCDE	3.77 (88 ± 63) bBC
Graphite	10.02 (26,425 ± 7,842) abBCDE	8.07 (3,843 ± 1,215) aABCD	3.76 (61 ± 31) bBC
BM-T	8.79 (28,740 ± 21,716) bcEFG	6.49 (3,526 ± 2,681) aDE	3.88 (79 ± 43) bBC
Green liquid	7.75 (11,148 ± 6,304) bcG	5.8 (1,691 ± 969) aE	2.25 (25 ± 17) bCD
Bio-Green granule	8.42 (7,245 ± 2,987) bcEFG	6.61 (1,280 ± 442) aDE	2.95 (25 ± 10) bCD
DT-200	9.72 (32,174 ± 15,414) abCDEF	7.48 (3,321 ± 1,562) aBCDE	4.27 (100 ± 51) bBC
RV42-20	7.05 (5,492 ± 4,610) cG	5.32 (792 ± 662) aE	3.00 (46 ± 22) bCD
Velum® 1GR	8.08 (23,092 ± 25,035) bcEFG	5.34 (6,009 ± 7,122) aE	3.41 (51 ± 16) bBCD
Untreated (control)	12.19 (222,578 ± 57,225) aA	10.20 (35,586 ± 11,083) aA	8.56 (9,744 ± 5008) aA
<i>P</i> -value	0.0161*	0.064	0.00*
<i>F</i> -value	2.92	2.15	6.16
112 DAP			
Oligo-5	12.10 ¹ (196,633 ² ± 52,671 ³) aA	9.37 (12,871 ± 3,510) aABC	4.08 (242 ± 155) aBC
Graphite	11.09 (136,400 ± 54,095) aABCD	8.49 (8,987 ± 3,485) aABCD	2.70 (51 ± 28) aCD
BM-T	11.77 (214,499 ± 90,379) aAB	8.98 (9,745 ± 3,359) aABC	2.19 (44 ± 30) aCD
Green liquid	11.48 (161,194 ± 68,465) aABC	9.46 (14,975 ± 3,726) aABC	3.21 (56 ± 21) aBCD
Bio-Green granule	11.99 (181,913 ± 56,063) aAB	9.57 (15,587 ± 3,891) aAB	3.90 (69 ± 36) aBC
DT-200	11.96 (191,525 ± 61,802) aAB	9.36 (12,750 ± 2,912) aABC	4.20 (75 ± 25) aBC
RV42-20	11.12 (131,431 ± 55,060) aAD	8.42 (8,675 ± 3,182) aABCD	1.37 (9 ± 6) aD
Velum® 1GR	9.79 (62,743 ± 44,094) aABCD	8.01 (4,264 ± 2,984) aBCD	4.41 (414 ± 329) aB
Untreated (control)	12.32 (238,545 ± 44,340) aA	9.02 (9,124 ± 2,100) aABC	5.34 (252 ± 91) aB
<i>P</i> -value	0.719	0.339	0.057
<i>F</i> -value	0.66	1.19	2.22
Interaction: Treatments			
<i>P</i> -value	0.03*	0.109	0.001*
<i>F</i> -value	2.95	1.86	4.93

Interaction: Sampling intervals			
<i>P</i> -value	0.00*	0.00*	3.50
<i>F</i> -value	62.36	31.06	0.74
Interaction: Treatments x sampling intervals			
<i>P</i> -value	0.16	0.095	0.052
<i>F</i> -value	1.61	1.92	2.25

¹Log (x+1) transformed.

²Non-transformed means.

³Standard error.

*Significant difference.

Means in the same column per sampling date followed by the same lowercase letter and in the same column followed by the same capital letter do not differ significantly ($P \leq 0.05$) according to Tukey's honest significant difference (HSD) test

4.3 Tunnel trials - *in vivo* experiment: effect of Velum® 1G nematicide and Bio-Green bio-fertilizer on nematode populations in controlled conditions

4.3.1 Egg numbers

Egg numbers showed no significant differences at 14 ($P=0.47$) and 28 DAP ($P=0.15$) for the three treatments (Table 4.10). A significantly lower number ($P=0.00$) of eggs between the untreated control and Velum® 1GR as well as Velum® 1GR + Bio-Green were observed at 56 DAP. At 112 DAP, all treatments had significantly lower ($P=0.00$) numbers compared to the untreated control, while Velum® 1GR + Bio-Green granule also had significantly lower egg numbers than Velum® 1GR and Bio-Green granule treatments (Fig. 4.3 A).

4.3.2 Total *Meloidogyne* spp. numbers

The total *M. incognita* numbers did not differ significantly among the treatments at 14 ($P=0.49$), 28 ($P=0.17$) and 56 DAP ($P=0.30$) but showed significant differences at 112 DAP ($P=0.00$). The untreated control had significantly higher total *M. incognita* numbers compared to the other three treatments while Velum® 1GR + Bio-Green granule had significantly lower *M. incognita* numbers than Velum® 1GR and Bio-Green granule treatments (Table 4.10; Fig. 4.3B).

4.3.3 Interaction data

Significant interactions were observed between all nematode numbers and treatments ($P=0.05$), as well as sampling times ($P=0.00$) and treatments x sampling times ($P=0.03$) (Table 4.10).

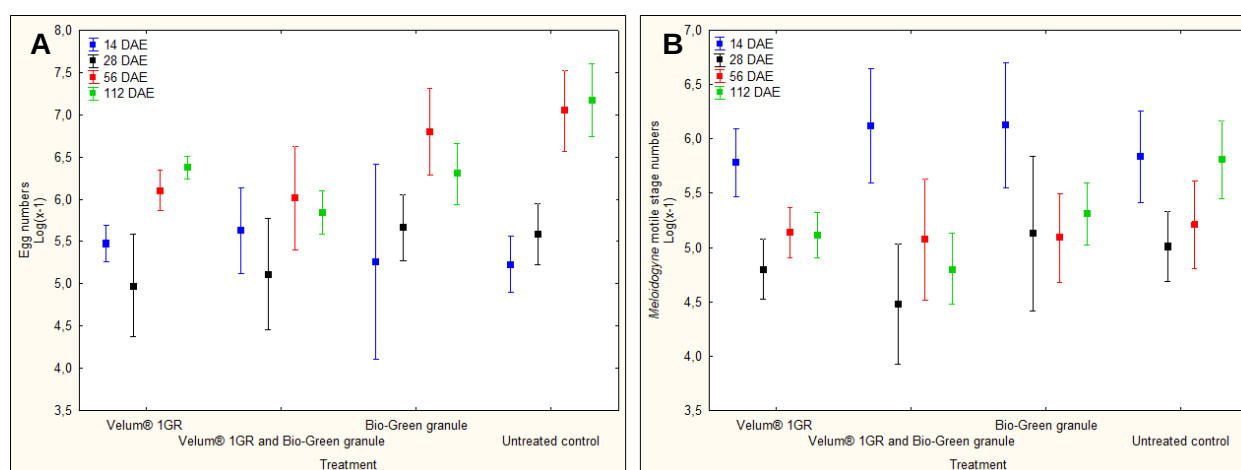


Figure 4.3. The effect of three experimental treatments, as well as an untreated control in a tunnel experiment. The log transformed ($\text{Log}(x-1)$) number of eggs (A) and *Meloidogyne incognita* motile stage numbers (B) in roots of a susceptible soybean cultivar (DM 6.9 RR) for four sampling intervals, 14, 28, 56 and 112 DAP (DAP = Days after planting). Vertical bars indicate the confidence interval (CI = 95%)

Table 4.10. Tunnel experiment. Effect of three products and a negative control on the nematode population density (individuals/20 g roots) extracted from roots of soybean cultivar DM 6.9 RR after inoculation 7 DAP (days after planting) with 2,000 eggs of *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*), 14, 28, 56 and 112 DAP (days after planting)

Treatment	<i>Meloidogyne</i> eggs per 20 g roots	<i>Meloidogyne</i> motile stages per 20 g roots
14 DAP		
Velum® 1GR	5.5 ¹ (262 ² ± 24 ³) aEFG	5.8 (391 ± 50) aBCDE
Velum® 1GR and Bio-Green granule	5.6 (294 ± 42) aDEFG	6.1 (494 ± 84) aAB
Bio-Green granule	5.3 (273 ± 111) aFG	6.2 (512 ± 100) aAB
Untreated control	5.2 (208 ± 35) aFG	5.9 (424 ± 92) aBCDE
<i>P</i> -value	0.47	0.49
<i>F</i> -value	0.87	0.82
28 DAP		
Velum® 1GR	5.2 (209 ± 32) aFG	4.8 (143 ± 16) abHI
Velum® 1GR and Bio-Green granule	5.1 (188 ± 47) aG	4.5 (95 ± 15) aI
Bio-Green granule	5.7 (300 ± 46) aDEF	5.2 (203 ± 51) aGH
Untreated control	5.6 (297 ± 45) aDEFG	5.0 (168 ± 22) aGH
<i>P</i> -value	0.15	0.17
<i>F</i> -value	1.86	1.76
56 DAP		
Velum® 1GR	6.1 (506 ± 62) bCD	5.5 (271 ± 25) aDEFG
Velum® 1GR and Bio-Green granule	6.0 (458 ± 125) bCD	5.5 (258 ± 65) aCDEF
Bio-Green granule	6.8 (953 ± 161) aAB	5.4 (243 ± 41) aEFG
Untreated control	7.0 (1361 ± 236) aA	5.8 (357 ± 53) aBCDE
<i>P</i> -value	0.000*	0.30
<i>F</i> -value	8.52	1.26
112 DAP		
Velum® 1GR	6.4 (610 ± 39) bBC	6.0 (395 ± 23) bABCD
Velum® 1GR and Bio-Green granule	5.8 (351 ± 34) cCDE	5.6 (290 ± 35) cCDEF
Bio-Green granule	6.8 (564 ± 71) bBC	6.0 (412 ± 44) bABC
Untreated control	7.2 (1499 ± 265) aA	6.4 (615 ± 41) aA
<i>P</i> -value	0.00*	0.00*
<i>F</i> -value	15.77	12.60
Interaction: Sampling times		
<i>P</i> -value	0.00*	0.05*
<i>F</i> -value	57.46	2.84
Interaction: Treatments		
<i>P</i> -value	0.00*	0.00*
<i>F</i> -value	10.56	28.80
Interaction: Sampling times x Treatments		
<i>P</i> -value	0.0002*	0.03*
<i>F</i> -value	4.06	2.14

¹Log (x+1) transformed.

²Non-transformed means.

³Standard error.

*Significant difference.

Means in the same column per sampling date followed by the same lowercase letter and in the same column followed by the same capital letter do not differ significantly ($P \leq 0.05$) according to Tukey's honest significant difference (HSD) test.

4.4 Field trials - *in vivo* experiment: effect of Velum® 1G nematicide and Bio-Green bio-fertilizer on nematode populations in natural conditions

4.4.1 Eggs numbers

No eggs were observed at 14 DAP. At 28 and 56 DAP, the Velum® 1GR + Bio-Green and the Bio-Green treatments had significantly lower egg numbers ($P=0.00$) than the untreated control, while at 112 DAP, the untreated control had significantly higher ($P=0.00$) egg numbers compared to the other treatments (Table 4.11; Fig. 4.4A).

4.4.2 Total *Meloidogyne* spp. numbers

No significant differences were observed at 14 ($P=0.09$) and 28 DAP ($P=0.39$) among the treatments. At 56 DAP, the total *M. incognita* numbers were significantly higher ($P=0.00$) for the untreated control compared to Velum® 1G and Bio-Green treatments (Table 4.11). At 112 DAP, the untreated control had significantly higher ($P=0.00$) numbers than the other three treatments (Fig. 4.4B).

4.4.3 Total *Pratylenchus* spp. numbers

At 14 DAP, Bio-Green granule treatment had significantly lower ($P=0.03$) *Pratylenchus* motile stage numbers than the untreated control. No significant differences ($p=0.21$) were observed among the treatments at 28 DAP. A significantly lower ($P=0.00$) *Pratylenchus* motile stage numbers were obtained at 56 DAP for the Velum® 1GR treatment, compared to the other treatments. (Table 4.11). No *Pratylenchus* was observed at 112 DAP.

4.4.4 Interaction data

Significant interactions ($P=0.00$) were observed between the sampling intervals and the treatments of the egg numbers, total *M. incognita* numbers as well as the total *Pratylenchus* spp. numbers ($P=0.00$). A significant interaction between sampling time x treatment was only observed for egg numbers ($P=0.00$) (Table 4.11).

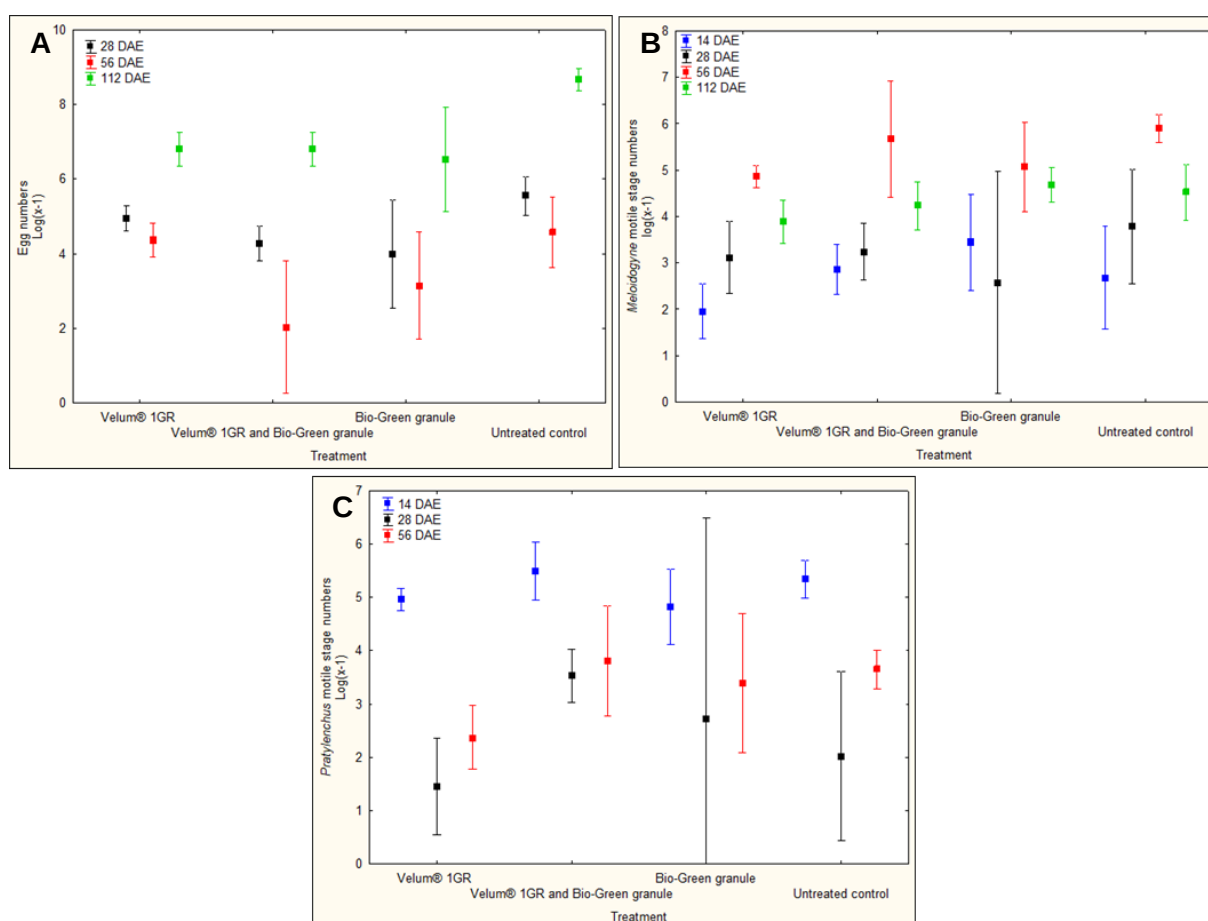


Figure 4.4. The effect of three experimental treatments as well as an untreated control in a field experiment. The log transformed ($\text{Log}(x+1)$) number of eggs (A), *Meloidogyne incognita* motile stages (B) and *Pratylenchus* spp. motile stages (C) in roots of the susceptible soybean cultivar (DM 6.9 RR) for four sampling intervals, 14, 28, 56 and 112 DAP (DAP = Days after planting). Vertical bars indicate the confidence interval (CI = 95%)

Table 4.11. Field experiment. Effect of three products and a negative control on nematode population density (individuals/20 g roots) obtained from roots of soybean cultivar DM 6.9 RR infected with *Meloidogyne* species (*M. incognita*, *M. javanica* and *M. enterolobii*) and *Pratylenchus* (unknown species), 14, 28, 56 and 112 DAP (days after planting)

Treatment	Eggs per 20 g roots	<i>Meloidogyne</i> motile stages per 20 g roots.	<i>Pratylenchus</i> motile stages per 20 g roots
14 DAP			
Velum® 1GR	0 H	2.0 ($12^1 \pm 3^2$) aF	5.0 (158 ± 18) bcAB
Velum® 1GR and Bio-Green	0 H	2.9 (19 ± 5) aDEF	5.5 (263 ± 56) aA
Bio-Green granule	0 H	3.5 (40 ± 12) aDE	4.8 (142 ± 36) cABC
Untreated control	0 H	2.7 (27 ± 7) aEF	3.5 (235 ± 43) abA
P-value	-	0.09	0.03*
F-value	-	2.31	3.13
28 DAP			
Velum® 1GR	5.0 ¹ ($185^2 \pm 35^3$) abCD	3.2 (59 ± 15) aDE	1.5 (36 ± 8) aF
Velum® 1GR and Bio-Green	4.3 (76 ± 13) bcDE	3.3 (28 ± 5) aDE	3.6 (41 ± 6) aBCD
Bio-Green granule	4.0 (89 ± 43) cEF	2.6 (39 ± 21) aEF	2.8 (101 ± 100) aDEF
Untreated control	5.6 (331 ± 89) aC	4.0 (105 ± 34) aCD	2.0 (36 ± 14) aEF

<i>P</i> -value	0.002*	0.39	0.21
<i>F</i> -value	5.82	1.04	1.60
56 DAP			
Velum® 1GR	4.4 (111 ± 21) abDE	4.9 (157 ± 18) cBC	2.4 (19 ± 5) bDEF
Velum® 1GR and Bio-Green	2.1 (17 ± 16) cG	5.7 (240 ± 78) abAB	3.8 (61 ± 22) aBCD
Bio-Green granule	3.2 (37 ± 12) bcF	5.2 (232 ± 80) bcAB	3.4 (30 ± 11) abCDE
Untreated control	4.6 (172 ± 66) aC	6.0 (412 ± 48) aAB	3.7 (44 ± 7) aBCD
<i>P</i> -value	0.0006*	0.0005*	0.0076*
<i>F</i> -value	7.39	7.44	4.65
112 DAP			
Velum® 1GR	6.8 (1388 ± 345) bB	5.6 (310 ± 34) bAB	0
Velum® 1GR and Bio-Green	6.8 (953 ± 306) bB	5.5 (243 ± 18) bAB	0
Bio-Green granule	6.5 (1017 ± 1708) bB	5.4 (230 ± 64) bAB	0
Untreated control	8.7 (6202 ± 630) aA	6.3 (577 ± 65) aA	0
<i>P</i> -value	0.00*	0.00*	-
<i>F</i> -value	13.72	9.34	-
<i>Interaction: Sampling times</i>			
<i>P</i> -value	0.00*	0.00*	0.00*
<i>F</i> -value	576.63	88.13	50.55
<i>Interaction: Treatments</i>			
<i>P</i> -value	0.00*	0.00*	0.00*
<i>F</i> -value	20.27	5.27	5.33
<i>Interaction: Sampling times x Treatments</i>			
<i>P</i> -value	0.00*	0.243	0.491
<i>F</i> -value	5.15	1.30	0.91

¹Log (x+1) transformed.

²Non-transformed means.

³Standard error.

*Significant difference.

Means in the same column followed per sampling date by the same lowercase letter and in the same column followed by the same capital letter do not differ significantly ($P \leq 0.05$) according to Tukey's honest significant difference (HSD) test.

5 Discussion

5.1 *In vitro* experiment: effect of several experimental biological products on the motility and viability of J2 *Meloidogyne* spp.

Several products were tested in *in vitro* bioassays to evaluate their nematocidal efficacy. Among those products most were able to immobilize the J2 of the mixed *Meloidogyne* spp. community used (*M. incognita*, *M. javanica* and *M. enterolobii*) after 72 h exposure for more than 78%, with most being higher than 95%. Some of the products were already effective after 48 h.

The T3C product

For product T3C, different concentrations of garlic oil extracts were tested, and all concentrations seemed to be effective in significantly reducing nematode motility. In a study conducted by Eder *et al.* (2021), a commercial nematocide NEMgaured® with garlic as the active ingredient was evaluated for its effects on the motility of *Meloidogyne incognita*. After 96 h, at a

garlic oil concentration of 45%, the nematodes showed 3.6% immotility and 96.4% were dead, thus confirming that results in the current study showed a similar trend to those of the study by Eder *et al.* (2021). A compound known as organic sulphur can be found in garlic with antimicrobial effects. Diallyl disulfide is a compound derived from organic sulphur and this compound has been shown to interfere with metal homeostasis in nematodes as well as an imbalance in redox reaction and might cause cell damage to nematodes (Eder *et al.*, 2021).

The Oligo product

Chitosan oligosaccharide is the active ingredient of the Oligo product and can reduce nematode populations inhibiting reproduction through chitinolytic micro-organisms (Fan *et al.*, 2022). Autofluorescence was used in *M. incognita* eggs and J2 to detect lipofuscin accumulation. This accumulation can increase the process of aging, and damage cell tissue and other cellular structures while it also negatively affects the reactive oxygen species' (ROS) metabolism (Fan *et al.*, 2022). The autofluorescence increased significantly in *M. incognita* J2 when it was exposed to a derivative of chitosan oligosaccharide (synthesis of 6-((5-((3,4,4-trifluorobut-3-en-1-yl) thio)-1,3,4-oxadiazol-2-yl) methoxy), thus explaining the mode of action of chitosan oligosaccharide (Fan *et al.*, 2022). During the same study, J2 hatching of *M. incognita* was inhibited by 80 to 100% at different concentrations after the derivative treatment, while J2 immotility reached 80% after 48 h of exposure and increased to 100% over time (Fan *et al.*, 2022). Our results showed that the percentage of immotility was higher than 96% for all concentrations tested.

The Ses-A product

Sesame oil extracts were the active ingredient of the product Ses-A. A comparable study was conducted by McCarthy *et al.* (2010) where Neotrol with sesame extracts was tested to determine immotility of J2 of *Meloidogyne javanica* after an exposure time of 48 h on a solid medium and using an aqueous solution. The study concluded that high concentrations of Neotrol can cause some immotility in nematodes when using an aqueous solution; the solid medium illustrated that the nematodes would move away from the Neotrol. This would indicate that sesame extract might have repellent effects rather than killing the nematodes (McCarthy *et al.*, 2010). In our bioassay, good results were obtained with all concentrations and J2 immotility of more than 89% was evident from 24 h after exposure.

The Aba-5 product

Abamectin is the active ingredient of the Aba-5 product. Abamectin is a secondary metabolite excreted by the bacteria *Streptomyces avermitilis*, which is commonly found in a variety of soils. Studies have shown that abamectin causes irreversible immotility of PPN and can be used to control *Meloidogyne* spp. effectively with increased concentrations of abamectin

leading to elevated nematode mortality (Qiao *et al.*, 2012). Laboratory experiments showed a mortality rate of 91.9% after 48 h exposure (Qiao *et al.*, 2012), while further studies under greenhouse conditions with tomato plants showed a significant reduction in root galls with different concentrations of abamectin. In the study, plants treated with 10 L/ha had a root gall index of 2.1, while plants treated with 5 L/ha had a root gall index of 3.3, which were substantially lower compared to the control plants with an index of 5.2 (Qiao *et al.*, 2012). The greenhouse experiments also showed increased plant height and vigour. When the experiment was repeated in two field trials similar results were obtained (Qiao *et al.*, 2012). It therefore compares very well to the results obtained in our study where J2 immotility of 89% was observed after 24 h, with the J2 immotility rate increasing over time.

The Graphite product

The Graphite product contains crushed graphite particles and research has shown that graphene as a pure carbon compound extracted from graphite resulted in a 94% reduction of nematode galls, and 99% reduction in egg masses and immotility of 98.5% of J2 of *Meloidogyne* spp. (Gareeb *et al.*, 2019). Our study confirmed these results and showed a 94% reduction in J2 motility after 24 h of exposure.

The products BM-T and Green liquid

Both these products are known as bio-fertilizers containing a variety of soil microbes, amino acids, and carbon. Although no statistical data could be found on the reduction of PPN using bio-fertilizers such as BM-T and Green liquid, an understanding of the interaction between PPN and their micro-environment may lead to the development of synthetic microbial communities to facilitate disease suppression and biocontrol of PPN (Topalovic *et al.*, 2020). These products which contain amino acids, sugars, carbon, and other organic compounds can lead to modifications in the nematode surfaces and influence the microbial attachment (Topalovic *et al.*, 2020). According to Topalovic *et al.* (2020), the interaction between crops and beneficial microbiota can lead to decreased nematode infection.

The product Oxa-40

This product contains 40% oxamyl and 60% Green liquid. Oxamyl is a chemical compound known for its efficiency on nematode mortality, however, this chemical has been banned in both the United States of America (USA) and Europe (Alvares *et al.*, 2022). Due to high toxicity levels and residues on crops, combining the oxamyl with a bio-fertilizer may lower the oxamyl concentration while improving plant growth. Ali and El-Ashry (2021) studied the effects of oxamyl in combination with three surfactants. Throughout the study, improved growth and development of tomato plants was observed as well as a 90% inhibition of *M. incognita* J2 hatching.

A consideration of *in vitro* bioassays, where the product is applied in a 1 to 30% solution, is that it is easier to obtain good results since the other variables are all controlled, and no interactions are present. A controlled environment cannot account for environmental interactions such as UV radiation and prevailing climatic conditions. It is therefore necessary to test these products under more natural conditions such as microplots or in the field. Future *in vitro* studies can also evaluate the effect of the products on J2 hatching and its effect on different nematode species such as *Pratylenchus* spp. and *Heterodera* spp.

5.2 Microplot - *in vivo* experiment: effect of bio-fertilizers on the motility of *Meloidogyne* spp. J2's

The microplot experiment included three products from the *in-vitro* experiment that showed the highest efficacy on the immotility of the J2 of *Meloidogyne* spp. These products were Oligo-5, Graphite, BM-T and Green liquid. Other products of interest that is comparable to those from the *in-vitro* experiment were also included in this experiment. These products included Bio-Green granule, DT-200, RV42-20 while a positive standard, Velum® 1GR (an inorganic, commercially used nematicide registered locally on soybean) was included.

The Bio-Green granule product

This biostimulant/biofertilizer added in the microplot experiment consists of vermicast that includes the biological agents *Trichoderma* and mycorrhizae. *Trichoderma* does not have a direct effect on nematode motility; however, it does have a direct effect on the plant and increases the defence mechanisms against nematodes (Poveda *et al.*, 2020). Plants can have transcriptomic, proteomic, and metabolomic modifications when *Trichoderma* is applied, which can lead to an optimal immune response and resistance to nematode infections (Poveda *et al.*, 2020). On the other hand, application of mycorrhiza can increase plant health by increasing moisture and nutrient uptake as it forms a symbiotic relationship with the plant, and structures of this beneficial fungus has the ability to extend further into the soil than the plant roots. Such extension acts as a root extension and increases the surface area of the roots resulting in improved accessibility to water and nutrients (Schouteden *et al.*, 2015). Although several studies have confirmed the reduction of PPN densities due to the presence of mycorrhiza, the mechanism of this interaction is still unknown (Schouteden *et al.*, 2015). Recent advances in genome sequencing of PPN species might lead to more insight into this interaction and could yield valuable insights in the future (Schouteden *et al.*, 2015). Our results showed significant control of *Pratylenchus* and egg numbers at 56 DAP when using Bio-Green granule confirming that the components are able to reduce nematode numbers. However, at 112 DAP, no control was visible. It might be interesting to conduct further studies with this product to identify the mode of action and maybe extend the efficacy period.

The product DT-200

DT-200 is commercially known as DiTera™ and contains the fungus *Myrothecium verrucaria* (90 g/kg). According to Dong *et al.* (2015), the fungus parasitizes all life stages of various PPN and it is suspected but not yet proven that *M. verrucaria* produces proteases, chitinases and toxins that facilitate the parasitism of these nematodes. A study done by Hagag (2021) determined the effectiveness of DT-200 on *M. incognita* populations on sugar beet in the laboratory as well as under greenhouse conditions. In our study, the effectiveness of DT-200 on *Meloidogyne* and *Pratylenchus* was tested in a microplot experiment, and the results obtained from the experiment indicated no significant reduction of nematode densities at both time intervals. The opposite results were found in, *in vivo* studies where a 92% J2 hatching reduction as well as 96% immotility of J2 was reported after 72 h of exposure (Hagag, 2021). The product in the greenhouse trials also resulted in reduced number of galls and final nematode population densities (86%) at a rate of 0.2 g/pot (Hagag, 2021). The opposite results can be attributed to controlled and non-controlled environments and the environmental adaptability of the active ingredient *M. verrucaria*.

The RV42-20 product

Application of RV-20 resulted in the reduction of *Pratylenchus* spp. densities which can be attributed to the application of *Bacillus amyloliquefaciens* (2.5×10^{10} spores/ml) which is the active ingredient of this product. These bacteria are known to survive extreme environmental conditions and cause nematode immotility (Souto *et al.*, 2019). A study was done by Souto *et al.* (2019) whereby *B. amyloliquefaciens* (3×10^9 spores/ml) was added to seed treatments and after evaluating its effect on *Pratylenchus brachyurus* (Godfrey, 1929) Filipjev and Schuurmans Stekhoven, 1941, 45 and 60 days after applying the treatments, reductions from 44 to 100% were recorded and a dose of 0.5 L/ha^{-1} resulted in 10 and 3 *P. brachyurus* individuals per g of roots, respectively (Souto *et al.*, 2019).

The product Velum® 1GR

The success of Velum® 1GR in the commercial market can be attributed to the active ingredient fluopyram as it inhibits the mitochondrial complex II as well as the succinate dehydrogenase (SDH) of *Meloidogyne* spp. (Scheleker *et al.*, 2022). SDH is an enzyme complex that can be found on the inner membrane of the mitochondria of the nematode cell and inhibition thereof reduces oxygen uptake and impact adversely on nematode respiration and motility; subsequently leading to mortality. This enzyme complex transports electrons and participates in the citric acid cycle in the organisms (Scheleker *et al.*, 2022). The inhibition of these systems can cause damage to the *Meloidogyne* spp.; however, they are able to recover, although the

nematode populations, reproduction potential and respiration would be greatly affected (Scheleker *et al.*, 2022). According to Han *et al.* (2023), fluopyram also affects the motility of *Pratylenchus penetrans* (Cobb, 1917) Filipjev and Schuurmans Stekhoven, 1941, however, the sensitivity of J2 was much higher compared to the J4 developmental stage. In our experiment, results were not convincing using this registered nematicide since only for eggs and *Pratylenchus* numbers at 56 DAP could significant differences be observed.

5.3 Tunnel and field trials - *in vivo* experiments: effect of Velum® 1GR nematicide and Bio-Green bio-fertilizer on nematode populations

Velum® 1GR and its combination with Bio-Green provided the best results in both tunnel and field trials concerning nematode control. These results are probably due to the nematicidal effects of Velum® 1GR as discussed in paragraph 5.2. Similar results were found in the field trial although the nematode numbers were substantially lower compared to densities in the tunnel trial where the pots were artificially inoculated. The Bio-Green product on its own did not efficiently control nematodes in the tunnel trial.

In general, several products were tested with good nematicidal properties. Some were evaluated further in field and tunnel trials, while others were only evaluated in bioassays. It is important to continue testing new products for nematicidal activity, especially if they are more environmentally friendly, easy to apply and affordable. Other application methods such as seed coatings could also be considered as they provide other beneficial characteristics in effective and more friendly nematode control. It is also important to evaluate the products on alternative crops such as maize, tomato, potatoes, and others. Another important aspect is testing it against other *Meloidogyne* and *Pratylenchus* species, as both genera consist of many economically important species. Finally, when testing new biological products, it is also important to investigate the effect of soil type and climatic conditions such as temperature and rainfall on the efficacy of the products.

An experimental limitation of field experiments can be the spot occurrence of nematodes and low population densities of the target nematode species. Although the required nematode species were present and had been confirmed as high in the preceding season when it caused severe damage in the planted soybean crop, the nematode population density was lower than expected at the termination of the trial. The reason for this phenomenon is not known although it is known that no application of nematicides was done during the previous summer season and winter season.

6 Conclusion

Plant-parasitic nematodes (PPN) are a significant problem in agriculture, causing major yield loss. Globally the most damaging nematode pest is the root-knot nematode (RKN) which can cause up to 44% loss in seed yield of soybean (Korayem and Mohamed, 2018). PPN are known to reduce plant growth, resulting in lower crop yields, and therefore several methods have been developed to control these pests (Sasaneli *et al.*, 2021). These methods include nematicides, botanicals, biological control as well as cultural control strategies (Subedi *et al.*, 2020). Chemical nematicides remain among the most used methods to control nematodes because of their high efficacy and easy application. However, in the last decade, multiple chemical nematicide groups have been banned due to the negative effects they exhibit on the environment and other non-target organisms (Oka, 2020). Therefore, new generation nematicides and other biocontrol products are being developed to keep up with the increased abundance of PPN in agricultural farmlands (Desaeger *et al.*, 2020). In this study several biological products (T3C, Oligo, Ses-A, Aba-5, Graphite, BM-T, Green liquid, and Oxa-40) as well as two commercial products with nematicidal activity were tested to evaluate their efficacy against *Meloidogyne* (RKN) and *Pratylenchus* (RLN) populations.

In vitro bioassays showed promising results since the percentage immotility of *M. incognita* for all the products evaluated was significant, compared to sterilized tap water as a standard. The product Oligo showed the highest percentage of immotility at different concentrations after 24 h.

The micro-plot experiments showed no significant differences between the different products tested and the untreated control, 112 DAP (days after planting), although Velum® 1GR and RV42-20 had the lowest nematode numbers when compared to the other products. However, at 56 DAP, the untreated control had significantly higher nematode populations than all products tested except for Graphite.

The two commercially available products (Velum® 1GR and Bio-Green granule) were also evaluated under tunnel and field conditions. At 112 DAP, the combination of Velum® 1GR and Bio-Green granule resulted in significantly lower nematode numbers compared to these products applied solo while the three treatments provided significantly lower nematode numbers than the untreated control.

We can conclude that the experimental bio-nematicide products from Elim Kunsmis negatively impacted the biology (motility of second-stage juveniles: J2) of *M. incognita* in an *in vitro* environment. However, the efficacy of these products under increased natural conditions such as the micro-plots did not result in extended nematode control since no significant changes in nematode population densities at 112 DAP were apparent.

The two commercially available products reduced RKN and RLN population densities significantly in soybean rhizospheres under tunnel and field conditions when they were applied in combination as well as separately. Farmers should consider both input costs and the percentage increase in yield before deciding on applying a combination of products or rather using one.

It is obvious that new products have the potential to reduce target PPN populations that has the potential to increase soybean yields. It is however important to develop nematode control strategies including chemical, biological, and cultural control methods such as resistant cultivars, crop rotations with resistant crops, to reduce nematode populations in a sustainable manner (Mwamula, 2022).

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